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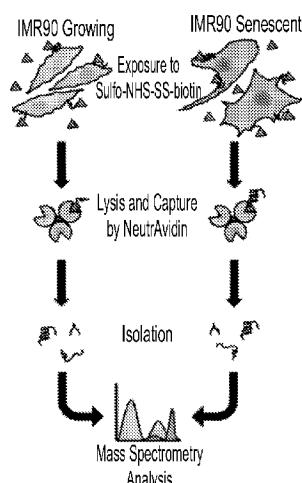


FIG. 1B

(57) Abstract: A method of targeting a pharmaceutical agent to a senescent cell is disclosed. The method comprises administering the pharmaceutical agent to the subject, wherein said pharmaceutical agent is attached to an affinity moiety, said affinity moiety being capable of binding specifically to a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1. The targeting may be for therapeutics or diagnostics.



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METHOD OF TARGETING SENESCENT CELLS

FIELD AND BACKGROUND OF THE INVENTION

5 The present invention, in some embodiments thereof, relates to methods of targeting senescent cells for treating and diagnosing diseases.

Cellular senescence, a stable form of cell cycle arrest, is a mechanism limiting the proliferative potential of cells. Senescence can be triggered in many cell types in response to diverse forms of cellular stress. It is a potent barrier to tumorigenesis and 10 contributes to the cytotoxicity of certain anti-cancer agents. While senescence limits tumorigenesis and tissue damage in a cell autonomous manner, senescent cells induce inflammation, tissue ageing, tissue destruction and promote tumorigenesis and metastasis in a cell non-autonomous manner in the sites of their presence. Therefore, 15 their elimination might lead to tumor prevention and inhibition of tissue ageing. Indeed, elimination of senescent cells was shown to slow down tissue ageing in an animal model (Baker et al., 2011).

Organisms may have developed elaborate mechanisms to eliminate senescent cells in order to avoid their deleterious effects on the microenvironment. However, their fate in tissue is not well characterized. On one hand, benign melanocytic nevi (moles) 20 are highly enriched for senescent cells yet can exist in skin throughout a lifetime, implying that senescent cells can be stably incorporated into tissues. On the other hand, it has been previously shown that components of the innate immune system specifically recognize and eliminate senescent cells *in vitro* and target senescent cells *in vivo* leading to tumor regression and reversion of liver fibrosis (Krizhanovsky et al., 2008b; Sagiv et 25 al., 2012; Xue et al., 2007). Therefore, senescent cells can turn over *in vivo* and the immune system contributes to this turnover. The effort that the immune system invests in recognition and elimination of senescent cells suggests, although not directly, that senescent cells are deleterious for the organism and their elimination is beneficial.

Background art includes WO2014/174511, WO2013/152038, WO2014/089124, 30 and Garnacho et al., Journal of Pharmacology and Experimental Therapeutics, March 2012 vol. 340 no. 3 638-647.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of targeting a pharmaceutical agent to a senescent cell in a subject comprising administering the pharmaceutical agent to the subject, wherein the pharmaceutical agent is attached to an affinity moiety, the affinity moiety being capable of binding specifically to a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1, thereby targeting the pharmaceutical agent to the senescent cell.

According to an aspect of some embodiments of the present invention there is provided a method of treating a disease associated with cell senescence in a subject in need thereof comprising administering to the agent a therapeutically effective amount of a cytotoxic agent attached to an affinity moiety, the affinity moiety being capable of binding specifically to a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1, thereby treating the disease.

According to an aspect of some embodiments of the present invention there is provided a particle having a senescent cell affinity moiety attached to an outer surface thereof, the senescent cell affinity moiety capable of specifically binding a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1.

According to an aspect of some embodiments of the present invention there is provided a composition of matter comprising an affinity moiety attached to a therapeutic agent, wherein the affinity moiety specifically binds to a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1.

According to an aspect of some embodiments of the present invention there is provided a pharmaceutical composition comprising the composition of matter or particle described herein as the active agent and a pharmaceutically acceptable carrier.

According to an aspect of some embodiments of the present invention there is provided a method of diagnosing a disease associated with cell senescence in a subject comprising analyzing the amount of at least one polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3

and a polypeptide set forth in Table 1 on the membrane of cells of the subject, wherein a level of the at least one polypeptide above a predetermined amount is indicative of the disease.

According to an aspect of some embodiments of the present invention there is
5 provided a method of identifying senescent cells in a cell population comprising
analyzing the amount of at least one polypeptide selected from the group consisting of
HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a
polypeptide set forth in Table 1 on the membrane of the cells of the cell population,
wherein a level of the at least one polypeptide above a predetermined amount is
10 indicative of senescent cells.

According to an aspect of some embodiments of the present invention there is
provided a composition of matter comprising senescent cells, wherein a polypeptide of
the cells is attached to an affinity moiety, the polypeptide being selected from the group
consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3
15 and a polypeptide set forth in Table 1.

According to an aspect of some embodiments of the present invention there is
provided a method of eliciting or boosting an immune response to a senescent cell in a
subject comprising administering to the subject a pharmaceutical composition
comprising at least one polypeptide or a polynucleotide encoding same selected from the
20 group consisting of HSP90B1, DBN1, PRKCSH, SPTBN1, NPM1 and a polypeptide set
forth in Table 1, wherein the composition does not comprise senescent cells or
membranes thereof, thereby eliciting or boosting the immune response to the senescent
cell.

According to an aspect of some embodiments of the present invention there is
25 provided a vaccine comprising at least one polypeptide or a polynucleotide encoding
same as an active agent, the polypeptide being selected from the group consisting of
HSP90B1, DBN1, PRKCSH, SPTBN1, NPM1, an adjuvant, wherein the vaccine does
not comprise senescent cells or membranes thereof and an immunologically acceptable
carrier.

According to an aspect of some embodiments of the present invention there is
30 provided a vaccine comprising cells expressing a heterogeneous polypeptide, as an

active agent and an immunologically acceptable carrier, the polypeptide being selected from the group consisting of HSP90B1, DBN1, PRKCSH, SPTBN1, NPM1.

According to some embodiments of the invention, the pharmaceutical agent is a therapeutic agent.

5 According to some embodiments of the invention, the therapeutic agent is a cytotoxic agent.

According to some embodiments of the invention, the pharmaceutical agent is a diagnostic agent.

10 According to some embodiments of the invention, the cytotoxic agent is directly attached to the affinity moiety.

According to some embodiments of the invention, the cytotoxic agent is indirectly attached to the affinity moiety.

According to some embodiments of the invention, the cytotoxic agent is comprised in a particle.

15 According to some embodiments of the invention, the affinity moiety is attached to the outer surface of the particle.

According to some embodiments of the invention, the cytotoxic agent comprises a polynucleotide agent.

20 According to some embodiments of the invention, the cytotoxic agent comprises an RNA silencing agent.

According to some embodiments of the invention, the cytotoxic agent down-regulates an activity and/or an amount of an apoptosis related polypeptide.

According to some embodiments of the invention, the apoptosis related polypeptide is selected from the group consisting of Bcl-xL, Bcl-w and p21.

25 According to some embodiments of the invention, the cytotoxic agent is selected from the group consisting of ABT-737, ABT-263, Gossypol, AT-101, TW-37 and Obatoclax.

According to some embodiments of the invention, the affinity moiety is selected from the group consisting or an antibody, an aptamer and a peptide.

30 According to some embodiments of the invention, the polypeptide is HSP90B1.

According to some embodiments of the invention, the disease is a fibrotic disease or an inflammatory disease.

According to some embodiments of the invention, the inflammatory disease is cancer.

According to some embodiments of the invention, the method further comprises administering to the subject at least one agent selected from the group consisting of a sebum-regulating agent, an antibacterial and/or antifungal agent, a keratolytic agent and/or keratoregulating agent, an astringent, an anti-inflammatory and/or anti-irritant, an antioxidant and/or free-radical scavenger, a cicatrizing agent, an anti-aging agent and a moisturizing agent.

According to some embodiments of the invention, the at least one agent is an anti-aging agent.

According to some embodiments of the invention, the affinity moiety is an antibody or an aptamer.

According to some embodiments of the invention, the particle is attached to or encapsulating a therapeutic agent or a diagnostic agent.

According to some embodiments of the invention, the therapeutic agent comprises a cytotoxic moiety.

According to some embodiments of the invention, the cytotoxic moiety comprises a polynucleotide agent.

According to some embodiments of the invention, the polynucleotide agent comprises an RNA silencing agent.

According to some embodiments of the invention, the cytotoxic agent down-regulates an activity and/or an amount of an apoptosis related polypeptide.

According to some embodiments of the invention, the apoptosis related polypeptide is selected from the group consisting of Bcl-xL, Bcl-w and p21.

According to some embodiments of the invention, the pharmaceutical composition is formulated for topical administration.

According to some embodiments of the invention, the pharmaceutical composition further comprises at least one agent selected from the group consisting of a sebum-regulating agent, an antibacterial and/or antifungal agent, a keratolytic agent and/or keratoregulating agent, an astringent, an anti-inflammatory and/or anti-irritant, an antioxidant and/or free-radical scavenger, a cicatrizing agent, an anti-aging agent and a moisturizing agent.

According to some embodiments of the invention, the at least one agent is an anti-aging agent.

According to some embodiments of the invention, the senescent cells are lysed cells.

5 According to some embodiments of the invention, the senescent cells are non-lysed cells.

According to some embodiments of the invention, the disease is a fibrotic disease or an inflammatory disease.

10 According to some embodiments of the invention, the inflammatory disease is cancer.

According to some embodiments of the invention, the identifying is effected in vivo.

According to some embodiments of the invention, the identifying is effected ex vivo.

15 According to some embodiments of the invention, the identifying is effected in vitro.

According to some embodiments of the invention, the at least one polypeptide is HSP90B1.

20 According to some embodiments of the invention, the analyzing is effected using an antibody that selectively binds the at least one polypeptide.

According to some embodiments of the invention, the antibody is attached to a detectable moiety.

According to some embodiments of the invention, the vaccine further comprises an adjuvant.

25 According to some embodiments of the invention, the polypeptide is expressed on a cell surface.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent

specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

5 Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings and images. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in 10 the art how embodiments of the invention may be practiced.

In the drawings:

FIGs. 1A-E. Isolation and identification of senescence-specific membrane proteins. (A) Images of senescent IMR90 cells 9 days after Etoposide (DIS), and 15 senescent IMR90 cells 9 days after infection with H-Ras (OIS). Scale bar - 100µm. (B)

Schematic description of isolation and identification of membrane proteins. (C-E) Integrative analysis of senescence restricted cell surface proteins. (C) Distribution of identified proteins in the two experiments (each experiment in at least 3 repeats). The upper part shows distribution of proteins exclusively detected in each senescent cell type – DIS and OIS. The lower part shows primary sub-cellular location of cell-surface 20 proteome of senescent cells combined from both experiments. (D) Immune-related canonical pathways attributed to the identified proteins. (E) Candidate upstream regulators potentially responsible for the changes observed in identified proteins as revealed by analysis using Ingenuity.

FIGs. 2A-I. Grp94 is upregulated on the surface of senescent cells. (A) 25 Intersection of senescence specific cell-surface proteins from DIS and OIS experiments, and the list of the nine shared proteins. (B) Main features obtained from MS analysis regarding ICAM1, ITGA3, and Grp94. (C) Location of unique peptides detected in MS analysis on the sequence of Grp94 protein (in blue).

(D) Western blot analysis of Grp94, GAPDH and HLA-A,B,C from Qiagen surface-proteins isolation kit comparing to total 30 cell-lysate. (E) Flow cytometry histogram for the frequency of cell-surface Grp94 on live IMR90 cells. (F) Quantitative analysis of cell-surface Grp94 on live IMR90 cells. (G) Flow cytometry histogram for the frequency of cell-surface ICAM1 on live IMR90

cells. (H) Quantitative analysis of cell-surface ICAM1 on live IMR90 cells. (I) Immunofluorescent staining of live IMR90 cells for Grp94 (Alexa-647) against cell-surface marker HLA-A,B,C (Alexa-488). Scale bar - 100 μ m.

FIGs. 3A-F. Grp94 translocates to cell-surface of senescent cells. (A) Flow cytometry histogram for the frequency cell-surface Grp94 of live IMR90 cells 0, 3, 7, 14, or 21 days after etoposide treatment. (B) Quantitative analysis of cell-surface Grp94 on live IMR90 cells 0, 3, 7, 14, or 21 days after etoposide treatment. (C) Scheme describing the mechanism of GPM1-mediated blockage of Grp94 translocation to cell-surface. (D) PrestoBlue viability assay for IMR90 growing or senescent Etoposide-treated cells after 24hrs treatment with 1, 10, or 100 μ M GPM1. GPM1 does not affect cell viability. (E) Flow cytometry histogram for the frequency of cell-surface Grp94 of live senescent etoposide-treated IMR90 cells after 24hrs treatment with 10 or 100 μ M GPM1. (F) Quantitative analysis of cell-surface Grp94 on live senescent etoposide-treated IMR90 cells after 24hrs treatment with 10 or 100 μ M GPM1.

FIGs. 4A-E. Extracellular Grp94 on senescent cells mediate cytotoxicity of innate components in-vitro. (A) Western blot analysis for Grp94/ β -Tubulin in Etoposide-treated IMR90 cells 4 days after transfection with siControl or siHSP90B1 (HSP90B1 is a gene coding for Grp94). (B) PrestoBlue viability assay for Etoposide-treated IMR90 4 days after Transfection with siControl or siHSP90B1. (C) Cytotoxicity of NK92 cells in cultures of IMR90 cells treated with siControl or siHSP90B1. NK92 were added for 1 hour at a ratio of 1:3 (D) Crystal Violet staining for IMR90 cells after co-culturing with MM6 monocytes in the presence of GPM1. (E) Quantitative analysis of cell-surface Grp94 of Mouse embryonic fibroblasts (MEFs) 14 days after Etoposide treatment compared to growing MEFs.

FIGs. 5A-D illustrate that GPM1 administration prevents immune surveillance of senescent hepatic stellate cells *in vivo*. Mice treated with 12 intraperitoneal (i.p.) injections of CCl₄ (1ml/kg, twice a week) to induce liver fibrosis were subsequently subjected to 12 daily i.p. injections of Vehicle or GPM1 (30mg/kg/day), n=5. (A) IF staining on frozen sections of fibrotic livers treated with Vehicle or GPM1, for Grp94 (orange) and cell surface marker β -Catenin (green). Scale bar - 20 μ m. (B) SA- β -Gal staining (upper panel), H&E staining (middle panel), and Sirius Red staining (lower panel) of frozen sections from control liver, fibrotic liver, and fibrotic livers after 12

days of Vehicle or GPM1. Scale bar of SA- β -Gal images - 100 μ m. Scale bar of Sirius Red images - 200 μ m (C) Quantification of SA- β -Gal positive cells in the livers. Values are means + standard error of the mean (SEM). (D) Quantification of fibrosis based on Sirius Red staining. Values are means + standard error of the mean (SEM). Fibrotic area in GPM1 treated mice was compared to the one of Vehicle treated mice using Student's t test (**p < 0.01).

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of targeting senescent cells for treating and diagnosing diseases.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Cellular senescence, a stable irreversible cell-cycle arrest, prevents propagation of damaged cells in the organism. Senescent cells can be found in fibrotic or inflammatory diseases of skin, liver, lung, pancreas, prostate, as well as in articular cartilage, atherosclerotic plaques and other age-related diseases. Moreover, senescent cells were shown to accumulate in normal tissues, especially skin, with age and suggested to contribute to tissue ageing.

The immune system recognizes and eliminates senescent cells to facilitate their removal from tissues. In order to unravel the molecular mechanisms behind this process, the present inventors evaluated the cell surface proteome specific to human senescent fibroblasts. Nine proteins were identified that were expressed exclusively on the surface of senescent cells (Figure 2A). One of these proteins is glucose regulated protein 94 (Grp94), an ER chaperone which translocates to cell surface, and acts there as a potent regulator of the immune response. Cell-surface Grp94 was shown to accumulate in senescent cells in a time-dependent manner (Figure 3A). This accumulation was inhibited by GPM1, a small-molecule which specifically promote Grp94 dimerization and retention in the ER.

Whilst reducing the present invention to practice, the present inventors validated the presence of the full-size Grp94 protein on the surface of senescent cells (Figure 2D),

and evaluated its functional role in the interaction with immune cells. The present inventors showed that down-regulation of cell-surface Grp94 decreases NK-cell mediated cytotoxicity toward senescent cells. In a similar manner, GPM1 has decreased susceptibility of senescent cells for elimination by monocytes.

5 The present inventors propose that the identified surface proteins specific for senescent cells may provide a target not only for the immune system, but also for delivering specific agents to these cells for their labeling or elimination. The elimination of senescent cells might be valuable strategy to prevent cancer, treat cancer and treat variety of age-related diseases where senescent cells are present.

10 Thus, according to a first aspect of the present invention there is provided a method of targeting an agent to a senescent cell in a subject comprising administering the agent to the subject, wherein the agent is attached to an affinity moiety, the affinity moiety being capable of binding specifically to a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1, thereby targeting the agent to the senescent cell.

15 The term "senescent cells" refers to cells that exhibit cell cycle arrest, generally during the G1 transition of the cell cycle or in few cases in G2, elicited by replicative exhaustion due to telomere attrition or in response to stresses such as DNA damage, chemotherapeutic drugs, or aberrant expression of oncogenes.

20 According to a particular embodiment, the senescent cells are characterized by at least one or more of the following characteristics:

1. activation of the p53/p21CIP1 and/or pRb/p16INK4A tumor suppressor pathways;
2. cells whose proliferation is irreversibly arrested;
3. shortening of telomere size;
4. expression of senescent-associated beta-galactosidase activity;
5. Specific chromatin modification;
6. Specific secretome;
7. Increase in reactive oxygen species and altered overall mitochondrial activity.

25 Irreversible cell cycle arrest may be assessed by FACS or BrdU incorporation assay. Shortening of telomere size may be characterized by evaluating the mean terminal restriction fragment (TRF) length for example by Southern blot analysis.

Other methods of ascertaining whether a cell is senescent are described in US Patent No. 20140056860, the contents of which are incorporated herein by reference.

Agents which may be targeted to the senescent cells include but are not limited to therapeutic agents and diagnostic agents.

Exemplary therapeutic agents include nucleic acid, polypeptides e.g. antibodies, anticancer agent (e.g., chemotherapy, radioisotopes, immunotherapy), antibiotic, enzyme, antioxidant, lipid intake inhibitor, hormone, anti-inflammatory, steroid, vasodilator, angiotensin converting enzyme inhibitor, angiotensin receptor antagonist, inhibitor for smooth muscle cell growth and migration, platelet aggregation inhibitor, anticoagulant, inhibitor for release of chemical mediator, promoter or inhibitor for endothelial cell growth, aldose reductase inhibitor, inhibitor for mesangium cell growth, lipoxygenase inhibitor, immunosuppressive, immunostimulant, antiviral agent, Maillard reaction suppressor, amyloidosis inhibitor, nitric oxide synthetic inhibitor, AGEs (Advanced glycation end-products) inhibitor, radical scavenger, protein, peptide; glycosaminoglycan and derivatives thereof; and oligosaccharide, polysaccharide, and derivatives thereof.

According to a particular embodiment, the pharmaceutical agent is a cytotoxic agent.

As used herein, the term “cytotoxic agent” refers to refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., ²¹¹At, ¹³¹I, ¹²⁵I, ³²P, ³⁵S and radioactive isotopes of Lu, including ¹⁷⁷Lu, ⁸⁶Y, ⁹⁰Y, ¹¹¹In, ¹⁷⁷Lu, ²²⁵Ac, ²¹²Bi, ²¹³Bi, ⁶⁶Ga, ⁶⁷Ga, ⁶⁸Ga, ⁶⁴Cu, ⁶⁷Cu, ⁷¹As, ⁷²As, ⁷⁶As, ⁷⁷As, ⁶⁵Zn, ⁴⁸V, ²⁰³Pb, ²⁰⁹Pb, ²¹²Pb, ¹⁶⁶Ho, ¹⁴⁹Pm, ¹⁵³Sm, ²⁰¹Tl, ¹⁸⁸Re, ¹⁸⁶Re and ⁹⁹mTc), anticancer agents as otherwise described herein, including chemotherapeutic (anticancer drugs e.g. methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), taxol, doxorubicin, cisplatin, 5-fluorouridine, melphalan, ethidium bromide, mitomycin C, chlorambucil, daunorubicin and other intercalating agents, enzymes and fragments thereof such as nucleolytic enzymes, antibiotics, therapeutic RNA molecules (e.g., siRNA, antisense oligonucleotides, microRNA, ribozymes, RNA decoys, aptamers), DNAzymes, antibodies, proteins and polynucleotides encoding same, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal

origin, such as pokeweed antiviral protein (PAP), ricin toxin A, abrin, gelonin, saporin, cholera toxin A, diphtheria toxin, *Pseudomonas* exotoxin, and alpha-sarcin, including fragments and/or variants thereof.

As mentioned, the present invention contemplates the use of RNA silencing agents as pharmaceutical agents and more specifically as cytotoxic agents. The RNA silencing agents may be directed against anti-apoptotic proteins including but not limited to Bcl-xL, Bcl-w and/or p21. Other targets for RNA silencing are described in WO2013152038 and WO2014089124, the contents of which are incorporated herein by reference.

