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(54) **Title**: COMPOSITIONS AND METHODS FOR USE OF HIGH MOLECULAR WEIGHT HYALURONIC ACID FOR CANCER THERAPY

(57) **Abstract**: The present invention provides compositions and methods for treating or preventing cancer in a subject in need thereof. In certain embodiments, the invention comprises compositions and methods for increasing the level of high molecular weight hyaluronic acid (HMW-HA) in a subject, thereby treating or preventing cancer.

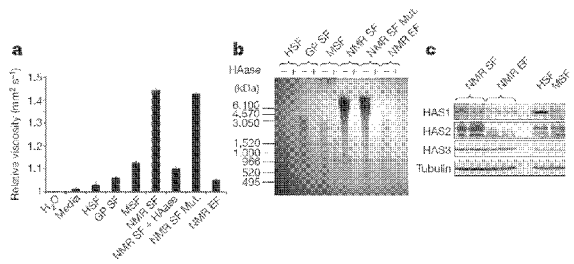

Figure 1A – Figure 1C

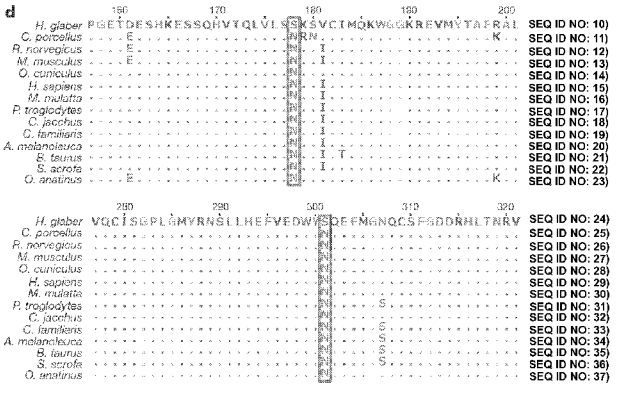

Figure 1D

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TITLE OF THE INVENTION

COMPOSITIONS AND METHODS FOR USE OF HIGH MOLECULAR WEIGHT HYALURONIC ACID FOR CANCER THERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 61/956,952 filed June 19, 2013, the contents of which are incorporated by reference herein in their entirety.

BACKGROUND OF THE INVENTION

Cancer remains one of the most common and most deadly afflictions in the world, accounting for approximately 8.2 million deaths in 2012 (World Cancer Report, 2014, World Health Organization). While there have been numerous efforts to identify anti-cancer therapies and prophylactics, the incidence of cancer continues to increase.

Previous studies identified a novel anticancer mechanism in the naked mole-rat, termed early contact inhibition (ECI) (Seluanov, A. et al., *Proc. Natl. Acad. Sci. USA, 2009,* 106:19207-19208). Contact inhibition is a process of arresting cell growth when cells come in contact with each other or the extracellular matrix. Contact inhibition is a powerful anticancer mechanism that is lost in cancer cells (Abercrombie, M., *Nature, 1979,* 281:259-262). Naked mole-rat cells arrest at a much lower density than mouse cells, and the loss of the ECI makes cells more susceptible to malignant transformation (Seluanov, A. et al., *Proc. Natl. Acad. Sci. USA, 2009,* 106:19207-19208). However, the signals triggering ECI in naked mole-rats remained unknown.

Thus, there is a need in the art for understanding anti-cancer mechanisms in the naked mole-rat and compositions and methods for treating cancer associated therewith. The present invention satisfies this unmet need.

SUMAMRY OF THE INVENTION

The present invention provides a method of treating or preventing cancer in a subject. The method comprises administering to the subject an effective amount of a composition which increases the level of high molecular weight hyaluronic acid (HMW-HA) in the subject. In one embodiment, the HMW-HA has a

molecular weight of at least 6,000 kDa. In one embodiment, the composition comprises HMW-HA.

In one embodiment, the composition comprises an isolated nucleic acid encoding mole-rat hyaluronic acid synthase 2 (HAS2). In one embodiment, the composition comprises mole-rat HAS2. In one embodiment, mole-rat HAS2 comprises the amino acid sequence of SEQ ID NO: 1.

In one embodiment, the composition comprises a cell which produces HMW-HA. In one embodiment, the cell is genetically modified to produce HMW-HA.

In one embodiment, the composition comprises a polymeric substrate. In one embodiment, the substrate comprises HMW-HA. In one embodiment, the substrate is administered to the subject in conjunction with surgical resection of a tumor.

The present invention provides a composition for treating or preventing cancer, wherein the composition comprises an agent which increases the level of high molecular weight hyaluronic acid (HMW-HA) in a subject. In one embodiment, the HMW-HA has a molecular weight of at least 6,000 kDa. In one embodiment, the composition comprises HMW-HA.

In one embodiment, the composition comprises an isolated nucleic acid encoding mole-rat hyaluronic acid synthase 2 (HAS2). In one embodiment, the composition comprises mole-rat HAS2. In one embodiment, mole-rat HAS2 comprises the amino acid sequence of SEQ ID NO: 1.

In one embodiment, the composition comprises a cell which produces HMW-HA. In one embodiment, the cell is genetically modified to produce HMW-HA.

In one embodiment, the composition comprises a polymeric substrate. In one embodiment, the substrate comprises HMW-HA.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of preferred embodiments of the invention will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiments which are presently preferred. It should be understood,

however, that the invention is not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

Figure 1, comprising Figure 1A through Figure 1D, depicts the results of experiments demonstrating that naked mole-rat cells secrete HA of exceptionally high molecular weight. (Figure 1A) Naked mole-rat cells make the culture media viscous. The viscosity of water, media or media conditioned with human skin (HSF), guinea pig skin (GP SF), mouse skin (MSF) or naked mole-rat skin (NMR SF) fibroblasts for 20 days. The NMR SF + HAase bar shows naked mole-rat conditioned media digested with HAase to specifically digest HA. The NMR SF Mut are naked mole-rat skin fibroblasts that spontaneously lost the ECI phenotype (Seluanov, A. et al., *Proc. Natl. Acad. Sci. USA, 2009,* 106:19207-19208). The NMR EF are naked mole-rat embryonic fibroblasts that do not show ECI. The experiment was repeated three times; error bars show standard deviation (s.d.) (Figure 1B) Purified HA separated on pulse-field gel. Each sample was either run intact or pre-digested with HAase. The experiment was repeated five times, using both skin and lung fibroblasts (Figure 13), and a representative gel is shown. (Figure 1C) Western blot showing the levels of HA synthases in naked mole-rat adult skin fibroblasts, naked mole-rat embryonic fibroblasts, human skin fibroblasts or mouse skin fibroblasts. (Figure 1D) Conserved catalytic domain of mammalian HAS2 proteins. The top sequence is the naked mole-rat HAS2. Dots indicate amino acids identical to the naked mole-rat sequence. The two amino acid changes unique to the naked mole-rat are indicated in the boxes.

Figure 2, comprising Figure 2A through Figure 2D, depicts the results of experiments demonstrating that naked mole-rat tissues contain high levels of HA. (Figure 2A) Naked mole-rat HAS2 overexpressed in human HEK293 cells secretes HMW-HA. Small panel on the right shows immunoblot with anti HAS2 antibodies on whole cell extracts from the control and HAS2-transfected cells. (Figure 2B) Tissues from the naked mole-rat, mouse, and guinea pig stained with Alcian Blue. The control samples treated with HAase do not show blue staining, demonstrating that the staining is specific to HA. Staining was performed on three different animals and representative skin and heart images are shown. Brain and kidney are shown in Figure 6. (Figure 2C) Naked mole-rat fibroblasts have low HAase activity. Naked mole-rat skin fibroblasts (NMR SF), guinea pig skin fibroblasts (GP SF), human skin fibroblasts (HSF), mouse skin fibroblasts (MSF) or HeLa cells were incubated with

the media containing HMW-HA for four days. The levels of HA were then analyzed by pulse-field gel. Control samples were incubated in the absence of cells. The experiments were repeated four times (all samples except GP), and three times for GF, error bars show s.d.; asterisk indicates $P<0.01$ by *t*-test. (Figure 2D) Naked mole-rat tissues have low HAase activity. Media containing HMW-HA was incubated with corresponding tissue fragments from naked mole-rats (NMR) or mice for six hours and HA levels analyzed by pulse-field gel. The experiments were repeated three times and error bars show s.d.; asterisk indicates $P<0.05$ by *t*-test.

Figure 3, comprising Figure 3A through Figure 3D, depicts the results of experiments demonstrating that HMW-HA is required for ECI. (Figure 3A) Naked mole rat cells (NMR SF) grown in the presence of HAase do not display ECI and proliferate to high cell density. (Figure 3B) Quantification of cell growth, showing the maximum cell number per plate achieved under indicated growth conditions. The last bar shows naked mole-rat cells grown in the presence of CD44-blocking antibody. The experiments were repeated four times (except the last bar, which was repeated three times) and error bars show s.d.; asterisk indicates $P<0.001$ by *t*-test. (Figure 3C) Naked mole-rat cells were grown in the presence of HAase for 12 days, then HAase was removed. (Figure 3D) Naked mole-rat cells have higher affinity to HA. Cells were incubated with fluorescein-labeled HA, and the average fluorescence was plotted. Experiment was repeated four times; error bars are standard error of the mean (s.e.m.).; asterisk indicates $P<0.001$ by *t*-test.

Figure 4, comprising Figure 4A and Figure 4B, depicts the results of experiments demonstrating that the removal of HMW-HA makes naked mole-rat cells susceptible to malignant transformation. (Figure 4A) Soft agar assays of anchorage-independent growth. Mouse (MSF) or naked mole-rat (NMR SF) cells were transfected with vectors encoding SV40 LT (LT) or its mutant derivatives K1 or Δ434 and H-Ras V12 (Ras), and plated in soft agar. Cells were cultured with or without HAase. The image shows colonies after 3 weeks of growth at 200× magnification. The experiment was repeated three times. (Figure 4B) Mouse xenograft experiment with naked mole-rat cells in which HMW-HA was abolished. NIH-III immunodeficient mice were injected with mouse (MSF) cells expressing SV40LT and H-Ras V12 as a positive control, or naked mole rat (NMR SF) cells expressing SV40LT and H-Ras V12 and either control shRNA, Hyal2 cDNA, or shRNA to

HAS2. All xenografts with mouse cells formed large tumors. Xenografts with naked mole-rat cells expressing control shRNA did not form tumors, while naked mole-rat cells overexpressing Hyal2 or HAS2 shRNA formed tumors in mice. The images show xenograft sites (arrows) and representative tumors. The number of xenografts resulting in tumor formation per the total number of xenografts with each cell type is shown on the right.

Figure 5, comprising Figure 5A and Figure 5B, depicts the results of experiments demonstrating that embryonic naked mole-rat fibroblasts do not undergo ECI. Embryonic naked mole-rat fibroblasts (NMR EF) were isolated from eight mid-gestation embryos. All the eight cultures showed similar growth characteristics. NMR EF cells did not undergo ECI and formed a dense monolayer on culture plates. (Figure 5A) Images of adult naked mole-rat skin fibroblasts (NMR SF), naked mole-rat embryonic fibroblasts, adult naked mole-rat lung fibroblasts (NMR LF), and mouse lung fibroblasts (MLF) at maximum cell density. (Figure 5B) Maximum cell density attained by fibroblasts from mouse and naked mole rat. For both mouse and naked mole rat, skin fibroblasts reach higher numbers on the plate than lung fibroblasts due to slightly smaller cell size. Naked mole-rat embryonic fibroblasts reach higher cell density than both skin and lung naked mole-rat cells. The experiments were repeated five times and error bars show s.d.

Figure 6 depicts the results of experiments demonstrating that naked mole-rat brain and kidneys contain high levels of HA. Tissues from the naked mole rat, mouse, and Guinea-pig were stained with Alcian Blue at pH 2.5. The control samples treated with HAase do not show blue staining, demonstrating that the staining is specific to HA. Staining was performed on three different animals and representative images are shown.

Figure 7 depicts the results of experiments demonstrating that naked mole-rat tissues contain high-molecular-weight HA. HA was purified from naked mole-rat (NMR) and mouse tissues, separated on pulse-field gel and stained with StainsAll solution. Samples were either run intact or pre-digested with HAase. In all the tissues, naked mole-rat HA had higher molecular mass than mouse HA.

Figure 8, comprising Figure 8A and Figure 8B, depicts the results of experiments analyzing cell death in the naked mole-rat cells after withdrawal of HAase. Naked mole-rat cells were cultured in the presence of HAase for 12 days, then HAase was removed and cells were cultured for additional four days. (Figure 8A)

Total cell number was decreased. The experiment was repeated three times and error bars show s.d.; asterisk indicates P=0.01 by t-test. (Figure 8B) Withdrawal of HAase caused some cells to die by apoptosis. In the cells described above, apoptosis was analyzed by Annexin V staining. The experiment was repeated 3 times and error bars show s.d.; asterisk indicates P=0.005 by t-test.

Figure 9 depicts the results of experiments demonstrating that NF2 and $p16^{Ink4a}$ status changes in response to cell density and the presence of HA. Western blot showing NF2 phosphorylation status and $p16^{Ink4a}$ levels in ECI (corresponding to $6x10^5$ cells per 10 cm plate), growing (g, corresponding to $3x10^5$ cells per 10 cm plate) and confluent (c, corresponding to $2x10^6$ cells per 10 cm plate) naked mole-rat cells. HSF, human skin fibroblasts; MSF, mouse skin fibroblasts; NMR SF, naked mole-rat skin fibroblasts; NMR SF Mut, naked mole-rat fibroblasts that lost ECI; HAase, enzyme that specifically digests HA.

Figure 10 depicts the results of experiments demonstrating that naked mole-rat cells cultured with CD44 antibody and embryonic naked mole-rat cells from colonies in soft agar. Adult naked mole-rat skin fibroblasts (NMR SF, left and middle columns), or embryonic naked mole-rat fibroblasts (NMR EF, right column) were transfected with SV40 Large T antigen (LT) or its derivatives K1 (inactivates p53 only) or Δ434 (inactivates Rb only) and H-Ras V12, and plated in soft agar. NMR SF cells were either cultured in the standard media (left column), or in the media supplemented with CD44-blocking antibody (Monoclonal Mouse IgG_{2A} Clone #2C5, R&D Systems). Adult naked mole-rat fibroblasts did not form colonies in soft agar, while blocking HA signaling with CD44 antibody made naked mole-rat cells susceptible to transformation with LT and oncogenic Ras. Embryonic naked mole-rat cells which do not secrete HMW-HA (Figure 1) readily formed colonies in soft agar upon introduction of LT and oncogenic Ras. Photographs were taken at 200× magnification.

Figure 11, comprising Figure 11A through Figure 11C, depicts the results of experiments demonstrating that knock-down of *HAS2* and overexpression of Hyal2 reduce HMW-HA production. (Figure 11A) shRNA to the naked mole-rat HAS2 gene was integrated in the genome of naked mole-rat skin fibroblasts (NMR SF) stably expressing SV40 Large T antigen and H-Ras V12. The resulting cell line (NMR SF +LT +Ras +shRNA HAS2) had strongly reduced HAS2 expression as determined by real-time RT-PCR. The experiments were repeated three times and

error bars show s.d. (Figure 11B) Hyal2 cDNA under CMV promoter was stably integrated in the naked mole-rat cells already expressing SV40 Large T and H-Ras V12 (NMR SF + LT +Ras). Western blot with Hyal2 antibodies is shown. (Figure 11C) Hyal2 expression or shRNA to *HAS2* reduce viscosity of the naked mole-rat conditioned media. Viscosity of the media conditioned with naked mole-rat cells expressing SV40 Large T antigen and H-Ras V12, and either shRNA to *HAS2* or Hyal2 was measured after 7 days using Ostwald Viscometer. The experiments were repeated three times and error bars show s.d.

Figure 12 depicts the results of experiments demonstrating that naked mole-rat cells in which HMW-HA is abolished by Hyal2 overexpression or shRNA to *HAS2* form colonies in soft agar assay. Mouse skin fibroblasts expressing SV40 Large T antigen and H-Ras V12 (MSF +LT +Ras), naked mole-rat skin fibroblasts expressing SV40 Large T antigen and H-Ras V12 (NMR SF +LT +Ras) were plated in soft agar. SV40 Large T antigen and H-Ras V12 are sufficient to transform mouse cells, but not the naked mole-rat cells. However, when Hyal2 cDNA or shRNA to *HAS2* were expressed in the naked mole-rat cells in addition to SV40 Large T antigen and H-Ras V12 these cells formed colonies in soft agar. Images were taken at 40× magnification.

Figure 13 depicts the results of experiments demonstrating that cells from another subterranean rodent, the blind mole rat (*Spalax judaei*) secrete HMW-HA. HA was purified from the media conditioned by mouse skin fibroblasts (MSF), naked mole-rat skin fibroblasts (NMR SF), naked mole-rat mutant skin fibroblasts (NMR SF Mut), naked mole-rat embryonic fibroblasts (NMR EF), naked mole-rat lung fibroblasts (NMR LF), or blind mole-rat skin fibroblasts (BMR SF), run on pulse field gel, and stained with StainsAll solution. Each sample was either run intact or was digested with 1 U/ml of HAase enzyme to show that the staining is specific for HA.

Figure 14 depicts the results of experiments demonstrating that HMW-HA containing media inhibits the growth of human cancer cells such as HeLa (left) and HT1080 (right).

DETAILED DESCRIPTION

The present invention provides compositions and methods for treating and preventing cancer in a subject diagnosed with cancer, suspected of having cancer,

or at risk for developing cancer. The present invention is based, in part, upon the finding that high molecular weight hyaluronic acid (HMW-HA) produced by the mole-rat confers an anti-cancer phenotype to the mole-rat. Further, it was found that hyaluronic acid synthase 2 (HAS2) of the mole-rat, differs from the HAS2 observed in other organisms, and is responsible for producing the HMW-HA produced in the mole-rat. Thus, the present invention provides compositions and methods for treating or preventing cancer in the subject by increasing the level of HMW-HA in a subject. For example, in certain aspects the present invention comprises the use of HMW-HA, an agent that increases production of HMW-HA, an isolated nucleic acid encoding HAS2, and a peptide comprising HAS2 to treat and prevent cancer in a subject in need thereof.

Definitions

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. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

As used herein, each of the following terms has the meaning associated with it in this section.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

"About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20%, ±10%, ±5%, ±1%, or ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

The term "abnormal" when used in the context of organisms, tissues, cells or components thereof, refers to those organisms, tissues, cells or components thereof that differ in at least one observable or detectable characteristic (e.g., age, treatment, time of day, etc.) from those organisms, tissues, cells or components thereof that display the "normal" (expected) respective characteristic. Characteristics which are normal or expected for one cell or tissue type, might be abnormal for a different cell or tissue type.

The term "cancer" as used herein is defined as disease characterized by the rapid and uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of various cancers include but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, head and neck cancers, lymphoma, leukemia, lung cancer and the like.

A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate.

In contrast, a "disorder" in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

A disease or disorder is "alleviated" if the severity of a symptom of the disease or disorder, the frequency with which such a symptom is experienced by a patient, or both, is reduced.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

An "effective amount" or "therapeutically effective amount" of a compound is that amount of compound which is sufficient to provide a beneficial effect to the subject to which the compound is administered. An "effective amount" of a delivery vehicle is that amount sufficient to effectively bind or deliver a compound.

"Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a

nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses (*e.g.*, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

"Homologous" refers to the sequence similarity or sequence identity between two polypeptides or between two nucleic acid molecules. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of positions compared X 100. For example, if 6 of 10 of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology. Generally, a comparison is made when two sequences are aligned to give maximum homology.

"Isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

The terms "patient," "subject," "individual," and the like are used interchangeably herein, and refer to any animal, or cells thereof whether *in vitro* or *in*

situ, amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a human.

"Parenteral" administration of a composition includes, *e.g.*, subcutaneous (s.c.), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, or infusion techniques.

The term "polynucleotide" as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into the monomeric "nucleotides." The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation, recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and PCR™, and the like, and by synthetic means.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

The term "promoter" as used herein is defined as a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

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 tissue specific manner.

A "constitutive" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

A "tissue-specific" promoter is a nucleotide sequence which, when operably linked with a polynucleotide encodes or specified by a gene, causes the gene product to be produced in a cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs of pathology, for the purpose of diminishing or eliminating those signs.

As used herein, "treating a disease or disorder" means reducing the frequency with which a symptom of the disease or disorder is experienced by a patient. Disease and disorder are used interchangeably herein.

The phrase "therapeutically effective amount," as used herein, refers to an amount that is sufficient or effective to prevent or treat (delay or prevent the onset of, prevent the progression of, inhibit, decrease or reverse) a disease or condition, including alleviating symptoms of such diseases.

To "treat" a disease as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject.

As used herein, the phrase "a tumor site" refers to any site or region within a subject which a tumor has formed, may be expected to form, or was previously located. In certain embodiments, the tumor site is in need of anti-tumor activity.

A "vector" is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Description

The present invention provides compositions and methods for the treatment and prevention of cancer. In certain embodiments, the invention relates to compositions and methods for increasing the level of high molecular weight hyaluronic acid (HMW-HA) for the treatment or prevention of cancer in a subject. In certain instances, HMW-HA is produced by a hyaluronic synthase (HAS2) protein. In one embodiment, the HAS2 protein is mole rat HAS2. It is described herein that mole rat HAS2 is distinct from HAS2 in other organisms, including other rodents. In certain instances, mole rat HAS2 is responsible for the production of HMW-HA that

confers an anti-cancer phenotype of mole rats. A representative sequence of HAS2, which is the naked mole rat HAS2 amino acid sequence, is presented in SEQ ID NO: 1. It is noted that the mole rat HAS2 comprises a Serine residue at positions 178 and 301 (underlined), which differs from HAS2 in other rodents.

SEQ ID NO:1 is:

MHCERFLCILRIIGTTLFGVSLLLGITAAYIVGYQFIQTDNYYFSFGLYGAFLAS
HLIIQSLFAFLEHRKMKKSLETPIKLNKTVALCIAAYQEDPDYLRKCLQSVKRL
TYPGIKVVMVIDGNSDDDLYMMDIFSEVMGRDKSATYIWKNNFHEKGPGET
DESHKESSQHVTQLVLS**S**KSVCIMQKWGGKREVMYTAFRALGRSVDYVQVC
DSDTMLDPASSVEMVKVLEEDPMVGGVGGDVQILNKYDSWISFLSSVRYWM
AFNIERACQSYFGCVQCISGPLGMYRNSLLHEFVEDWY**S**QEFMGNQCSFGDD
RHLTNRVLSLGYATKYTARSKCLTETPIEYLRWLNQQTRWSKSYFREWLYN
AMWFHKHHLWMTYEAVITGFFPFFLIATVIQLFYRGKIWNILLFLLTVQLVGL
IKSSFASCLRGNIIMVFMSLYSVLYMSSLLPAKMFAIATINKAGWGTSGRKTIV
VNFIGLIPVSVWFTILLGGVIFTIYKESKKPFSESKQTVLIVGTLLYACYWVML
LTLYVVLINKCGRRKKGQQYDMVLDV (SEQ ID NO: 1)

In one aspect, the present invention provides a composition comprising an isolated nucleic acid encoding HAS2. In one embodiment, the present invention provides a composition comprises a peptide comprising HAS2. The present invention also relates to methods of using an isolated nucleic acid encoding HAS2 or an HAS2 peptide for the treatment or prevention of cancer.

The present invention provides a method of treating or preventing cancer in a subject in need thereof. For example, the subject may be diagnosed with cancer, suspected of having cancer, or at risk for developing cancer. The present method may be used to treat or prevent any time of cancer, including, but not limited to, a solid tumor, blood cancer, skin cancer, breast cancer, pancreatic cancer, glioblastoma, prostate cancer, epithelial tumors, and the like.

In certain embodiments, the method comprises administering to the subject an effective amount of a composition which increases the level of HMW-HA in the subject. Exemplary compositions may comprise, for example, HMW-HA, an isolated nucleic acid encoding HAS2, an isolated HAS2 peptide, a cell which produces HMW-HA, a genetically modified cell which produces HMW-HA, an agent that increased production of HMW-HA, an inhibitor of a negative regulator of HMW-

HA, an agent that increased HAS2, an inhibitor of a negative regulator of HAS2 or a combination thereof.

Compositions

The present invention comprises compositions for treating and preventing cancer. In one embodiment, the composition comprises HMW-HA. In certain embodiments, the molecular weight of HMW-HA is greater than 6,000 kDa as determined on agarose gel electrophoresis. In various embodiments, the molecular weight of the HMW-HA is greater than 7,000, 8,000, 9,000, 10,000, 11,000, 12,000, 13,000, 14,000, and 15,000 kDa (including molecular weight in kDa of all integers between 6,000 and 15,000). Thus, in embodiments, when a reference is made to a HMW-HA, it means HA which has a molecular weight of at least 6,000 kDa.

The HMW-HA is an unbranched disaccharide glucuronic acid/N-acetylglucosamine polymer. HA is one of the major components of the extracellular matrix. Biological responses triggered by HA depend on the HA polymer length. HMW-HA represses mitogenic signaling and has anti-inflammatory properties, while low molecular weight HA promotes proliferation and inflammation.

In one embodiment, HMW-HA can be obtained from a cell which produces HMW-HA. In certain embodiments, the cell is cultured from a mole rat cell. In one embodiment, the cell is cultured from an adult mole rat fibroblast. As described elsewhere herein, mole rats produce significant amounts of HMW-HA. In one embodiment, the cell is a recombinant cell genetically modified to express HAS2 and produce HMW-HA. HMW-HA can be conveniently collected in the conditioned medium obtained of these cells.

In certain embodiments, HMW-HA is obtained from a cell which is cultured from a mole rat cell. The mole rat cell can be obtained from any mole rat. For example, mole rats belonging to family Bathyergidea, family Spalacidae, or family Geomyidae can be used. Any mole rat species from these families can be used. For example, useful species from the Bathyergidea family include Naked Mole Rat (*Heterocephalus glaber*), Cape Dune Mole Rat (*Bathyergus suillus*), Namaqua Dune Mole Rat (*Bathyergus janetta*), Slivery Blesmol (*Heliophobius argenteocinereus*), Ghana Blemol (*Fukomys zechi*), Malawian Blesmol (*Fukomys whytei*), Ochre Blemol (*Fukomys ochraceocinereus*), Kataba Blesmol (*Fukomys micklemi*), Mechow's Blesmol (*Fuomys mechowii*), Kafue Blesmol (*Fuomys kafuensis*), Nigerian Blesmol

(*Fukomys foxi*), Mashona Blesmol (*Fukomys darlingi*), Damaraland Blesmol (*Fukomys damarensis*), Bocage's Blesmol (*Fukomys bocagei*), Ansell's Blesmol (*Fukomys anselli*), Zambian Blesmol (*Fukomys amatus),* Matabeleland Mole Rat (*Cryptomys nimrodi*), Cape Blesmol (*Georychus capensis*). Examples of useful species from the Spalacidae family include Middle East Blind Mole Rat (*Spalax ehrenbergi*) (Superspecies), Sandy Mole Rat (*Spalax aenarius*), Mt. Carmel Blind Mole Rat (*Spalax carmeli*), Upper Galilee Mountains Blind Mole Rat (*Spalax galili*), Giant Mole Rat (*Spalax giganteus*), Golan Heights Blind Mole Rat (*Spalaz golani*), Balkan Mole Rat (*Spalax graecus*), Judean Mountains Blind Mole Rat (*Spalax judaei*), Lesser Mole Rat (*Spalax graecus*), Greater Mole Rat (*Spalax microphthalmus*), Munzur Mole Rat (*Spalax munzuri*), Nehring's Blind Mole Rat (*Spalax nehringi*), Kazakhstan Bline Mole Rat (*Spalax auralensis*), Podolsk Mole Rat (*Spalaz zemni*). Examples of useful species from Geomyidae family include Pocket gophers (*Geomys, Heterogeomys, Orthogeomys, Pappogeomys, Thomomys, and Zygogeomys*). Examples of useful species from the genus Chinchilla include (*Chinchilla lanigera*). Examples of useful species from the Talpidae family include star-nose mole (*Condylura cristata*). Examples of useful species from the Ctenomyidae family include tuco-tuco (*Ctenomys sp.)*

In one embodiment, non-embryonic cells are obtained from the mole rat. For example, cells can be obtained from adult mole rats. While any cells can be obtained and cultured from the mole rats, it is convenient to obtain fibroblasts (such as skin fibroblasts) because these can be conveniently maintained in culture.

In one embodiment, the mole rat is the naked mole rat (NMR) (*Heterocephalus glaber*). It is known for eusocial colony structure and behavioral characteristics. It is a small rodent with exceptionally long lifespan (up to 30 years) and resistance to cancer. It is observed that cells obtained from the NMR are unable to form robust anchorage-independent growth when transfected with oncogenic Ras and SV40 Large T, while this combination readily transforms mouse fibroblasts. It was found that this resistance to oncogenic transformation is possibly due to NMR fibroblasts displaying hypersensitivity to contact inhibition, a phenomenon termed "early contact inhibition" ("ECI"; also referred to herein as "E.C.I."), as their fibroblasts arrest at a much lower density than those from a mouse or human. This early contact inhibition requires the activity of p53 and pRb tumor suppressor pathways and is associated with the induction of $p16^{Ink4a}$. It has been determined that

upstream signaling of early contact inhibition is induced by NMR cells producing an excess of HMW-HA (>6,000 kDa).

NMR cells that undergo early contact inhibition overexpress HMW-HA, its synthase HAS2 and also CD44, when compared to early contact inhibition null embryonic NMR cells. This HMW-HA then may stimulate the ECI phenotype through interaction with an NMR specific isoforms or posttranslational modification of CD44 and results in maintaining the tumor suppressor NF2 in a growth prohibitive/anti-cancer dephosphorylated form. Removal of HMW-HA from NMR skin fibroblast (SF) cultures resulted in the loss of ECI, the phosphorylation of NF2 and the ability of NMRSF cells to be oncogenically transformed and form robust colony growth in an anchorage independent soft agar assay. The HMW-HA secreted by NMR cells proved to offer resistance to both internal and external oxidative stress. NMRSF derived HMW-HA was effective at providing resistance to oxidative stress to human cells. Due to the ease of harvesting culture media from NMRSF cells and obtaining HMW-HA in solution, these cells can be used for the production of highly desirable and clinically effective HMW-HA.

The HMW-HA can be obtained from the cells of any mole rat, such as a naked mole rat or a blind mole rat. A convenient cell type as a source of HMW-HA from these mole rats is fibroblasts. The fibroblasts can be obtained from mole rats of any age by techniques well known in the art. Generally, skin fibroblasts are easily obtained and methods for obtaining skin fibroblasts are well known in the art.

In one embodiment, primary fibroblasts or cell lines established from the primary fibroblasts can be used. In some instances, the cell lines are immortalized. For example, primary fibroblasts can be cultured from the skin of mole rats. An exemplary protocol for obtaining skin fibroblasts is now described.

Skin is obtained from mole rats by standard process. A convenient area for obtaining skin is the underarm area as the skin. Generally, the naked mole rats do not have fur, but if fur is present, it can be shaved first. The skin sample is then cut into fragments (such as 1 cm^2). The skin fragments are further cut into smaller fragments (such as about 1 mm pieces). The fragments are then exposed to protease mixture to loosen the cells (such as Liberase Blendzyme 3, and 1X antibiotic/antimycotic) generally in a tissue culture medium (such as DMEM/F12). Digestion can be carried out at 37°C for a desirable period (such as 30-90 minutes). Digestion can be stopped by removing the digestion medium or by adding enzyme

inhibitors. Released cells can be collected, centrifuged and re-suspended in a suitable medium (such as DMEM/F12 with 10-15% FBS). The primary fibroblast cultures can be incubated at 37°C, 5% CO_2 ad 3% O_2).

In one embodiment, fast proliferating transformed mole-rat cell lines are produced. Transformed cell lines can be obtained in at least two ways: (1) spontaneous transformation is sometimes observed when the primary cells are cultured for over 100 days; (2) by stably transfecting primary cells with SV40 large T antigen (Simian Vacuolating Virus 40 Tag; "LT"). LT is derived from polyomavirus SV40. Its amino acid sequence and nucleotide sequences encoding it are known in the art. It is a hexamer protein that can transform a variety of cell types. The mechanism by which it can be used to transform cells is known (see, for example, Ali SH, DeCaprio JA. Cellular transformation by SV40 large T antigen: interaction with host proteins. Semin Cancer Biol. 2001 Feb;11(1):15-23) and relates to inactivation of two major and well characterized tumor suppressor pathways, Rb and p53. Polynucleotide vectors encoding LT that can be used to transform cells according to the method of the invention are available to the public from, for example, Addgene, Cambridge, Massachusetts, USA, but the invention includes transforming cells using any suitable polynucleotide that encodes LT, or any derivative of LT that can transform eukaryotic cells. Several exemplary cell lines have successfully been produced by transformation using LT-encoding expression vectors (plasmid 13970 from Addgene, Inc.).

The cultured cells, such primary, secondary or any passage cells as well as long-term cultures of mole rat cells or from stably transformed mole rat cells such as fibroblasts may be cultured in any suitable method. The mole rat cells can be cultured in monolayer, beads (i.e., two-dimensions) or in three-dimensional culture systems. The cells can be cultured by standard methods using aseptic processing and handling. The cells are cultured in the desired culture medium. In various embodiments the cells can be cells from the NMR or the BMR.

The cell culture medium in which the NMR cells are cultured can be any standard cell culture medium which provides adequate nutrition to the cells. Before being conditioned (such as before being added to the cells), the medium is termed as "pre-conditioned medium". Suitable cell media include, but are not limited to EMEM, Dulbecco's Modified Eagle's Medium (DMEM), Ham's F12, RPMI 1640, Iscove's, McCoy's and other media formulations readily apparent to those skilled in the art. Such media can be easily prepared or obtained from commercial sources.

Details of cell culture media and methods can be found in Methods For Preparation of Media, Supplements and Substrate For Serum-Free Animal Cell Culture Alan R. Liss, New York (1984) and Cell &Tissue Culture: Laboratory Procedures, John Wiley & Sons Ltd., Chichester, England 1996. The medium may be supplemented, with components, such as vitamins, growth factors, proteins, sugars, anti-oxidants, etc. as necessary to support the desired cell culture. Additionally serum, such as bovine serum, which is a complex solution of albumins, globulins and growth factors may be added if desired. For human use, if desired, animal serum be avoided. Instead, serum-free medium can be used or human plasma can be added in the same amount as animal serum. Hormones, growth factors or other agents may be added into the medium.

The cells can be used in continuous cultures or can be frozen for later use. Techniques for freezing and reculturing frozen cells are well known in the art.

Conditioned medium can be obtained after suitable incubation times (such as 3 hours to 30 days and all times therebetween) and HA can be purified from the conditioned media to the extent desired or the conditioned medium can be used as such.

HMW-HA can be purified from the conditioned media by, for example, the following process comprising treating the conditioned medium with proteases, ethanol precipitation, resuspension of the precipitate in buffer and gel electrophoresis wherein HMW-HA can be identified by doing treatment with hyaluronidase.

The conditioned media obtained from the cells should be processed under sterile conditions or sterilized as needed. When appropriate (i.e., once the medium is conditioned so that hyaluronic acid or some other marker such as growth factors have reached desirable levels in the medium) the "conditioned" medium can be collected. In one embodiment, the conditioned media can be collected anytime from 3 hours to 30 days of conditioning (i.e., after plating). In one embodiment, the conditioned medium is collected after 2, 3, 6, 12, 18, 24, 30, 36, 42, 48 hours, 3, 4, 5, 10, 15, 20, 25 or 30 days and all days and hours therebetween, and all ranges of time between 2 hours and 30 days. In one embodiment, it is collected anytime between 2 hours and 60 hours of incubation.

The mole rat cell derived conditioned medium is unique in that it contains a high level of HMW-HA. Therefore in one embodiment, this invention

provides a neat (unprocessed) conditioned medium from the cells provided herein or partially or fully purified fractions of the medium comprising high molecular weight hyaluronic acid. In one embodiment, the conditioned medium from the cells comprises at least 50 ng/ml of HA having a molecular weight of at least 6,000 kDa. In various embodiments, the conditioned medium has at least 50 ng to 5 mg/ml of HMW-HA and all integer values and ranges in nanograms therebetween. In various embodiments, one ml of conditioned medium has at least 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 ng, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 µg of HMW-HA. In another embodiment, one ml of conditioned medium has 1 to 5 µg of HMW-HA.

If desired, the conditioned medium can be processed to concentrate selected components. For example, the medium may be concentrated 10 to 20 fold using a positive pressure concentration device (such as a device having a filter with a 0.2 or 0.45 µm cut-off (Amicon, Beverly, Mass.). Also, the conditioned medium may be further processed for HA isolation and purification to remove unwanted proteases. Methods of purification include ethanol precipitation followed by suspension in desired buffers including PBS, HEPES, TRIS and the like; gel chromatography, ion exchange, affinity chromatography HPLC purification and the like.

In one embodiment, the conditioned medium from the long term cultured or stably transformed fibroblasts can be collected once the cells are over 50% confluent. In various embodiments, conditioned medium can be collected when the cells are between 50 to 100% (and all integers therebetween) confluent. In one embodiment, the cells are 80-90% confluent. Once collected, the conditioned medium is filtered to remove debris. As discussed above, the filtered medium can be used neat or can be processed to remove and/or concentrate desired components. The conditioned medium with or without processing can be used fresh or can be stored (at refrigerator or freezer temperatures) for later use.

HMW-HA can be produced using the recombinant expression systems described herein or those that are otherwise apparent to those skilled in the art given the benefit of the present disclosure, or commercially available systems engineered to express the HAS2 described herein.

HMW-HA made using a recombinant expression system, which comprises a nucleic acid encoding the novel HAS2 described herein, can be purified from the expression system using any suitable technique, method and/or apparatus. In one embodiment, the HMW-HA is secreted into media by cells into which an

expression vector described herein has been introduced. Thus, in one embodiment, the invention provides cell culture media which contains the HMW-HA. The invention also includes compositions comprising HMW-HA that has been separated from, for example, conditioned media in which cells which express the HAS2 of the invention are cultured. The conditioned media can be, for example, treated with proteases, ethanol precipitation, resuspension of the precipitate in buffer and gel The HMW-HA can be purified to any desirable degree of purity. The conditioned media obtained from the cells should be processed under sterile conditions or sterilized as needed. When appropriate (i.e., once the medium is conditioned so that hyaluronic acid or some other marker such as growth factors have reached desirable levels in the medium) the "conditioned" medium can be collected. In one embodiment, the conditioned media can be collected anytime from 3 hours to 30 days of conditioning (i.e., after plating cells which express the recombinant HAS2 as described herein). In one embodiment, the conditioned medium is collected after 2, 3, 6, 12, 18, 24, 30, 36, 42, 48 hours, 3, 4, 5, 10, 15, 20, 25 or 30 days and all days and hours therebetween, and all ranges of time between 2 hours and 30 days. In one embodiment, it is collected anytime between 2 hours and 60 hours of incubation.

The recombinant cell-derived conditioned medium is unique in that it contains a high level of high molecular weight hyaluronic acid. Therefore in one embodiment, this invention provides a neat (unprocessed) conditioned medium from the cells provided herein or partially or fully purified fractions of the medium comprising high molecular weight hyaluronic acid. In one embodiment, the conditioned medium from the cells comprises at least 50 ng/ml of HA having a molecular weight of at least 6,000 kDa. In various embodiments, the conditioned medium has at least 50 ng to 5 mg/ml of HMW-HA and all integer values and ranges in nanograms therebetween. In various embodiments, one ml of conditioned medium has at least 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 ng, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 μg of HMW-HA. In another embodiment, one ml of conditioned medium has 1 to 5 μg of HMW-HA.

If desired, the conditioned medium can be processed to concentrate selected components. For example, the medium may be concentrated 10 to 20 fold using a positive pressure concentration device (such as a device having a filter with a 0.2 or 0.45 μm cut-off (Amicon, Beverly, Mass.). Also, the conditioned medium may be further processed for HA isolation and purification to remove unwanted proteases.

The conditioned medium with or without processing can be used fresh or can be stored (at refrigerator or freezer temperatures) for later use.

In one embodiment, the present invention provides a composition comprising an isolated nucleic acid encoding HAS2, or a biologically functional fragment thereof. In one embodiment, the isolated nucleic acid sequence encodes mole rat HAS2. In one embodiment, the isolated nucleic acid sequence encodes HAS2 comprising an amino acid sequence of SEQ ID NO: 1. Further, the invention encompasses an isolated nucleic acid encoding a peptide having substantial homology to HAS2 disclosed herein. In certain embodiments, the isolated nucleic acid sequence encodes HAS2 having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence homology with the amino acid sequence of SEQ NO: 1.

The isolated nucleic acid sequence encoding HAS2 can be obtained using any of the many recombinant methods known in the art, such as, for example by screening libraries from cells expressing the gene, by deriving the gene from a vector known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, the gene of interest can be produced synthetically, rather than cloned.

A representative nucleic acid sequence encoding the peptide of SEQ ID NO:1 is presented in SEQ ID NO:2. However, the present invention includes all nucleic acid sequences which can encode the amino acid sequence of SEQ ID NO:1.

In certain embodiments, the composition increases the expression of a biologically functional fragment of HAS2. For example, in one embodiment, the composition comprises an isolated nucleic acid sequence encoding a biologically functional fragment of HAS2. As would be understood in the art, a biologically functional fragment is a portion or portions of a full length sequence that retain the biological function of the full length sequence. Thus, a biologically functional fragment of HAS2 comprises a peptide that retains the function of full length HAS2.

The isolated nucleic acid may comprise any type of nucleic acid, including, but not limited to DNA and RNA. For example, in one embodiment, the composition comprises an isolated DNA molecule, including for example, an isolated cDNA molecule, encoding HAS2, or functional fragment thereof. In one embodiment, the composition comprises an isolated RNA molecule encoding HAS2, or a functional fragment thereof.

The nucleic acid molecules of the present invention can be modified to improve stability in serum or in growth medium for cell cultures. Modifications can be added to enhance stability, functionality, and/or specificity and to minimize immunostimulatory properties of the nucleic acid molecule of the invention. For example, in order to enhance the stability, the 3'-residues may be stabilized against degradation, e.g., they may be selected such that they consist of purine nucleotides, particularly adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine by 2'-deoxythymidine is tolerated and does not affect function of the molecule.

In one embodiment of the present invention the nucleic acid molecule may contain at least one modified nucleotide analogue. For example, the ends may be stabilized by incorporating modified nucleotide analogues.

Non-limiting examples of nucleotide analogues include sugar- and/or backbone-modified ribonucleotides (i.e., include modifications to the phosphate-sugar backbone). For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. In preferred backbone-modified ribonucleotides the phosphoester group connecting to adjacent ribonucleotides is replaced by a modified group, e.g., of phosphothioate group. In preferred sugar-modified ribonucleotides, the 2' OH-group is replaced by a group selected from H, OR, R, halo, SH, SR, NH_2, NHR, NR_2 or ON, wherein R is C_1-C_6 alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I.