The term "Bcl-xL" refers to the human protein also known as B-cell lymphoma-extra large, having a sequence as set forth in SEQ ID NO: 1 and homologs and orthologs thereof. The cDNA sequence of human Bcl-xL is set forth in SEQ ID NO: 2.

The term "Bcl-w" refers to the human protein also known as Bcl-2-like protein 2, having a sequence as set forth in SEQ ID NO: 3 and homologs and orthologs thereof. The cDNA sequence of human Bcl-w is set forth in SEQ ID NO: 4.

The term "p21" also known as "cyclin-dependent kinase inhibitor 1" refers to the human protein having a sequence as set forth in SEQ ID NO: 5 and homologs and orthologs thereof. The cDNA sequence of human p21 is set forth in SEQ ID NO: 6.

As used herein, the phrase "RNA silencing" refers to a group of regulatory mechanisms [e.g. RNA interference (RNAi), transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), quelling, co-suppression, and translational repression] mediated by RNA molecules which result in the inhibition or "silencing" of the expression of a corresponding protein-coding gene. RNA silencing has been observed in many types of organisms, including plants, animals, and fungi.

As used herein, the term "RNA silencing agent" refers to an RNA which is capable of inhibiting or "silencing" the expression of a target gene. In certain embodiments, the RNA silencing agent is capable of preventing complete processing (e.g., the full translation and/or expression) of an mRNA molecule through a post-transcriptional silencing mechanism. RNA silencing agents include noncoding RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. Exemplary RNA silencing agents include dsRNAs such as siRNAs, miRNAs and shRNAs. In one

embodiment, the RNA silencing agent is capable of inducing RNA interference. In another embodiment, the RNA silencing agent is capable of mediating translational repression.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla. Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex.

Accordingly, the present invention contemplates use of dsRNA to downregulate protein expression from mRNA.

According to one embodiment, the dsRNA is greater than 30 bp. The use of long dsRNAs (i.e. dsRNA greater than 30 bp) has been very limited owing to the belief that these longer regions of double stranded RNA will result in the induction of the interferon and PKR response. However, the use of long dsRNAs can provide numerous advantages in that the cell can select the optimal silencing sequence alleviating the need to test numerous siRNAs; long dsRNAs will allow for silencing libraries to have less

complexity than would be necessary for siRNAs; and, perhaps most importantly, long dsRNA could prevent viral escape mutations when used as therapeutics.

Various studies demonstrate that long dsRNAs can be used to silence gene expression without inducing the stress response or causing significant off-target effects - see for example [Strat et al., Nucleic Acids Research, 2006, Vol. 34, No. 13 3803–3810; Bhargava A et al. Brain Res. Protoc. 2004;13:115–125; Diallo M., et al., Oligonucleotides. 2003;13:381–392; Paddison P.J., et al., Proc. Natl Acad. Sci. USA. 2002;99:1443–1448; Tran N., et al., FEBS Lett. 2004;573:127–134].

In particular, the present invention also contemplates introduction of long dsRNA (over 30 base transcripts) for gene silencing in cells where the interferon pathway is not activated (e.g. embryonic cells and oocytes) see for example Billy et al., PNAS 2001, Vol 98, pages 14428-14433 and Diallo et al, Oligonucleotides, October 1, 2003, 13(5): 381-392. doi:10.1089/154545703322617069.

The present invention also contemplates introduction of long dsRNA specifically designed not to induce the interferon and PKR pathways for down-regulating gene expression. For example, Shinagwa and Ishii [*Genes & Dev.* 17 (11): 1340-1345, 2003] have developed a vector, named pDECAP, to express long double-strand RNA from an RNA polymerase II (Pol II) promoter. Because the transcripts from pDECAP lack both the 5'-cap structure and the 3'-poly(A) tail that facilitate ds-RNA export to the cytoplasm, long ds-RNA from pDECAP does not induce the interferon response.

Another method of evading the interferon and PKR pathways in mammalian systems is by introduction of small inhibitory RNAs (siRNAs) either via transfection or endogenous expression.

The term "siRNA" refers to small inhibitory RNA duplexes (generally between 18-30 basepairs) that induce the RNA interference (RNAi) pathway. Typically, siRNAs are chemically synthesized as 21mers with a central 19 bp duplex region and symmetric 2-base 3'-overhangs on the termini, although it has been recently described that chemically synthesized RNA duplexes of 25-30 base length can have as much as a 100-fold increase in potency compared with 21mers at the same location. The observed increased potency obtained using longer RNAs in triggering RNAi is theorized to result

from providing Dicer with a substrate (27mer) instead of a product (21mer) and that this improves the rate or efficiency of entry of the siRNA duplex into RISC.

It has been found that position of the 3'-overhang influences potency of a siRNA and asymmetric duplexes having a 3'-overhang on the antisense strand are generally more potent than those with the 3'-overhang on the sense strand (Rose et al., 2005). This can be attributed to asymmetrical strand loading into RISC, as the opposite efficacy patterns are observed when targeting the antisense transcript.

It will be appreciated that more than one siRNA agent may be used to down-regulate a target gene. Thus, for example, the present invention contemplates use of at least two siRNAs that target Bcl-xL, at least three siRNAs that target Bcl-xL, or even at least four siRNAs that target Bcl-xL, each targeting a different sequence in the Bcl-xL gene. Further, the present invention contemplates use of at least two siRNAs that target Bcl-w, at least three siRNAs that target Bcl-w, or even at least four siRNAs that target Bcl-w, each targeting a different sequence in the Bcl-w gene. Further, the present invention contemplates use of at least two siRNAs that target p21, at least three siRNAs that target p21, or even at least four siRNAs that target p21, each targeting a different sequence in the p21 gene.

The strands of a double-stranded interfering RNA (e.g., a siRNA) may be connected to form a hairpin or stem-loop structure (e.g., a shRNA). Thus, as mentioned the RNA silencing agent of the present invention may also be a short hairpin RNA (shRNA).

The term "shRNA", as used herein, refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The number of nucleotides in the loop is a number between and including 3 to 23, or 5 to 15, or 7 to 13, or 4 to 9, or 9 to 11. Some of the nucleotides in the loop can be involved in base-pair interactions with other nucleotides in the loop. Examples of oligonucleotide sequences that can be used to form the loop include 5'-UUCAAGAGA-3' (SEQ ID NO: 7; Brummelkamp, T. R. et al. (2002) Science 296: 550) and 5'-UUUGUGUAG-3' (SEQ ID NO: 8; Castanotto, D. et al.

(2002) RNA 8:1454). It will be recognized by one of skill in the art that the resulting single chain oligonucleotide forms a stem-loop or hairpin structure comprising a double-stranded region capable of interacting with the RNAi machinery.

According to another embodiment the RNA silencing agent may be a miRNA. 5 miRNAs are small RNAs made from genes encoding primary transcripts of various sizes. They have been identified in both animals and plants. The primary transcript (termed the "pri-miRNA") is processed through various nucleolytic steps to a shorter precursor miRNA, or "pre-miRNA." The pre-miRNA is present in a folded form so that the final (mature) miRNA is present in a duplex, the two strands being referred to as the 10 miRNA (the strand that will eventually basepair with the target). The pre-miRNA is a substrate for a form of dicer that removes the miRNA duplex from the precursor, after which, similarly to siRNAs, the duplex can be taken into the RISC complex. It has been demonstrated that miRNAs can be transgenically expressed and be effective through expression of a precursor form, rather than the entire primary form (Parizotto et al. 15 (2004) Genes & Development 18:2237-2242 and Guo et al. (2005) Plant Cell 17:1376-1386).

Unlike, siRNAs, miRNAs bind to transcript sequences with only partial complementarity (Zeng et al., 2002, Molec. Cell 9:1327-1333) and repress translation without affecting steady-state RNA levels (Lee et al., 1993, Cell 75:843-854; Wightman 20 et al., 1993, Cell 75:855-862). Both miRNAs and siRNAs are processed by Dicer and associate with components of the RNA-induced silencing complex (Hutvagner et al., 2001, Science 293:834-838; Grishok et al., 2001, Cell 106: 23-34; Ketting et al., 2001, Genes Dev. 15:2654-2659; Williams et al., 2002, Proc. Natl. Acad. Sci. USA 99:6889-6894; Hammond et al., 2001, Science 293:1146-1150; Mourlatos et al., 2002, Genes 25 Dev. 16:720-728). A recent report (Hutvagner et al., 2002, Scienceexpress 297:2056-2060) hypothesizes that gene regulation through the miRNA pathway versus the siRNA pathway is determined solely by the degree of complementarity to the target transcript. It is speculated that siRNAs with only partial identity to the mRNA target will function in translational repression, similar to a miRNA, rather than triggering RNA degradation.

30 A suitable siRNA capable of downregulating Bcl-xL can be the siRNA of SEQ ID NO: 9, 10 or 11. A suitable siRNA capable of downregulating Bcl-w can be the

siRNA of SEQ ID NO: 12, 13 or 14. A suitable siRNA capable of downregulating p21 can be the siRNA of SEQ ID NO: 15, 16 or 17.

It will be appreciated that the RNA silencing agent of the present invention need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

Other cytotoxic agents contemplated by the present invention are polynucleotides that encode pro-apoptotic proteins.

Non-limiting examples of pro-apoptotic genes include caspases, Bik, Puma, Bim, Bax, Bak, Bid, Bad, Bmf, Noxa, and Hrk.

The term "caspase" refers to proteases that play essential roles in apoptosis (programmed cell death) and necrosis. At least 12 caspases have been identified in humans. There are two types of apoptotic caspases: initiator (apical) caspases and effector (executioner) caspases. Initiator caspases (e.g., CASP2 (Genbank Accession: NM001224.4), CASP8 (Genbank Accession: NM001080124.1), CASP9 (Genbank Accession: NM001229.3), and CASP10 (Genbank Accession: NM001206524.1)) cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases (e.g., CASP3 (Genbank Accession: NM004346.3), CASP6 (Genbank Accession: NM001226.3), CASP7 (Genbank Accession: NM001227.3)) in turn cleave other protein substrates within the cell, to trigger the apoptotic process. The initiation of this cascade reaction is regulated by caspase inhibitors.

Polynucleotide agents (e.g. encoding pro-apoptotic polypeptides or encoding an RNA silencing agent targeted against anti-apoptotic polypeptides) are typically administered as part of an expression construct. In this case, the polynucleotide agent is ligated in a nucleic acid construct under the control of a cis-acting regulatory element (e.g. promoter) capable of directing an expression of the cytotoxic agent in a constitutive or inducible manner. An exemplary promoter which is active in senescent cells is the p16 promoter - see for example US Patent No. 20150064137, the contents of which are incorporated herein by reference.

The nucleic acid agent may be delivered using an appropriate gene delivery vehicle/method (transfection, transduction, etc.). Optionally an appropriate expression system is used. Examples of suitable constructs include, but are not limited to, pcDNA3,

pcDNA3.1 (+/-), pGL3, PzeoSV2 (+/-), pDisplay, pEF/myc/cyto, pCMV/myc/cyto each of which is commercially available from Invitrogen Co. (www.invitrogen.com).

The expression construct may also be a virus. Examples of viral constructs include but are not limited to adenoviral vectors, retroviral vectors, vaccinia viral vectors, adeno-associated viral vectors, polyoma viral vectors, alphaviral vectors, rhabdoviral vectors, lenti viral vectors and herpesviral vectors.

A viral construct such as a retroviral construct includes at least one transcriptional promoter/enhancer or locus-defining element(s), or other elements that control gene expression by other means such as alternate splicing, nuclear RNA export, or post-transcriptional modification of messenger. Such vector constructs also include a packaging signal, long terminal repeats (LTRs) or portions thereof, and positive and negative strand primer binding sites appropriate to the virus used, unless it is already present in the viral construct. In addition, such a construct typically includes a signal sequence for secretion of the peptide from a host cell in which it is placed. Preferably, the signal sequence for this purpose is a mammalian signal sequence or the signal sequence of the peptide variants of the present invention. Optionally, the construct may also include a signal that directs polyadenylation, as well as one or more restriction site and a translation termination sequence. By way of example, such constructs will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second-strand DNA synthesis, and a 3' LTR or a portion thereof.

Preferably the viral dose for infection is at least 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , 10^{12} , 10^{13} , 10^{14} , 10^{15} or higher pfu or viral particles.

Double stranded RNA may be synthesized by adding two opposing promoters to the ends of the gene segments, wherein one promoter is placed immediately 5' to the gene and the opposing promoter is placed immediately 3' to the gene segment. The dsRNA may then be transcribed with the appropriate polymerase.

Other cytotoxic agents known to down-regulate anti-apoptotic proteins are also contemplated by the present inventors – these include for example ABT-737, ABT-263, Gossypol, AT-101, TW-37 and Obatoclax.

According to another embodiment, the pharmaceutical agent is a diagnostic agent.

Exemplary diagnostic drugs include in vivo diagnostics such as an X ray contrast medium, a diagnostic agent for ultrasound, an isotope-labeled agent for diagnosis by nuclear medicine, and an agent for diagnosis by nuclear magnetic resonance.

As mentioned, the pharmaceutical agents of this aspect of the present invention are attached either directly or indirectly to an affinity moiety.

The affinity moiety may comprise a chemical (non-peptide) molecule, an aptamer, a peptide or an antibody (e.g. antibody-derived epitope binding domain) which is capable of specifically binding to one of the following proteins:

HSP90B1, also referred to herein as Grp94, Refseq no: NM_003299.2, (SEQ ID NO: 18), DNAJB4 Refseq no: NM_007034.3 (SEQ ID NO: 19), PI4K2A Refseq no: NM_018425.3 (SEQ ID NO: 20), DBN1 Refseq no: NM_004395.3 or NM_080881.2 (SEQ ID NOs: 21), PRKCSH NM_001001329.2 or NM_001289102.1 (SEQ ID NO: 22), SPTBN1 Refseq no: NM_178313.2 (SEQ ID NO: 23), NPM1 Refseq no: NM_001037738.2 (SEQ ID NO: 24), ITGA3 NM_002204.2 (SEQ ID NO: 25) and any of the polypeptides listed in Table 1 herein below.

For any of the aspects of the present invention, the polypeptide may be selected from the group consisting of HSP90B1, also referred to herein as Grp94, Refseq no: NM_003299.2, (SEQ ID NO: 18), DNAJB4 Refseq no: NM_007034.3 (SEQ ID NO: 19), PI4K2A Refseq no: NM_018425.3 (SEQ ID NO: 20), DBN1 Refseq no: NM_004395.3 or NM_080881.2 (SEQ ID NOs: 21), PRKCSH NM_001001329.2 or NM_001289102.1 (SEQ ID NO: 22), SPTBN1 Refseq no: NM_178313.2 (SEQ ID NO: 23), NPM1 Refseq no: NM_001037738.2 (SEQ ID NO: 24), ITGA3 NM_002204.2 (SEQ ID NO: 25).

For any of the aspects of the present invention, the polypeptide may be selected from the group consisting of HSP90B1, also referred to herein as Grp94, Refseq no: NM_003299.2, (SEQ ID NO: 18), DBN1 Refseq no: NM_004395.3 or NM_080881.2 (SEQ ID NOs: 21), PRKCSH NM_001001329.2 or NM_001289102.1 (SEQ ID NO: 22), SPTBN1 Refseq no: NM_178313.2 (SEQ ID NO: 23) and NPM1 Refseq no: NM_001037738.2 (SEQ ID NO: 24).

For any of the aspects of the present invention, the polypeptide may be HSP90B1, also referred to herein as Grp94, Refseq no: NM_003299.2, (SEQ ID NO: 18).

In one embodiment binding or specifically binding means a binding affinity 5 (KD) of 10^{-8} mol/l or less, preferably 10^{-9} M to 10^{-13} mol/l.

In one embodiment, the affinity moiety binds to one of the above proteins with at least 5 fold higher affinity, 10 fold higher affinity or even 20 fold higher affinity than to a non-related protein.

The term "antibody" as used in this invention includes intact molecules as well 10 as functional fragments thereof.

As used herein, the phrase "antibody fragment" refers to a functional fragment of an antibody (such as Fab, F(ab')2, Fv, scFv, dsFv, or single domain molecules such as VH and VL) that is capable of binding to an epitope of an antigen.

Suitable antibody fragments for practicing some embodiments of the invention 15 include a complementarity-determining region (CDR) of an immunoglobulin light chain (referred to herein as "light chain"), a complementarity-determining region of an immunoglobulin heavy chain (referred to herein as "heavy chain"), a variable region of a light chain, a variable region of a heavy chain, a light chain, a heavy chain, an Fd fragment, and antibody fragments comprising essentially whole variable regions of both 20 light and heavy chains such as a Fv, a single chain Fv (scFv), a disulfide-stabilized Fv (dsFv), an Fab, an Fab', and an F(ab')2.

Functional antibody fragments comprising whole or essentially whole variable regions of both light and heavy chains are defined as follows:

(i) Fv, defined as a genetically engineered fragment consisting of the variable 25 region of the light chain (VL) and the variable region of the heavy chain (VH) expressed as two chains;

(ii) single chain Fv ("scFv"), a genetically engineered single chain molecule including the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain 30 molecule.

(iii) disulfide-stabilized Fv (“dsFv”), a genetically engineered antibody including the variable region of the light chain and the variable region of the heavy chain, linked by a genetically engineered disulfide bond.

5 (iv) Fab, a fragment of an antibody molecule containing a monovalent antigen-binding portion of an antibody molecule which can be obtained by treating whole antibody with the enzyme papain to yield the intact light chain and the Fd fragment of the heavy chain which consists of the variable and CH1 domains thereof;

10 (v) Fab’, a fragment of an antibody molecule containing a monovalent antigen-binding portion of an antibody molecule which can be obtained by treating whole antibody with the enzyme pepsin, followed by reduction (two Fab’ fragments are obtained per antibody molecule);

15 (vi) F(ab’)2, a fragment of an antibody molecule containing a monovalent antigen-binding portion of an antibody molecule which can be obtained by treating whole antibody with the enzyme pepsin (i.e., a dimer of Fab’ fragments held together by two disulfide bonds); and

(vii) Single domain antibodies are composed of a single VH or VL domains which exhibit sufficient affinity to the antigen.

Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art (See for example, Harlow and Lane, 20 Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

Antibody fragments according to some embodiments of the invention can be prepared by proteolytic hydrolysis of the antibody or by expression in E. coli or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression 25 systems) of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab’)2. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulphydryl groups resulting 30 from cleavage of disulfide linkages, to produce 3.5S Fab’ monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab’ fragments and an Fc fragment directly. These methods are described, for example, by

Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R. [Biochem. J. 73: 119-126 (1959)]. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar et al. [Proc. Nat'l Acad. Sci. USA 69:2659-62 (19720]. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by [Whitlow and Filpula, Methods 2: 97-105 (1991); Bird et al., Science 242:423-426 (1988); Pack et al., Bio/Technology 11:1271-77 (1993); and U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry [Methods, 2: 106-10 (1991)].

Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab').sub.2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form

a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature* 332:323-327 (1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies

(Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., Bio/Technology 10,: 779-783 (1992); Lonberg et al., Nature 368: 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild et al., Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14: 826 (1996); and Lonberg and Huszar, Intern. Rev. Immunol. 13, 65-93 (1995).

Antibodies directed against any of the proteins HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and those listed in Table 1 are commercially available from Pierce (e.g. anti-Grp94; PA5-24824).

As mentioned, the affinity moiety of this aspect of the present invention may also be an aptamer.

As used herein, the term "aptamer" refers to a nucleic acid that specifically binds to a target, such as a protein, through interactions other than Watson-Crick base pairing. In a particular embodiment, the aptamer specifically binds to one or more targets (e.g., a protein or protein complex) to the general exclusion of other molecules in a sample. The aptamer may be a nucleic acid such as an RNA, a DNA, a modified nucleic acid, or a mixture thereof. The aptamer may also be a nucleic acid in a linear or circular form and may be single stranded or double stranded. The aptamer may comprise oligonucleotides that are at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40 or more nucleotides in length. Aptamers may comprise sequences that are up to 40, up to 60, up to 80, up to 100, up to 150, up to 200 or more nucleotides in length. Aptamers may be from about 5 to about 150 nucleotides, from about 10 to about 100 nucleotides, or from about 20 to about 75 nucleotides in length. While aptamers are discussed herein as nucleic acid molecules (e.g., oligonucleotides) aptamers, aptamer equivalents may also be used in place of the nucleic acid aptamers, such as peptide aptamers.

According to one embodiment, the pharmaceutical agent and the affinity moiety (e.g. antibody or aptamer) are attached directly to one another.

Thus according to another aspect of the present invention there is provided a composition of matter (e.g. complex) comprising an affinity moiety attached to a therapeutic agent, wherein the affinity moiety specifically binds to a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and any of the polypeptides listed in Table 1, herein below.

Affinity moieties have been described herein above.

The pharmaceutical agent of the invention may be attached or conjugated to the affinity moiety of the invention in various ways, depending on the context, application and purpose.

When both the pharmaceutical agent and the affinity moiety are polypeptides, the conjugate may be produced by recombinant means. For example, the nucleic acid sequence encoding a toxin (e.g., PE38KDEL) or a fluorescent protein [e.g., green fluorescent protein (GFP), red fluorescent protein (RFP) or yellow fluorescent protein (YFP)] may be ligated in-frame with the nucleic acid sequence encoding an antibody of the invention and be expressed in a host cell to produce a recombinant conjugated antibody. Alternatively, at least one of the affinity moiety or pharmaceutical agent may be chemically synthesized by, for example, the stepwise addition of one or more amino acid residues in defined order such as solid phase peptide synthetic techniques.

A pharmaceutical agent may also be attached to the affinity moiety of the invention using standard chemical synthesis techniques widely practiced in the art [see e.g., [hypertexttransferprotocol://worldwideweb.chemistry.org/portal/Chemistry](http://worldwideweb.chemistry.org/portal/Chemistry)], such as using any suitable chemical linkage, direct or indirect, as via a peptide bond (when the functional moiety is a polypeptide), or via covalent bonding to an intervening linker element, such as a linker peptide or other chemical moiety, such as an organic polymer. Chimeric peptides may be linked via bonding at the carboxy (C) or amino (N) termini of the peptides, or via bonding to internal chemical groups such as straight, branched or cyclic side chains, internal carbon or nitrogen atoms, and the like.

Exemplary methods for conjugating peptide pharmaceutical agent to polypeptide affinity moieties (e.g. antibodies) are described herein below:

5 **SPDP conjugation** – A non-limiting example of a method of SPDP conjugation is described in Cumber et al. (1985, Methods of Enzymology 112: 207-224). Briefly, a peptide, such as a detectable or therapeutic moiety (e.g., 1.7 mg/ml) is mixed with a 10-fold excess of SPDP (50 mM in ethanol); the antibody is mixed with a 25-fold excess of
10 SPDP in 20 mM sodium phosphate, 0.10 M NaCl pH 7.2 and each of the reactions is incubated for about 3 hours at room temperature. The reactions are then dialyzed against PBS. The peptide is reduced, e.g., with 50 mM DTT for 1 hour at room temperature. The reduced peptide is desalted by equilibration on G-25 column (up to 5 % sample/column volume) with 50 mM KH₂PO₄ pH 6.5. The reduced peptide is combined with the SPDP-antibody in a molar ratio of 1:10 antibody:peptide and
15 incubated at 4 °C overnight to form a peptide-antibody conjugate.

15 **Glutaraldehyde conjugation** - A non-limiting example of a method of glutaraldehyde conjugation is described in G.T. Hermanson (1996, "Antibody Modification and Conjugation, in Bioconjugate Techniques, Academic Press, San Diego). Briefly, the antibody and the peptide (1.1 mg/ml) are mixed at a 10-fold excess with 0.05 % glutaraldehyde in 0.1 M phosphate, 0.15 M NaCl pH 6.8, and allowed to react for 2 hours at room temperature. 0.01 M lysine can be added to block excess sites. After-the reaction, the excess glutaraldehyde is removed using a G-25 column equilibrated with PBS (10 % v/v sample/column volumes).

20 **Carbodiimide conjugation** - Conjugation of a peptide with an antibody can be accomplished using a dehydrating agent such as a carbodiimide, e.g., in the presence of 4-dimethyl aminopyridine. Carbodiimide conjugation can be used to form a covalent bond between a carboxyl group of peptide and an hydroxyl group of an antibody (resulting in the formation of an ester bond), or an amino group of an antibody (resulting in the formation of an amide bond) or a sulphydryl group of an antibody (resulting in the formation of a thioester bond). Likewise, carbodiimide coupling can be used to form analogous covalent bonds between a carbon group of an antibody and an hydroxyl, amino or sulphydryl group of the peptide [see, J. March, Advanced Organic Chemistry: Reaction's, Mechanism, and Structure, pp. 349-50 & 372-74 (3d ed.), 1985].
25 For example, the peptide can be conjugated to an antibody via a covalent bond using a carbodiimide, such as dicyclohexylcarbodiimide [B. Neises et al. (1978), Angew
30 Chem. Int. Ed. Engl. 17: 111-113].

Chem., Int. Ed. Engl. 17:522; A. Hassner et al. (1978, Tetrahedron Lett. 4475); E.P. Boden et al. (1986, J. Org. Chem. 50:2394) and L.J. Mathias (1979, Synthesis 561)].

When both the pharmaceutical agent and the affinity moiety are antibodies, the present invention contemplates generation of bispecific antibodies wherein each arm of
5 the antibody recognizes a different antigen.

It will be appreciated that the affinity moiety and the pharmaceutical agent of this aspect of the present invention may be attached indirectly - e.g. via a particle, wherein the pharmaceutical agent is inside the particle or on the outer surface thereof and the affinity moiety is on the outer surface of the particle.

10 Thus, according to another aspect of the present invention there is provided a particle having a senescent cell affinity moiety attached to an outer surface thereof, the senescent cell affinity moiety capable of specifically binding a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and any of the polypeptides listed in Table 1, herein below.

15 Senescent cell affinity moieties have been described herein above.

As used herein, "particles" refers to nano - micro structures which are not biological cells.

20 The particle may be a synthetic carrier, gel or other object or material having an external surface which is capable of being loadable with (e.g., encapsulating) a pharmaceutical agent. The particle may be either polymeric or non-polymeric preparations.

Exemplary particles that may be used according to this aspect of the present invention include, but are not limited to polymeric particles, microcapsules, liposomes, microspheres, microemulsions, nanoparticles, nanocapsules, nano-spheres, nano-liposomes, nano-emulsions and nanotubes.
25

According to a particular embodiment, the particles are nanoparticles.

As used herein, the term "nanoparticle" refers to a particle or particles having an intermediate size between individual atoms and macroscopic bulk solids. Generally, nanoparticle has a characteristic size (e.g., diameter for generally spherical nanoparticles, or length for generally elongated nanoparticles) in the sub-micrometer range, e.g., from about 1 nm to about 500 nm, or from about 1 nm to about 200 nm, or of the order of 10 nm, e.g., from about 1 nm to about 100 nm. The nanoparticles may

be of any shape, including, without limitation, elongated particle shapes, such as nanowires, or irregular shapes, in addition to more regular shapes, such as generally spherical, hexagonal and cubic nanoparticles. According to one embodiment, the nanoparticles are generally spherical.

5 The particles of this aspect of the present invention may have a charged surface (i.e., positively charged or negatively charged) or a neutral surface.

Agents which are used to fabricate the particles may be selected according to the desired charge required on the outer surface of the particles.

Thus, for example if a negatively charged surface is desired, the particles may
10 be fabricated from negatively charged lipids (i.e. anionic phospholipids) such as described herein below.

When a positively charged surface is desired, the particles may be fabricated from positively charged lipids (i.e. cationic phospholipids), such as described herein below.

15 As mentioned, non charged particles are also contemplated by the present invention. Such particles may be fabricated from neutral lipids such as phosphatidylethanolamine or dioleoylphosphatidylethanolamine (DOPE).

It will be appreciated that combinations of different lipids may be used to fabricate the particles of the present invention, including a mixture of more than one cationic lipid, a mixture of more than one anionic lipid, a mixture of more than one neutral lipid, a mixture of at least one cationic lipid and at least one anionic lipid, a mixture of at least one cationic lipid and at least one neutral lipid, a mixture of at least one anionic lipid and at least one neutral lipid and additional combinations of the above. In addition, polymer-lipid based formulations may be used.

25 There are numerous polymers which may be attached to lipids. Polymers typically used as lipid modifiers include, without being limited thereto: polyethylene glycol (PEG), polysialic acid, polylactic (also termed polylactide), polyglycolic acid (also termed polyglycolide), poly-(lactic-co-glycolic)poly-(vinyl-alcohol), polyvinylpyrrolidone, polyethyloxazoline, polyhydroxyethylloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide, polydimethylacrylamide, polyvinylmethylether, polyhydroxyethyl acrylate, derivatized celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

The polymers may be employed as homopolymers or as block or random copolymers.

The particles may also include other components. Examples of such other components includes, without being limited thereto, fatty alcohols, fatty acids, and/or cholesterol esters or any other pharmaceutically acceptable excipients which may affect the surface charge, the membrane fluidity and assist in the incorporation of the biologically active lipid into the lipid assembly. Examples of sterols include cholesterol, cholesterol hemisuccinate, cholesterol sulfate, or any other derivatives of cholesterol. Preferred lipid assemblies according the invention include either those which form a micelle (typically when the assembly is absent from a lipid matrix) or those which form a liposome (typically, when a lipid matrix is present).

The particles of the present invention may be modified. According modified to enhance their circulatory half-life (e.g. by PEGylation) to reduce their clearance, to prolong their scavenging time-frame and to allow antibody binding. The PEG which is incorporated into the articles may be characterized by of any of various combinations of chemical composition and/or molecular weight, depending on the application and purpose.

Methods of coupling affinity moieties (e.g. antibodies) on particle's outer surface (e.g., liposomes) are known in the art.

As used herein "coupling" or "coupled on" refers to covalent or non-covalent attachment of the affinity moiety to the particle.

Antibody conjugation methods which can be used in accordance with the teachings of the present invention can be divided to direct binding or indirect binding. Some methods are provided hereinbelow and are summarized in Ansell, Supra. While specifically referring to liposomes, the procedures described hereinbelow may be applied to a variety of particles, while using modified protocols simply applied by the ordinary artisan.

Direct conjugation methods are well known to those of skill in the art. See for example, G. Gregoriadis, (1984) "Liposome Technology" CRC Press, Boca Raton, Fla. and D. D. Lasic, "Liposomes: from physics to applications" (1993) Elsevier, Amsterdam; N.Y. Particularly preferred is conjugation through a thioether linkage. This may be accomplished by reacting the antibody with a maleimide derivatized lipid such

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as maleimide derivatized phosphatidylethanolamine (M-PE) or dipalmitoylethanolamine (M-DEP). This approach is described in detail by Martin et al. J. Biol. Chem., 257: 286-288 (1982) which is incorporated herein by reference.

In another preferred embodiment, the antibody can be coupled to a hydrophilic 5 polymer (e.g., a PEG). Means of attaching targeting molecules to polymer linkers are well known to those of skill in the art (see, e.g., chapter 4 in Monoclonal Antibodies: Principles and Applications, Birch and Lennox, eds., John Wiley & Sons, Inc., New York (1995); and Blume et al. Biochem. Biophys. Acta. 1149: 180-184 (1993). In a particularly preferred embodiment, an antibody or a fragment thereof (e.g., Fab' 10 fragment) is linked to a maleimide derivatized PEG through the --SII group of the antibody. The maleimide-derivative of PEG-PE is included in the liposome preparation as described above and below and the antibody can be conjugated with the liposome via the sulphydryl group at pH 7.2.

Amine modifications making use of cross-linking agents such as EDC are taught 15 in Endoh et al. 1981 J. Immun. Meth. 44:79-85; Dunnick 1975 J. Nuclear. Med. 16:483-487; Alternatively, direct modification of antibodies with activated fatty acids, such as N-hydroxysuccinimide (NHS) ester or palmitic acid, prior to incorporation into a liposome membrane, typically by detergent dialysis procedures (Huang et al. 1980, J. Biol. Chem. 255:8015-8018. Reagents, such as EDC, are used in conjunction with 20 NHS to activate acidic functions on liposomes, which are then conjugated to the amino groups on antibodies. Better control of the conjugation reaction can be achieved using heterobifunctional cross-linkers which efficiently introduce a unique and selective reactive function, such as a protected thiol or maleimide group. Examples of these crosslinkers are SPDP (Barbet et al. 1981 J. Supramolec. Struct. Cell. Biochem. 16:243-258), S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA, Jones 1993 Biochim. Biophys. Acta. 1152:23:1-32; Schwendener 1990 Biochim. Biophys. Acta. 1026:69-79 and 4-(p-maleimidophenyl)butyric acid N-hydroxysuccinimide ester(SMPB (Hansen 1995 Biochim. Biophys. Acta. 1239:133-144). Antibodies which have been 25 activated by these crosslinkers can, after deprotection where appropriate, react with activated lip- ids in liposome bilayers. Maleimide and protected thiol-derivatized lipids 30 are available from commercial sources for this purpose.

Deprotection of 3-pyridyl disulfides is usually effected by DTT and occasionally by some other mercaptan. Once deprotected, sulfhydryl groups can react with maleimide (for example SMPB-modified conjugates) or iodo (for example, iodoacetic acid N-hydroxysuccinimide ester (SIAA)-modified conjugates) groups. Maleimide groups are recommended since iodo functions can react with amino groups in either of the substrates, leading to undesirable side products. Deprotection is not required for these reagents.

5 Indirect conjugation methods:

Biotin-avidin - For example, a biotin conjugated antibody may be bound to a particle (e.g., liposome) containing a streptavidin. Alternatively, the biotinylated antibody may be conjugated to a biotin derivatized liposome by an avidin or streptavidin linker. Ahmad et al., Cancer Res., 52: 4817-4820 (1992) which is herein incorporated by reference, describes such a mode of coupling. When monovalent Fab molecules are used, typically about 30 to 125 and more typically about 50 to 100 Fab' molecules per liposome are used.

10 Binding via protein A/G/L-liposome conjugates targeted to the Fc chain of antibodies is taught in Matthay et al. 1986 Cancer Res. 46:4904-4910; Machy et al. 1983 Biochem. Biophys. Acta. 901:157-160.

15 Loading of the particle with the pharmaceutical agent can be effected concomitant with, or following particle assembly.

20 Thus, in one preferred embodiment, for example, when the pharmaceutical agent is a nucleic acid, e.g., DNA, RNA, siRNA, plasmid DNA, short-hairpin RNA, small temporal RNA (stRNA), microRNA (miRNA), RNA mimetics, or heterochromatic siRNA, the nucleic acid agent of interest has a charged backbone that prevents efficient encapsulation in the lipid particle. Accordingly, the nucleic acid agent of interest may be condensed with a cationic polymer, e.g., PEI, polyamine spermidine, and spermine, or cationic peptide, e.g., protamine and polylysine, prior to encapsulation in the lipid particle. In one embodiment, the agent is not condensed with a cationic polymer.

25 In another embodiment, the agent of interest is encapsulated in the lipid particle in the following manner. The particle is provided lyophilized. The agent of interest is in an aqueous solution. The agent of interest in aqueous solution is utilized to rehydrate the

lyophilized lipid particle. Thus, the agent of interest is encapsulated in the rehydrated lipid particle.

In one embodiment, two agents of interest may be delivered by the particles (e.g., lipid based particle). One agent is hydrophobic and the other is hydrophilic. The 5 hydrophobic agent may be added to the lipid particle during formation of the lipid particle. The hydrophobic agent associates with the lipid portion of the lipid particle. The hydrophilic agent is added in the aqueous solution rehydrating the lyophilized lipid particle. In an exemplary embodiment of two agent delivery a condensed siRNA is 10 encapsulated in a liposome and wherein a drug that is poorly soluble in aqueous solution is associated with the lipid portion of the lipid particle. As used herein, "poorly soluble in aqueous solution" refers to a composition that is less than 10% soluble in water.

As used herein "loading" refers to encapsulating or absorbing.

The term "encapsulated" as used herein refers to the pharmaceutical agent being distributed in the interior portion of the particles. Preferably, the pharmaceutical agents 15 are homogenously distributed. Homogeneous distribution of a pharmaceutical agent in polymer particles is known as a matrix encapsulation. However, due to the manufacturing process it is foreseen that minor amounts of the pharmaceutical agent may also be present on the outside of the particle and/or mixed with the polymer making up the shell of the particle.

20 As used herein "absorbed" refers to binding of the pharmaceutical agent to the outer surface of the particle.

Since pharmaceutical agents described herein (e.g. cytotoxic agents) are attached to affinity moieties which target senescent cells, the present inventors propose that these complexes may be used to treat subjects having diseases associated with cell 25 senescence.

As used herein, the term "subject" refers to a mammalian subject, preferably a human.

A number of diseases and conditions, which involve an inflammatory response can be treated using the methodology described hereinabove. Examples of such 30 diseases and conditions are summarized infra.

Inflammatory diseases - Include, but are not limited to, chronic inflammatory diseases and acute inflammatory diseases.

Inflammatory diseases associated with hypersensitivity

Examples of hypersensitivity include, but are not limited to, Type I hypersensitivity, Type II hypersensitivity, Type III hypersensitivity, Type IV hypersensitivity, immediate hypersensitivity, antibody mediated hypersensitivity, 5 immune complex mediated hypersensitivity, T lymphocyte mediated hypersensitivity and DTH.

Type I or immediate hypersensitivity, such as asthma.

Type II hypersensitivity include, but are not limited to, rheumatoid diseases, rheumatoid autoimmune diseases, rheumatoid arthritis (Krenn V. *et al.*, Histol Histopathol 2000 Jul;15 (3):791), spondylitis, ankylosing spondylitis (Jan Voswinkel *et al.*, Arthritis Res 2001; 3 (3): 189), systemic diseases, systemic autoimmune diseases, systemic lupus erythematosus (Erikson J. *et al.*, Immunol Res 1998;17 (1-2):49), sclerosis, systemic sclerosis (Renaudineau Y. *et al.*, Clin Diagn Lab Immunol. 1999 Mar;6 (2):156); Chan OT. *et al.*, Immunol Rev 1999 Jun;169:107), glandular diseases,

15 glandular autoimmune diseases, pancreatic autoimmune diseases, diabetes, Type I diabetes (Zimmet P. Diabetes Res Clin Pract 1996 Oct;34 Suppl:S125), thyroid diseases, autoimmune thyroid diseases, Graves' disease (Orgiazzi J. Endocrinol Metab Clin North Am 2000 Jun;29 (2):339), thyroiditis, spontaneous autoimmune thyroiditis (Braley-Mullen H. and Yu S, J Immunol 2000 Dec 15;165 (12):7262), Hashimoto's

20 thyroiditis (Toyoda N. *et al.*, Nippon Rinsho 1999 Aug;57 (8):1810), myxedema, idiopathic myxedema (Mitsuma T. Nippon Rinsho. 1999 Aug;57 (8):1759); autoimmune reproductive diseases, ovarian diseases, ovarian autoimmunity (Garza KM. *et al.*, J Reprod Immunol 1998 Feb;37 (2):87), autoimmune anti-sperm infertility (Diekman AB. *et al.*, Am J Reprod Immunol. 2000 Mar;43 (3):134), repeated fetal loss

25 (Tincani A. *et al.*, Lupus 1998;7 Suppl 2:S107-9), neurodegenerative diseases, neurological diseases, neurological autoimmune diseases, multiple sclerosis (Cross AH. *et al.*, J Neuroimmunol 2001 Jan 1;112 (1-2):1), Alzheimer's disease (Oron L. *et al.*, J Neural Transm Suppl. 1997;49:77), myasthenia gravis (Infante AJ. And Kraig E, Int Rev Immunol 1999;18 (1-2):83), motor neuropathies (Kornberg AJ. J Clin Neurosci.

30 2000 May;7 (3):191), Guillain-Barre syndrome, neuropathies and autoimmune neuropathies (Kusunoki S. Am J Med Sci. 2000 Apr;319 (4):234), myasthenic diseases, Lambert-Eaton myasthenic syndrome (Takamori M. Am J Med Sci. 2000 Apr;319

(4):204), paraneoplastic neurological diseases, cerebellar atrophy, paraneoplastic cerebellar atrophy, non-paraneoplastic stiff man syndrome, cerebellar atrophies, progressive cerebellar atrophies, encephalitis, Rasmussen's encephalitis, amyotrophic lateral sclerosis, Sydenham chorea, Gilles de la Tourette syndrome,
5 polyendocrinopathies, autoimmune polyendocrinopathies (Antoine JC. and Honnorat J. Rev Neurol (Paris) 2000 Jan;156 (1):23); neuropathies, dysimmune neuropathies (Nobile-Orazio E. et al., Electroencephalogr Clin Neurophysiol Suppl 1999;50:419); neuromyotonia, acquired neuromyotonia, arthrogryposis multiplex congenita (Vincent A. et al., Ann N Y Acad Sci. 1998 May 13;841:482), cardiovascular diseases,
10 cardiovascular autoimmune diseases, atherosclerosis (Matsuura E. et al., Lupus. 1998;7 Suppl 2:S135), myocardial infarction (Vaarala O. Lupus. 1998;7 Suppl 2:S132), thrombosis (Tincani A. et al., Lupus 1998;7 Suppl 2:S107-9), granulomatosis, Wegener's granulomatosis, arteritis, Takayasu's arteritis and Kawasaki syndrome (Praprotnik S. et al., Wien Klin Wochenschr 2000 Aug 25;112 (15-16):660); anti-factor
15 VIII autoimmune disease (Lacroix-Desmazes S. et al., Semin Thromb Hemost.2000;26 (2):157); vasculitises, necrotizing small vessel vasculitises, microscopic polyangiitis, Churg and Strauss syndrome, glomerulonephritis, pauci-immune focal necrotizing glomerulonephritis, crescentic glomerulonephritis (Noel LH. Ann Med Interne (Paris).
2000 May;151 (3):178); antiphospholipid syndrome (Flamholz R. et al., J Clin Apheresis 1999;14 (4):171); heart failure, agonist-like beta-adrenoceptor antibodies in heart failure (Wallukat G. et al., Am J Cardiol. 1999 Jun 17;83 (12A):75H), thrombocytopenic purpura (Moccia F. Ann Ital Med Int. 1999 Apr-Jun;14 (2):114); hemolytic anemia, autoimmune hemolytic anemia (Efremov DG. et al., Leuk Lymphoma 1998 Jan;28 (3-4):285), gastrointestinal diseases, autoimmune diseases of
25 the gastrointestinal tract, intestinal diseases, chronic inflammatory intestinal disease (Garcia Herola A. et al., Gastroenterol Hepatol. 2000 Jan;23 (1):16), celiac disease (Landau YE. and Shoenfeld Y. Harefuah 2000 Jan 16;138 (2):122), autoimmune diseases of the musculature, myositis, autoimmune myositis, Sjogren's syndrome (Feist E. et al., Int Arch Allergy Immunol 2000 Sep;123 (1):92); smooth muscle autoimmune disease (Zauli D. et al., Biomed Pharmacother 1999 Jun;53 (5-6):234), hepatic diseases, hepatic autoimmune diseases, autoimmune hepatitis (Manns MP. J Hepatol 2000
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Aug;33 (2):326) and primary biliary cirrhosis (Strassburg CP. *et al.*, Eur J Gastroenterol Hepatol. 1999 Jun;11 (6):595).