Other examples of modifications are nucleobase-modified ribonucleotides, i.e., ribonucleotides, containing at least one non-naturally occurring nucleobase instead of a naturally occurring nucleobase. Bases may be modified to block the activity of adenosine deaminase. Exemplary modified nucleobases include, but are not limited to, uridine and/or cytidine modified at the 5-position, e.g., 5-(2-amino)propyl uridine, 5-bromo uridine; adenosine and/or guanosines modified at the 8 position, e.g., 8-bromo guanosine; deaza nucleotides, e.g., 7-deaza-adenosine; O- and N-alkylated nucleotides, e.g., N6-methyl adenosine are suitable. It should be noted that the above modifications may be combined.

In some instances, the nucleic acid molecule comprises at least one of the following chemical modifications: 2'-H, 2'-O-methyl, or 2'-OH modification of one or more nucleotides. In certain embodiments, a nucleic acid molecule of the invention can have enhanced resistance to nucleases. For increased nuclease

resistance, a nucleic acid molecule, can include, for example, 2'-modified ribose units and/or phosphorothioate linkages. For example, the 2' hydroxyl group (OH) can be modified or replaced with a number of different "oxy" or "deoxy" substituents. For increased nuclease resistance the nucleic acid molecules of the invention can include 2'-O-methyl, 2'-fluorine, 2'-O-methoxyethyl, 2'-O-aminopropyl, 2'-amino, and/or phosphorothioate linkages. Inclusion of locked nucleic acids (LNA), ethylene nucleic acids (ENA), *e.g.*, 2'-4'-ethylene-bridged nucleic acids, and certain nucleobase modifications such as 2-amino-A, 2-thio (e.g., 2-thio-U), G-clamp modifications, can also increase binding affinity to a target.

In one embodiment, the nucleic acid molecule includes a 2'-modified nucleotide, *e.g.*, a 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA). In one embodiment, the nucleic acid molecule includes at least one 2'-O-methyl-modified nucleotide, and in some embodiments, all of the nucleotides of the nucleic acid molecule include a 2'-O-methyl modification.

In certain embodiments, the nucleic acid molecule of the invention preferably has one or more of the following properties:

Nucleic acid agents discussed herein include otherwise unmodified RNA and DNA as well as RNA and DNA that have been modified, *e.g.*, to improve efficacy, and polymers of nucleoside surrogates. Unmodified RNA refers to a molecule in which the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are the same or essentially the same as that which occur in nature, preferably as occur naturally in the human body. The art has referred to rare or unusual, but naturally occurring, RNAs as modified RNAs, see, *e.g.*, Limbach *et al.* (*Nucleic Acids Res.*, 1994, 22:2183-2196). Such rare or unusual RNAs, often termed modified RNAs, are typically the result of a post-transcriptional modification and are within the term unmodified RNA as used herein. Modified RNA, as used herein, refers to a molecule in which one or more of the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are different from that which occur in nature, preferably different from that which occurs in the human body. While they are referred to as "modified RNAs" they will of course, because of the modification, include molecules that are not, strictly speaking, RNAs. Nucleoside surrogates are

molecules in which the ribophosphate backbone is replaced with a non-ribophosphate construct that allows the bases to be presented in the correct spatial relationship such that hybridization is substantially similar to what is seen with a ribophosphate backbone, *e.g.*, non-charged mimics of the ribophosphate backbone.

Modifications of the nucleic acid of the invention may be present at one or more of, a phosphate group, a sugar group, backbone, N-terminus, C-terminus, or nucleobase.

The present invention also includes a vector in which the isolated nucleic acid of the present invention is inserted. The art is replete with suitable vectors that are useful in the present invention.

In brief summary, the expression of natural or synthetic nucleic acids encoding HAS2 is typically achieved by operably linking a nucleic acid encoding the HAS2 or portions thereof to a promoter, and incorporating the construct into an expression vector. The vectors to be used are suitable for replication and, optionally, integration in eukaryotic cells. Typical vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence.

The vectors of the present invention may also be used for nucleic acid immunization and gene therapy, using standard gene delivery protocols. Methods for gene delivery are known in the art. *See, e.g.*, U.S. Pat. Nos. 5,399,346, 5,580,859, 5,589,466, incorporated by reference herein in their entireties. In another embodiment, the invention provides a gene therapy vector.

The isolated nucleic acid of the invention can be cloned into a number of types of vectors. For example, the nucleic acid can be cloned into a vector including, but not limited to a plasmid, a phagemid, a phage derivative, an animal virus, and a cosmid. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors, and sequencing vectors.

Further, the vector may be provided to a cell in the form of a viral vector. Viral vector technology is well known in the art and is described, for example, in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno- associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a

promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers, (e.g., WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193).

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. A number of retroviral systems are known in the art. In some embodiments, adenovirus vectors are used. A number of adenovirus vectors are known in the art. In one embodiment, lentivirus vectors are used.

For example, vectors derived from retroviruses such as the lentivirus are suitable tools to achieve long-term gene transfer since they allow long-term, stable integration of a transgene and its propagation in daughter cells. Lentiviral vectors have the added advantage over vectors derived from onco-retroviruses such as murine leukemia viruses in that they can transduce non-proliferating cells, such as hepatocytes. They also have the added advantage of low immunogenicity. In one embodiment, the composition includes a vector derived from an adeno-associated virus (AAV). Adeno-associated viral (AAV) vectors have become powerful gene delivery tools for the treatment of various disorders. AAV vectors possess a number of features that render them ideally suited for gene therapy, including a lack of pathogenicity, minimal immunogenicity, and the ability to transduce postmitotic cells in a stable and efficient manner. Expression of a particular gene contained within an AAV vector can be specifically targeted to one or more types of cells by choosing the appropriate combination of AAV serotype, promoter, and delivery method

In certain embodiments, the vector also includes conventional control elements which are operably linked to the transgene in a manner which permits its transcription, translation and/or expression in a cell transfected with the plasmid vector or infected with the virus produced by the invention. As used herein, "operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest. Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e.,

Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A great number of expression control sequences, including promoters which are native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized.

Additional promoter elements, e.g., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. Another example of a suitable promoter is Elongation Growth Factor -1α (EF-1α). However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter. Further, the invention should not be limited to the use of constitutive promoters. Inducible promoters are also contemplated as part of the invention. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is operatively linked when such expression is desired, or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

Enhancer sequences found on a vector also regulates expression of the gene contained therein. Typically, enhancers are bound with protein factors to enhance the transcription of a gene. Enhancers may be located upstream or downstream of the gene it regulates. Enhancers may also be tissue-specific to enhance transcription in a specific cell or tissue type. In one embodiment, the vector of the present invention comprises one or more enhancers to boost transcription of the gene present within the vector.

In order to assess the expression of HAS2, the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other aspects, the selectable marker may be carried on a separate piece of DNA and used in a co- transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic-resistance genes, such as neo and the like.

Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (e.g., Ui-Tei et al., 2000 FEBS Letters 479: 79-82). Suitable expression systems are well known and may be prepared using known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter- driven transcription.

Methods of introducing and expressing genes into a cell are known in the art. In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast, or insect cell by any method in the

art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook et al. (2012, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York). A preferred method for the introduction of a polynucleotide into a host cell is calcium phosphate transfection.

Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome (e.g., an artificial membrane vesicle).

In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (*in vitro, ex vivo* or *in vivo*). In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply

be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma, St. Louis, MO; dicetyl phosphate ("DCP") can be obtained from K & K Laboratories (Plainview, NY); cholesterol ("Choi") can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Chloroform is used as the only solvent since it is more readily evaporated than methanol. "Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh et al., 1991 Glycobiology 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

Regardless of the method used to introduce exogenous nucleic acids into a host cell, in order to confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; "biochemical" assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs

and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

In one embodiment, the composition of the present invention comprises a peptide comprising HAS2, or biologically functional fragment thereof. The peptide of the present invention may be made using chemical methods. For example, peptides can be synthesized by solid phase techniques (Roberge J Y et al (1995) Science 269: 202-204), cleaved from the resin, and purified by preparative high performance liquid chromatography. Automated synthesis may be achieved, for example, using the ABI 431 A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

The invention should also be construed to include any form of a peptide having substantial homology to HAS2 disclosed herein. Preferably, a peptide which is "substantially homologous" is about 50% homologous, more preferably about 70% homologous, even more preferably about 80% homologous, more preferably about 90% homologous, even more preferably, about 95% homologous, and even more preferably about 99% homologous to amino acid sequence of HAS2 disclosed herein.

The peptide may alternatively be made by recombinant means or by cleavage from a longer polypeptide. The composition of a peptide may be confirmed by amino acid analysis or sequencing.

The variants of the peptides according to the present invention may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, (ii) one in which there are one or more modified amino acid residues, e.g., residues that are modified by the attachment of substituent groups, (iii) one in which the peptide is an alternative splice variant of the peptide of the present invention, (iv) fragments of the peptides and/or (v) one in which the peptide is fused with another peptide, such as a leader or secretory sequence or a sequence which is employed for purification (for example, His-tag) or for detection (for example, Sv5 epitope tag). The fragments include peptides generated via proteolytic cleavage (including multi-site proteolysis) of an original sequence. Variants may be post-translationally, or chemically modified. Such variants are deemed to be within the scope of those skilled in the art from the teaching herein.

As known in the art the "similarity" between two peptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to a sequence of a second polypeptide. Variants are defined to include peptide sequences different from the original sequence, preferably different from the original sequence in less than 40% of residues per segment of interest, more preferably different from the original sequence in less than 25% of residues per segment of interest, more preferably different by less than 10% of residues per segment of interest, most preferably different from the original protein sequence in just a few residues per segment of interest and at the same time sufficiently homologous to the original sequence to preserve the functionality of the original sequence and/or the ability to produce HMW-HA. The present invention includes amino acid sequences that are at least 60%, 65%, 70%, 72%, 74%, 76%, 78%, 80%, 90%, or 95% similar or identical to the original amino acid sequence. The degree of identity between two peptides is determined using computer algorithms and methods that are widely known for the persons skilled in the art. The identity between two amino acid sequences is preferably determined by using the BLASTP algorithm [BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990)].

The peptides of the invention can be post-translationally modified. For example, post-translational modifications that fall within the scope of the present invention include signal peptide cleavage, glycosylation, acetylation, isoprenylation, proteolysis, myristoylation, protein folding and proteolytic processing, etc. Some modifications or processing events require introduction of additional biological machinery. For example, processing events, such as signal peptide cleavage and core glycosylation, are examined by adding canine microsomal membranes or Xenopus egg extracts (U.S. Pat. No. 6,103,489) to a standard translation reaction.

The peptides of the invention may include unnatural amino acids formed by post-translational modification or by introducing unnatural amino acids during translation. A variety of approaches are available for introducing unnatural amino acids during protein translation.

A peptide or protein of the invention may be conjugated with other molecules, such as proteins, to prepare fusion proteins. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins provided that the resulting fusion protein retains the functionality of the HAS2 comprising peptide.

A peptide or protein of the invention may be phosphorylated using conventional methods such as the method described in Reedijk et al. (The EMBO Journal 11(4):1365, 1992).

Cyclic derivatives of the peptides of the invention are also part of the present invention. Cyclization may allow the peptide to assume a more favorable conformation for association with other molecules. Cyclization may be achieved using techniques known in the art. For example, disulfide bonds may be formed between two appropriately spaced components having free sulfhydryl groups, or an amide bond may be formed between an amino group of one component and a carboxyl group of another component. Cyclization may also be achieved using an azobenzene-containing amino acid as described by Ulysse, L., et al., J. Am. Chem. Soc. 1995, 117, 8466-8467. The components that form the bonds may be side chains of amino acids, non-amino acid components or a combination of the two. In an embodiment of the invention, cyclic peptides may comprise a beta-turn in the right position. Beta-turns may be introduced into the peptides of the invention by adding the amino acids Pro-Gly at the right position.

It may be desirable to produce a cyclic peptide which is more flexible than the cyclic peptides containing peptide bond linkages as described above. A more flexible peptide may be prepared by introducing cysteines at the right and left position of the peptide and forming a disulphide bridge between the two cysteines. The two cysteines are arranged so as not to deform the beta-sheet and turn. The peptide is more flexible as a result of the length of the disulfide linkage and the smaller number of hydrogen bonds in the beta-sheet portion. The relative flexibility of a cyclic peptide can be determined by molecular dynamics simulations.

The invention also relates to peptides comprising HAS2 fused to, or integrated into, a target protein, and/or a targeting domain capable of directing the chimeric protein to a desired cellular component or cell type or tissue. The chimeric proteins may also contain additional amino acid sequences or domains. The chimeric proteins are recombinant in the sense that the various components are from different sources, and as such are not found together in nature (i.e. are heterologous).

In one embodiment, the targeting domain can be a membrane spanning domain, a membrane binding domain, or a sequence directing the protein to associate with for example vesicles or with the nucleus. In one embodiment, the targeting domain can target a peptide to a particular cell type or tissue. For example, the

targeting domain can be a cell surface ligand or an antibody against cell surface antigens of a target tissue (e.g. cancerous tissue). A targeting domain may target the peptide of the invention to a cellular component. In certain embodiments, the targeting domain targets a tumor-specific antigen or tumor-associated antigen.

A peptide of the invention may be synthesized by conventional techniques. For example, the peptides or chimeric proteins may be synthesized by chemical synthesis using solid phase peptide synthesis. These methods employ either solid or solution phase synthesis methods (see for example, J. M. Stewart, and J. D. Young, Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Co., Rockford Ill. (1984) and G. Barany and R. B. Merrifield, The Peptides: Analysis Synthesis, Biology editors E. Gross and J. Meienhofer Vol. 2 Academic Press, New York, 1980, pp. 3-254 for solid phase synthesis techniques; and M Bodansky, Principles of Peptide Synthesis, Springer-Verlag, Berlin 1984, and E. Gross and J. Meienhofer, Eds., The Peptides: Analysis, Synthesis, Biology, suprs, Vol 1, for classical solution synthesis.) By way of example, a RLP or chimeric protein may be synthesized using 9-fluorenyl methoxycarbonyl (Fmoc) solid phase chemistry with direct incorporation of phosphothreonine as the N-fluorenylmethoxy-carbonyl-O-benzyl-L-phosphothreonine derivative.

N-terminal or C-terminal fusion proteins comprising a peptide or chimeric protein of the invention conjugated with other molecules may be prepared by fusing, through recombinant techniques, the N-terminal or C-terminal of the peptide or chimeric protein, and the sequence of a selected protein or selectable marker with a desired biological function. The resultant fusion proteins contain the HAS2 comprising peptide or chimeric protein fused to the selected protein or marker protein as described herein. Examples of proteins which may be used to prepare fusion proteins include immunoglobulins, glutathione-S-transferase (GST), hemagglutinin (HA), and truncated myc.

Peptides of the invention may be developed using a biological expression system. The use of these systems allows the production of large libraries of random peptide sequences and the screening of these libraries for peptide sequences that bind to particular proteins. Libraries may be produced by cloning synthetic DNA that encodes random peptide sequences into appropriate expression vectors. (see Christian et al 1992, J. Mol. Biol. 227:711; Devlin et al, 1990 Science 249:404; Cwirla et al 1990, Proc. Natl. Acad, Sci. USA, 87:6378). Libraries may also be

constructed by concurrent synthesis of overlapping peptides (see U.S. Pat. No. 4,708,871).

The peptides and chimeric proteins of the invention may be converted into pharmaceutical salts by reacting with inorganic acids such as hydrochloric acid, sulfuric acid, hydrobromic acid, phosphoric acid, etc., or organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, succinic acid, malic acid, tartaric acid, citric acid, benzoic acid, salicylic acid, benezenesulfonic acid, and toluenesulfonic acids.

The present invention includes a composition comprising a cell which produces HMW-HA, comprises a nucleic acid encoding HAS2, comprises HAS2 protein, or a combination thereof. For example, in one embodiment, the composition comprises a cell which comprises mole-rat HAS2 protein, or a nucleic acid encoding mole-rat HAS2. In one embodiment, the cell is derived from a mole-rat. In one embodiment, the cell is genetically modified to comprise HAS2 protein and/or a nucleic acid encoding HAS2. In one embodiment, the cell is modified to express mole-rat HAS2 protein. For example, in one embodiment, the cell is genetically modified using an isolated nucleic acid encoding mole-rat HAS2.

In certain embodiments, genetically modified cell is autologous to a subject being treated with the composition of the invention. Alternatively, the cells can be allogeneic, syngeneic, or xenogeneic with respect to the subject.

The genetically modified cell may be modified in vivo or ex vivo, using techniques standard in the art. Genetic modification of the cell may be carried out using an expression vector or using a naked isolated nucleic acid construct.

In one embodiment, the cell is obtained and modified ex vivo, using an isolated nucleic acid encoding HAS2, thereby modifying the cell to produce HMW-HA. In one embodiment, the cell is obtained from a subject, genetically modified to produce HMW-HA, and is re-administered to the subject. In certain embodiments, the cell is expanded ex vivo or in vitro to produce a population of cells, wherein at least a portion of the population is administered to a subject in need.

In one embodiment, the cell is genetically modified to stably express HAS2 protein and produce HMW-HA. In another embodiment, the cell is genetically modified to transiently express HAS2 protein and produce HMW-HA.

In one embodiment, the cell is genetically modified using the clustered, regularly interspaced, short palindromic repeat (CRISPR)-Cas technology. The

CRISPR-Cas technology has the ability to provide targeted alteration of nucleic acid sequences, including insertions deletions and single base pair alterations with high fidelity (see for example, Horvath et al., 2010, Science, 327(5962):167-170).

For example, in one embodiment, the cell is modified by administering to the cell a Cas9 peptide, or nucleic acid encoding the Cas9 peptide, along with a targeting RNA molecule to modify the endogenous HAS2 gene of the cell. For example, the system may be used to introduce modifications into the endogenous genome such that HAS2 expressed in the cell is mole-rat HAS2 comprising the amino acid sequence of SEQ ID NO: 1.

The present invention provides a scaffold or substrate composition comprising HMW-HA, a cell producing HMW-HA, or a combination thereof. For example, in one embodiment, HMW-HA, a cell producing HMW-HA, or a combination thereof is incorporated within a scaffold. In another embodiment, HMW-HA, a cell producing HMW-HA, or a combination thereof, is applied to the surface of a scaffold. The scaffold of the invention may be of any type known in the art. Non-limiting examples of such a scaffold includes a, hydrogel, electrospun scaffold, foam, mesh, sheet, patch, and sponge.

(a) Hydrogels

In one embodiment, the present invention provides a hydrogel comprising HMW-HA, a cell producing HMW-HA, or a combination thereof. Hydrogels can generally absorb a great deal of fluid and, at equilibrium, typically are composed of 60-90% fluid and only 10-30% polymer. In a preferred embodiment, the water content of hydrogel is about 70-80%. Hydrogels are particularly useful due to the inherent biocompatibility of the cross-linked polymeric network (Hill-West, et al.,1994, Proc. Natl. Acad. Sci. USA 91:5967-5971). Hydrogel biocompatibility may be attributed to hydrophilicity and ability to imbibe large amounts of biological fluids (Brannon-Peppas. Preparation and Characterization of Cross-linked Hydrophilic Networks in Absorbent Polymer Technology, Brannon-Peppas and Harland, Eds. 1990, Elsevier: Amsterdam, pp 45-66; Peppas and Mikos. Preparation Methods and Structure of Hydrogels in Hydrogels in Medicine and Pharmacy, Peppas, Ed. 1986, CRC Press: Boca Raton, Fla., pp 1-27).

In one embodiment, the composition comprises a hydrogel comprising HMW-HA. An HMW-HA hydrogel may comprise one or more other biopolymer or

synthetic polymer. The hydrogels may be prepared by crosslinking hydrophilic biopolymers or synthetic polymers. Examples of the hydrogels formed from physical or chemical crosslinking of hydrophilic biopolymers, include but are not limited to, hyaluronans, chitosans, alginates, collagen, dextran, pectin, carrageenan, polylysine, gelatin or agarose. (see.: W. E. Hennink and C. F. van Nostrum, 2002, Adv. Drug Del. Rev. 54, 13-36 and A. S. Hoffman, 2002, Adv. Drug Del. Rev. 43, 3-12). These materials consist of high-molecular weight backbone chains made of linear or branched polysaccharides or polypeptides. Examples of hydrogels based on chemical or physical crosslinking synthetic polymers include but are not limited to (meth)acrylate-oligolactide-PEO-oligolactide-(meth)acrylate, poly(ethylene glycol) (PEO), poly(propylene glycol) (PPO), PEO-PPO-PEO copolymers (Pluronics), poly(phosphazene), poly(methacrylates), poly(N-vinylpyrrolidone), PL(G)A-PEO-PL(G)A copolymers, poly(ethylene imine), poly(ethylene glycol) diacrylate (PEGDA), etc. (see A. S Hoffman, 2002Adv. Drug Del. Rev, 43, 3-12)..

In certain embodiments, the hydrogel is modified to comprise one or more therapeutic agents. Hydrogels may be modified with functional groups for covalently attaching a variety of compounds such as therapeutic agents. In one embodiment, compounds, such as therapeutic agents, may be incorporated into the hydrogel matrix. Exemplary compounds include, but are not limited to, vitamins and other nutritional supplements; glycoproteins (e.g., collagen); fibronectin; peptides and proteins; carbohydrates (both simple and/or complex); proteoglycans; antigens; oligonucleotides (sense and/or antisense DNA and/or RNA); antibodies (for example, to infectious agents, tumors, drugs or hormones); chemotherapeutic agents; cells; and gene therapy reagents.