Type IV or T cell mediated hypersensitivity, include, but are not limited to, rheumatoid diseases, rheumatoid arthritis (Tisch R, McDevitt HO. Proc Natl Acad Sci U S A 1994 Jan 18;91 (2):437), systemic diseases, systemic autoimmune diseases,

5 systemic lupus erythematosus (Datta SK., Lupus 1998;7 (9):591), glandular diseases, glandular autoimmune diseases, pancreatic diseases, pancreatic autoimmune diseases, Type 1 diabetes (Castano L. and Eisenbarth GS. Ann. Rev. Immunol. 8:647); thyroid

diseases, autoimmune thyroid diseases, Graves' disease (Sakata S. *et al.*, Mol Cell Endocrinol 1993 Mar;92 (1):77); ovarian diseases (Garza KM. *et al.*, J Reprod Immunol 1998 Feb;37 (2):87), prostatitis, autoimmune prostatitis (Alexander RB. *et al.*, Urology 1997 Dec;50 (6):893), polyglandular syndrome, autoimmune polyglandular syndrome,

10 Type I autoimmune polyglandular syndrome (Hara T. *et al.*, Blood. 1991 Mar 1;77 (5):1127), neurological diseases, autoimmune neurological diseases, multiple sclerosis, neuritis, optic neuritis (Soderstrom M. *et al.*, J Neurol Neurosurg Psychiatry 1994

15 May;57 (5):544), myasthenia gravis (Oshima M. *et al.*, Eur J Immunol 1990 Dec;20 (12):2563), stiff-man syndrome (Hiemstra HS. *et al.*, Proc Natl Acad Sci U S A 2001 Mar 27;98 (7):3988), cardiovascular diseases, cardiac autoimmunity in Chagas' disease

(Cunha-Neto E. *et al.*, J Clin Invest 1996 Oct 15;98 (8):1709), autoimmune thombocytopenic purpura (Semple JW. *et al.*, Blood 1996 May 15;87 (10):4245), anti-

20 helper T lymphocyte autoimmunity (Caporossi AP. *et al.*, Viral Immunol 1998;11 (1):9), hemolytic anemia (Sallah S. *et al.*, Ann Hematol 1997 Mar;74 (3):139), hepatic diseases, hepatic autoimmune diseases, hepatitis, chronic active hepatitis (Franco A. *et al.*, Clin Immunol Immunopathol 1990 Mar;54 (3):382), biliary cirrhosis, primary

25 biliary cirrhosis (Jones DE. Clin Sci (Colch) 1996 Nov;91 (5):551), nephric diseases, nephric autoimmune diseases, nephritis, interstitial nephritis (Kelly CJ. J Am Soc Nephrol 1990 Aug;1 (2):140), connective tissue diseases, ear diseases, autoimmune

connective tissue diseases, autoimmune ear disease (Yoo TJ. *et al.*, Cell Immunol 1994 Aug;157 (1):249), disease of the inner ear (Glodde B. *et al.*, Ann N Y Acad Sci 1997

30 Dec 29;830:266), skin diseases, cutaneous diseases, dermal diseases, bullous skin diseases, pemphigus vulgaris, bullous pemphigoid and pemphigus foliaceus.

Examples of delayed type hypersensitivity include, but are not limited to, contact dermatitis and drug eruption.

Examples of types of T lymphocyte mediating hypersensitivity include, but are not limited to, helper T lymphocytes and cytotoxic T lymphocytes.

5 Examples of helper T lymphocyte-mediated hypersensitivity include, but are not limited to, $T_{h}1$ lymphocyte mediated hypersensitivity and $T_{h}2$ lymphocyte mediated hypersensitivity.

Autoimmune diseases

Include, but are not limited to, cardiovascular diseases, rheumatoid diseases, 10 glandular diseases, gastrointestinal diseases, cutaneous diseases, hepatic diseases, neurological diseases, muscular diseases, nephric diseases, diseases related to reproduction, connective tissue diseases and systemic diseases.

Examples of autoimmune cardiovascular diseases include, but are not limited to atherosclerosis (Matsuura E. *et al.*, Lupus. 1998;7 Suppl 2:S135), myocardial infarction 15 (Vaarala O. Lupus. 1998;7 Suppl 2:S132), thrombosis (Tincani A. *et al.*, Lupus 1998;7 Suppl 2:S107-9), Wegener's granulomatosis, Takayasu's arteritis, Kawasaki syndrome (Praprotnik S. *et al.*, Wien Klin Wochenschr 2000 Aug 25;112 (15-16):660), anti-factor VIII autoimmune disease (Lacroix-Desmazes S. *et al.*, Semin Thromb Hemost.2000;26 (2):157), necrotizing small vessel vasculitis, microscopic polyangiitis, Churg and 20 Strauss syndrome, pauci-immune focal necrotizing and crescentic glomerulonephritis (Noel LH. Ann Med Interne (Paris). 2000 May;151 (3):178), antiphospholipid syndrome (Flamholz R. *et al.*, J Clin Apheresis 1999;14 (4):171), antibody-induced heart failure (Wallukat G. *et al.*, Am J Cardiol. 1999 Jun 17;83 (12A):75H), thrombocytopenic purpura (Moccia F. Ann Ital Med Int. 1999 Apr-Jun;14 (2):114; 25 Semple JW. *et al.*, Blood 1996 May 15;87 (10):4245), autoimmune hemolytic anemia (Efremov DG. *et al.*, Leuk Lymphoma 1998 Jan;28 (3-4):285; Sallah S. *et al.*, Ann Hematol 1997 Mar;74 (3):139), cardiac autoimmunity in Chagas' disease (Cunha-Neto E. *et al.*, J Clin Invest 1996 Oct 15;98 (8):1709) and anti-helper T lymphocyte autoimmunity (Caporossi AP. *et al.*, Viral Immunol 1998;11 (1):9).

30 Examples of autoimmune rheumatoid diseases include, but are not limited to rheumatoid arthritis (Krenn V. *et al.*, Histol Histopathol 2000 Jul;15 (3):791; Tisch R,

McDevitt HO. Proc Natl Acad Sci units S A 1994 Jan 18;91 (2):437) and ankylosing spondylitis (Jan Voswinkel *et al.*, Arthritis Res 2001; 3 (3): 189).

Examples of autoimmune glandular diseases include, but are not limited to, pancreatic disease, Type I diabetes, thyroid disease, Graves' disease, thyroiditis, spontaneous autoimmune thyroiditis, Hashimoto's thyroiditis, idiopathic myxedema, ovarian autoimmunity, autoimmune anti-sperm infertility, autoimmune prostatitis and Type I autoimmune polyglandular syndrome. Diseases include, but are not limited to autoimmune diseases of the pancreas, Type 1 diabetes (Castano L. and Eisenbarth GS. Ann. Rev. Immunol. 8:647; Zimmet P. Diabetes Res Clin Pract 1996 Oct;34 Suppl:S125), autoimmune thyroid diseases, Graves' disease (Orgiazzi J. Endocrinol Metab Clin North Am 2000 Jun;29 (2):339; Sakata S. *et al.*, Mol Cell Endocrinol 1993 Mar;92 (1):77), spontaneous autoimmune thyroiditis (Braley-Mullen H. and Yu S, J Immunol 2000 Dec 15;165 (12):7262), Hashimoto's thyroiditis (Toyoda N. *et al.*, Nippon Rinsho 1999 Aug;57 (8):1810), idiopathic myxedema (Mitsuma T. Nippon Rinsho. 1999 Aug;57 (8):1759), ovarian autoimmunity (Garza KM. *et al.*, J Reprod Immunol 1998 Feb;37 (2):87), autoimmune anti-sperm infertility (Diekman AB. *et al.*, Am J Reprod Immunol. 2000 Mar;43 (3):134), autoimmune prostatitis (Alexander RB. *et al.*, Urology 1997 Dec;50 (6):893) and Type I autoimmune polyglandular syndrome (Hara T. *et al.*, Blood. 1991 Mar 1;77 (5):1127).

Examples of autoimmune gastrointestinal diseases include, but are not limited to, chronic inflammatory intestinal diseases (Garcia Herola A. *et al.*, Gastroenterol Hepatol. 2000 Jan;23 (1):16), celiac disease (Landau YE. and Shoenfeld Y. Harefuah 2000 Jan 16;138 (2):122), colitis, ileitis and Crohn's disease.

Examples of autoimmune cutaneous diseases include, but are not limited to, autoimmune bullous skin diseases, such as, but are not limited to, pemphigus vulgaris, bullous pemphigoid and pemphigus foliaceus.

Examples of autoimmune hepatic diseases include, but are not limited to, hepatitis, autoimmune chronic active hepatitis (Franco A. *et al.*, Clin Immunol Immunopathol 1990 Mar;54 (3):382), primary biliary cirrhosis (Jones DE. Clin Sci (Colch) 1996 Nov;91 (5):551; Strassburg CP. *et al.*, Eur J Gastroenterol Hepatol. 1999 Jun;11 (6):595) and autoimmune hepatitis (Manns MP. J Hepatol 2000 Aug;33 (2):326).

Examples of autoimmune neurological diseases include, but are not limited to, multiple sclerosis (Cross AH. *et al.*, J Neuroimmunol 2001 Jan 1;112 (1-2):1), Alzheimer's disease (Oron L. *et al.*, J Neural Transm Suppl. 1997;49:77), myasthenia gravis (Infante AJ. And Kraig E, Int Rev Immunol 1999;18 (1-2):83; Oshima M. *et al.*, Eur J Immunol 1990 Dec;20 (12):2563), neuropathies, motor neuropathies (Kornberg AJ. J Clin Neurosci. 2000 May;7 (3):191); Guillain-Barre syndrome and autoimmune neuropathies (Kusunoki S. Am J Med Sci. 2000 Apr;319 (4):234), myasthenia, Lambert-Eaton myasthenic syndrome (Takamori M. Am J Med Sci. 2000 Apr;319 (4):204); paraneoplastic neurological diseases, cerebellar atrophy, paraneoplastic cerebellar atrophy and stiff-man syndrome (Hiemstra HS. *et al.*, Proc Natl Acad Sci units S A 2001 Mar 27;98 (7):3988); non-paraneoplastic stiff man syndrome, progressive cerebellar atrophies, encephalitis, Rasmussen's encephalitis, amyotrophic lateral sclerosis, Sydenham chorea, Gilles de la Tourette syndrome and autoimmune polyendocrinopathies (Antoine JC. and Honnorat J. Rev Neurol (Paris) 2000 Jan;156 (1):23); dysimmune neuropathies (Nobile-Orazio E. *et al.*, Electroencephalogr Clin Neurophysiol Suppl 1999;50:419); acquired neuromyotonia, arthrogryposis multiplex congenita (Vincent A. *et al.*, Ann N Y Acad Sci. 1998 May 13;841:482), neuritis, optic neuritis (Soderstrom M. *et al.*, J Neurol Neurosurg Psychiatry 1994 May;57 (5):544) and neurodegenerative diseases.

Examples of autoimmune muscular diseases include, but are not limited to, myositis, autoimmune myositis and primary Sjogren's syndrome (Feist E. *et al.*, Int Arch Allergy Immunol 2000 Sep;123 (1):92) and smooth muscle autoimmune disease (Zauli D. *et al.*, Biomed Pharmacother 1999 Jun;53 (5-6):234).

Examples of autoimmune nephric diseases include, but are not limited to, nephritis and autoimmune interstitial nephritis (Kelly CJ. J Am Soc Nephrol 1990 Aug;1 (2):140).

Examples of autoimmune diseases related to reproduction include, but are not limited to, repeated fetal loss (Tincani A. *et al.*, Lupus 1998;7 Suppl 2:S107-9).

Examples of autoimmune connective tissue diseases include, but are not limited to, ear diseases, autoimmune ear diseases (Yoo TJ. *et al.*, Cell Immunol 1994 Aug;157 (1):249) and autoimmune diseases of the inner ear (Gloddek B. *et al.*, Ann N Y Acad Sci 1997 Dec 29;830:266).

Examples of autoimmune systemic diseases include, but are not limited to, systemic lupus erythematosus (Erikson J. *et al.*, Immunol Res 1998;17 (1-2):49) and systemic sclerosis (Renaudineau Y. *et al.*, Clin Diagn Lab Immunol. 1999 Mar;6 (2):156); Chan OT. *et al.*, Immunol Rev 1999 Jun;169:107).

5 ***Infectious diseases***

Examples of infectious diseases include, but are not limited to, chronic infectious diseases, subacute infectious diseases, acute infectious diseases, viral diseases, bacterial diseases, protozoan diseases, parasitic diseases, fungal diseases, mycoplasma diseases and prion diseases.

10 ***Graft rejection diseases***

Examples of diseases associated with transplantation of a graft include, but are not limited to, graft rejection, chronic graft rejection, subacute graft rejection, hyperacute graft rejection, acute graft rejection and graft versus host disease.

15 ***Allergic diseases***

Examples of allergic diseases include, but are not limited to, asthma, hives, urticaria, pollen allergy, dust mite allergy, venom allergy, cosmetics allergy, latex allergy, chemical allergy, drug allergy, insect bite allergy, animal dander allergy, stinging plant allergy, poison ivy allergy and food allergy.

According to a particular embodiment, the agents (and combinations thereof) 20 are used to treat pre-malignant lesions.

As used herein, the phrase "pre-malignant lesion" refers to a mass of cells and/or tissue having increased probability of transforming into a malignant tumor. Examples of pre-malignant lesions include, but are not limited to, adenomatous polyps, Barrett's esophagus, Pancreatic Intraepithelial Neoplasia (PanIN), IPMN (Intraductal Papillary Mucinous Neoplasia), DCIS (Ductal Carcinoma in Situ) in the breast, leukoplakia and erythroplakia. Thus, the pre-malignant lesion which is treated using the agents of this aspect of the present invention can transform into a malignant solid or non-solid (e.g., hematological malignancies) cancer (or tumor). According to a particular embodiment, the pre-malignant lesion which is treated using the agents of the present invention is an adenomatous polyp of the colon, an adenomatous polyp of the rectum, an adenomatous polyp of the small bowel and Barrett's esophagus.

Examples of fibrotic diseases include diseases of an epithelial barrier tissue, diseases of the skin, lung or gut.

Contemplated fibrotic diseases which may be treated using the agents described herein include but are not limited to eosinophilic esophagitis, hypereosinophilic syndromes (HES), Loeffler's endomyocarditis, endomyocardial fibrosis, idiopathic pulmonary fibrosis, and scleroderma.

According to a particular embodiment the agents are used for treating liver fibrosis, wound healing, skin fibrosis, pulmonary disease, kidney fibrosis, prostatitis, atherosclerosis, arthritis, osteoporosis or pancreatitis.

An exemplary pulmonary disease contemplated by the present invention is chronic obstructive pulmonary disease (COPD) or Idiopathic pulmonary fibrosis.

According to still another embodiment, the disease is associated with cartilage degeneration – e.g. arthritis.

According to still another embodiment, the disease is associated with bone degeneration – e.g. osteoporosis.

According to still another embodiment, the disease is not cancer.

The complexes of the present invention may be provided per se or may be formulated in compositions intended for a particular use.

Since the complexes of the present invention selectively target senescent cells, the present inventors contemplate that another use thereof is in cosmetic compositions as anti-aging agents for rejuvenating the skin. Thus, the agents of the present invention may be formulated for cosmetics.

Such compositions typically comprise pharmaceutically acceptable excipient, notably dermatologically acceptable suitable for external topical application.

The cosmetic composition according to the present invention may further comprise at least one pharmaceutical adjuvant known to the person skilled in the art, selected from thickeners, preservatives, fragrances, colorants, chemical or mineral filters, moisturizing agents, thermal spring water, etc.

The composition may comprise at least one agent selected from a sebum-regulating agent, an antibacterial agent, an antifungal agent, a keratolytic agent, a keratoregulating agent, an astringent, an anti-inflammatory/anti-irritant, an

antioxidant/free-radical scavenger, a cicatrizing agent, an anti-aging agent and/or a moisturizing agent.

The term "sebum-regulating agent" refers, for example, to 5- α -reductase inhibitors, notably the active agent 5- α -Avocuta^{RTM} sold by Laboratoires Expanscience.

5 Zinc and gluconate salts thereof, salicylate and pyroglutamic acid, also have sebum-suppressing activity. Mention may also be made of spironolactone, an anti-androgen and aldosterone antagonist, which significantly reduces the sebum secretion rate after 12 weeks of application. Other extracted molecules, for example from seeds of the pumpkin Cucurbita pepo, and squash seed oil, as well as palm cabbage, limit sebum production by 10 inhibiting 5- α -reductase transcription and activity. Other sebum-regulating agents of lipid origin that act on sebum quality, such as linoleic acid, are of interest.

15 The terms "anti-bacterial agent" and "antifungal agent" refer to molecules that limit the growth of or destroy pathogenic microorganisms such as certain bacteria like P. acnes or certain fungi (*Malassezia furfur*). The most traditional are preservatives generally used in cosmetics or nutraceuticals, molecules with anti-bacterial activity (pseudo-preservatives) such as caprylic derivatives (capryloyl glycine, glyceryl caprylate, etc.), such as hexanediol and sodium levulinate, zinc and copper derivatives (gluconate and PCA), phytosphingosine and derivatives thereof, benzoyl peroxide, piroctone olamine, zinc pyrithione, selenium sulfide, econazole, ketoconazole, or local 20 antibiotics such as erythromycin and clindamycin, etc.

25 The terms "keratoregulating agent" and "keratolytic agent" refer to an agent that regulates or helps the elimination of dead cells of the stratum corneum of the epidermis. The most commonly used keratoregulating/keratolytic agents include: alpha-hydroxy acids (AHAs) of fruits (citric acid, glycolic acid, malic acid, lactic acid, etc.), AHA esters, combinations of AHAs with other molecules such as the combination of malic acid and almond proteins (Keratolite^{RTM}), the combination of glycolic acid or lactic acid with arginine or the combination of hydroxy acid with lipid molecules such as LHA^{RTM} (lipo-hydroxy acid), amphoteric hydroxy acid complexes (AHCare), willow bark (*Salix alba* bark extract), azelaic acid and salts and esters thereof, salicylic acid and derivatives 30 thereof such as capryloyl salicylic acid or in combination with other molecules such as the combination of salicylic acid and polysaccharide (beta-hydroxy acid, or BHA),

tazarotene, adapalene, as well as molecules of the retinoid family such as tretinoin, retinaldehyde, isotretinoin and retinol.

The term "astringent" refers to an agent that helps constrict pores, the most commonly used being polyphenols, zinc derivatives and witch hazel.

5 The term "anti-inflammatory/anti-irritant" refers to an agent that limits the inflammatory reaction led by cytokines or arachidonic acid metabolism mediators and has soothing and anti-irritating properties. The most traditional are glycyrrhetic acid (licorice derivative) and salts and esters thereof, alpha-bisabolol, Ginkgo biloba, Calendula, lipoic acid, beta-carotene, vitamin B3 (niacinamide, nicotinamide), vitamin
10 E, vitamin C, vitamin B12, flavonoids (green tea, quercetin, etc.), lycopene or lutein, avocado sugars, avocado oleodistillate, arabinogalactan, lupin peptides, lupin total extract, quinoa peptide extract, Cycloceramide^{RTM}. (oxazoline derivative), anti-glycation agents such as carnosine, N-acetyl-cysteine, isoflavones such as, for example, genistein/genistin, daidzein/daidzin, spring water or thermal spring water (eau d'Avene,
15 eau de la Roche Posay, eau de Saint Gervais, eau d'Uriage, eau de Gamarde), goji extracts (*Lycium barbarum*), plant amino acid peptides or complexes, topical dapson, or anti-inflammatory drugs.

The term "antioxidant" refers to a molecule that decreases or prevents the oxidation of other chemical substances. The antioxidants/free-radical scavengers that
20 may be used in combination are advantageously selected from the group comprised of thiols and phenols, licorice derivatives such as glycyrrhetic acid and salts and esters thereof, alpha-bisabolol, Ginkgo biloba extract, Calendula extract, Cycloceramide^{RTM} (oxazoline derivative), avocado peptides, trace elements such as copper, zinc and selenium, lipoic acid, vitamin B12, vitamin B3 (niacinamide, nicotinamide), vitamin C, vitamin E, coenzyme Q10, krill, glutathione, butylated hydroxytoluene (BHT), butylated
25 hydroxyanisole (BHA), lycopene or lutein, beta-carotene, the family of polyphenols such as tannins, phenolic acids, anthocyanins, flavonoids such as, for example, extracts of green tea, of red berries, of cocoa, of grapes, of *Passiflora incarnata* or of *Citrus*, or isoflavones such as, for example, genistein/genistin and daidzein/daidzin. The group of
30 antioxidants further includes anti-glycation agents such as carnosine or certain peptides, N-acetyl-cysteine, as well as antioxidant or free-radical scavenging enzymes such as

superoxide dismutase (SOD), catalase, glutathione peroxidase, thioredoxin reductase and agonists thereof.