Therapeutic agents which may be incorporated into the hydrogel scaffold include, but are not limited to, analgesics, anesthetics, antifungals, antibiotics, anti-inflammatories, anthelmintics, antidotes, antiemetics, antihistamines, antihypertensives, antimalarials, antimicrobials, antipsychotics, antipyretics, antiseptics, antiarthritics, antituberculotics, antitussives, antivirals, cardioactive drugs, cathartics, chemotherapeutic agents, a colored or fluorescent imaging agent, corticoids (such as steroids), antidepressants, depressants, diagnostic aids, diuretics, enzymes, expectorants, hormones, hypnotics, minerals, nutritional supplements, parasympathomimetics, potassium supplements, radiation sensitizers, a radioisotope, sedatives, sulfonamides, stimulants, sympathomimetics, tranquilizers, urinary anti-

infectives, vasoconstrictors, vasodilators, vitamins, xanthine derivatives, and the like. The therapeutic agent may also be other small organic molecules, naturally isolated entities or their analogs, organometallic agents, chelated metals or metal salts, peptide-based drugs, or peptidic or non-peptidic receptor targeting or binding agents. It is contemplated that linkage of the therapeutic agent to the matrix may be via a protease sensitive linker or other biodegradable linkage.

In certain embodiments, one or more multifunctional cross-linking agents known in the art may be utilized as reactive moieties that covalently link biopolymers or synthetic polymers.

(b) Electrospun scaffolds

In one embodiment, HMW-HA, a cell producing HMW-HA, or a combination thereof, may be incorporated into nanofibrous biocompatible electrospun matrices. In some embodiments, HMW-HA, a cell producing HMW-HA, or a combination thereof is blended with a synthetic polymer, such as poly(ethylene oxide) (PEO) to produce a tissue engineering scaffold.

The scaffolds of the invention may be produced in a variety of ways. In an exemplary embodiment, the scaffold may be produced by electrospinning. Electrospinning is an atomization process of a conducting fluid which exploits the interactions between an electrostatic field and the conducting fluid. When an external electrostatic field is applied to a conducting fluid (e.g., a semi-dilute polymer solution or a polymer melt), a suspended conical droplet is formed, whereby the surface tension of the droplet is in equilibrium with the electric field. Electrostatic atomization occurs when the electrostatic field is strong enough to overcome the surface tension of the liquid. The liquid droplet then becomes unstable and a tiny jet is ejected from the surface of the droplet. As it reaches a grounded target, the material may be collected as an interconnected web containing relatively fine, i.e. small diameter, fibers. The resulting films (or membranes) from these small diameter fibers have very large surface area to volume ratios and small pore sizes. A detailed description of electrospinning apparatus is provided in Zong, et al., 2002 Polymer 43: 4403-4412; Rosen et al., 1990 Ann Plast Surg 25: 375-87; Kim, K., Biomaterials 2003, 24: 4977-85; Zong, X., 2005 Biomaterials 26: 5330-8. After electrospinninng, extrusion and molding may be utilized to further fashion the polymers. To modulate fiber organization into aligned fibrous polymer scaffolds, the use of patterned

electrodes, wire drum collectors, or post-processing methods such as uniaxial stretching has been successful. Zong, X., 2005 Biomaterials 26: 5330-8; Katta, P., 2004 Nano Lett 4: 2215-2218; Li, D., 2005 Nano Lett 5: 913-6. Methods to produce a solution comprising HMW-HA suitable for electrospinning are well-known in the art.

The invention also includes combinations of natural materials, combinations of synthetic materials, and combinations of both natural and synthetic materials. For example, the HMW-HA, a cell producing HMW-HA, or a combination thereof may be combined with natural materials, synthetic materials, or both natural and synthetic materials to produce the scaffolds of the invention. Examples of combinations include, but are not limited to: blends of different types of collagen (e.g. Type I with Type II, Type I with Type III, Type II with Type III, etc.); blends of one or more types of collagen with fibrinogen, thrombin, elastin, PGA, PLA, and polydioxanone; and blends of fibrinogen with one or more types of collagen, thrombin, elastin, PGA, PLA, and polydioxanone.

In embodiments in which the matrix contains substances that are to be released from the matrix, incorporating electroprocessed synthetic components, such as biocompatible substances, can modulate the release of substances from an electroprocessed composition. For example, layered or laminate structures may be used to control the substance release profile. Unlayered structures may also be used, in which case the release is controlled by the relative stability of each component of the construct. For example, layered structures composed of alternating electroprocessed materials are prepared by sequentially electroprocessing different materials onto a target. The outer layers are, for example, tailored to dissolve faster or slower than the inner layers. Multiple agents may be delivered by this method, optionally at different release rates. Layers may be tailored to provide a complex, multi-kinetic release profile of a single agent over time. Using combinations of the foregoing provides for release of multiple substances released, each with its own profile. Complex profiles are possible.

In some embodiments, the electroprocessed material itself may provide a therapeutic effect. For example, electroprocessed HMW-HA provides a therapeutic effect in treating or preventing cancer. Non-limiting examples of a material that has a therapeutic effect is electroprocessed fibrinogen, thrombin, fibrin, or combinations thereof.

(c) Method for Forming Matrices or Scaffolds

A biocompatible scaffold may be shaped using methods such as, for example, solvent casting, compression molding, filament drawing, meshing, leaching, weaving, foaming, electrospinning and coating. In solvent casting, a solution of one or more proteins in an appropriate solvent, is cast as a branching pattern relief structure. After solvent evaporation, a thin film is obtained. In compression molding, a polymer is pressed at pressures up to 30,000 pounds per square inch into an appropriate pattern. Filament drawing involves drawing from the molten polymer and meshing involves forming a mesh by compressing fibers into a felt-like material. In leaching, a solution containing two materials is spread into a shape close to the final form of the artificial organ. Next a solvent is used to dissolve away one of the components, resulting in pore formation. (See U.S. Pat. No. 5,514,378 to Mikos).

The scaffold may be shaped into any number of desirable configurations to satisfy any number of overall system, geometry or space restrictions. For example, in the use of the scaffold for bladder, urethra, valve, or blood vessel reconstruction, the matrix or scaffold may be shaped to conform to the dimensions and shapes of the whole or a part of the tissue. The scaffold may be shaped in different sizes and shapes to conform to the organs of differently sized patients. The matrix or scaffold may also be shaped in other fashions to accommodate the special needs of the patient.

In one embodiment, the scaffolds are seeded with one or more populations of cells. Isolated cells may be cultured in vitro or ex vivo to increase the number of cells available for coating ore seeding the scaffold.

Exemplary types of cells which may be seeded in or on the scaffold include, but is not limited to, stem cells, progenitor cells, fibroblasts, somatic cells, and the like. In certain embodiments, the scaffold is seeded with a cell that is genetically modified, as described elsewhere herein. For example, in one embodiment, the scaffold is seeded with a cell that is genetically modified to produce HMW-HA.

Seeding of cells onto the matrix or scaffold may be performed according to standard methods. For example, the seeding of cells onto polymeric substrates for use in tissue repair has been reported (see, e.g., Atala, A. et al., J. Urol. 148(2 Pt 2): 658-62 (1992); Atala, A., et al. J. Urol. 150 (2 Pt 2): 608-12 (1993)). Cells grown in culture may be trypsinized to separate the cells, and the separated cells

may be seeded on the matrix. Alternatively, cells obtained from cell culture may be lifted from a culture plate as a cell layer, and the cell layer may be directly seeded onto the scaffold without prior separation of the cells.

In a preferred embodiment, in the range of 1 million to 700 50 million cells are suspended in medium and applied to each square centimeter of a surface of a scaffold. Preferably, between 1 million and 50 million cells, and more preferably, between 1 million and 10 million cells are suspended in media and applied to each square centimeter of a surface of a scaffold. The matrix or scaffold is incubated under standard culturing conditions, such as, for example, 37°C., 5% CO_2, for a period of time until the cells attached. However, it will be appreciated that the density of cells seeded onto the scaffold may be varied. For example, greater cell densities promote greater tissue regeneration by the seeded cells, while lesser densities may permit relatively greater regeneration of tissue by cells infiltrating the graft from the host. Other seeding techniques may also be used depending on the matrix or scaffold and the cells. For example, the cells may be applied to the matrix or scaffold by vacuum filtration. Selection of cell types, and seeding of cells onto a scaffold, will be routine to one of ordinary skill in the art in light of the teachings herein.

The present invention also provides pharmaceutical compositions comprising one or more of the compositions described herein. Formulations may be employed in admixtures with conventional excipients, i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for administration to the wound or treatment site. The pharmaceutical compositions may be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure buffers, coloring, and/or aromatic substances and the like. They may also be combined where desired with other active agents, e.g., other analgesic agents.

Administration of the compositions of this invention may be carried out, for example, by parenteral, by intravenous, intratumoral, subcutaneous, intramuscular, or intraperitoneal injection, or by infusion or by any other acceptable systemic method.

As used herein, “additional ingredients” include, but are not limited to, one or more of the following: excipients; surface active agents; dispersing agents; inert diluents; granulating and disintegrating agents; binding agents; lubricating

agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents; emulsifying agents, demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants; antibiotics; antifungal agents; stabilizing agents; and pharmaceutically acceptable polymeric or hydrophobic materials. Other "additional ingredients" that may be included in the pharmaceutical compositions of the invention are known in the art and described, for example in Genaro, ed. (1985, Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA), which is incorporated herein by reference.

The composition of the invention may comprise a preservative from about 0.005% to 2.0% by total weight of the composition. The preservative is used to prevent spoilage in the case of exposure to contaminants in the environment. Examples of preservatives useful in accordance with the invention included but are not limited to those selected from the group consisting of benzyl alcohol, sorbic acid, parabens, imidurea and combinations thereof. A particularly preferred preservative is a combination of about 0.5% to 2.0% benzyl alcohol and 0.05% to 0.5% sorbic acid.

In an embodiment, the composition includes an anti-oxidant and a chelating agent that inhibits the degradation of one or more components of the composition. Preferred antioxidants for some compounds are BHT, BHA, alpha-tocopherol and ascorbic acid in the preferred range of about 0.01% to 0.3% and more preferably BHT in the range of 0.03% to 0.1% by weight by total weight of the composition. Preferably, the chelating agent is present in an amount of from 0.01% to 0.5% by weight by total weight of the composition. Particularly preferred chelating agents include edetate salts (e.g. disodium edetate) and citric acid in the weight range of about 0.01% to 0.20% and more preferably in the range of 0.02% to 0.10% by weight by total weight of the composition. The chelating agent is useful for chelating metal ions in the composition that may be detrimental to the shelf life of the formulation. While BHT and disodium edetate are the particularly preferred antioxidant and chelating agent respectively for some compounds, other suitable and equivalent antioxidants and chelating agents may be substituted therefore as would be known to those skilled in the art.

Liquid suspensions may be prepared using conventional methods to achieve suspension of the HMW-HA or other composition of the invention in an aqueous or oily vehicle. Aqueous vehicles include, for example, water, and isotonic

saline. Oily vehicles include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin. Liquid suspensions may further comprise one or more additional ingredients including, but not limited to, suspending agents, dispersing or wetting agents, emulsifying agents, demulcents, preservatives, buffers, salts, flavorings, coloring agents, and sweetening agents. Oily suspensions may further comprise a thickening agent. Known suspending agents include, but are not limited to, sorbitol syrup, hydrogenated edible fats, sodium alginate, polyvinylpyrrolidone, gum tragacanth, gum acacia, and cellulose derivatives such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose. Known dispersing or wetting agents include, but are not limited to, naturally-occurring phosphatides such as lecithin, condensation products of an alkylene oxide with a fatty acid, with a long chain aliphatic alcohol, with a partial ester derived from a fatty acid and a hexitol, or with a partial ester derived from a fatty acid and a hexitol anhydride (e.g., polyoxyethylene stearate, heptadecaethyleneoxycetanol, polyoxyethylene sorbitol monooleate, and polyoxyethylene sorbitan monooleate, respectively). Known emulsifying agents include, but are not limited to, lecithin, and acacia. Known preservatives include, but are not limited to, methyl, ethyl, or n-propyl-para- hydroxybenzoates, ascorbic acid, and sorbic acid.

Treatment methods

The present invention provides a method for the treatment or prevention of cancer by increasing the level of HMW-HA in a subject in need thereof. For example, in certain aspects, the method prevents the development of cancer, prevents the metastasis of a cancer, prevents the recurrence of a cancer, reduces the aggressiveness of a cancer, reduces the size of a cancerous tumor, and the like.

Cancers that may be treated include tumors that are not vascularized, or not yet substantially vascularized, as well as vascularized tumors. The cancers may comprise non-solid tumors (such as hematological tumors, for example, leukemias and lymphomas) or may comprise solid tumors. Types of cancers to be treated with a composition of the invention include, but are not limited to, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant

tumors, and malignancies *e.g.*, sarcomas, carcinomas, and melanomas. Adult tumors/cancers and pediatric tumors/cancers are also included.

Hematologic cancers are cancers of the blood or bone marrow. Examples of hematological (or hematogenous) cancers include leukemias, including acute leukemias (such as acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia), chronic leukemias (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia.

Solid tumors are abnormal masses of tissue that usually do not contain cysts or liquid areas. Solid tumors can be benign or malignant. Different types of solid tumors are named for the type of cells that form them (such as sarcomas, carcinomas, and lymphomas). Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteosarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as a glioma (such as brainstem glioma and mixed gliomas), glioblastoma (also known as glioblastoma multiforme) astrocytoma, CNS lymphoma, germinoma, medulloblastoma, Schwannoma craniopharyogioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, neuroblastoma, retinoblastoma and brain metastases).

In certain embodiments, the method comprises administering an effective amount of a composition described herein to a subject diagnosed with cancer, suspected of having cancer, or at risk for developing cancer. In certain

aspects, the composition is contacted to a cell or tissue where cancer is present or at risk for developing. In one embodiment, the composition is administered systemically to the subject.

In one embodiment, the method comprises genetically modifying a cell to produce HMW-HA. For example, in one embodiment, the method comprises contacting a cell with an isolated nucleic acid encoding HAS2, for example mole-rat HAS2, thereby inducing the cell to produce HAS2 protein. In one embodiment, the cell is genetically modified in vivo in the subject being treated.

In certain aspects, for in vivo, delivery the nucleic acid is injected directly into the subject. For example, in one embodiment, the nucleic acid is delivered at the site where the composition is required.

In vivo nucleic acid transfer techniques include, but is not limited to, transfection with viral vectors such as adenovirus, Herpes simplex I virus, adeno-associated virus), lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example), naked DNA, and transposon-based expression systems. Exemplary gene therapy protocols see Anderson et al., Science 256:808-813 (1992). See also WO 93/25673 and the references cited therein. In certain embodiments, the method comprises administering of RNA, for example mRNA, directly into the subject (see for example, Zangi et al., 2013 Nature Biotechnology, 31: 898-907).

For ex vivo treatment, an isolated cell is modified in an ex vivo or in vitro environment. In one embodiment, the cell is autologous to a subject being treated with the composition of the invention. Alternatively, the cell can be allogeneic, syngeneic, or xenogeneic with respect to the subject. The modified cells may then be administered to the subject directly, or within a scaffold as described elsewhere herein. For example, the modified cell may be seeded on or within a scaffold to be administered to the subject.

One skilled in the art recognizes that different methods of delivery may be utilized to administer an isolated nucleic acid into a cell. Examples include: (1) methods utilizing physical means, such as electroporation (electricity), a gene gun (physical force) or applying large volumes of a liquid (pressure); and (2) methods wherein the nucleic acid or vector is complexed to another entity, such as a liposome, aggregated protein or transporter molecule.

Furthermore, the actual dose and schedule can vary depending on whether the compositions are administered in combination with other pharmaceutical compositions, or depending on interindividual differences in pharmacokinetics, drug disposition, and metabolism. Similarly, amounts can vary in *in vitro* applications depending on the particular cell line utilized (e.g., based on the number of vector receptors present on the cell surface, or the ability of the particular vector employed for gene transfer to replicate in that cell line). Furthermore, the amount of vector to be added per cell will likely vary with the length and stability of the therapeutic gene inserted in the vector, as well as also the nature of the sequence, and is particularly a parameter which needs to be determined empirically, and can be altered due to factors not inherent to the methods of the present invention (for instance, the cost associated with synthesis). One skilled in the art can easily make any necessary adjustments in accordance with the exigencies of the particular situation.

Genetically modified cells may also contain a suicide gene i.e., a gene which encodes a product that can be used to destroy the cell. In many gene therapy situations, it is desirable to be able to express a gene for therapeutic purposes in a host, cell but also to have the capacity to destroy the host cell at will. The therapeutic agent can be linked to a suicide gene, whose expression is not activated in the absence of an activator compound. When death of the cell in which both the agent and the suicide gene have been introduced is desired, the activator compound is administered to the cell thereby activating expression of the suicide gene and killing the cell. Examples of suicide gene/prodrug combinations which may be used are herpes simplex virus-thymidine kinase (HSV-tk) and ganciclovir, acyclovir; oxidoreductase and cycloheximide; cytosine deaminase and 5-fluorocytosine; thymidine kinase thymidilate kinase (Tdk::Tmk) and AZT; and deoxycytidine kinase and cytosine arabinoside.

In one embodiment, the cell is genetically modified using the clustered, regularly interspaced, short palindromic repeat (CRISPR)-Cas technology. The CRISPR-Cas technology has the ability to provide targeted alteration of nucleic acid sequences, including insertions deletions and single base pair alterations with high fidelity (see for example, Horvath et al., 2010, Science, 327(5962):167-170 and U.S. Patent No. 8,697,359).

For example, in one embodiment, the method comprises administering to an isolated cell a Cas9 peptide, or nucleic acid encoding the Cas9 peptide, along

with a CRISPR-Cas guide RNA molecule to modify the endogenous HAS2 gene of the cell. For example, the system may be used to introduce modifications into the endogenous genome such that HAS2 expressed in the cell is mole-rat HAS2 comprising the amino acid sequence of SEQ ID NO: 1.

The composition of the invention may be administered to a patient or subject in need in a wide variety of ways. Modes of administration include intraoperatively intravenous, intravascular, intramuscular, subcutaneous, intracerebral, intraperitoneal, soft tissue injection, surgical placement, arthroscopic placement, and percutaneous insertion, e.g. direct injection, cannulation or catheterization. Any administration may be a single application of a composition of invention or multiple applications. Administrations may be to single site or to more than one site in the individual to be treated. Multiple administrations may occur essentially at the same time or separated in time.

In certain embodiments, the composition of the invention is administered during surgical resection or debulking of a tumor. For example, in subjects undergoing surgical treatment of a tumor, the composition may be administered to the tumor site in order to further treat the tumor, prevent the growth of the tumor, or prevent the formation of additional tumors. For example, a scaffold comprising HMW-HA, cell producing HMW-HA, or combination thereof, may be administered to the tumor site.

Subjects to which administration of the pharmaceutical compositions of the invention is contemplated include, but are not limited to, humans and other primates, mammals including commercially relevant mammals such as non-human primates, cattle, pigs, horses, sheep, cats, and dogs.

Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the subject, and the type and severity of the subject's disease, although appropriate dosages may be determined by clinical trials.

When "an immunologically effective amount", "an anti-tumor effective amount", "an tumor-inhibiting effective amount", or "therapeutic amount" is indicated, the precise amount of the compositions of the present invention to be administered can be determined by a physician with consideration of individual

differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject).

The administration of the subject compositions may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (*i.v.*) injection, or intraperitoneally. In one embodiment, the compositions of the present invention are administered to a patient by intradermal or subcutaneous injection. In another embodiment, the compositions of the present invention are preferably administered by *i.v.* injection. The compositions of T cells may be injected directly into a tumor.

In certain embodiments of the present invention the composition is administered to a subject in conjunction with (*e.g.*, before, simultaneously or following) any number of relevant treatment modalities, including but not limited surgery, chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAM PATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludaribine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, cytokines, and irradiation. In a further embodiment, the cell compositions of the present invention are administered to a patient in conjunction with (*e.g.*, before, simultaneously or following) bone marrow transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. In another embodiment, the cell compositions of the present invention are administered following B-cell ablative therapy such as agents that react with CD20, *e.g.*, Rituxan. For example, in one embodiment, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In certain embodiments, following the transplant, subjects receive an infusion of the expanded immune cells of the present invention. In an additional embodiment, the composition is administered before or following surgery.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of

illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

Example 1: High-molecular-weight hyaluronan mediates the cancer resistance of the naked mole-rat

The naked mole-rat displays exceptional longevity, with a maximum lifespan exceeding 30 years (Buffenstein, R. et al., *Sci Aging Knowledge Environ,* 2002, pe7; Buffenstein, R., *J Comp Physiol [B]*, 2008, 178:439-445 and Kim, E. B. et al., *Nature*, 2011, doi:10.1038/nature10533). This is the longest reported lifespan for a rodent species and is especially striking considering the small body mass of the naked mole-rat. In comparison, a similarly sized house mouse has a maximum lifespan of 4 years (Turturro, A. et al., *J Gerontol A Biol Sci Med Sci, 1999,* 54, B492-501 and de Magalhaes, J. P., et al., *Nucleic Acids Res, 2005,* 33, D537-543). In addition to their longevity, naked mole-rats show an unusual resistance to cancer. Multi-year observations of large naked mole-rat colonies did not detect a single incidence of cancer (Buffenstein, R., *J Comp Physiol [B]*, 2008, 178:439-445 and Delaney, M. A., et al., *Vet Pathol*, 2013, doi:10.1177/0300985812471543). The results presented herein identify a mechanism responsible for the naked mole-rat's cancer resistance. It was found that naked mole-rat fibroblasts secrete extremely high molecular weight hyaluronan (HA), which is over five times larger than human or mouse HA. This high molecular weight HA accumulates abundantly in naked mole rat tissues due to the decreased activity of HA-degrading enzymes and a unique sequence of hyaluronan synthase 2 (*HAS2*). Furthermore, the naked mole-rat cells are more sensitive to HA signaling, as the naked mole rat cells have a higher affinity to HA than the mouse or human cells. Perturbation of the signaling pathways sufficient for malignant transformation of mouse fibroblasts fails to transform naked mole-rat cells. However,

once high molecular weight HA is removed by either knocking down HAS2 or overexpressing the HA-degrading enzyme, Hyal2, naked mole-rat cells become susceptible to malignant transformation and readily form tumors in mice. While not wishing to be bound by any particular theory, it is speculated that naked mole-rats have evolved a higher concentration of HA in the skin to provide skin elasticity needed for life in underground tunnels. This trait may have then been co-opted to provide cancer resistance and longevity to this species.

Mice and rats are standard animal models for cancer research due in part to their short lifespan and high incidence of cancer. However, these traits imply that mice and rats have fewer anticancer mechanisms, and novel tumor resistance mechanisms are less likely to be discovered using these models. Here, experiments were focused on a small rodent, the naked mole-rat, which in contrast to mice and rats, is long-lived and cancer resistant.

The materials and methods employed in these experiments are now described.

Animals

Naked mole-rats were from the University of Rochester colonies. C57BL/6 mice and NIH III nude mice (NIH-Lyst bg-JFoxn1nu Btk xid) were purchased from Charles River Labs. Non-albino guinea pigs were obtained from Elm Hill Labs. Cells and tissues were obtained from at least three different animals.

Cell culture

Primary mouse, guinea pig, blind mole-rat and naked mole-rat cells were isolated from lung and underarm skin. Cells were obtained from five naked mole-rats, three mice, three guinea pigs, and three blind mole-rats. The growth characteristics, and HA secretion did not differ between the cell lines from different animals therefore the experiments were performed on three skin cell lines from three animals. All cell lines were used at early passage (<12-15 PDs). Human primary skin fibroblasts HCA2 were obtained. Embryonic naked mole rat fibroblasts were isolated from eight mid-gestation embryos.

Mouse, human, guinea pig and blind mole-rat cells were cultured at 37°C, 5% CO_2, 3% O_2; naked mole-rat cells were cultured at 32 °C, 5% CO_2, 3% O_2 on treated polystyrene culture dishes (Corning) in EMEM media (ATCC)

supplemented with 15% fetal bovine serum (Gibco), nonessential amino acids, sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco).

Viscosity Assay

To determine relative kinematic viscosity, 3 ml of distilled H_2O, unused complete EMEM media, or media conditioned with naked mole-rat, mouse, or human cells were run through a 0.6 mm capillary Ostwald viscometer (Barnstead International) at 22°C and timed for the passage of the media or distilled H_2O through the capillary. For HAase control, naked mole-rat media was treated with hyaluronidase 1 U/ml HAase from *Streptomyces hyalurolyticus* (Sigma-Aldrich). The relative viscosity of unused and conditioned media was determined by comparing times required to pass through the capillary to that of distilled H_2O. Samples were run three times to determine an average relative viscosity.

Cell growth analysis

To measure cell proliferation and the confluent density, cells were seeded on 60 mm gridded plates (Corning). Three 2x2 mm squares were marked on each plate and the number of cells in those squares was counted each day for 20 days. For cell growth in the presence of HAase, 24 hours post plating the media was changed to media containing 3 U/ml HAase (Sigma). Media was then replaced with fresh media containing the enzyme every 48 hours. Images of the squares were taken using SPOT Advanced (Diagnostic Instruments) and analyzed using the colony counting program on ImageQuant TL (GE). The average count of the three squares for each day was multiplied by 458.33 to give the total cell count per 10 cm plate. Cell count data was analyzed using Microsoft Excel.

To calculate maximum cell number cells were harvested from the confluent plates and counted using Z2 Coulter counter (Beckman Coulter). Every sample was counted three times and the averages were used to calculate the maximum cell number from at least three independent experiments.

HA analysis by pulse field gel electrophoresis

HA was purified from conditioned media (typically at day 20) by first treating 2 ml of conditioned media with 500 μg of Proteinase K (Roche) at 50 °C for 45 minutes to remove proteins. Samples were then precipitated by adding 2 ml of

100% ethanol. The pellet was dissolved in 500 µl TE Buffer and incubated overnight at 4 °C. The following day, aliquots were removed and control samples treated with 1 U/ml of HAase from *Streptomyces hyalurolyticus* (Sigma-Aldrich). Twenty-five µl of each sample was mixed with 5 µl 4M sucrose loading solution and loaded to a 0.4% pulse field SeaKem Gold agarose gel (Cambrex). Ten µl of HA molecular size markers; HiLadder (~500 kDA to ~1,500 kDa) and Mega-HA Ladder (1,500 kDa to 6,000 kDa) (from Hyalose) were run to determine the size of HA from each sample. Samples were run overnight at 4 °C at 75 volts with a 1 to 10 running ratio in TBE buffer using CHEF-DRII system (Bio-Rad). The gel was next stained in a method adapted from (Lee, H. G. et al., *Anal Biochem, 1994,* 219:278-287). Briefly, the gel was placed in a 0.005% (w/v) Stains-All (Sigma-Aldrich) in 50% ethanol solution overnight. To de-stain, the gel was placed in distilled H_2O for 18-hours in the dark and then placed under ambient light in distilled H_2O for 1 hour to complete the final de-staining stages and then photographed under white light. The amount of HA was quantified by counting pixels using Image J software.

HA extraction from tissues

Tissues were excised immediately after sacrificing the animals and weighed. Tissues were chopped and the same amount of tissues were digested at 50 °C overnight in the digestion buffer containing 10 mM Tris-Cl, 25 mM EDTA, 100 mM NaCl, 0.5% SDS and 0.1 mg/ml proteinase K (Roche). Then 27 mM $MgCl_2$ was added to chelate EDTA and Pefabloc SC was added to inhibit Proteinase K. 500-unit Benzonase® endonuclease (Sigma-Aldrich) was added to remove nucleic acid. The complete digestion of DNA and RNA was confirmed by running the agarose gel and staining with ethidium bromide (Bio-Rad). Total polysaccharide extraction was performed by phenol/chloroform extraction followed by ethanol precipitation. Finally, corresponding volume (100µl/100mg) of 10 mM Tris-Cl (pH 8.5) was added to dissolve the pellet.

Expression of naked mole rat HAS2 gene in human cells

HEK293 cells were transfected with an expression vector containing HAS2 under the CMV promoter and allowed to express HAS2 for 2 days, after which

HA secreted into the media was analyzed by pulse-field gel. Control cells were transfected with a GFP expression vector.

HAase activity assay

Culture media containing HMW-HA secreted by naked mole-rat cells was mixed 1:1 with fresh media and incubated with 2 x 10^5 naked mole-rat fibroblasts, human diploid fibroblasts, mouse fibroblasts, or HeLa cells for four days. Then the media was harvested, HA was extracted and analyzed on a pulse-field gel as described above. HA levels before and after incubation were compared as a measure of HAase activity. For analysis of tissues, corresponding tissues were chopped into 1 mm cubes and washed twice with PBS. HMW-HA containing media was incubated with equal amounts (by weight) of tissue fragments of each tissue type for six hours and HA levels were analyzed as described elsewhere herein.

Antibodies

The following antibodies were used: HAS1 (ab104864 Abcam), HAS2 (sc-66916 Santa Cruz), HAS3 (sc-66917 Santa Cruz), α-tubulin (ab4074 Abcam), CD44 (Monoclonal Mouse IgG_{2A} Clone #2C5, Catalog Number BBA10, R&D Systems), NF2/Merlin (ab30329 Abcam), p16 (ab14244 Abcam), Hyal2 (ab68608bAbcam).

Tissue staining

HA detection in tissues was done as follows. Tissue samples from young animals (3 year old naked mole-rats, 3-5 months old mice, 1 year old guinea pigs) were fixed in 10% buffered neutral formalin, embedded in paraffin and quadruple sections cut at 5 μm were mounted on glass slides. Slides were deparaffinized and hydrated in distilled H_2O. They were then placed in Coplin jars containing 40 ml of hyaluronidase digestion solution (40 U hyaluronidase from *Streptomyces hyalurolyticus* (Sigma) in 40 mL of PBS) for the samples to be digested or in 40 ml of PBS for the non-digested samples. The jars were microwaved for one minute at 60 W and then transferred to a 37 °C oven for 1 hour. The slides were rinsed four times in distilled H_2O, followed by three rinses with 3% acetic acid. Next, slides were placed in new Coplin jars containing 1.0% filtered alcian blue solution at pH 2.5 (Alcian Blue 8GX, C.I. 74240 (Leica) in 3% acetic acid and microwaved at 60

W for 3 minutes followed by an additional 5 minute incubation in the hot 1.0% alcian blue solution. Slides were rinsed three times in distilled H_2O, dehydrated in graded alcohols and rinsed three times in xylene. Images were taken by light microscopy.

Naked mole-rat cell growth assays with CD44 antibody

Naked mole-rat cells were seeded 50 cells/square onto cell culture treated 6 cm polystyrene gridded tissue culture plates (Corning). Twenty-four hours post plating, the media was changed to contain 5 μg/ml of CD44 specific antibody (Monoclonal Mouse IgG_{2A} Clone #2C5, Catalog Number BBA10, R&D Systems) or no antibody control. Media was changed every 24-hours and images were taken daily using SPOT Advanced imaging software (Diagnostic Instruments). Images from three different squares from two independent plates were counted from both the CD44 antibody treated or control groups.

HA affinity assay

Naked mole-rat and mouse cells were harvested at subconfluent exponential phase. One hundred thousand (10^5) cells of each type were incubated for 45 min on ice in 210 μl PBS containing 1.5% Fetal Calf Serum and 35 μg/ml fluorescein-labeled HA (Fluorescein-Labeled HA from Bovine Trachea, sc-221733, Santa Cruz Biotech, Santa Cruz, CA). Five thousand cells from each replicate were analyzed by FACS. The experiment was repeated four times.

Transfections

Naked mole-rat skin fibroblasts were seeded at $2x10^5$ cells/ 100 mm plate seven days prior to transfection. Mouse skin fibroblasts were seeded at $5x10^5$ cells/ 100 mm plate two days prior to transfection. For transfection, cells were harvested, counted and 10^6 cells were transfected with 5 μg of plasmid DNA using Amaxa Nucleofector II on program U-020 and solution NHDF (Amaxa). After transfection, cells were seeded at $2x10^5$ live cells per 10 cm plate for apoptosis analysis and $7x10^4$ live cells per 6 cm grid plates (Corning) for cell growth analysis in the same media as stated above. Media was replaced 24 hours post transfection to remove dead cells due to electroporation.

Anchorage-independent soft agar growth assay

One million mouse, naked mole-rat wild type and naked mole-rat mutant exponentially growing skin fibroblast cells were transfected by Amaxa with the following plasmid DNA mixtures: 5 µg pEGFP-N1 (Clontech) and 5 µg pSG5 Large T (Addgene 9053), 5 µg pRas-V12 (Clontech) and 5µg pSG5 Large T, 5 µg pRas-V12 and 5µg pSG5 Large T K1 (Addgene 9055), or 5µg pRas-V12 and 5µg pSG5 Large T Δ434-444 (Addgene 9054). After transfection, cells were seeded and allowed to recover for 24 hours on 10 cm treated polystyrene plates (Corning) in 1X Minimum Essential Medium, Eagle with Earle's Balances salt Solution supplemented with 15% fetal calf serum and antibiotics (Gibco). The following day, a 2 ml final solution of 0.5% Difco Agar Noble (BD Bioscience) and 1X media mixture was poured into 6 cm treated polystyrene plates (Corning) and allowed to solidify in incubators at 37 °C. After harvesting and counting cells transfected 24 hours previous, 50 to 50,000 cells were serially diluted and resuspended in 1 ml of 2X media. This cell suspension was then quickly mixed with 1 ml 0.7% liquid Difco Agar Noble, making a final 0.35% agar/1X media solution, and seeded on top of the solidified 0.5%/1X media. Plates were incubated at 32 °C, 5% CO_2, ad 3% O_2 for twenty-four hours before the addition of 1ml of EMEM media with or without HAase from *Streptomyces hyalurolyticus* (Sigma) at 3 U/ml. To test the effect of CD44 antibody, 5 µg/ml of CD44 antibody (BBA10, R&D Systems) was added and changed daily. Cells were grown for six weeks, with the removal of old liquid EMEM media and the addition of 1 ml of new EMEM with or without 3 U/ml HAase every 48 hours to ensure efficient digestion of HMW-HA. Plates were monitored every 48 hours and photographed at week 3 (mouse cells) and week 6 (naked mole-rat cells) after plating at 200x on a Nikon TS100 phase contract microscope using SPOT software (Diagnostic Instruments).

shRNA-mediated HAS knockdown and Hyal2 overexpression

shRNAs were designed by Integrated DNA Technology (IDT) with shRNA Design Tool:
5'GATCCGCCAGCTGCCTCAGAGGAATTCAAGAGATTCCTCTGAGGCAGCT GGCTTTTTTGGAAA-3' (SEQ ID NO: 3)
5'AGCTTTTCCAAAAAAGCCAGCTGCCTCAGAGGAATCTCTTGAATTCCTCT GAGGCAGCTGGCG-3' (SEQ ID NO: 4).

The corresponding 63bp DNA oligonucleotides harboring the 19-mer hairpin sequence, loop sequence, polythymidine tract (U6 terminator), BamHI and HindIII restriction site overhangs were designed according to the user manual of pSilencer™ 2.1-U6 neo kit (Life Technologies) and chemically synthesized by IDT. The complementary oligonucleotides were annealed and ligated to the pre-cut pSilencer2.1-U6 neo vector using the rapid DNA ligation kit (Roche). Following transformation into Top10 competent cells (Life Technologies), successful ligation was confirmed using restriction digestion and DNA sequencing with M13F primer (5'GTAAAACGACGGCCAGT-3'; SEQ ID NO: 5).

Human Hyal2 cDNA was amplified from pCMV6-HYAL2 (sc117754 OriGene) using the primers 5'CCG*GAATTC*GCCACCATGCGGGCAGGCCCAGGCCCCACCG-3' (SEQ ID NO: 6) and 5'ATAAGAAT*GCGGCCGC*CTACAAGGTCCAGGTAAAGGCCAGGGC-3' (SEQ ID NO: 7) and cloned into pEGFP-N1-neo plasmid to replace EGFP fragment using EcoR1 and NotI restriction enzymes.

Transfection grade plasmids were prepared with EndoFree plasmid maxi kit (Qiagen) and linearized with ScaI. One μg linearized plasmid was transfected into ~$1x10^6$ cells by Nucleofector (Amaxa) with U20 program, followed by G418 selection at 1 mg/ml for 2 weeks. Clones that stably expressed the shRNA were picked and expanded to characterize the knockdown efficiency. Clones with highest levels of HAS knockdown efficiency or the best Hyal2 expression were used for the *in vivo* xenograft assay.

Quantitative RT-PCR

Total RNA was extracted from cells at 80% confluence (2 days after splitting) using RNeasy Mini Kit (Qiagen). cDNA was generated using SuperScript®III reverse transcriptase (Life Technologies) with Oligo(dT)18 primer. First-strand cDNA was amplified using FastStart Universal SYBR Green Master (Roche; 04913850001) with corresponding primers in which QuantumRNA™ beta-actin internal standards (Life Technologies) were used as reference. Quantitative PCR was conducted with Applied Biosystems 7300 real-time PCR systems at 95°C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The standard curves

for the quantitative PCR were set using 2, 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256 µl cDNA generated with RNA from NSF-LT-RAS cells. qPCR Primers:

HAS2-Forward: 5'-GAAAAGGGTCCTGGTGAGACGGATGAG-3' (SEQ ID NO: 8);

HAS2-Reverse: 5'-TTCACCATCTCCACAGATGAGGCAGG-3' (SEQ ID NO: 9)

Tumor xenograft assay

NIH-III nude mice (Crl:NIH-Lyst^{bg-J} Foxn1^{nu}Btk^{xid}) were purchased from Charles river Laboratories Inc. (Wilmington, MA, USA). Seven-week-old female mice were used to establish xenografts. For each injection, 4×10^6 cells were harvested and resuspended in 100 µl of ice-cold 20% matrigel (BD Bioscience, Franklin Lakes, NJ) in PBS (Gibco). This 100 µl solution was injected subcutaneously close to the base of the external ear or into the flank just in front of the hind legs with 22 gauge needle. Transplantations of MSF-LT-Ras cells were allowed to grow for 2-3 weeks, while xenografts with NMR cells were allowed to grow for 65 days before sacrifice. Tumors were excised and size and weight were recorded. The mice were dissected and tumor metastasis was examined for each organ.

The results of the experiments are now described.

Anti-cancer properties of high molecular weight hyaluronic acid (HMW-HA)

While culturing multiple lines of naked mole-rat fibroblasts it was observed that that the culture media became very viscous after a few days. Viscosity measurements confirmed that the media conditioned by the naked mole-rat cells was more viscous than the media conditioned by human, guinea pig, or mouse cells (Figure 1A). The guinea pig was included in this study because it is phylogenetically closer to the naked mole-rat than the mouse. The viscous "substance" secreted by the naked mole-rat fibroblasts was identified as high molecular weight HA (HMW-HA). Treatment with hyaluronidase (HAase) that specifically digests HA reduced the media viscosity to background levels (Figure 1A). Naked mole-rat embryonic fibroblasts, which do not display ECI, did not increase viscosity of the culture media (Figure 1A and Figure 5).

HA is an unbranched disaccharide glucuronic acid/N-acetylglucosamine polymer and is one of the major components of the extracellular matrix (Toole, B. P., *Nat Rev Cancer, 2004,* 4:528-539). Biological responses triggered by HA depend on the HA polymer length. HMW-HA represses mitogenic signaling and has anti-inflammatory properties (Kothapalli, D. *et al., J Cell Biol, 2007,* 176:535-544), while low molecular weight HA promotes proliferation and inflammation (Pure, E. et al., *Cell Signal, 2009,* 21:651-655).

Analysis of HA from tissue culture media using pulse field electrophoresis showed that the HA secreted by naked mole-rat cells has a molecular weight of 6-12 MDa, while mouse and guinea pig HA ranges from 0.5-3 MDa (Figure 1B); human HA has a molecular weight of 0.5-2 MDa (Holmes, M. W. et al., *Biochem J, 1988,* 250:435-441). Naked mole-rat embryonic fibroblasts did not secrete HMW-HA (Figure 1B).

Importantly, a mutated clone NMR SF Mut, which spontaneously lost the ECI phenotype and $p16^{INK4a}$ expression (Seluanov, A. et al., *Proc. Natl. Acad. Sci. USA, 2009,* 106:19207-19208), still produced HMW-HA (Figure 1A and Figure 1B), indicating that the physical presence of HMW-HA is not sufficient for the ECI phenotype; rather the intact signaling pathway leading from HMW-HA to induction of p16 is required. These experiments establish HMW-HA as the extracellular signal that triggers ECI.

In vertebrate cells HA is produced by HA synthases HAS1, HAS2, and HAS3, which differ in tissue distribution and the size of HA produced (Jiang, D., et al., *Annu Rev Cell Dev Biol, 2007,* 23:435-461). Naked mole-rat skin fibroblasts overexpressed HAS2, the enzyme responsible for the synthesis of HMW-HA in comparison with mouse and human fibroblasts (Figure 1C). Naked mole-rat embryonic fibroblasts, which do not secrete HMW-HA, did not show increased levels of HAS2. The levels of HAS1 and HAS3 were similar between mouse, human, and naked mole-rat cells (Figure 1C). Collectively, these results show that naked mole-rat cells, which display ECI, secrete HA of exceptionally high molecular weight.

Hyaluronan synthases are highly conserved in vertebrates. The HAS2 protein has 98.7% identity and 100% similarity between human and mouse. HAS2 cDNA was cloned and sequenced from the naked mole-rat and it was compared to other mammalian *HAS2* genes (Figure 1D). Two Asparagines that are 100% conserved among mammals were replaced with Serines in the naked mole-rat HAS2.

This change occurs in no other mammalian *HAS2* genes deposited in GenBank, including the naked mole-rat's close relative, the guinea pig. HAS2 contains seven putative transmembrane domains and a cytoplasmic loop (Watanabe, K. et al., *J Biol Chem, 1996,* 271:22945-22948). The conserved regions carrying Asparagine to Serine substitutions correspond to the cytoplasmic loop containing the enzyme's active site. These unique amino acid changes may be responsible for the high processivity of the naked mole-rat HAS2. Indeed, when the cDNA for the naked mole-rat HAS2 was overexpressed in human HEK293 cells, they began secreting HMW-HA (Figure 2A).

It was then examined whether naked mole-rat tissues contain high levels of HA in comparison to mouse and guinea pig. Tissue sections were stained with alcian blue, and control samples were treated with HAase prior to staining to show that the staining is specific to HA. Naked mole-rat skin, heart, brain and kidney were highly enriched for HA (Figure 2B and Figure 6). Furthermore, the HA extracted from naked mole-rat tissues had a higher molecular weight than HA from mouse tissues (Figure 7). These results indicate that production of HMW-HA by naked mole-rat cells is not an artifact of tissue culture, but a unique *in vivo* property of this species.