The agents that cicatrize/repair the barrier function which may be used in combination are advantageously vitamin A, panthenol (vitamin B5), Avocadofurane.RTM., avocado sugars, lupeol, maca peptide extract, quinoa peptide extract, arabinogalactan, zinc oxide, magnesium, silicon, madecassic or asiatic acid, dextran sulfate, coenzyme Q10, glucosamine and derivatives thereof, chondroitin sulfate and on the whole glycosaminoglycans (GAGs), dextran sulfate, ceramides, cholesterol, squalane, phospholipids, fermented or unfermented soya peptides, plant peptides, marine, plant or biotechnological polysaccharides such as algae extracts or fern extracts, trace elements, extracts of tannin-rich plants such as tannins derived from gallic acid called gallic or hydrolysable tannins, initially found in oak gall, and catechin tannins resulting from the polymerization of flavan units whose model is provided by the catechu (Acacia catechu). The trace elements that may be used are advantageously selected from the group comprised of copper, magnesium, manganese, chromium, selenium, silicon, zinc and mixtures thereof.

Anti-aging agents that can act in combination to treat acne in mature subjects are antioxidants and in particular vitamin C, vitamin A, retinol, retinal, hyaluronic acid of any molecular weight, Avocadofurane^{RTM}, lupin peptides and maca peptide extract.

The most commonly used moisturizers/emollients are glycerin or derivatives thereof, urea, pyrrolidone carboxylic acid and derivatives thereof, hyaluronic acid of any molecular weight, glycosaminoglycans and any other polysaccharides of marine, plant or biotechnological origin such as, for example, xanthan gum, Fucogel.RTM., certain fatty acids such as lauric acid, myristic acid, monounsaturated and polyunsaturated omega-3, -6, -7 and -9 fatty acids (linoleic acid, palmitoleic acid, etc.), sunflower oleodistillate, avocado peptides and cupuacu butter.

For treatment of diseases, the agents of the present invention may be formulated in pharmaceutical compositions.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the therapeutic agent (as described herein above) accountable for the biological effect which is attached directly or indirectly to the targeting agent. It will be appreciated that the pharmaceutical compositions may comprise additional active agents known to be useful in treating a particular disease. Thus, for example for treatment of skin fibrotic diseases, the present inventors contemplate pharmaceutical compositions comprising the above described agents together with at least one sebum-regulating agent, an antibacterial agent, an antifungal agent, a keratolytic agent, a keratoregulating agent, an astringent, an anti-inflammatory/anti-irritant, an antioxidant/free-radical scavenger, a cicatrizing agent, an anti-aging agent and/or a moisturizing agent, as described herein above.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the 5 common coronary artery, intravenous, intraperitoneal, intranasal, or intraocular injections.

According to a particular embodiment, the route of administration is via topical delivery.

Alternately, one may administer the pharmaceutical composition in a local rather 10 than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or 15 lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper 20 formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the 25 formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, 30 and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain

tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be 5 suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection 10 suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which 15 increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of the present invention may also be 20 formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective 25 amount means an amount of active ingredients (e.g. siRNA agents together with targeting agents) effective to prevent, alleviate or ameliorate symptoms of a disorder (e.g., fibrotic or inflammatory disease) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability 30 of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated from animal models (e.g. mouse models of

liver fibrosis induced by CCl₄, mouse model of pancreatitis induced by Caerulein, mouse model of COPD) to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can
5 be determined by standard pharmaceutical procedures in experimental animals. The data obtained from these animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See
10 e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide cell numbers sufficient to induce normoglycemia (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and
15 route of administration. Detection assays can be used to determine plasma concentrations.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions of the present invention may, if desired, be presented in a pack or
20 dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be
25 accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product
30 insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate

container, and labeled for treatment of an indicated condition, as if further detailed above.

Since the present inventors have found that the polypeptides HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and any of the 5 polypeptides listed in Table 1, herein below are selectively upregulated on the surface of senescent cells as compared to non-senescent cells, the present inventors propose that measuring the level of such proteins may be used to identify senescent cells.

Thus, according to still another aspect of the present invention there is provided a method of identifying senescent cells in a cell population comprising analyzing the 10 amount of at least one polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and any of the polypeptides listed in Table 1 on the membrane of the cells of the cell population, wherein a level of the at least one polypeptide above a predetermined amount is indicative of senescent cells.

15 The identifying may be part of a method of diagnosing a disease associated with senescent cells as further described herein above.

The term "diagnosing" as used herein refers to determining the presence of a disease, classifying a disease, staging a disease, determining a severity of a disease, monitoring disease progression, forecasting an outcome of the disease, predicting 20 survival and/or prospects of recovery (i.e. prognosis).

The subject may be a healthy animal or human subject undergoing a routine well-being check up. Alternatively, the subject may be at risk of having the disease (e.g., a genetically predisposed subject, a subject with medical and/or family history of cancer, a subject who has been exposed to carcinogens, occupational hazard, 25 environmental hazard] and/or a subject who exhibits suspicious clinical signs of the disease [e.g., blood in the stool or melena, unexplained pain, sweating, unexplained fever, unexplained loss of weight up to anorexia, changes in bowel habits (constipation and/or diarrhea), tenesmus (sense of incomplete defecation, for rectal cancer specifically), anemia and/or general weakness). Still alternatively, the subject may be diagnosed as having a disease associated with senescent cells, but the stage is being 30 evaluated.

Determining an expression of any of the polypeptides listed above may be effected on the RNA or protein level as detailed below.

According to one embodiment, the determining is effected ex vivo.

According to another embodiment, the determining is effected in vivo.

5 Diseases which may be diagnosed are listed herein above. According to a particular embodiment, the disease is cancer or a premalignant disease (e.g. Pancreatic Intraepithelial Neoplasia (PanIN)).

Methods of detecting expression of the polypeptides on the RNA level

When the polypeptide is expressed solely on the membrane (e.g. ITGA3) and not 10 in other cell compartments, RNA based methods on whole cell extracts may be used. In this scenario lysed cells may be used for the detection of the polypeptides.

Preferably, when the polypeptide is not expressed solely on the membrane and is expressed in other cell compartments, RNA based methods are performed on membrane extracts.

15 In order to detect expression of the polypeptides on the RNA level, typically polynucleotide probes (e.g. oligonucleotides or primers) are used that are capable of specifically hybridizing to their RNA or cDNA generated therefrom.

Preferably, the oligonucleotide probes and primers utilized by the various 20 hybridization techniques described hereinabove are capable of hybridizing to their targets under stringent hybridization conditions.

By way of example, hybridization of short nucleic acids (below 200 bp in length, e.g. 17-40 bp in length) can be effected by the following hybridization protocols depending on the desired stringency; (i) hybridization solution of 6 x SSC and 1 % SDS or 3 M TMACl, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS, 25 100 µg/ml denatured salmon sperm DNA and 0.1 % nonfat dried milk, hybridization temperature of 1 - 1.5 °C below the Tm, final wash solution of 3 M TMACl, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS at 1 - 1.5 °C below the Tm (stringent hybridization conditions) (ii) hybridization solution of 6 x SSC and 0.1 % SDS or 3 M TMACl, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS, 30 100 µg/ml denatured salmon sperm DNA and 0.1 % nonfat dried milk, hybridization temperature of 2 - 2.5 °C below the Tm, final wash solution of 3 M TMACl, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS at 1 - 2.5 °C below the Tm (moderate hybridization conditions).

1.5 °C below the Tm, final wash solution of 6 x SSC, and final wash at 22 °C (stringent to moderate hybridization conditions); and (iii) hybridization solution of 6 x SSC and 1 % SDS or 3 M TMACl, 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 7.6), 0.5 % SDS, 100 µg/ml denatured salmon sperm DNA and 0.1 % nonfat dried milk, 5 hybridization temperature at 2.5-3 °C below the Tm and final wash solution of 6 x SSC at 22 °C (moderate hybridization solution).

Northern Blot analysis: This method involves the detection of a particular RNA in a mixture of RNAs. An RNA sample is denatured by treatment with an agent (e.g., formaldehyde) that prevents hydrogen bonding between base pairs, ensuring that all the 10 RNA molecules have an unfolded, linear conformation. The individual RNA molecules are then separated according to size by gel electrophoresis and transferred to a nitrocellulose or a nylon-based membrane to which the denatured RNAs adhere. The membrane is then exposed to labeled DNA probes. Probes may be labeled using radio-isotopes or enzyme linked nucleotides. Detection may be using autoradiography, 15 colorimetric reaction or chemiluminescence. This method allows both quantitation of an amount of particular RNA molecules and determination of its identity by a relative position on the membrane which is indicative of a migration distance in the gel during electrophoresis.

RT-PCR analysis: This method uses PCR amplification of relatively rare RNAs 20 molecules. First, RNA molecules are purified from the cells and converted into complementary DNA (cDNA) using a reverse transcriptase enzyme (such as an MMLV-RT) and primers such as, oligo dT, random hexamers or gene specific primers. Then by applying gene specific primers and Taq DNA polymerase, a PCR amplification reaction is carried out in a PCR machine. Those of skills in the art are capable of 25 selecting the length and sequence of the gene specific primers and the PCR conditions (i.e., annealing temperatures, number of cycles and the like) which are suitable for detecting specific RNA molecules. It will be appreciated that a semi-quantitative RT-PCR reaction can be employed by adjusting the number of PCR cycles and comparing the amplification product to known controls.

30 **RNA *in situ* hybridization stain:** In this method DNA or RNA probes are attached to the RNA molecules present in the cells. Generally, the cells are first fixed to microscopic slides to preserve the cellular structure and to prevent the RNA molecules

from being degraded and then are subjected to hybridization buffer containing the labeled probe. The hybridization buffer includes reagents such as formamide and salts (e.g., sodium chloride and sodium citrate) which enable specific hybridization of the DNA or RNA probes with their target mRNA molecules *in situ* while avoiding non-specific binding of probe. Those of skills in the art are capable of adjusting the hybridization conditions (*i.e.*, temperature, concentration of salts and formamide and the like) to specific probes and types of cells. Following hybridization, any unbound probe is washed off and the slide is subjected to either a photographic emulsion which reveals signals generated using radio-labeled probes or to a colorimetric reaction which reveals signals generated using enzyme-linked labeled probes.

In situ RT-PCR stain: This method is described in Nuovo GJ, et al. [Intracellular localization of polymerase chain reaction (PCR)-amplified hepatitis C cDNA. Am J Surg Pathol. 1993, 17: 683-90] and Komminoth P, et al. [Evaluation of methods for hepatitis C virus detection in archival liver biopsies. Comparison of histology, immunohistochemistry, *in situ* hybridization, reverse transcriptase polymerase chain reaction (RT-PCR) and *in situ* RT-PCR. Pathol Res Pract. 1994, 190: 1017-25]. Briefly, the RT-PCR reaction is performed on fixed cells by incorporating labeled nucleotides to the PCR reaction. The reaction is carried on using a specific *in situ* RT-PCR apparatus such as the laser-capture microdissection PixCell I LCM system available from Arcturus Engineering (Mountainview, CA).

Oligonucleotide microarray – In this method oligonucleotide probes capable of specifically hybridizing with the polynucleotides of the present invention are attached to a solid surface (e.g., a glass wafer). Each oligonucleotide probe is of approximately 20-25 nucleic acids in length. To detect the expression pattern of the polynucleotides of the present invention in a specific cell sample (e.g., blood cells), RNA is extracted from the cell sample using methods known in the art (using e.g., a TRIZOL solution, Gibco BRL, USA). Hybridization can take place using either labeled oligonucleotide probes (e.g., 5'-biotinylated probes) or labeled fragments of complementary DNA (cDNA) or RNA (cRNA). Briefly, double stranded cDNA is prepared from the RNA using reverse transcriptase (RT) (e.g., Superscript II RT), DNA ligase and DNA polymerase I, all according to manufacturer's instructions (Invitrogen Life Technologies, Frederick, MD, USA). To prepare labeled cRNA, the double stranded cDNA is subjected to an *in vitro*

transcription reaction in the presence of biotinylated nucleotides using e.g., the BioArray High Yield RNA Transcript Labeling Kit (Enzo, Diagnostics, Affymetrix Santa Clara CA). For efficient hybridization the labeled cRNA can be fragmented by incubating the RNA in 40 mM Tris Acetate (pH 8.1), 100 mM potassium acetate and 30 mM magnesium acetate for 35 minutes at 94 °C. Following hybridization, the microarray is washed and the hybridization signal is scanned using a confocal laser fluorescence scanner which measures fluorescence intensity emitted by the labeled cRNA bound to the probe arrays.

For example, in the Affymetrix microarray (Affymetrix®, Santa Clara, CA) each gene on the array is represented by a series of different oligonucleotide probes, of which, each probe pair consists of a perfect match oligonucleotide and a mismatch oligonucleotide. While the perfect match probe has a sequence exactly complimentary to the particular gene, thus enabling the measurement of the level of expression of the particular gene, the mismatch probe differs from the perfect match probe by a single base substitution at the center base position. The hybridization signal is scanned using the Agilent scanner, and the Microarray Suite software subtracts the non-specific signal resulting from the mismatch probe from the signal resulting from the perfect match probe.

Methods of detecting the polypeptides on the protein level

When the polypeptide is expressed solely on the membrane (e.g. ITGA3) and not in other cell compartments, whole cell extracts may be analyzed. In this scenario lysed cells may be used for the detection of the polypeptides.

Preferably, when the polypeptide is not expressed solely on the membrane and is expressed in other cell compartments, membrane extracts are used for methods which require the generation of cellular extracts. In situ methods, such as immunostaining and FACS may be used regardless whether the polypeptide is expressed solely on the membrane or not.

Determining expression of the polypeptides on the protein level is typically effected using an antibody capable of specifically interacting with same. Methods of detecting the above described proteins include immunoassays which include but are not limited to competitive and non-competitive assay systems using techniques such as Western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay),

"sandwich" immunoassays, and immunoprecipitation assays and immunohistochemical assays as detailed herein below.

Below is a list of techniques which may be used to determine the level of the proteins described herein above on the protein level.

5 **Enzyme linked immunosorbent assay (ELISA):** This method involves fixation of a sample (e.g., fixed cells or a proteinaceous solution) containing a protein substrate to a surface such as a well of a microtiter plate. A substrate specific antibody coupled to an enzyme is applied and allowed to bind to the substrate. Presence of the antibody is then detected and quantitated by a colorimetric reaction employing the enzyme coupled to the antibody. Enzymes commonly employed in this method include horseradish peroxidase and alkaline phosphatase. If well calibrated and within the linear range of response, the amount of substrate present in the sample is proportional to the amount of color produced. A substrate standard is generally employed to improve quantitative accuracy.

15 **Western blot:** This method involves separation of a substrate from other protein by means of an acrylamide gel followed by transfer of the substrate to a membrane (e.g., nylon or PVDF). Presence of the substrate is then detected by antibodies specific to the substrate, which are in turn detected by antibody binding reagents. Antibody binding reagents may be, for example, protein A, or other antibodies. Antibody binding 20 reagents may be radiolabeled or enzyme linked as described hereinabove. Detection may be by autoradiography, colorimetric reaction or chemiluminescence. This method allows both quantitation of an amount of substrate and determination of its identity by a relative position on the membrane which is indicative of a migration distance in the acrylamide gel during electrophoresis.

25 **Radio-immunoassay (RIA):** In one version, this method involves precipitation of the desired protein (*i.e.*, the substrate) with a specific antibody and radiolabeled antibody binding protein (e.g., protein A labeled with I¹²⁵) immobilized on a precipitable carrier such as agarose beads. The number of counts in the precipitated pellet is proportional to the amount of substrate.

30 In an alternate version of the RIA, a labeled substrate and an unlabelled antibody binding protein are employed. A sample containing an unknown amount of substrate is

added in varying amounts. The decrease in precipitated counts from the labeled substrate is proportional to the amount of substrate in the added sample.

Fluorescence activated cell sorting (FACS): This method involves detection of a substrate *in situ* in cells by substrate specific antibodies. The substrate specific antibodies are linked to fluorophores. Detection is by means of a cell sorting machine which reads the wavelength of light emitted from each cell as it passes through a light beam. This method may employ two or more antibodies simultaneously. It will be appreciated that when the protein is not expressed selectively on the cell membrane, care should be taken to avoid the antibody penetrating the cell membrane.

Immunohistochemical analysis: This method involves detection of a substrate *in situ* in fixed cells by substrate specific antibodies. The substrate specific antibodies may be enzyme linked or linked to fluorophores. Detection is by microscopy and subjective or automatic evaluation. If enzyme linked antibodies are employed, a colorimetric reaction may be required. It will be appreciated that immunohistochemistry is often followed by counterstaining of the cell nuclei using for example Hematoxyline or Giemsa stain. It will be appreciated that when the protein is not expressed selectively on the cell membrane, care should be taken to avoid the antibody penetrating the cell membrane.

In situ activity assay: According to this method, a chromogenic substrate is applied on the cells containing an active enzyme and the enzyme catalyzes a reaction in which the substrate is decomposed to produce a chromogenic product visible by a light or a fluorescent microscope.

As mentioned, the identifying/diagnosing/staging is carried out by analyzing an amount or activity of the polypeptides in a cell sample of the subject, wherein a difference in an amount or activity thereof beyond a predetermined threshold with respect to a control cell sample is indicative of the disease. It will be appreciated that the amount of change may correspond with a degree or a stage of the disease. Thus, larger differences may indicate a later stage of the disease with a poorer prognosis, whereas lower differences may indicate an early stage of the disease with a better prognosis.

The patient sample typically comprises cells. It may be part of a tissue sample, retrieved during a biopsy. Alternatively, the sample may be a bodily fluid, e.g. blood, urine, saliva, CSF, plasma etc.

For diagnosis of cancer, the cell sample may comprise cells of the primary tumor and/or metastatic effusion thereof.

5 The predetermined level may be established based on results from control (non-diseased) cells.

10 The control cell sample typically depends on the patient sample being analyzed. Thus, for example, in the case of colon cancer, the control sample may comprise colon cells of a healthy individual (or at least one not suffering from colon cancer) or from a known stage of colon cancer (e.g. non-metastatic stage). In the case of breast cancer, the control sample may comprise breast cells of a healthy individual (or at least one not suffering from breast cancer) or from a known stage of breast cancer.

15 The control cells are typically normally differentiated, non-senescent cells, preferably of the same tissue and specimen as the tested cells. Typically, the amount of change in expression of the polypeptides is statistically significant.

Preferably, the difference is at least 10 %, 20 %, 30 %, 40 %, 50 %, 80 %, 100 % (i.e., two-fold), 3 fold, 5 fold or 10 fold different as compared to the control cells.

20 It will be appreciated that the control data may also be taken from databases and literature.

On obtaining the results of the analysis, the subject is typically informed. Additional diagnostic tests may also be performed so as to corroborate the results of the diagnosing (e.g. gold standard tests, assessing the aggressiveness of the tumor, the patient's health and susceptibility to treatment, etc.).

25 Imaging studies such as CT and/or MRI may be obtained to further diagnose the disease.

In addition, when the disease is cancer, the diagnosis or choice of therapy may be determined by further assessing the size of the tumor, or the lymph node stage or both, optionally together or in combination with other risk factors.

30 The present inventors propose that based on the results of the diagnosis, a suitable therapy may be selected – i.e. personalized medicine.

As mentioned, the present inventors showed that down-regulation of cell-surface Grp94 decreases NK-cell mediated cytotoxicity toward senescent cells and decreased susceptibility of senescent cells for elimination by monocytes.

Thus, the present inventors propose that polypeptides which are expressed on the surface of senescent cells may be used to elicit or boost an immune response to a senescent cell.

Thus, according to another aspect of the present invention there is provided a method of eliciting or boosting an immune response to a senescent cell in a subject comprising administering to the subject a pharmaceutical composition comprising at least one polypeptide or a polynucleotide encoding same selected from the group consisting of HSP90B1, DBN1, PRKCSH, SPTBN1, NPM1 and a polypeptide set forth in Table 1, wherein the pharmaceutical composition does not comprise senescent cells, thereby eliciting or boosting the immune response to the senescent cell.

The immune response may comprise clearance of the senescent cell by the immune response (e.g. a helper T cell or a cytotoxic T-cell response).

The present inventors contemplate administering at least one, two, three, four, five, six, seven, eight, nine, ten or more of the polypeptides of this aspect of the present invention.

The polypeptides may be provided as full length polypeptides or as antigenic fragments thereof.

As used herein, the term "antigenic fragment" refers to an immunogenic portion of the full-length polypeptide. Such antigenic fragments may comprise at least 5, 10, 20, 25, 30, 35, 40, 45, 50, 55, or 60 or more contiguous amino acids (or any number of contiguous amino acids between 5-60, including 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, 45-50, 40-55, or 55-60, or more than 60 contiguous amino acids) of the polypeptides. In a more particular embodiment, antigenic fragment comprises at least 20 contiguous amino acids. An antigenic fragment of a mature or full-length polypeptide has one or more epitopes that induce a specific immune response, which may comprise production of antibodies that specifically bind to the antigenic fragment and to the immunogenic portion within the mature and full-length polypeptide from which the antigenic peptide is derived, and to a senescent cell that expresses the polypeptide.