HA levels are regulated by HA-degrading enzymes, HAases (Stern, R. et al., *Chemical reviews, 2006,* 106:818-839). HAase activity was measured in naked mole-rat, mouse and human cells by quantifying HA degradation after incubation with these cells. HAase activity of the naked mole-rat cells was much lower than that of human, mouse or guinea pig cells (Figure 2C). Similarly, HAase activity was lower in the naked mole-rat tissues than in the mouse tissues (Figure 2D). These results indicate that two mechanisms contribute to accumulation of HMW-HA in the naked mole-rat: more robust synthesis and slower degradation.

It was previously demonstrated that ECI contributes to cancer resistance of the naked mole-rat by arresting cell cycle via the induction of $p16^{INK4a}$ (Seluanov, A. et al., *Proc. Natl. Acad. Sci. USA, 2009,* 106:19207-19208). To determine the role of HMW-HA in ECI naked mole-rat fibroblasts were cultured in the presence of bacterial HAase. Enzymatic digestion of HMW-HA abrogated the ECI phenotype and caused naked mole-rat cells to grow to complete confluence (Figure 3A and Figure 3B). Upon subsequent removal of HAase from the culture media, a

fraction of cells detached from the plate and died by apoptosis (Figure 8), while the remaining cells re-acquired the ECI phenotype (Figure 3C).

CD44 is a major HA receptor in human and mouse cells (Toole, B. P., *Nat Rev Cancer, 2004,* 4:528-539; Pure, E. et al., *Cell Signal, 2009,* 21:651-655 and Ponta, H., et al., *Nat Rev Mol Cell Biol, 2003,* 4:33-45). To confirm that HA signaling triggers ECI via the CD44 receptor naked mole-rat cells were cultured in the presence of a CD44-blocking antibody. Naked mole-rat cells grown with CD44 antibodies reached a higher cell density (Figure 3B) indicating that the ECI signal from HMW-HA is in part transmitted via the CD44 receptor.

Flow cytometric assay was then used to measure the affinity of the naked mole-rat cells to fluorescently labeled HA. Naked mole-rat cells displayed a two-fold higher affinity to HA than mouse or human cells (Figure 3D), which can contribute to higher sensitivity of naked mole rat cells to HA signaling.

On the cytoplasmic face, the CD44 receptor interacts with NF2 (merlin), which mediates contact inhibition (Morrison, H. et al., *Genes Dev, 2001,* 15:968-980). The phosphorylated, growth promoting, form of NF2 appeared in naked mole-rat cells grown in the presence of HAase, while cells cultured without HAase contained mainly the unphosphorylated growth-inhibitory form of NF2 (Figure 9). It was previously shown that ECI is associated with induction of $p16^{INK4a}$, while the NMR SF Mut cells that do not display ECI have lost $p16^{INK4a}$ expression (Seluanov, A. et al., *Proc. Natl. Acad. Sci. USA, 2009,* 106:19207-19208)[7]. Accordingly, naked mole-rat cells grown in the presence of HAase displayed reduced levels of $p16^{INK4a}$ (Figure 9). Collectively these results establish that ECI is controlled by the HA/CD44/NF2 pathway.

The role of HMW-HA in the resistance of naked mole-rat cells to malignant transformation was then tested in a soft agar assay. SV40 Large T antigen (SV40 LT) is a viral oncoprotein that binds and inactivates p53 and pRb. The mutant derivative LTK1 (K1) inactivates only p53, while LTΔ434-444 (Δ434) inactivates only pRb and its family members (Hahn, W. C. et al., *Mol Cell Biol, 2002,* 22:2111-2123). A combination of H-Ras V12 and SV40 LT is sufficient to transform mouse fibroblasts (Rangarajan, A., et al., *Cancer Cell, 2004,* 6:171-183), but, as shown earlier, is not sufficient to confer anchorage independent growth to naked mole-rat cells (Seluanov, A. et al., *Proc. Natl. Acad. Sci. USA, 2009,* 106:19207-19208). To

test the role of HMW-HA in the naked mole-rat's resistance to transformation, naked mole-rat fibroblasts were transfected with H-Ras V12 combined with SV40 LT or its mutants K1 or Δ434 and cultured them in soft agar in the presence of HAase. Under these conditions, cells transfected with H-Ras V12 and SV40 LT, or H-Ras V12 and Δ434 formed robust colonies (Figure 4A). Similarly, naked mole rat cells cultured in the presence of CD44 blocking antibody formed colonies in soft agar (Figure 10). These results demonstrate that if HMW-HA is degraded by HAase or HA signaling is blocked by a CD44 antibody, naked mole-rat cells become susceptible to anchorage-independent growth triggered by H-Ras V12 and SV40 LT. In embryonic naked mole-rat fibroblasts, which do not secrete HMW-HA, H-Ras V12 and SV40 LT or Δ434 were sufficient to trigger anchorage-independent growth (Figure 10).

H-Ras V12 and SV40 LT expressing naked mole-rat cells were then generated, in which HMW-HA was abolished by either integrating shRNA targeting HAS2 (Figure 11A) or overexpressing an HA-degrading enzyme Hyal2 (Figure 11B). These cells no longer increased the viscosity of their culture media (Figure 11C and readily formed colonies in soft agar (Figure 12). To confirm that HMW-HA inhibits tumor formation *in vivo*, xenograft experiments were performed with naked mole-rat cells containing a knockdown of HAS2, or overexpressing Hyal2 (Figure 4B). In the positive control, mouse cells expressing H-Ras V12 and SV40 LT readily formed tumors in mice. Naked mole-rat cells expressing H-Ras V12 and SV40 LT did not form tumors, consistent with an earlier report (Liang, S., et al., *Aging Cell, 2010,* 9:626-635). Remarkably, naked mole-rat cells expressing H-Ras V12 and SV40 LT and shRNA to HAS2 or overexpressing Hyal2 formed tumors in mice. This experiment establishes HMW-HA, produced by HAS2, as a key component responsible for the elevated cancer resistance of the naked mole-rat.

Experiments were conducted to examine if media comprising HMW-HA secreted by naked mole rat skin fibroblasts had any effect on the growth of cancerous cells. Figure 14 demonstrates that HMW-HA containing media inhibits the growth of human cancer cells, HeLa and HT1080, demonstrating that HMW-HA can be used to treat cancer.

The HMW-HA in the naked mole-rat could have evolved as an adaptation to subterranean lifestyle to provide flexible skin needed to squeeze through underground tunnels. Interestingly, it was found that cells of a different subterranean

rodent, the blind mole-rat, which is phylogenetically closer to mice and rats than to the naked mole-rat also secreted HMW-HA (Figure 13). In summary, the results presented herein demonstrate that extremely HMW-HA, its binding to the CD44 receptor, and lower HAase activity play a key role in mediating the cancer resistance of the naked mole-rat. Using naked mole-rat HMW-HA in the clinic or targeting Hyal2, or the HA-CD44 signaling pathway opens new avenues for cancer prevention and life extension.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. A method of treating or preventing cancer in a subject, the method comprising administering to the subject an effective amount of a composition which increases the level of high molecular weight hyaluronic acid (HMW-HA) in the subject.

2. The method of claim 1, wherein the HMW-HA has a molecular weight of at least 6,000 kDa.

3. The method of claim 1, wherein the composition comprises HMW-HA.

4. The method of claim 1, wherein the composition comprises an isolated nucleic acid encoding mole-rat hyaluronic acid synthase 2 (HAS2).

5. The method of claim 1, wherein the composition comprises mole-rat HAS2.

6. The method of claim 5, wherein mole-rat HAS2 comprises the amino acid sequence of SEQ ID NO: 1.

7. The method of claim 1, wherein the composition comprises a cell which produces HMW-HA.

8. The method of claim 7, wherein the cell is genetically modified to produce HMW-HA.

9. The method of claim 1, wherein the composition comprises a polymeric substrate.

10. The method of claim 9, wherein the substrate comprises HMW-HA.

11. The method of claim 9, wherein the substrate is administered to the subject in conjunction with surgical resection of a tumor.

12. A composition for treating or preventing cancer, wherein the composition comprises an agent which increases the level of high molecular weight hyaluronic acid (HMW-HA) in a subject.

13. The composition of claim 12, wherein the HMW-HA has a molecular weight of at least 6,000 kDa.

14. The composition of claim 12, wherein the composition comprises HMW-HA.

15. The composition of claim 12, wherein the composition comprises an isolated nucleic acid encoding mole-rat hyaluronic acid synthase 2 (HAS2).

16. The composition of claim 12, wherein the composition comprises mole-rat HAS2.

17. The composition of claim 16, wherein mole-rat HAS2 comprises the amino acid sequence of SEQ ID NO: 1.

18. The composition of claim 12, wherein the composition comprises a cell which produces HMW-HA.

19. The composition of claim 18, wherein the cell is genetically modified to produce HMW-HA.

20. The composition of claim 12, wherein the composition comprises a polymeric substrate.

21. The composition of claim 20, wherein the substrate comprises HMW-HA.

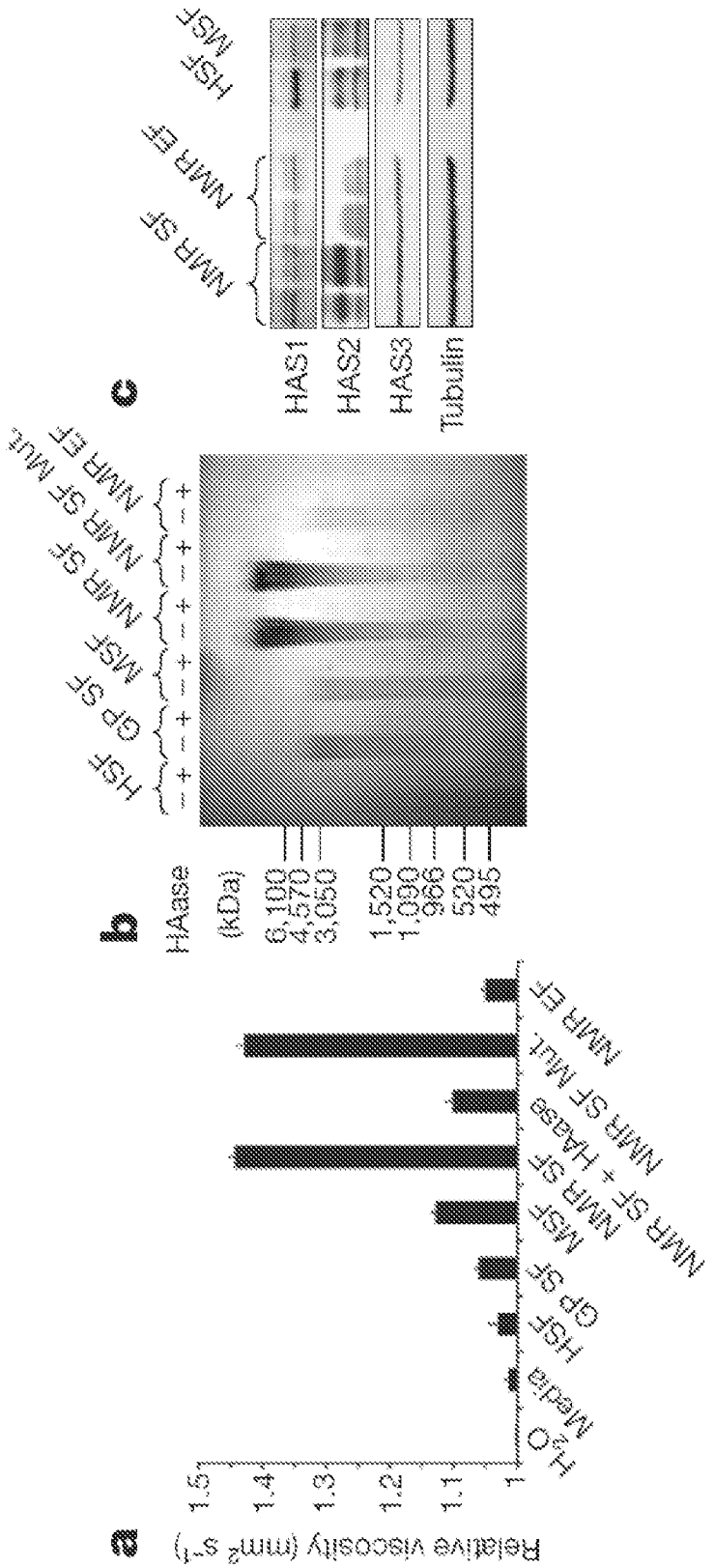


Figure 1A – Figure 1C

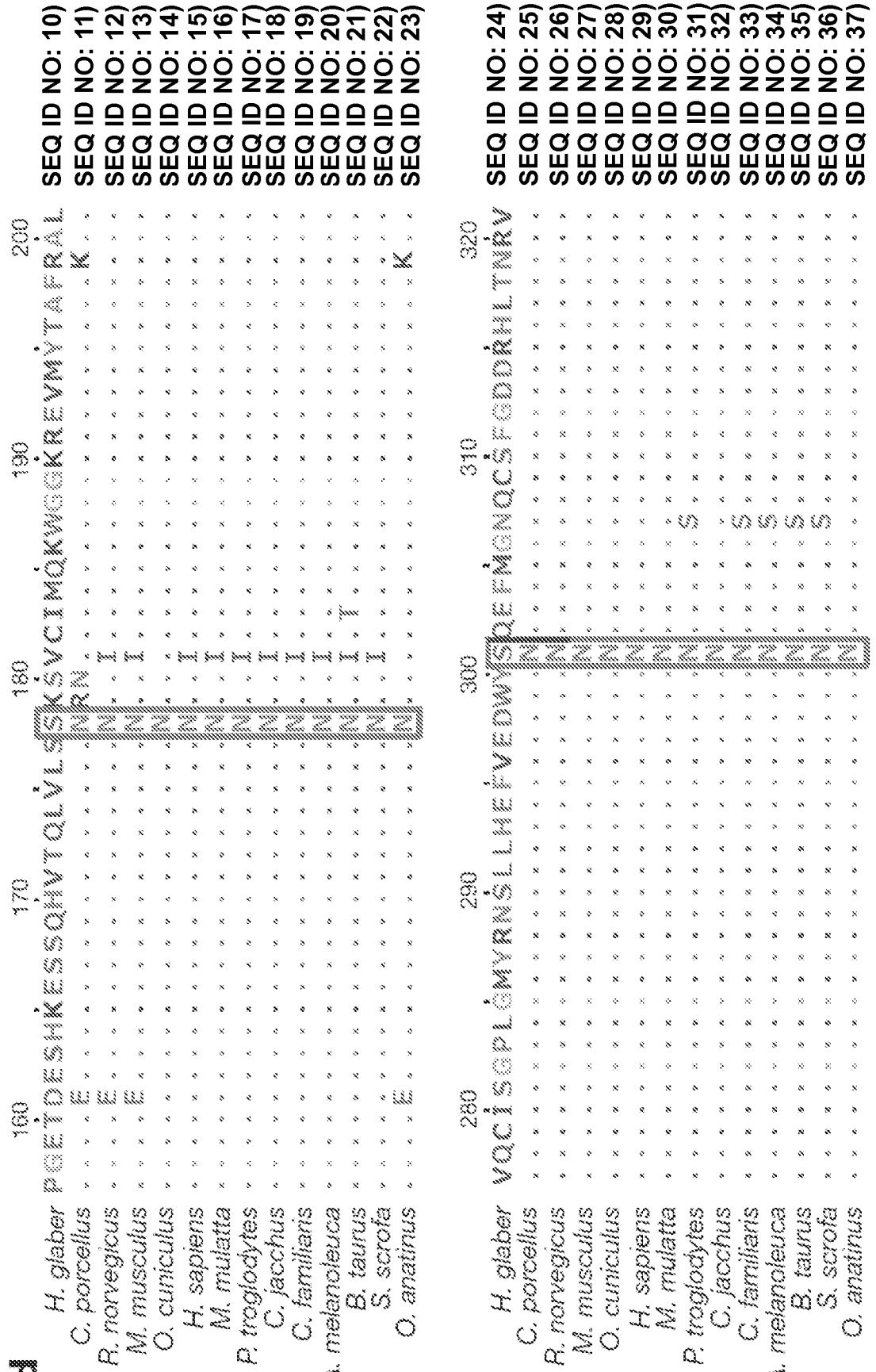

d
160
170
180
190
200
H. glaber PGETDESHKESSQHVTQLVLSKSVCIMQKWGGKREVMYTAFRALK SEQ ID NO: 10)
C. porcellus E N N K SEQ ID NO: 11)
R. norvegicus E N I SEQ ID NO: 12)
M. musculus E N I SEQ ID NO: 13)
O. cuniculus N I SEQ ID NO: 14)
H. sapiens N I SEQ ID NO: 15)
M. mulatta N I SEQ ID NO: 16)
P. troglodytes N I SEQ ID NO: 17)
C. jacchus N I SEQ ID NO: 18)
C. familiaris N I SEQ ID NO: 19)
A. melanoleuca N I SEQ ID NO: 20)
B. taurus N I T SEQ ID NO: 21)
S. scrofa N I SEQ ID NO: 22)
O. anatinus E N K SEQ ID NO: 23)
280
290
300
310
320
H. glaber VQCISGPLGMYRNSLHEFVEDWYSPEFMGNQCSFGDDRHLTNRV SEQ ID NO: 24)
C. porcellus N SEQ ID NO: 25)
R. norvegicus N SEQ ID NO: 26)
M. musculus N SEQ ID NO: 27)
O. cuniculus N SEQ ID NO: 28)
H. sapiens N SEQ ID NO: 29)
M. mulatta N SEQ ID NO: 30)
P. troglodytes N S SEQ ID NO: 31)
C. jacchus N SEQ ID NO: 32)
C. familiaris N S SEQ ID NO: 33)
A. melanoleuca N S SEQ ID NO: 34)
B. taurus N S SEQ ID NO: 35)
S. scrofa N S SEQ ID NO: 36)
O. anatinus N SEQ ID NO: 37)

Figure 1D

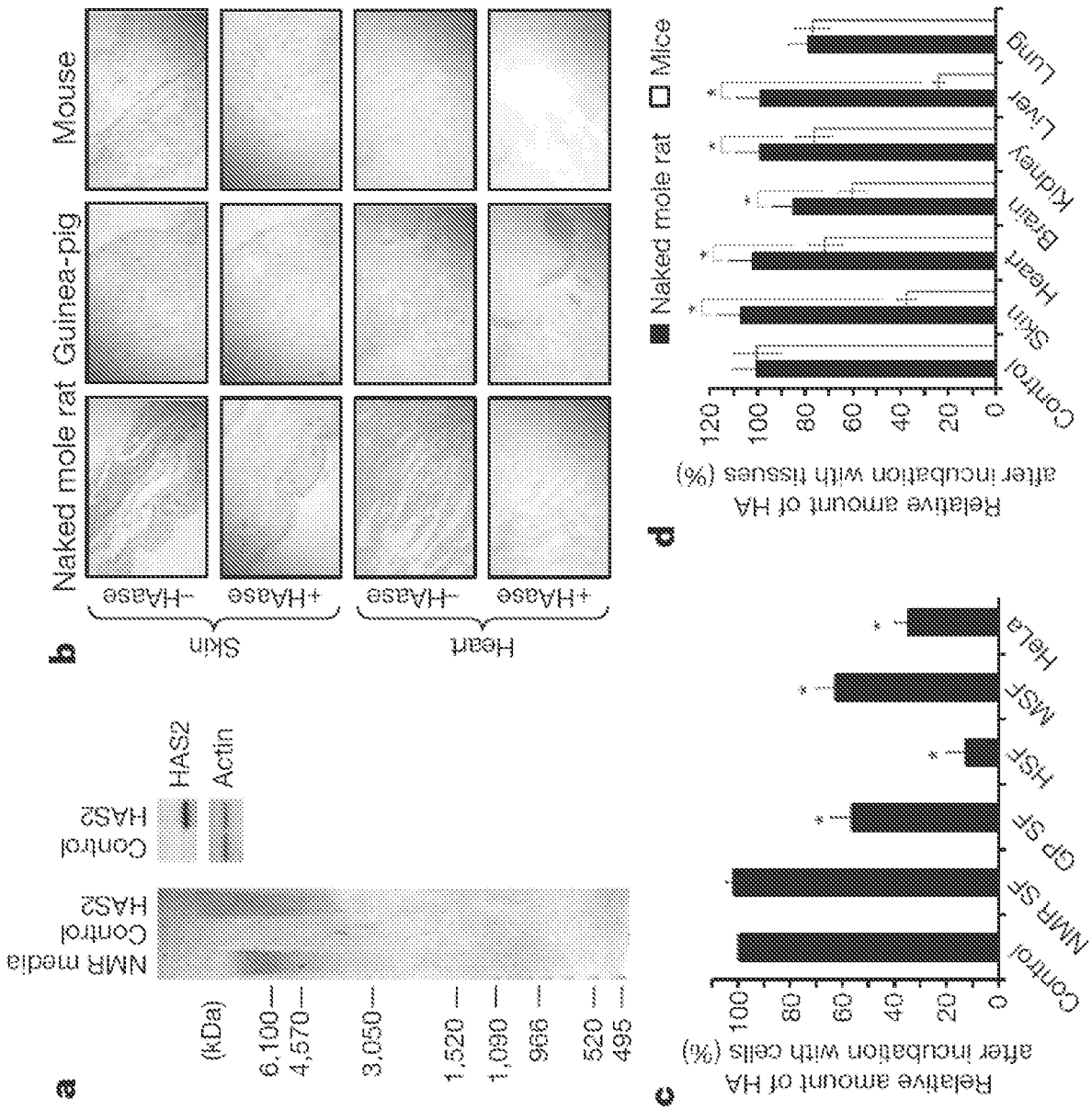

a
NMR media
Control
HAS2
Control
HAS2
HAS2
Actin
(kDa)
6,100
4,570
3,050
1,520
1,090
966
520
495
b
Naked mole rat
Guinea-pig
Mouse
Skin
Heart
-HAase
+HAase
-HAase
+HAase
c
Relative amount of HA
after incubation with cells (%)
Control
NMR SF
GP SF
HSF
MSF
HeLa
d
Naked mole rat
Mice
Relative amount of HA
after incubation with tissues (%)
Control
Skin
Heart
Brain
Kidney
Liver
Lung