The polypeptides or fragments thereof of the present invention may be administered per se to induce an immune response, or alternatively, as part of a composition i.e. vaccine, which comprises an immunologically acceptable carrier.

It will be appreciated that the polypeptides may be administered in the form of an expression construct which comprises the corresponding nucleic acid sequence to the polypeptide. The expression construct may be administered instead of the polypeptides themselves (e.g. in a prime boost protocol) or in addition to the polypeptides of the present invention. It will be further appreciated that the polypeptides may be expressed in a cell population (e.g. dendritic cells) and the cell population may be provided. According to a particular embodiment, the polypeptides are not administered as part of a senescent cell population or membrane fraction thereof.

Suitable agents that provide a target antigen include recombinant vectors, for example, bacteria, viruses, and naked DNA. Recombinant vectors are prepared using standard techniques known in the art, and contain suitable control elements operably linked to the nucleotide sequence encoding the target antigen. See, for example, Plotkin, et al. (eds.) (2003) *Vaccines*, 4.sup.th ed., W.B. Saunders, Co., Phila., Pa.; Sikora, et al. (eds.) (1996) *Tumor Immunology* Cambridge University Press, Cambridge, UK; Hackett and Ham (eds.) *Vaccine Adjuvants*, Humana Press, Totowa, N.J.; Isaacson (eds.) (1992) *Recombinant DNA Vaccines*, Marcel Dekker, NY, N.Y.; Morse, et al. (eds.) (2004) *Handbook of Cancer Vaccines*, Humana Press, Totowa, N.J.), Liao, et al. (2005) *Cancer Res.* 65:9089-9098; Dean (2005) *Expert Opin. Drug Deliv.* 2:227-236; Arlen, et al. (2003) *Expert Rev. Vaccines* 2:483-493; Dela Cruz, et al. (2003) *Vaccine* 21:1317-1326; Johansen, et al. (2000) *Eur. J. Pharm. Biopharm.* 50:413-417; Excler (1998) *Vaccine* 16:1439-1443; Disis, et al. (1996) *J. Immunol.* 156:3151-3158). Peptide vaccines are described (see, e.g., McCabe, et al. (1995) *Cancer Res.* 55:1741-1747; Minev, et al. (1994) *Cancer Res.* 54:4155-4161; Snyder, et al. (2004) *J. Virology* 78:7052-7060. Virus-derived vectors include viruses, modified viruses, and viral particles (see, e.g., US patent No. 8,926,993, incorporated herein by reference). The virus-derived vectors can be administered directly to a mammalian subject, or can be introduced ex vivo into an antigen presenting cell (APC), where the APC is then administered to the subject.

Viral vectors may be based on, e.g., Togaviruses, including alphaviruses and flaviviruses; alphaviruses, such as Sindbis virus, Sindbis strain SAAR86, Semliki Forest

virus (SFV), Venezuelan equine encephalitis (VEE), Eastern equine encephalitis (EEE), Western equine encephalitis, Ross River virus, Sagiyami virus, O'Nyong-nyong virus, Highlands J virus. Flaviviruses, such as Yellow fever virus, Yellow fever strain 17D, Japanese encephalitis, St. Louis encephalitis, Tick-borne encephalitis, Dengue virus, 5 West Nile virus, Kunjin virus (subtype of West Nile virus); arterivirus such as equine arteritis virus; and rubivirus such as rubella virus, herpesvirus, modified vaccinia Ankara (MVA); avipox viral vector; fowlpox vector; vaccinia virus vector; influenza virus vector; adenoviral vector, human papilloma virus vector; bovine papilloma virus vector, and so on. Viral vectors may be based on an orthopoxvirus such as variola virus 10 (smallpox), vaccinia virus (vaccine for smallpox), Ankara (MVA), or Copenhagen strain, camelpox, monkeypox, or cowpox. Viral vectors may be based on an avipoxvirus virus, such as fowlpox virus or canarypox virus.

Adenoviral vectors and adeno-associated virus vectors (AAV) are available, where adenoviral vectors include adenovirus serotype 5 (adeno5; Ad5), adeno6, 15 adeno11, and adeno35. Available are at least 51 human adenovirus serotypes, classified into six subgroups (subgroups A, B, C, D, E, and F). Adenovirus proteins useful, for example, in assessing immune response to an "empty" adenoviral vector, include hexon protein, such as hexon 3 protein, fiber protein, and penton base proteins, and human immune responses to adenoviral proteins have been described (see, e.g., Wu, et al. 20 (2002) *J. Virol.* 76:12775-12782; Mascola (2006) *Nature* 441:161-162; Roberts, et al. (2006) *Nature* 441:239-243).

General methods to prepare immunogenic or vaccine compositions are described in Remington's Pharmaceutical Science; Mack Publishing Company Easton, Pa. (latest edition). To increase immunogenicity, the polypeptides of the present invention may be 25 adsorbed to or conjugated to beads such as latex or gold beads, ISCOMs, and the like. Immunogenic compositions may comprise adjuvants, which are substance that can be added to an immunogen or to a vaccine formulation to enhance the immune-stimulating properties of the immunogenic moiety. Liposomes are also considered to be adjuvants (Gregoriades, G. et al., *Immunological Adjuvants and Vaccines*, Plenum Press, New 30 York, 1989) Examples of adjuvants or agents that may add to the effectiveness of proteinaceous immunogens include aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-in-oil

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emulsions, and oil-in-water emulsions. A preferred type of adjuvant is muramyl dipeptide (MDP) and various MDP derivatives and formulations, e.g., N-acetyl-D-glucosaminyl-(β .1-4)-N-acetylmuramyl-L-alanyl-D-isoglutamine (GMDP) (Hornung, R L et al. Ther Immunol 1995 2:7-14) or ISAF-1 (5% squalene, 2.5% pluronic L121, 0.2% Tween 80 in phosphate-buffered solution with 0.4 mg of threonylmuramyl dipeptide; see Kwak, L W et al. (1992) N. Engl. J. Med., 327:1209-1238). Other useful adjuvants are, or are based on, cholera toxin, bacterial endotoxin, lipid X, whole organisms or subcellular fractions of the bacteria Propionobacterium acnes or Bordetella pertussis, polyribonucleotides, sodium alginate, lanolin, lysolecithin, vitamin A, saponin and saponin derivatives such as QS21 (White, A. C. et al. (1991) Adv. Exp. Med. Biol., 303:207-210) which is now in use in the clinic (Helling, F et al. (1995) Cancer Res., 55:2783-2788; Davis, T A et al. (1997) Blood, 90: 509), levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. A number of adjuvants are available commercially from various sources, for example, Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.) or Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Mich.), Amphigen (oil-in-water), Alhydrogel (aluminum hydroxide), or a mixture of Amphigen and Alhydrogel. Aluminum is approved for human use.

The present invention also contemplates antiserum induced in one subject using the polypeptides or fragments thereof of the present invention, removed from that subject and used to treat another subject by passive immunization or transfer of the antibodies. For disclosure of such passive immunization with patient sera, neutralizing antisera or mAbs, see Nishimura Y et al. (2003) Proc Natl Acad Sci USA 100:15131-36; Mascola J R (2003) Curr Mol Med. 3:209-16; Ferrantelli F et al. (2003) AIDS 17:301-9; Ferrantelli F et al (2002) Curr Opin Immunol. 14:495-502; Xu W et al. (2002) Vaccine 20:1956-60; Nichols C N et al. (2002) AIDS Res Hum Retrovir. 8:49-56; Cho M W et al. (2000) J. Virol. 74:9749-54; Mascola J R et al. (2000) Nat Med. 6:207-10; Andrus. L et al. (1998) J. Inf. Dis. 77: 889-897; Parren P W (1995) AIDS 9:F1-6; Hinkula J et al. (1994) J Acquir Immune Defic Syndr. 7:940-51; Prince A M et al. (1991) AIDS Res Hum Retrovir 7:971-73; Emini E A et al. (1990) J. Virol. 64:3674-84, all incorporated by reference.

The amount of polypeptide or fragment to be administered to induce an immune response depends on the precise polypeptide selected, the health and weight of the recipient, the route of administration, the existence of other concurrent treatment, if any, the frequency of treatment, the nature of the effect desired, and the judgment of the skilled practitioner.

An exemplary dose for treating a subject is an amount of up to about 100 milligrams of active polypeptide per kilogram of body weight. A typical single dosage of the polypeptide or chimeric protein is between about 1 ng and about 100 mg/kg body weight, and preferably from about 10 µg to about 50 mg/kg body weight. A total daily dosage in the range of about 0.1 milligrams to about 7 grams is preferred for intravenous administration. A useful dose of an antibody for passive immunization is between 10-100 mg/kg. It has been suggested (see references cited above for passive immunity) that an effective in vivo dose of an antibody/antiserum is between about 10- and 100-fold more than an effective neutralizing concentration or dose in vitro. These dosages can be determined empirically in conjunction with the present disclosure and state-of-the-art. The polypeptides of the present invention may be administered alone or in conjunction with other therapeutics directed to the treatment of the disease or condition.

The subject vaccines find use in methods for eliciting or boosting a cellular immune response, e.g., a helper T cell or a cytotoxic T-cell response to senescent cells. The vaccine of the present invention may be used, for example, both for immunization and to boost immunity after exposure. As such, the subject vaccines find use as both prophylactic and therapeutic vaccines to induce immune responses that are specific for senescent cells that are relevant to various disease conditions.

The vaccine may contain other antigenic polypeptides, e.g. comprised in a tumor cell lysate, an irradiated tumor cell, an antigen-presenting cell pulsed with peptides of the target antigen (e.g. a dendritic cell).

The vaccine of this aspect of the present invention may also comprise an agent which enhances the immunogenicity of the immunogen e.g. a helper antigen or carrier moiety. A helper antigen includes a T cell helper antigen, which is an antigen that is recognized by a T helper cell and evokes an immune response in a T helper cell. T helper cells are lymphocytes that are involved in activating and directing other immune cells such as cytotoxic T cells, B cells, and/or macrophages. Carrier moieties have been

long known in the immunology art and include without limitation, keyhole limpet hemocyanin, bovine serum albumin, cationized BSA, or ovalbumin. For human use, toxoids of bacterial proteins (e.g., tetanus toxoid, diphtheria toxoid, cholera toxoid, and the like) are typically employed as carrier proteins.

5 In certain embodiments, the immunogen comprises at least one senescent cell associated antigen or at least one antigenic fragment thereof and a helper antigen or carrier moiety that is linked, conjugated, or attached to the antigen or antigenic fragment thereof. The helper antigen or carrier moiety may be recombinantly expressed in frame and directly linked to a senescent cell associated antigen or fragment thereof. In certain
10 embodiments, a fusion protein comprising at least two senescent cell associated antigens or at least two antigenic fragments thereof or a combination of same may also comprise a helper antigen or carrier moiety. Alternatively, the helper antigen or carrier moiety may be chemically conjugated, linked, or attached to the senescent cell associated antigen or fragment thereof. In still another embodiment, the helper antigen or carrier
15 moiety may be formulated together with any immunogen described herein but not covalently or non-covalently bound to the immunogen to form an immunogenic composition.

In another embodiment, the immunogenic compositions described herein include a co-stimulatory polypeptide. In certain embodiments, the immunogen comprises at least
20 one senescent cell associated antigen (as described herein) or at least one antigenic fragment thereof and a co-stimulatory molecule that is linked, conjugated, or attached to the antigen or antigenic fragment thereof. The co-stimulatory molecule may be recombinantly expressed in frame and directly linked to a senescent cell associated antigen or fragment thereof. In certain embodiments, a fusion protein comprising at least
25 two senescent cell associated antigen or at least two antigenic fragments thereof or a combination of same may also comprise a co-stimulatory molecule. Alternatively, the co-stimulatory molecule may be chemically conjugated, linked, or attached to the senescent cell associated antigen or fragment thereof. In still another embodiment, the co-stimulatory molecule may be formulated together with any immunogen described
30 herein but not covalently or non-covalently bound to the immunogen to form the immunogenic composition.

Exemplary co-stimulatory molecules include, by way of example, GM-CSF, IL-2, IL-4, IL-6, IL-7, IL-15, IL-21, IL-23, TNFa, B7.1 (CD80), B7.2 (CD86), 41BB, CD40 ligand (CD40L), drug-inducible CD40 (iCD40), and the like. When an immunogenic composition comprises a polynucleotide encoding the co-stimulatory molecule, or a recombinant expression virus comprising the polynucleotide, expression of the co-stimulatory molecule is typically under the control of one or more regulatory elements selected to direct the expression of the coding sequences in a cell of choice, such as a dendritic cell.

Recombinantly engineered antigen-presenting cells such as dendritic cells, for example, may be modified by recombinant technology to express increased levels of antigen presenting machinery, adhesion and/or co-stimulatory molecules, including MHC class I/antigen complexes, MHC class II/antigen complexes, CD1, hsp70-90, CD9, CD63, CD81, CD1 lb, CD1 lc, CD40, CD54 (ICAM-1), CD63, CD80, CD86, 41BBL, OX40L, chemokine receptor CCRL-10 and CXCR1-6, mannose-rich C-type lectin receptor DEC205 and Toll-like receptors TLR4 and TLR9 or membrane-bound TGF- β . The exosomes derived from these recombinantly engineered antigen presenting cells will express these additional molecules and can transfer them to the T helper cells, T regulatory cells, or dendritic cells upon absorption.

As mentioned, the present invention contemplates antigen-presenting cells (APCs), e.g., dendritic cells (DCs), that include senescent cell-associated antigens, as described herein above, for example, by being presented on the surface of the antigen-presenting cells.

Dendritic cells play a critical role in coordinating innate and adaptive immune responses. DCs are bone-marrow derived cells characterized by dendritic morphology and high mobility that are seeded in all tissues. DCs are specialized antigen presenting cells that are capable of capturing and processing antigens, migrating from the periphery to a lymphoid organ, and presenting the antigens in a MHC-restricted manner to naive T-cells (see, e.g., Banchereau & Steinman, 1998, Nature 392:245-252; Steinman et al., 2003, Ann. Rev. Immunol. 21:685-711). Immature DCs are capable of processing and presenting antigens, which leads to immune regulation and/or suppression. Maturation (activation) of DCs is required to induce differentiation of antigen-specific T cells into effector T cells (see, e.g., Palucka et al, 2012, Nat. Rev. Cancer 12:265-277). Mature

DCs express high levels of MHC-antigen complex and other co-stimulatory molecules, such as CD40, B7-1, B7-2, and CD 1a (see, e.g., Steinman, 1991, Ann. Rev. Immunol. 9:271-296; Banchereau & Steinman, 1998, Nature 392:245-252). These molecules play key roles in stimulating T cells. Due to their properties, DC-based vaccination strategies have been developed in cancer (see, e.g., Heiser et al, 2001, Cancer Res. 61:338; Heiser et al, 2001, J. Immunol. 166:2953; Milazzo et al, 2002, Blood 101:977; Zu et al, 2003, Cancer Res. 63:2127). Likewise, DC based immunogens (vaccines) may be able to elicit CD8⁺ T cells capable of recognizing peptide-MHC Class complexes on senescent cells and target them for destruction.

Dendritic cells may be obtained from various sources using methods known in the art. DC precursors may be purified from peripheral blood (see, e.g., Fong et al., 2003, Annu Rev. Immunol. 15: 138). DCs may be also be differentiated from peripheral blood monocytes or CD34⁺ hematopoietic progenitor cells ex vivo (see, e.g., Sallusto et al, 1994, J. Exp. Med. 179: 1109; Banchereau et al, 2001, Cancer Res. 61:6451; Makensen et al, 2000, Int. J. Cancer 86:385). Methods for in vitro proliferation of dendritic cells from DC precursors and their use as immunogens are described in U.S. Patents 5,851,756; 5,994,126; 6,475,483; and 8,283,163 each of which is incorporated herein by reference in its entirety. A method for isolating DCs from human peripheral blood is described in U.S. Patent 5,643,786, incorporated herein by reference in its entirety. U.S. Patent Publication 2006/0063255, U.S. Patent Publication 2006/0057129, and U.S. Patent 7,247,480, each of which is incorporated herein by reference in its entirety, describe methods for making dendritic cell vaccines from human embryonic stem cells.

Methods of isolating APCs, such as dendritic cells, are known in the art. Procedures such as repetitive density gradient separation, fluorescence activated cell sorting techniques, positive selection, negative selection, or a combination thereof are routinely used to obtain enriched populations of DCs. Methods for isolating DCs may be found in O'Doherty et al, 1993, J. Exp. Med. 178: 1067-78; Young and Steinman, 1990, J. Exp. Med. 171:1315-32; Freudenthal et al, 1990, Proc. Natl. Acad. Sci. USA 57:7698-7702; Markowicz and Engleman, 1990, J. Clin. Invest. 85:955-961; Mehta-Damani et al, 1994, J. Immunol. 153:996-1003; Thomas et al, 1993, J. Immunol. 151:6840-6852.

Dendritic cells may be loaded with specific antigens *ex vivo* and then administered to a subject (see, e.g., Banchereau et al, 2005, *Nat. Rev. Immunol.* 5:296-306; Figdor et al, 2004, *Nat. Med.* 10:475-480, each of which is incorporated herein by reference in its entirety). Various methods for loading antigens to DCs have been described and are known in the art. R A encoding a specific antigen may be pulsed into dendritic cells before administration to a subject by electroporation, cationic lipids, cationic peptides or using dendrimers (see, e.g., Boczkowski et al. 1996, *J. Exp. Med.* 184:465; Heiser et al, 2001, *Cancer Res.* 61:338; Heiser et al, *J. Immunol.* 2001, 166:2953; U.S. Patent Publication 2006/0063255; Choi et al, 2005, *Cell Cycle* 4:669).

DCs may also be loaded with protein or peptide that is purified or isolated from a target cell, chemically synthesized, or recombinantly expressed. Nucleic acid vectors encoding a specific antigen may also be used for DC loading (see, e.g., Frolkis et al., 2003, *Cancer Gene Ther.* 10:239). Exemplary vectors include plasmids, cationic lipid complexes, viral vectors, cDNA encoding antigen loaded onto dendrimers, or other small particulates that enhance uptake by phagocytic cells. U.S. Patent 6,300,090 and U.S. Patent 6,455,299 describe using non-replicating viral vectors comprising sequence encoding an antigen for infecting dendritic cells, resulting in antigen presentation on the DC surface.

Alternatively, DCs may be loaded with specific antigens *in vivo*.

Antigens may be delivered directly to DCs using chimeric proteins that are comprised of a DC receptor-specific antibody fused to a selected antigen (see, e.g., Bonifaz et al, 2004, *J. Exp. Med.* 199:815-824; Bonifaz et al, 2004, *J. Exp. Med.* 196: 1627-1638; Hawiger et al, 2001, *J. Exp. Med.* 194:769-780; each of which is incorporated herein by reference in its entirety). U.S. Patent Publication 2012/0070462, incorporated herein by reference in its entirety, describes targeted antigen delivery to dendritic cells using recombinant viral vectors comprising a polynucleotide encoding the antigen and a targeting molecule, which binds to a DC-specific surface marker (e.g., DC-SIGN).

Antigenic peptides useful for presentation by DCs for vaccination are peptides that stimulate a T cell mediated immune response (e.g., cytotoxic T cell response) by presentation to T cells on MHC molecules. Useful antigenic peptides and proteins for use in the present disclosure include those derived from senescent cells (e.g., senescent cell-associated antigens). Depending on the method of DC loading utilized, a senescent

cell-associated antigen may be presented in a variety of forms. In some embodiments, a senescent cell-associated antigen is presented as a senescent cell lysate to DCs. In other embodiments, senescent cell-associated antigens are obtained by acid elution of peptides presented on MHC molecules of the senescent cell surface. For example, senescent cells
5 are washed with an isotonic solution to remove media components. The cells are then treated with acid to dissociate peptides from surface MHCs, and the cells are removed from the solution containing the soluble peptides. Antigenic peptides may be obtained by chemical synthesis or produced using recombinant methods with host cells and vector expression systems. A senescent cell associated antigen may also be delivered as a
10 polynucleotides (RNA or DNA) to a DC directly or indirectly (e.g., via a plasmid or viral vector). The antigenic peptides presented on MHC molecules are typically short peptides and may be 5, 6, 7, 8, 9, or 10 amino acids, for example.

A senescent cell associated antigen introduced into DCs may also be designed as a fusion peptide, wherein the antigen is joined to a protein or peptide sequence that
15 enhances transport into endosomal and other intracellular compartments involved in Class II histocompatibility loading. For example, the N-terminus of such a fusion protein may comprise a suitable heterologous leader or signal sequence for the endosomal compartment and the C-terminus may comprise a transmembrane and luminal component of a member of the LAMP family for lysosomal targeting (see, e.g., U.S.
20 Patent 5,633,234; WO 02/080851; Sawada et al, 1993, J. Biol. Chem. 268:9014; each of which is incorporated by reference herein in its entirety). Endosomal and lysosomal sorting signals include tyrosine based signals, dileucine-based signals, acidic clusters, and transmembrane proteins labeled with ubiquitin (see, e.g., Bonifacino et al, 2003, Annu. Rev. Biochem. 72:395; U.S. Patent 6,248,565).

It is expected that during the life of a patent maturing from this application many relevant pharmaceutical agents will be developed and the scope of the term pharmaceutical agent is intended to include all such new technologies *a priori*.
25

As used herein the term “about” refers to ± 10 %.