Figure 2

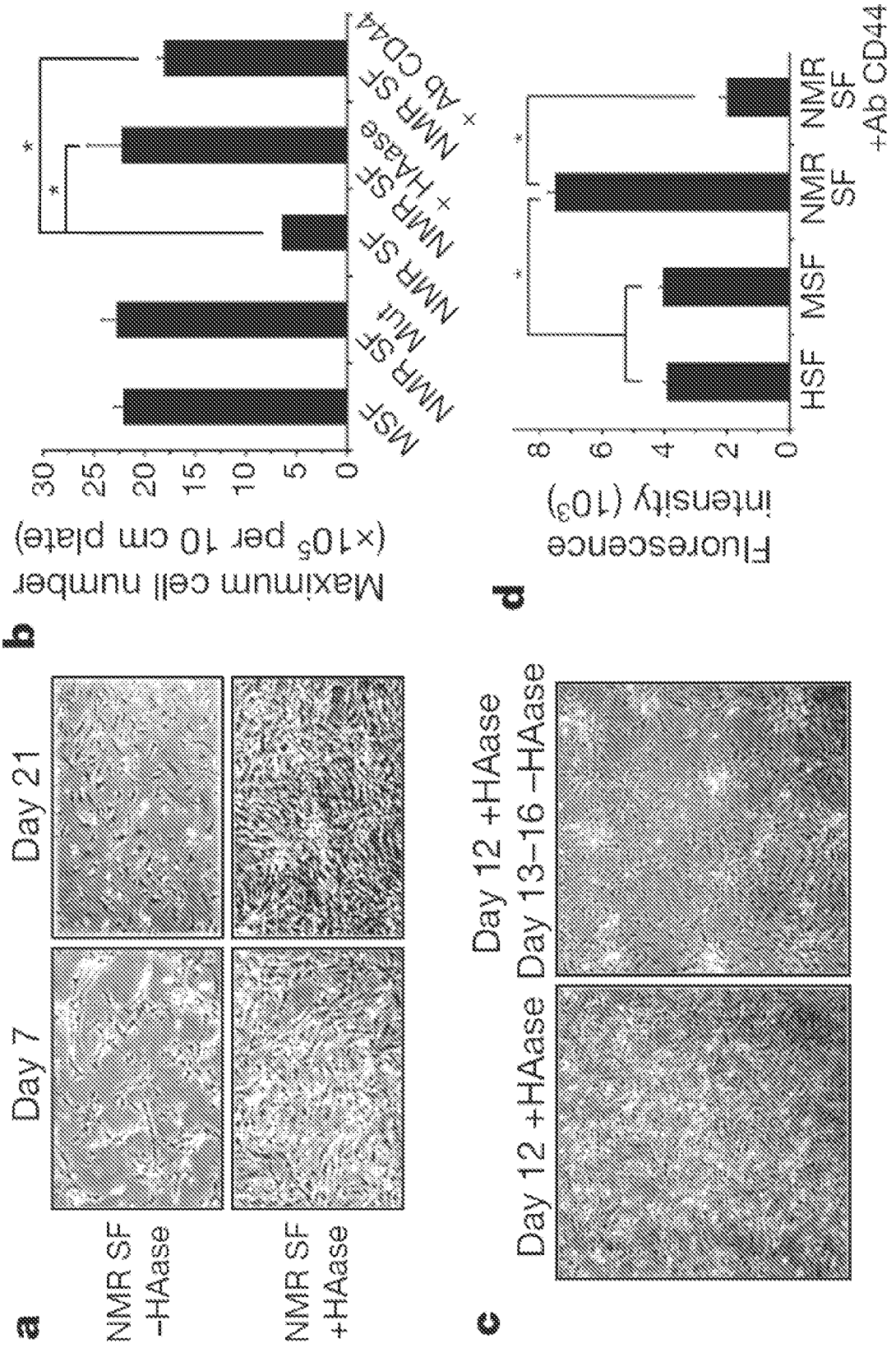

a
Day 7
Day 21
NMR SF −HAase
NMR SF +HAase
b
Maximum cell number (×10⁵ per 10 cm plate)
30
25
20
15
10
5
0
MSF
NMR SF
NMR SF + HAase
NMR SF + Ab CD44
c
Day 12 +HAase
Day 12 +HAase
Day 13–16 −HAase
d
Fluorescence intensity (10³)
8
6
4
2
0
HSF
MSF
NMR SF
NMR SF +Ab CD44

Figure 3

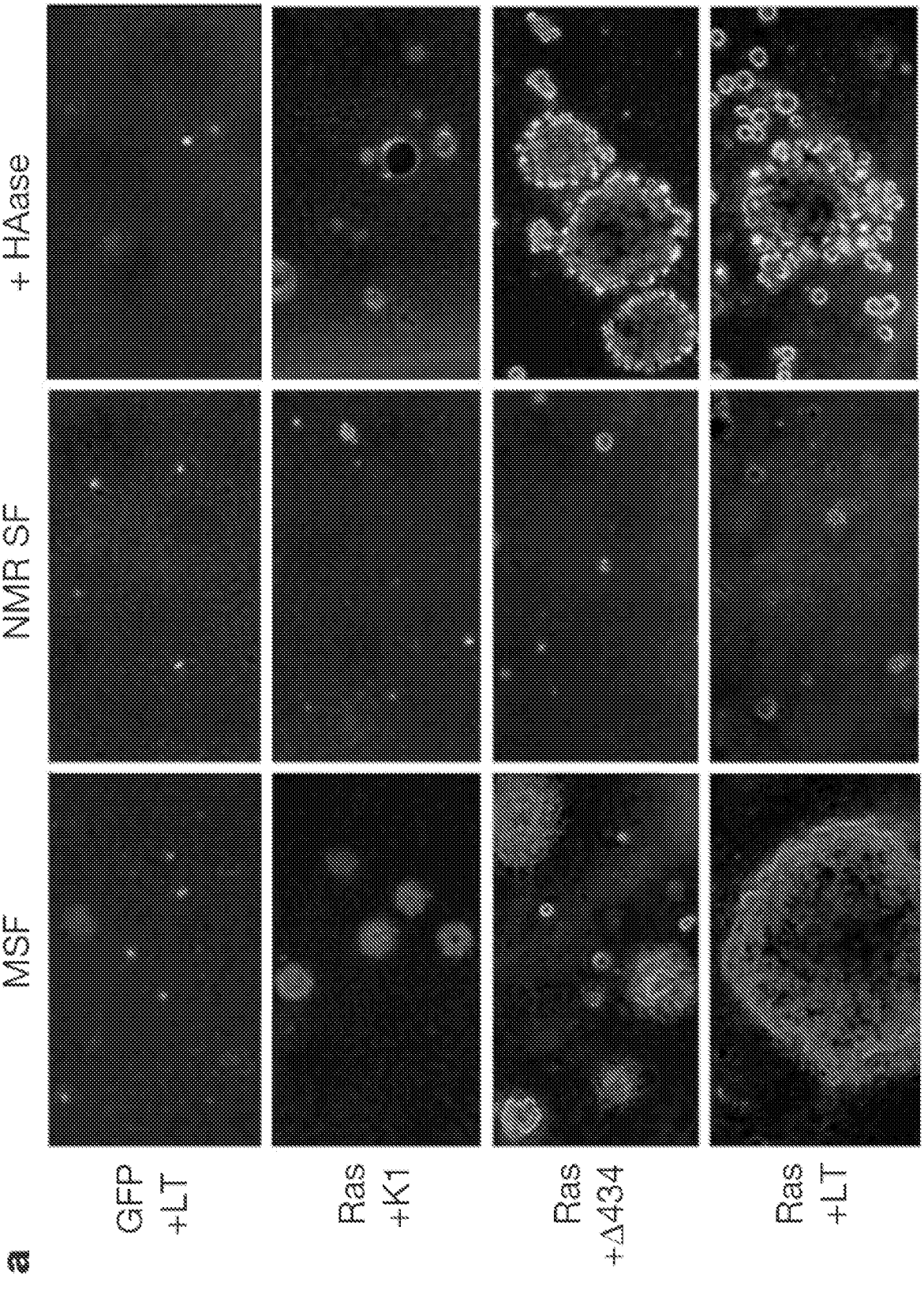


Figure 4A

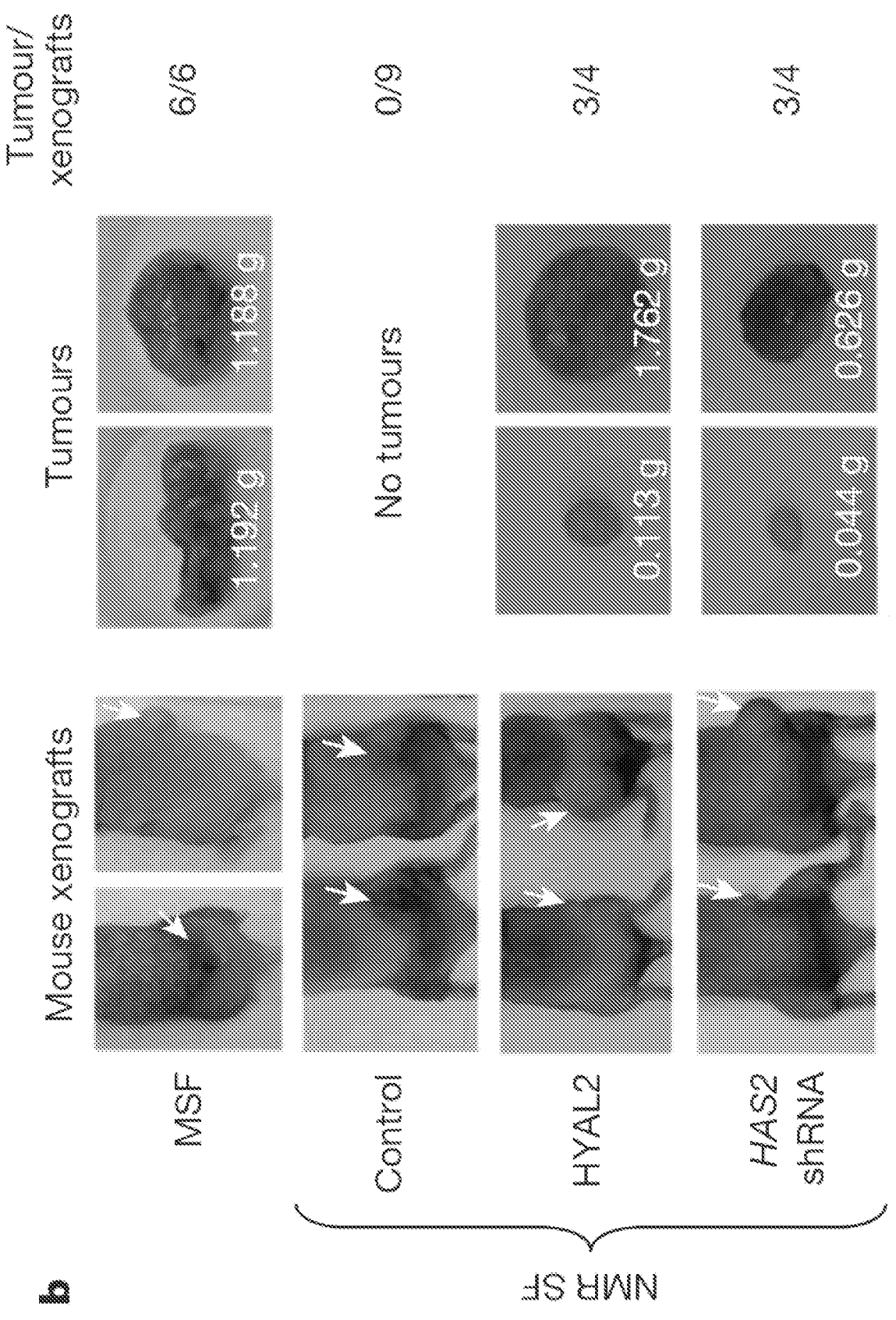

b
NMR SF
MSF
Control
HYAL2
HAS2 shRNA
Mouse xenografts
Tumours
No tumours
1.192 g
1.198 g
0.113 g
1.762 g
0.044 g
0.626 g
Tumour/ xenografts
6/6
0/9
3/4
3/4

Figure 4B

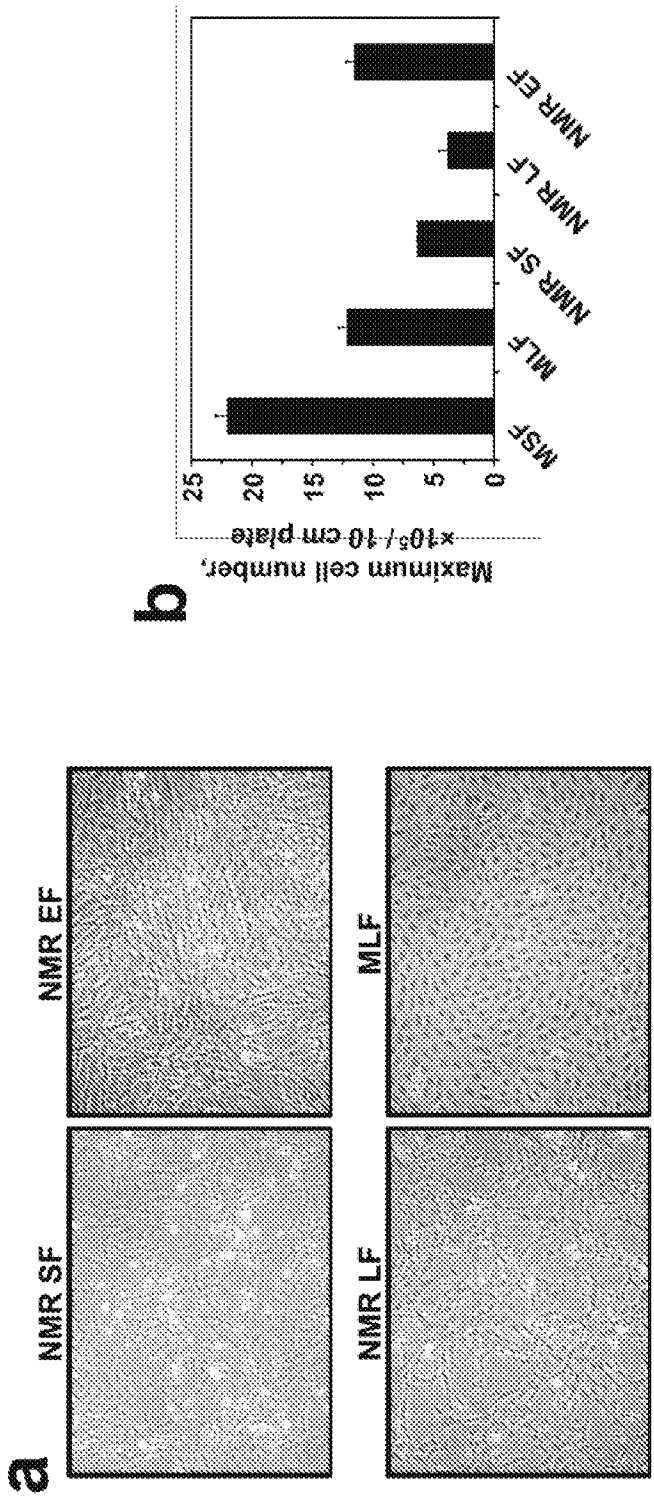

a
NMR SF
NMR EF
NMR LF
MLF
b
Maximum cell number, ×10⁵/ 10 cm plate
0
5
10
15
20
25
MSF
MLF
NMR SF
NMR LF
NMR EF

Figure 5

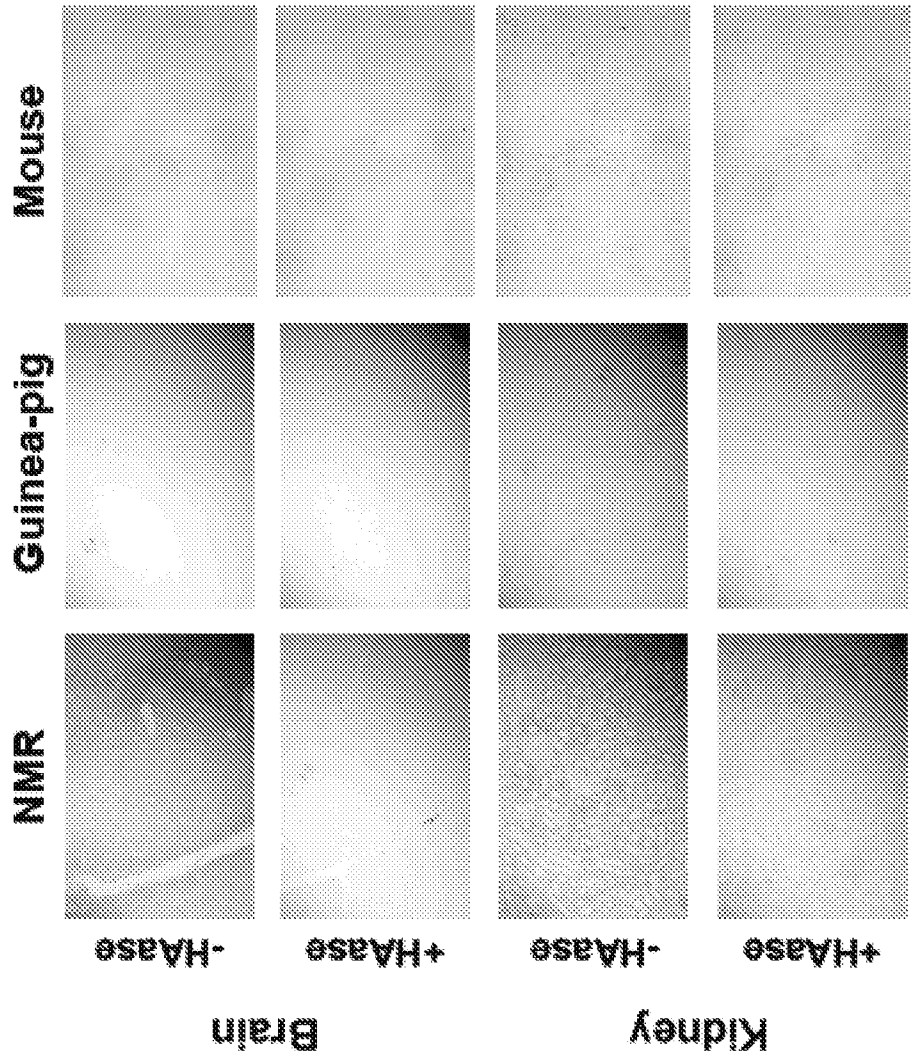


Figure 6

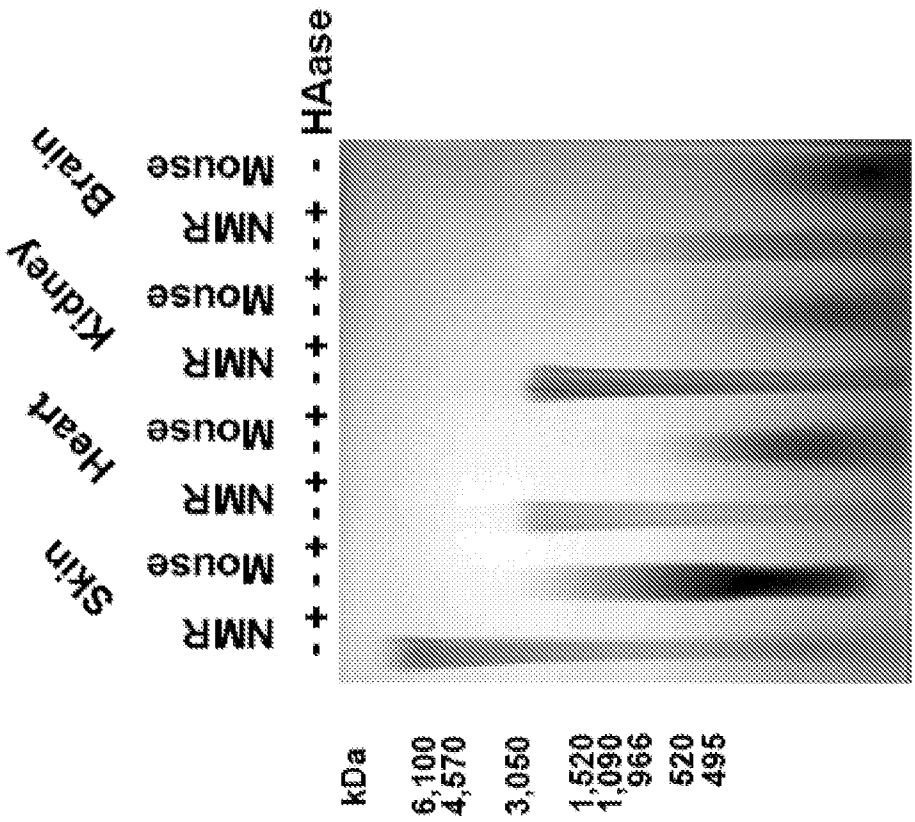

HAase
- + - + - + - + - + - +
NMR
Mouse
NMR
Mouse
NMR
Mouse
NMR
Mouse
NMR
Mouse
Skin
Heart
Kidney
Brain
kDa
6,100
4,570
3,050
1,520
1,090
966
520
495