The terms “comprises”, “comprising”, “includes”, “including”, “having” and
30 their conjugates mean “including but not limited to”.

The term “consisting of” means “including and limited to”.

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

5 As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof

10 Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

15 As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

20 As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

25 It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various 30 embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

5

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning"

Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

10 **MATERIALS AND METHODS**

Cell Culture: Human IMR-90 fibroblasts were purchased from ATCC, and Mouse embryonic fibroblasts (MEFs) were derived according to standard procedures (Manipulating the Mouse Embryo: A Laboratory Manual, 3rd ed. A. Nagy, et al., Cold Spring Harbor Laboratory Press, 2003). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM L-glutamine, 100 units/ml of penicillin, 100µg/ml of streptomycin, and 10% FBS, in a low oxygen incubator. DIS was induced by etoposide treatment (E1383, Sigma) at a concentration of 100µM for 48 hours as described previously (Krizhanovsky et al. *Cell* 134.4 (2008): 657-667). OIS was induced by infection of IMR-90 cells with pWZL plasmid containing oncogenic Hras^{V12}.

20 **Identification of cell surface proteins:** Cell-surface proteins were purified from IMR-90 cells, 9 days post insult using commercial kit (Pierce). Briefly, cells were exposed to Sulfo-NHS-SS-biotin, lysed and loaded on columns to capture membrane proteome. Following several washing steps, purified cell surface proteins were finally eluted. Samples were then analyzed by Label-free mass spectrometry at the proteomic unit at the Grand Israel National Center for Personalized Medicine. Integrative analysis was done by Ingenuity Pathway Analysis software (Qiagen). Cell-surface proteins were analyzed according to their original subcellular localization using the cellular component branch of GO by online DAVID v6.7 software.

25 **Validation of MS results:** Grp94 translocation to cell surface was first validated by purification of plasma membrane proteome (Qproteome Plasma Membrane Protein Kit, Qiagen). Equal amounts of cell-surface proteins or total cell lysates from growing

IMR-90 and senescent IMR-90 were separated on 12% SDS-polyacrylamide gels and transferred onto PVDF membrane (IPVH00010, Millipore). After blocking with 5% BSA in TBST (TBS with 0.01% Tween 20) for 1hr, the membranes were probed with antibodies against Grp94 (PA5-24824, Thermo Fisher Scientific), HLA-A,B,C (BioLegend), GAPDH and β -tubulin (both, Santa Cruz) overnight at 4°C. Antibodies were visualized with HRP-based chemiluminescence detection kit (34080, Thermo Fisher Scientific).

Immunofluorescence assay: Cells were incubated with anti-Grp94 antibody (diluted in DMEM) for 3h in 37 °C, washed 3 times with warm PBS and incubated with goat anti-rabbit Alexa647 conjugated antibody (111-605-003, Jackson immuno-research) for 30 minutes, washed 3 times with warm PBS and visualized in Olympus IX81 microscope and XM10 camera and processed using ImageJ v1.47 software.

Flow-cytometry assay

IMR-90 cells were gently dissociated from plates using TripLE express reagent (12604-013, Thermo Fisher Scientific), and maintained in cold FACS buffer (PBS containing 1% FCS and 0.1% Sodium Azid) throughout all procedure. Cells were incubated with anti-Grp94 antibody (1:20 in FACS buffer), followed by goat anti-rabbit Alexa647 conjugated antibody. DAPI was shortly introduced in order to exclude dead cells. Cells were analyzed in a SORP-LSRII instrument (BD Biosciences). Data was collected from at least 20,000 single-cell events. Cells were gated by their size (FSC/SSC), DAPI negative (live cells) and then analyzed for their fluorescence intensity at the wavelength of 647 (cell-surface Grp94 level), using FlowJo v10 software.

GPM1 inhibitor: GPM1 was synthesized by the Organic synthesis unit at the Weizmann institute as was described previously (Kim et al. 2012). Final product was characterized by NMR. GPM1 were dissolved in 5% DMSO in phosphate-buffered saline (PBS) and control vehicle was 5% DMSO in PBS.

Cytotoxicity assays: For in-vitro cytotoxicity assays target cells were plated in 12-well plates at 4×10^4 cells per well; 1×10^5 NK-92 cells were subsequently added to each well. Following 2h of co-incubation, NK-92 cells were washed gently and the cytotoxicity was determined based on the viability of remaining adherent cells. To block Grp94-mediated cytotoxicity, ON-TARGETplus SMARTpool small-interfering RNA

targeting HSP90B1 and the non-targeting (control) pool were transfected into senescent IMR-90 cells with Dharmafect 1 reagent (all from Dharmacon, Lafayette, CO, USA). Transfections were performed overnight, and 4 days later cytotoxicity assays were executed. Viability was determined by using PrestoBlue reagent kit (A13262, Life Technologies Ltd.), and was calculated relatively to control DMSO treated cells.

For phagocytosis assay Etopoide-treated IMR-90 cells were plated in 12-well plates at 4×10^4 cells per well. Cells were incubated 24hrs with GPM1 at the concentration of 0, 10 or 100 μ g/ml, and then co-cultures with MM6 cells for 6 days. Assessment of remaining cells was determined by crystal violet staining.

Statistical analysis: Statistical analysis of the results was analyzed using one-sided two-sample t-tests or one-way ANOVA followed by Tukey post-hoc test. Data are expressed as means \pm S.E.M, a P value <0.05 was considered significant.

RESULTS

Senescent cells are known to express several common ligands which are required for natural killer immune cells recognition and subsequent cytotoxicity [5]. In order to identify the fraction proteome which is being expressed on cell surface of senescent cells, the present inventors analyzed membrane proteins using high-throughput proteomics analysis. Normal human fibroblasts cells IMR90 were used to execute two distinct sets of experiments; exposure to the chemotherapeutic agent Etoposide for 48hrs, which results in DNA Damage Induced Senescence (DIS), and infection with oncogenic H-Ras^{V12}, leading to Oncogene-induced senescence (OIS). At day 9 post DNA damage or introduction of the oncogene, cells acquired senescent-like morphology and proliferative arrest (Figure 1A). At this time point, the cells were exposed to Sulfo-NHS-SS-biotin, lysed and loaded on columns to capture membrane proteome. Following a few rounds of washing, the proteins were eluted to obtain membrane protein samples. The samples were then analyzed by mass spectrometry (Figure 1B). 2198 different proteins were identified in the samples, 257 of them existed only in samples derived from senescent cells but not from growing cells (Figure 1C).

An integrative list of human hits with 5 peptides and more + 20% coverage and more is provided in Table 1, herein below.

Table 1

<i>GeneBank No.</i>	<i>Gene</i>	<i>Protein Names</i>	<i>Protein Description</i>	<i>Unique Peptides</i>	<i>Protein Sequence Coverage (%)</i>	<i>Average Ratio</i>
NM_001199 954.1.	ACTG1	ACTG_HUMAN, SW:P63261	Actin, cytoplasmic 2	6	70.7	8.47
NM_001150 .2.	ANPEP	B4DP96_HUMAN, TR:B4DP96	Uncharacterized protein	7	26.3	2.70
NM_001177 .5.	ARL1	B4DZG7_HUMAN, TR:B4DZG7	ADP- ribosylation factor-like protein 1	5	57.0	2.11
NM_001693 .3.	ATP6V1B2	VATB2_HUMAN, SW:P21281	V-type proton ATPase subunit B, brain isoform	8	28.8	4.37
NM_004859 .3.	CLTC	CLH1_HUMAN, SW:Q00610-2	Isoform 2 of Clathrin heavy chain 1	43	34.3	2.95
NM_004094 .4.	EIF2S1	H0YJS4_HUMAN, TR:H0YJS4	Eukaryotic translation initiation factor 2 subunit 1	5	23.8	2.52
NM_002056 .3.	GFPT1	GFPT1_HUMAN, SW:Q06210-2	Isoform 2 of Glucosamine- fructose-6- phosphate aminotransferase [isomerizing] 1	8	19.7	2.29
NM_000424 .3.	KRT5	K2C5_HUMAN, SW:P13647	Keratin, type II cytoskeletal 5	10	20.8	2.98
NM_001256 282.1	KRT8	K2C8_HUMAN, SW:P05787	Keratin, type II cytoskeletal 8	5	20.5	2.14
XM_005248	MCCC	MCCB_HUMAN,	Isoform 2 of	7	25.0	2.43

567.1.	2	SW:Q9HCC0-2	Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial			
NM_001114 614.1.	MFGE8	F5H7N9_HUMAN, TR:F5H7N9	Lactadherin short form	5	29.0	2.89
NM_001024 628.2.	NRP1	Q5T7F1_HUMAN, TR:Q5T7F1	Neuropilin 1	6	27.6	5.49
NM_201381 .2.	PLEC	PLEC_HUMAN, SW:Q15149-7	Isoform 7 of Plectin	106	45.8	1.93
NM_152132 .2.	PSMA3	PSA3_HUMAN, SW:P25788-2	Isoform 2 of Proteasome subunit alpha type-3	5	25.4	2.18
NM_001199 163.1.	PSMC5	PRS8_HUMAN, SW:P62195-2	Isoform 2 of 26S protease regulatory subunit 8	8	25.1	4.72
NM_012232 .5.	PTRF	PTRF_HUMAN, SW:Q6NZI2-2	Isoform 2 of Polymerase I and transcript release factor	5	28.3	2.01
NM_004637 .5.	RAB7A	RAB7A_HUMAN, SW:P51149	Ras-related protein Rab-7a	5	31.9	2.75
NM_001256 577.2.	RPL10	F8W7C6_HUMAN, TR:F8W7C6	60S ribosomal protein L10	6	31.9	3.38
NM_001042 576.1.	RRBP1	F8W7S5_HUMAN, TR:F8W7S5	Ribosome-binding protein 1	9	24.4	3.70
NM_001788 .5	SEPT7	H0Y3Y4_HUMAN, TR:H0Y3Y4	Septin-7	8	21.2	214.6 8
NM_006288 .3.	THY1	E9PIM6_HUMAN, TR:E9PIM6	Thy-1 membrane glycoprotein	6	45.8	3.91

In order to understand the nature of the identified proteins, integrative analysis of the identified proteins was performed using the data mining software, Ingenuity. The distribution of the protein's original subcellular origin was analyzed. Surprisingly, much of the cell-surface proteome was recognized as an intra-cellular (Figure 1C). Nonetheless, the vast majority of the proteins are presented by only 2-4 peptides, which could imply that many peptides identified do not stand for the full and functional

proteins, but rather for peptides presented by antigen presenting machineries. These machineries might include major histo-compatibility complexes and members of the heat shock protein families.

In a parallel analysis approach, the identified proteins were mapped to known canonical pathways. In the context of immune-related processes, integrin signaling was identified as the strongest hit (Figure 1D). Integrin signaling in senescent cells consist of at least 10 different integrins, predominantly ITGA3 and ITGB5. The second strongest immunological hit was Fc γ -receptor mediated phagocytosis. This finding is in-line with several studies which identify macrophages and monocytes as potentially important players in immune surveillance of senescent cells [16]. Another strong immunological hit was fMLP (f-Met-Leu-Phe) signaling. N-formylated peptides are believed to derive from mitochondrial proteins upon tissue damage and known to act mainly on neutrophils [17]. fMLP is a strong chemo-attractant, but also induces adherence, degranulation and production of tissue-destructive oxygen-derived free radicals in phagocytic cells [17, 18].

Finally, potential upstream regulators were identified (Figure 1E). Nrf2, the nuclear factor 2 transcription factor, was found to be the most activated. Although Nrf2 is constitutively expressed in all tissues, it may be further induced by cellular stressors including endogenous reactive-oxygen species and oncogene activation [19]. This single transcription factor mediates multiple avenues of cytoprotection by activating the transcription of more than 200 genes interacts with other important cell regulators such as p53 and NF- κ B and through their combined interactions is the guardian of health-span, protecting against many age-related diseases including cancer and neurodegeneration [20]. Conversely, RICTOR, the Rapamycin-insensitive companion of mTOR, was identified as being the most inhibited upstream regulator. RICTOR binds directly to mTOR to stabilize TORC2 which regulates cell growth and survival in response to hormonal signals and growth factors [21].

Next, the results from the two main experiments were intersected. This approach increased exclusivity of these proteins for the senescence program with no dependence on the exact trigger and increased the confidence in specific proteins. A short but unique list of 9 proteins was found to be common between both DIS and OIS (Figure 2A). Under these restrictions, 7 out of the 9 proteins were previously documented as being

found in the plasma membrane compartment, and 3 of them were previously reported to exist apically on cell surface (according to the UniProtKB/Swiss-Prot database). A closer look at the top 3 proteins revealed that one of them is ICAM1, a cell surface glycoprotein which is typically expressed on endothelial cells and cells of the immune system (Figure 2B). This protein is known to be up-regulated during cellular senescence, and therefore it can serve as internal control and provide independent confirmation for the results of this study [22]. Another hit is ITGA3, an integrin alpha subunit that together with integrin beta-1 composes $\alpha 3\beta 1$ integrin duplex. The $\alpha 3\beta 1$ complex is a receptor for variety of ECM components, known to play a role in neural migration and endothelial adhesion [23, 24]. The third hit, Grp94, also known as Endoplasmic or gp96, is the main ER-resident chaperone. Grp94 belongs to the heat-shock 90 family and encoded by the HSP90B1 gene in humans. The encoded protein is localized to the ER where it plays critical roles in folding proteins in the secretory pathway such as Toll-like receptors and integrins [25]. Unlike the first two hits, normally, this protein does not find its way to the cell-surface. Nevertheless, upon severe ER stress, Grp94 can be actively translocated to the extracellular interface, where it displays important modulatory effects on both the innate and adaptive immune response [26, 27]. Macrophages for instance, are a prominent GRP94 target, as GRP94 reported to activate several signaling pathways in both LPS-dependent and independent manner [28]. Second, Grp94 can activate dendritic cells (DCs), to enhance their immune-stimulatory capacities [29]. In parallel, Grp94 can undergo endocytosis to antigen-presenting cells (APCs), and to mediate the cross-presentation of Grp94-bound peptides on MHC class I molecules for activation of cytotoxic T cells [30, 31].

Despite its average size (92469Da), 42 different unique peptides were identified for this protein, covering almost 60% of protein sequence (Figure 2B). Analysis of the exact location of those sequences on the protein sequence map suggests that the hits represent the full length protein and not fragments or single peptides (Figure 2C).

In order to validate the existence of the full length Grp94 protein on cell-surface of senescent cells, an unbiased approach was implemented which consists of plasma membrane purification followed by Western Blot (WB) analysis. The results of this experiment show that Grp94 cell-surface expression is preferential or even exclusive to senescent cells, with a clear band in the size of ~96kDA (Figure 2D). In order to

quantitatively asses this elevation, immunofluorescence staining was performed on live cells under conditions which allow the staining of only the external Grp94 molecules, and the samples were analyzed by FACS (Figure 2E). The results indicate a 3-fold increase in Grp94 levels on senescent cells (Figure 2F). As a positive control the levels 5 of ICAM1 were examined which showed a 2-fold increase in senescent cells (Figures 2G-H). Finally, IMR90 growing or DIS cells were seeded on cover-slips and stained for cell-surface Grp94/HLA-A,B,C. In agreement with the results described above, the senescent IMR90 cells display a marked increase in the levels of cell-surface Grp94 (Figure 2I).

10 Stress in the ER might increase its promiscuity, enabling ER resident proteins to be found in other sites in the cell, such as cell-surface. In order to understand whether the existence of Grp94 on the surface of senescent cells is a direct consequence of the senescence program, or alternatively, a common result of stress conditions, the present inventors examined the kinetics of cell-surface Grp94 at different time-points after 15 treating propagating IMR90 cells with Etoposide (Figure 3A). Importantly, Grp94 levels were not elevated 3 days after etoposide treatment, indicating that the presence of Grp94 on cell surface is not a common stress response, but only after the establishment of the senescent program (Figure 3B). In addition, the data suggest that during cellular 20 senescence Grp94 gradually accumulates on the cell surface. The continuous recycling of escaped ER proteins such as Grp94 is mediated by retrograde transport from Golgi to ER through COPI-coated vesicles [32]. During this process, Grp94 localization is regulated through its C-terminal KDEL sequence, which is recognized by the KDEL receptor ERD2 [33]. Grp94 homodimerization potentially promote ERD2 binding [34, 35]. Consequently, ERD2-Grp94 complex returns to the ER where it dissociates, freeing 25 ERD2 for further cycling of transport. In order to examine the validity of this pathway to Grp94 during the senescence program, GPM1, a small molecule which has been shown to promote dimerization of Grp94, thus promoting recycling of Grp94 into the ER and blocking its translocation to cell-surface (Figure 3B) [36] was synthesized. After ensuring that GPM1 does not affect the cell's viability under different 30 concentrations (Figure 3C), it was found that GPM1 could reduce cell surface levels of Grp94 of senescent IMR90 cells (Figure 3D). Thus, it may be concluded that GPM1

may be used to manipulate cell-surface levels of Grp94, and accordingly to investigate the role of Grp94 in the interaction of senescent cells with the immune system.

Grp94 is known to have multiple effects on the immune system, a property for which it has received the title “the immune system’s Swiss army-knife” [27]. Studies from recent years have revealed close interactions between senescent cells and the immune system. Immune surveillance of senescent cells is mediated on many occasions by NK cells which are attracted to the site of the senescent cells where they are recognized and eliminated [7, 37]. In order to elucidate possible role for Grp94 in immune surveillance of NK cells the total levels of Grp94 in senescent IMR90 cells were down-regulated using specific siRNA for 4 days. The effect was validated by Western blot analysis (Figure 4A). A slight decrease in the viability of the siHSP90B1 treated cells was observed in comparison to siControl (Figure 4B). This result is in line with the role of ER-resident Grp94 in stabilization of unfolded proteins during stress conditions. Finally, those cells were co-cultured with NK-92 cells for 2 hours. The NK cells were then gently removed and cytotoxicity was calculated as the ratio between the IMR90 cell’s viability prior to and following co-culture. The results show a decreased cytotoxicity of NK-92 cells toward senescent cells that have low Grp94 expression levels (Figure 4C), suggesting that NK cell cytotoxicity is mediated, at least in part, by cell-surface Grp94.

In order to understand the functional importance of Grp94 for phagocytosis of macrophages, DIS IMR90 cells were treated with GPM1 or DMSO. The viability of senescent cells post co-culturing with human MM6 monocytes was examined. The preliminary data show that under the effective concentration of GPM1 (100 μ M) senescent cells have reduced viability following co-culturing with monocytes (Figure 4D), suggesting Grp94 as a regulator of immune surveillance of senescent cells.

The levels of cell surface Grp94 were also examined in mouse embryonic fibroblast cells (MEFs). A significant elevation in cell surface Grp94 was detected upon treatment with etoposide (Figure 4E). The relatively high correlation between localization of Grp94 in human and mouse cells implies that it may serve as a new marker for senescent cells.

In order to shed more light on the biological significance of cell surface Grp94 in senescent cells, mice were treated with GPM1 following the establishment of chronic

fibrosis in the liver. It was previously demonstrated that senescent hepatic stellate cells are gradually eliminated by immune cells until their full elimination around 10-20 days from the last CCl₄ injection. The administration of GPM1 dramatically increased intracellular levels of Grp94 and eliminated the positive staining at the plasma membrane regions (Figure 5A). Histological analyses indicate that GPM1 treatment blocks the clearance of senescent cells (Figure 5B). The increase in tissue-residing senescent cells was accompanied with increased fibrosis in the GPM1-treated animals compared to the Vehicle-treated animals (Figure 5B). Quantitative analysis confirmed these findings (Figure 5C,D).

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

References

1. Campisi, J. and F. d'Adda di Fagagna, *Cellular senescence: when bad things happen to good cells.* Nat Rev Mol Cell Biol, 2007. 8(9): p. 729-40.
2. Collado, M., M.A. Blasco, and M. Serrano, *Cellular senescence in cancer and aging.* Cell, 2007. 130(2): p. 223-33.
3. Adams, P.D., *Healing and hurting: molecular mechanisms, functions, and pathologies of cellular senescence.* Mol Cell, 2009. 36(1): p. 2-14.
4. Serrano, M., et al., *Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a.* Cell, 1997. 88(5): p. 593-602.
5. Xue, W., et al., *Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas.* Nature, 2007. 445(7128): p. 656-60.
6. Kang, T.W., et al., *Senescence surveillance of pre-malignant hepatocytes limits liver cancer development.* Nature, 2011. 479(7374): p. 547-51.
7. Krizhanovsky, V., et al., *Senescence of activated stellate cells limits liver fibrosis.* Cell, 2008. 134(4): p. 657-67.
8. Michaloglou, C., et al., *BRAFE600-associated senescence-like cell cycle arrest of human naevi.* Nature, 2005. 436(7051): p. 720-4.
9. Parrinello, S., et al., *Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation.* J Cell Sci, 2005. 118(Pt 3): p. 485-96.
10. Jeyapalan, J.C. and J.M. Sedivy, *Cellular senescence and organismal aging.* Mech Ageing Dev, 2008. 129(7-8): p. 467-74.
11. Collado, M. and M. Serrano, *Senescence in tumours: evidence from mice and humans.* Nat Rev Cancer, 2010. 10(1): p. 51-7.
12. Ovadya, Y. and V. Krizhanovsky, *Senescent cells: SASPected drivers of age-related pathologies.* Biogerontology, 2014. 15(6): p. 627-42.
13. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. 140(6): p. 883-99.
14. Baker, D.J., et al., *Opposing roles for p16Ink4a and p19Arf in senescence and ageing caused by BubR1 insufficiency.* Nat Cell Biol, 2008. 10(7): p. 825-36.