Figure 7

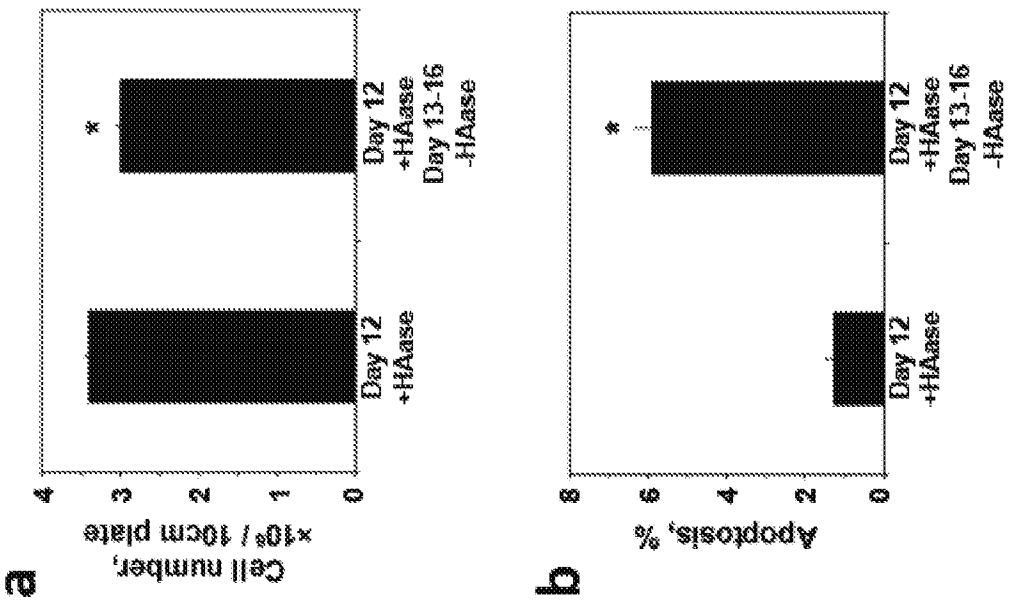

a
Cell number, ×10⁵ / 10cm plate
0
1
2
3
4
*
Day 12 +HAase
Day 13-16 -HAase
b
Apoptosis, %
0
2
4
6
8
*
Day 12 +HAase
Day 13-16 -HAase

Figure 8

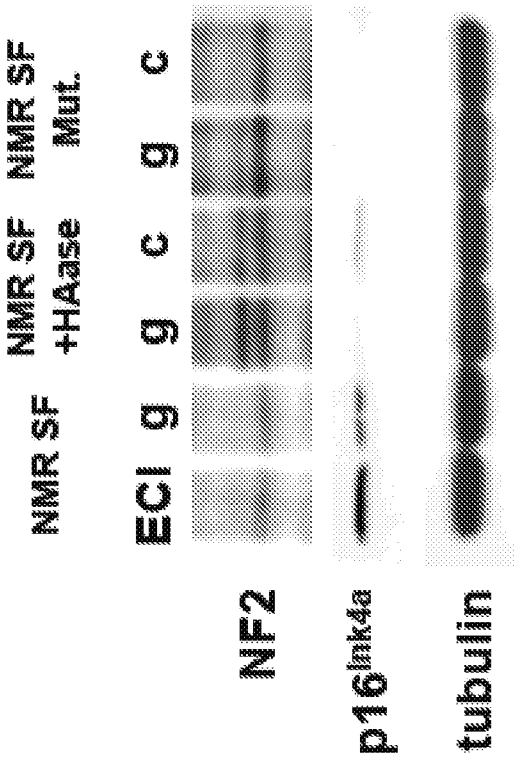


Figure 9

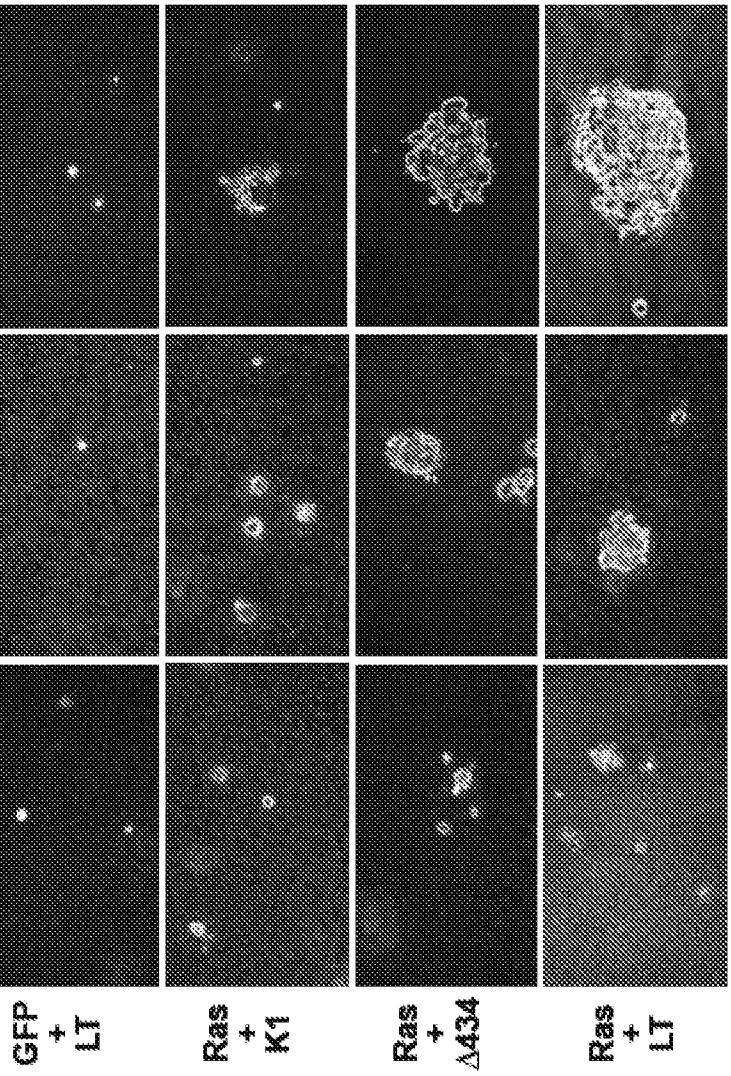


Figure 10

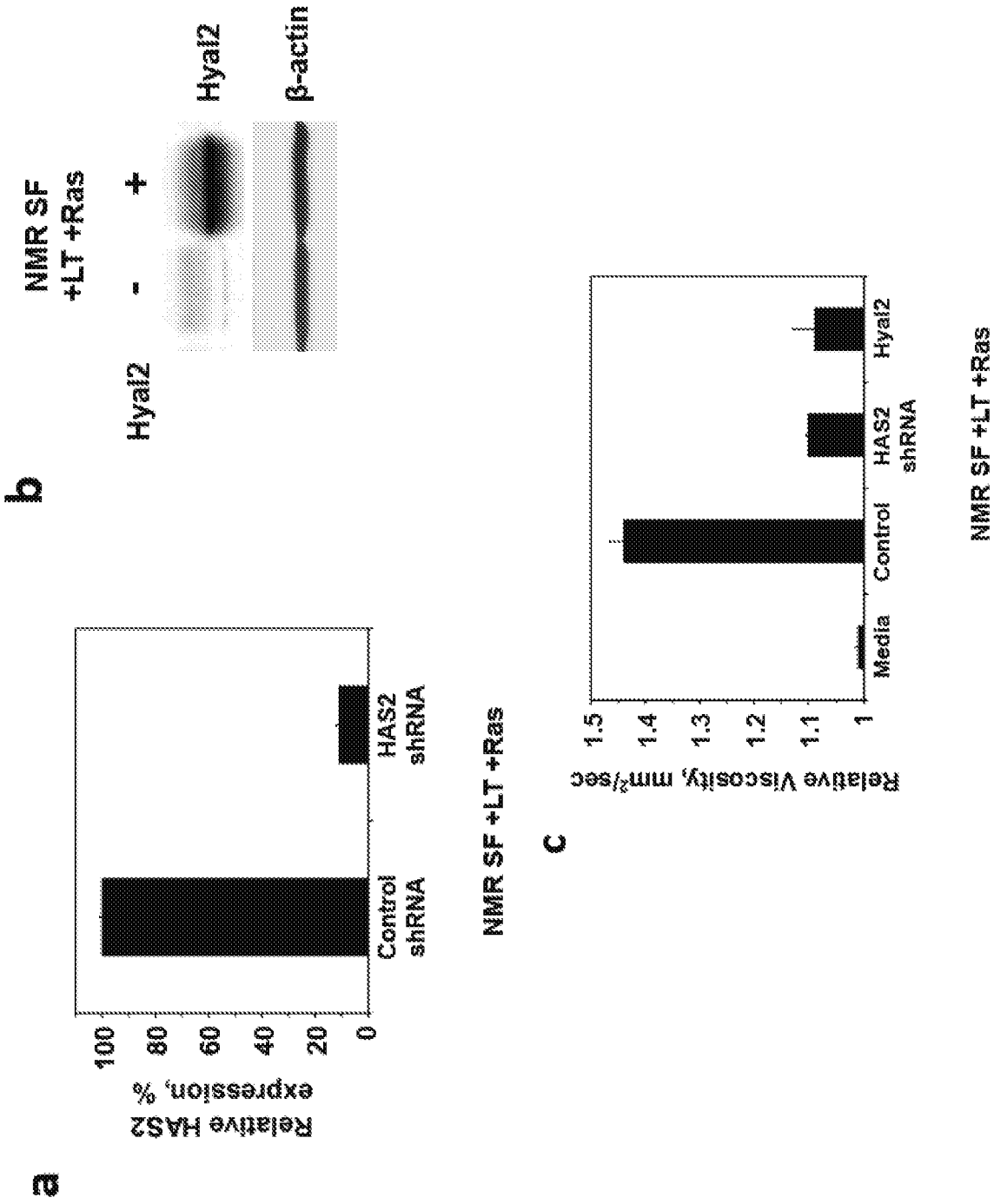

a
Relative HAS2 expression, %
100
80
60
40
20
0
Control shRNA
HAS2 shRNA
NMR SF +LT +Ras
b
NMR SF +LT +Ras
Hyal2
-
+
Hyal2
β-actin
c
Relative Viscosity, mm²/sec
1.5
1.4
1.3
1.2
1.1
1
Media
Control
HAS2 shRNA
Hyal2
NMR SF +LT +Ras

Figure 11

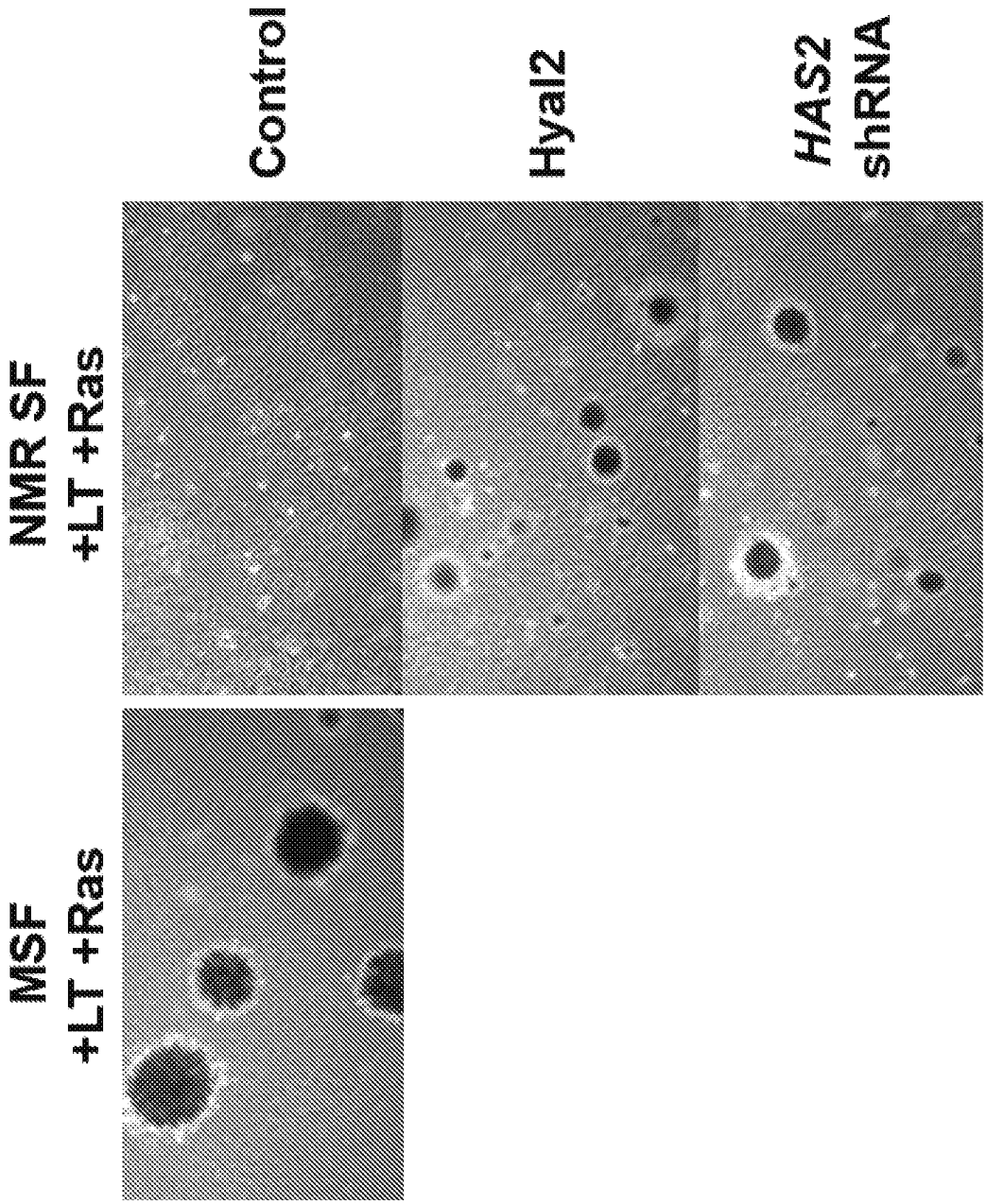


Figure 12

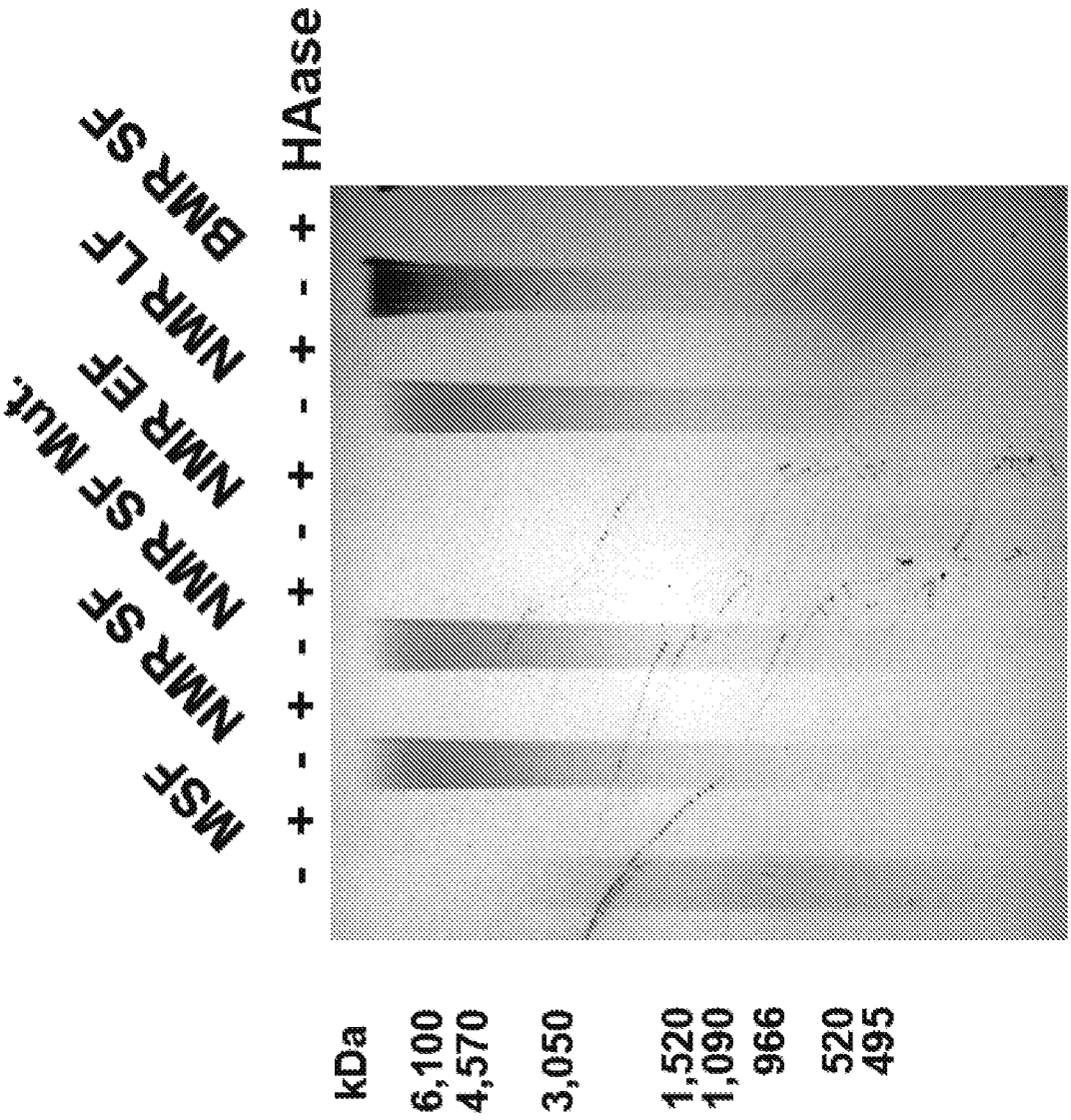
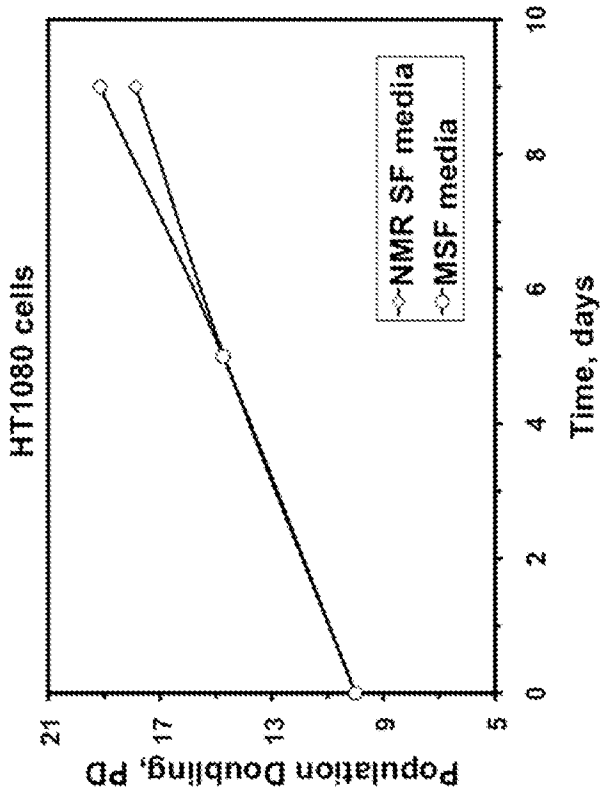

HAase
+ - + - + - + - + -
BMR SF
NMR LF
NMR EF
NMR SF Mult.
NMR SF
MSF
kDa
6,100
4,570
3,050
1,520
1,090
966
520
495

Figure 13


HT1080 cells
Population Doubling, PD
Time, days
NMR SF media
MSF media
21
17
13
9
5
0
2
4
6
8
10

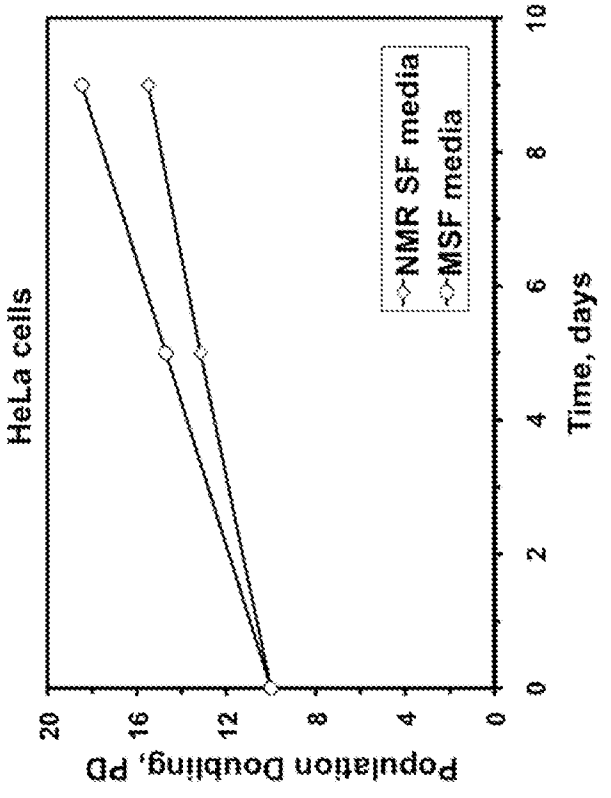

HeLa cells
Population Doubling, PD
Time, days
NMR SF media
MSF media
20
16
12
8
4
0
2
4
6
8
10

Figure 14