15. Baker, D.J., et al., *Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders*. Nature, 2011. 479(7372): p. 232-6.
16. Lujambio, A., et al., *Non-cell-autonomous tumor suppression by p53*. Cell, 2013. 153(2): p. 449-60.
17. Carp, H., *Mitochondrial N-formylmethionyl proteins as chemoattractants for neutrophils*. The Journal of experimental medicine, 1982. 155(1): p. 264-275.
18. Selvatici, R., et al., *Signal transduction pathways triggered by selective formylpeptide analogues in human neutrophils*. Eur J Pharmacol, 2006. 534(1-3): p. 1-11.
19. DeNicola, G.M., et al., *Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis*. Nature, 2011. 475(7354): p. 106-9.
20. Lewis, K.N., et al., *Nrf2, a guardian of healthspan and gatekeeper of species longevity*. Integr Comp Biol, 2010. 50(5): p. 829-43.
21. Shiota, C., et al., *Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability*. Dev Cell, 2006. 11(4): p. 583-9.
22. Gorgoulis, V.G., et al., *p53-dependent ICAM-1 overexpression in senescent human cells identified in atherosclerotic lesions*. Lab Invest, 2005. 85(4): p. 502-11.
23. Schmid, R.S., et al., *alpha3beta1 integrin modulates neuronal migration and placement during early stages of cerebral cortical development*. Development, 2004. 131(24): p. 6023-31.
24. Glinskii, O.V., et al., *Endothelial integrin alpha3beta1 stabilizes carbohydrate-mediated tumor/endothelial cell adhesion and induces macromolecular signaling complex formation at the endothelial cell membrane*. Oncotarget, 2014. 5(5): p. 1382-9.
25. Wu, S., et al., *The molecular chaperone gp96/GRP94 interacts with Toll-like receptors and integrins via its C-terminal hydrophobic domain*. J Biol Chem, 2012. 287(9): p. 6735-42.
26. Altmeyer, A., et al., *Tumor-specific cell surface expression of the-KDEL containing, endoplasmic reticular heat shock protein gp96*. Int J Cancer, 1996. 69(4): p. 340-9.

27. Schild, H. and H.G. Rammensee, *gp96--the immune system's Swiss army knife.* Nat Immunol, 2000. 1(2): p. 100-1.
28. Reed, R.C., et al., *GRP94/gp96 elicits ERK activation in murine macrophages. A role for endotoxin contamination in NF-kappa B activation and nitric oxide production.* J Biol Chem, 2003. 278(34): p. 31853-60.
29. Zheng, H., et al., *Cell surface targeting of heat shock protein gp96 induces dendritic cell maturation and antitumor immunity.* J Immunol, 2001. 167(12): p. 6731-5.
30. Dai, J., et al., *Cell surface expression of heat shock protein gp96 enhances cross-presentation of cellular antigens and the generation of tumor-specific T cell memory.* Cancer Immun, 2003. 3: p. 1.
31. Berwin, B., et al., *Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells.* EMBO J, 2003. 22(22): p. 6127-36.
32. Yamamoto, K., et al., *The KDEL receptor mediates a retrieval mechanism that contributes to quality control at the endoplasmic reticulum.* EMBO J, 2001. 20(12): p. 3082-91.
33. Semenza, J.C., et al., *ERD2, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway.* Cell, 1990. 61(7): p. 1349-57.
34. Dollins, D.E., et al., *Structures of GRP94-nucleotide complexes reveal mechanistic differences between the hsp90 chaperones.* Mol Cell, 2007. 28(1): p. 41-56.
35. Nemoto, T. and N. Sato, *Oligomeric forms of the 90-kDa heat shock protein.* Biochem J, 1998. 330 (Pt 2): p. 989-95.
36. Han, J.M., et al., *Identification of gp96 as a novel target for treatment of autoimmune disease in mice.* PLoS One, 2010. 5(3): p. e9792.
37. Sagiv, A. and V. Krizhanovsky, *Immunosurveillance of senescent cells: the bright side of the senescence program.* Biogerontology, 2013. 14(6): p. 617-28.
38. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets.* Nat Rev Immunol, 2011. 11(11): p. 723-37.

39. Binder, R.J. and P.K. Srivastava, *Essential role of CD91 in re-presentation of gp96-chaperoned peptides*. Proc Natl Acad Sci U S A, 2004. 101(16): p. 6128-33.
40. Binder, R.J., D.K. Han, and P.K. Srivastava, *CD91: a receptor for heat shock protein gp96*. Nat Immunol, 2000. 1(2): p. 151-5.
41. Naylor, R.M., D.J. Baker, and J.M. van Deursen, *Senescent cells: a novel therapeutic target for aging and age-related diseases*. Clin Pharmacol Ther, 2013. 93(1): p. 105-16.
42. Mantovani, A., et al., *Macrophage plasticity and polarization in tissue repair and remodelling*. J Pathol, 2013. 229(2): p. 176-85.

WHAT IS CLAIMED IS:

1. A method of targeting a pharmaceutical agent to a senescent cell in a subject comprising administering the pharmaceutical agent to the subject, wherein said pharmaceutical agent is attached to an affinity moiety, said affinity moiety being capable of binding specifically to a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1, thereby targeting the pharmaceutical agent to the senescent cell.
2. A method of treating a disease associated with cell senescence in a subject in need thereof comprising administering to the agent a therapeutically effective amount of a cytotoxic agent attached to an affinity moiety, said affinity moiety being capable of binding specifically to a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1, thereby treating the disease.
3. A cytotoxic agent attached to an affinity moiety, said affinity moiety being capable of binding specifically to a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1 for use in treating a disease associated with cell senescence.
4. The method of claim 1, wherein said pharmaceutical agent is a therapeutic agent.
5. The method of claim 4, wherein said therapeutic agent is a cytotoxic agent.
6. The method of claim 1, wherein said pharmaceutical agent is a diagnostic agent.

7. The method of any one of claims 1 or 5, wherein said cytotoxic agent is directly attached to the affinity moiety.

8. The method of any one of claims 1 or 5, wherein said cytotoxic agent is indirectly attached to the affinity moiety.

9. The method of any one of claims 1, 5 or 8, wherein said cytotoxic agent is comprised in a particle.

10. The method of claim 9, wherein said affinity moiety is attached to the outer surface of said particle.

11. The method of any one of claims 1 or 5, wherein said cytotoxic agent comprises a polynucleotide agent.

12. The method of claim 11, wherein said cytotoxic agent comprises an RNA silencing agent.

13. The method of any one of claims 1 or 5, wherein said cytotoxic agent down-regulates an activity and/or an amount of an apoptosis related polypeptide.

14. The method of claim 13, wherein said apoptosis related polypeptide is selected from the group consisting of Bcl-xL, Bcl-w and p21.

15. The method of claim 13, wherein said cytotoxic agent is selected from the group consisting of ABT-737, ABT-263, Gossypol, AT-101, TW-37 and Obatoclax.

16. The method of any one of claims 1-15, wherein said affinity moiety is selected from the group consisting of an antibody, an aptamer and a peptide.

17. The method of any one of claims 1-16, wherein said polypeptide is HSP90B1.

18. The method of any one of claims 2-17, wherein said disease is a fibrotic disease or an inflammatory disease.

19. The method of claim 18, wherein said inflammatory disease is cancer.

20. The method of any one of claims 2-19, further comprising administering to the subject at least one agent selected from the group consisting of a sebum-regulating agent, an antibacterial and/or antifungal agent, a keratolytic agent and/or keratoregulating agent, an astringent, an anti-inflammatory and/or anti-irritant, an antioxidant and/or free-radical scavenger, a cicatrizing agent, an anti-aging agent and a moisturizing agent.

21. The method of claim 20, wherein said at least one agent is an anti-aging agent.

22. A particle having a senescent cell affinity moiety attached to an outer surface thereof, the senescent cell affinity moiety capable of specifically binding a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1.

23. A composition of matter comprising an affinity moiety attached to a therapeutic agent, wherein said affinity moiety specifically binds to a polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1.

24. The composition of matter of claim 23, wherein said affinity moiety is an antibody or an aptamer.

25. The particle of claim 22, being attached to or encapsulating a therapeutic agent or a diagnostic agent.

26. The particle of claim 25, wherein said therapeutic agent comprises a cytotoxic moiety.

27. The composition of matter or particle of claims 23 or 26, wherein said cytotoxic moiety comprises a polynucleotide agent.

28. The composition of matter or particle of claim 27, wherein said polynucleotide agent comprises an RNA silencing agent.

29. The composition of matter or particle of claims 23 or 26, wherein said cytotoxic agent down-regulates an activity and/or an amount of an apoptosis related polypeptide.

30. The composition of matter or particle of claim 29, wherein said apoptosis related polypeptide is selected from the group consisting of Bcl-xL, Bcl-w and p21.

31. A pharmaceutical composition comprising the composition of matter or particle of claims 23-29 as the active agent and a pharmaceutically acceptable carrier.

32. The pharmaceutical composition of claim 31, being formulated for topical administration.

33. The pharmaceutical composition of any one of claims 31 or 32, further comprising at least one agent selected from the group consisting of a sebum-regulating agent, an antibacterial and/or antifungal agent, a keratolytic agent and/or keratoregulating agent, an astringent, an anti-inflammatory and/or anti-irritant, an antioxidant and/or free-radical scavenger, a cicatrizing agent, an anti-aging agent and a moisturizing agent.

34. The pharmaceutical composition of claim 33, wherein said at least one agent is an anti-aging agent.

35. A method of identifying senescent cells in a cell population comprising analyzing the amount of at least one polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1 on the membrane of the cells of the cell population, wherein a level of said at least one polypeptide above a predetermined amount is indicative of senescent cells.

36. A composition of matter comprising senescent cells, wherein a polypeptide of said cells is attached to an affinity moiety, said polypeptide being selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1.

37. The composition of matter of claim 36, wherein said senescent cells are lysed cells.

38. The composition of matter of claim 36, wherein said senescent cells are non-lysed cells.

39. A method of diagnosing a disease associated with cell senescence in a subject comprising analyzing the amount of at least one polypeptide selected from the group consisting of HSP90B1, DNAJB4, PI4K2A, DBN1, PRKCSH, SPTBN1, NPM1, ITGA3 and a polypeptide set forth in Table 1 on the membrane of cells of the subject, wherein a level of said at least one polypeptide above a predetermined amount is indicative of the disease.

40. The method of claim 39, wherein said disease is a fibrotic disease or an inflammatory disease.

41. The method of claim 40, wherein said inflammatory disease is cancer.

42. The method of any one of claims 35 or 39, wherein the identifying is effected in vivo.

43. The method of any one of claims 35 or 39, wherein the identifying is effected ex vivo.

44. The method of any one of claims 35 or 39, wherein the identifying is effected in vitro.

45. The method of any one of claims 35 or 39-44, wherein said at least one polypeptide is HSP90B1.

46. The method of any one of claims 35 or 39-45, wherein said analyzing is effected using an antibody that selectively binds to said at least one polypeptide.

47. The method of claim 46, wherein said antibody is attached to a detectable moiety.

48. A method of eliciting or boosting an immune response to a senescent cell in a subject comprising administering to the subject a pharmaceutical composition comprising at least one polypeptide or a polynucleotide encoding same selected from the group consisting of HSP90B1, DBN1, PRKCSH, SPTBN1, NPM1 and a polypeptide set forth in Table 1, wherein said composition does not comprise senescent cells or membranes thereof, thereby eliciting or boosting the immune response to the senescent cell.

49. A vaccine comprising at least one polypeptide or a polynucleotide encoding same as an active agent, said polypeptide being selected from the group consisting of HSP90B1, DBN1, PRKCSH, SPTBN1, NPM1, an adjuvant, wherein said vaccine does not comprise senescent cells or membranes thereof and an immunologically acceptable carrier.

50. A vaccine comprising cells expressing a heterogeneous polypeptide, as an active agent and an immunologically acceptable carrier, said polypeptide being

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selected from the group consisting of HSP90B1, DBN1, PRKCSH, SPTBN1, NPM1.

51. The vaccine of claim 50, further comprising an adjuvant.

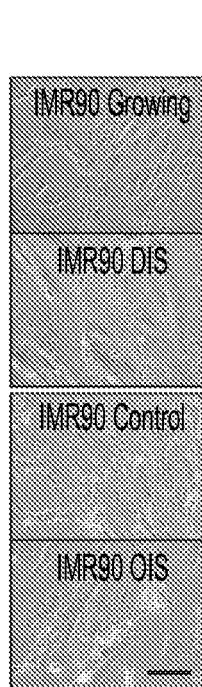


FIG. 1A

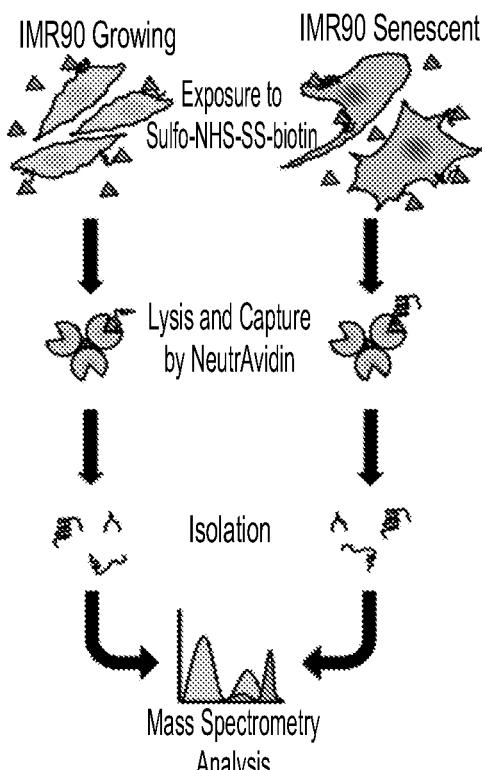


FIG. 1B

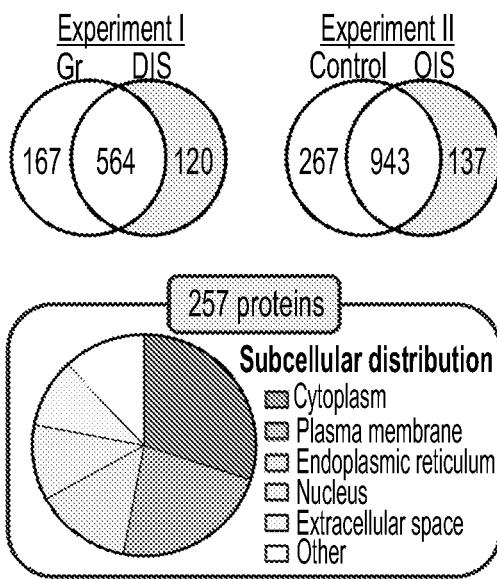


FIG. 1C

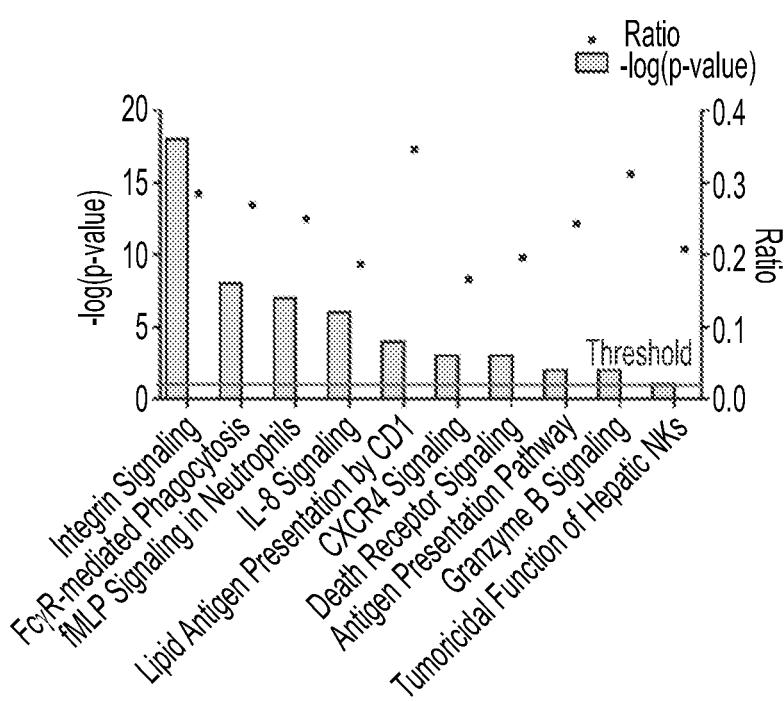


FIG. 1D

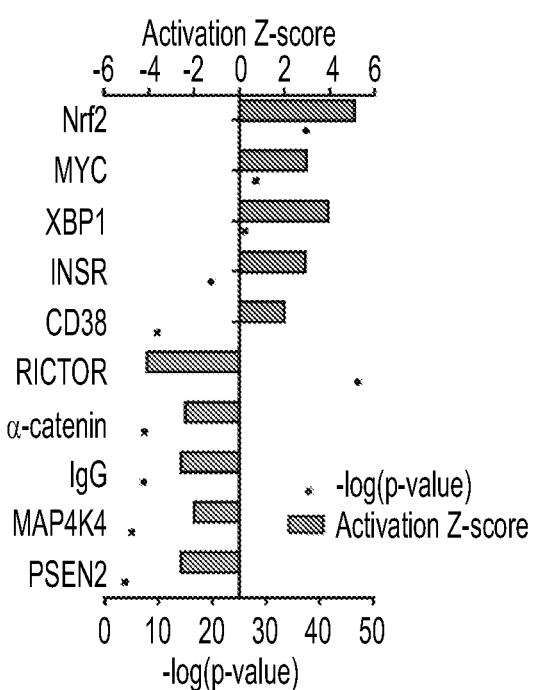
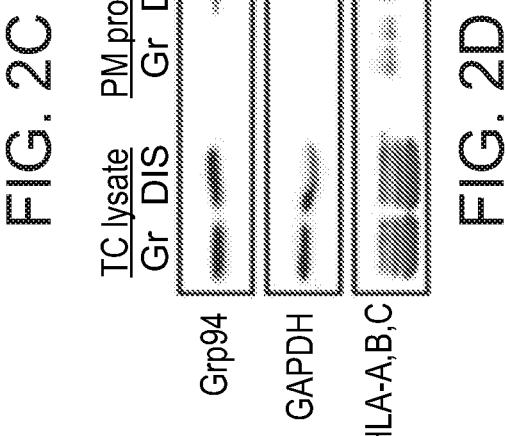
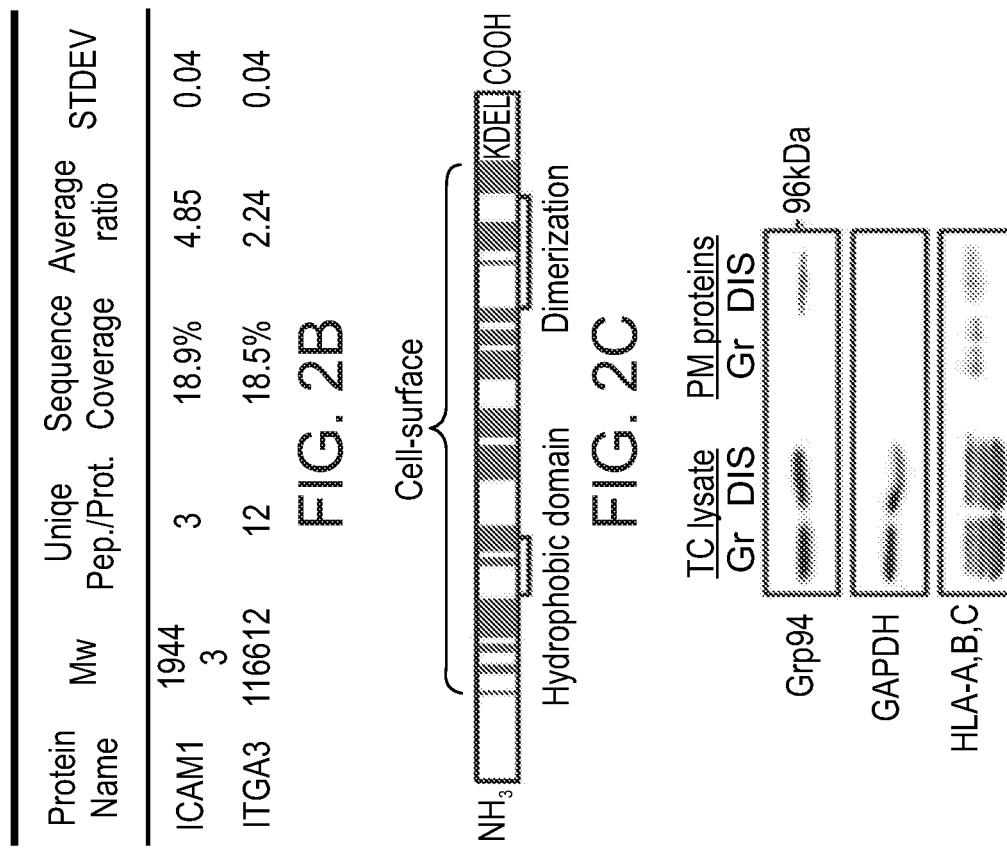
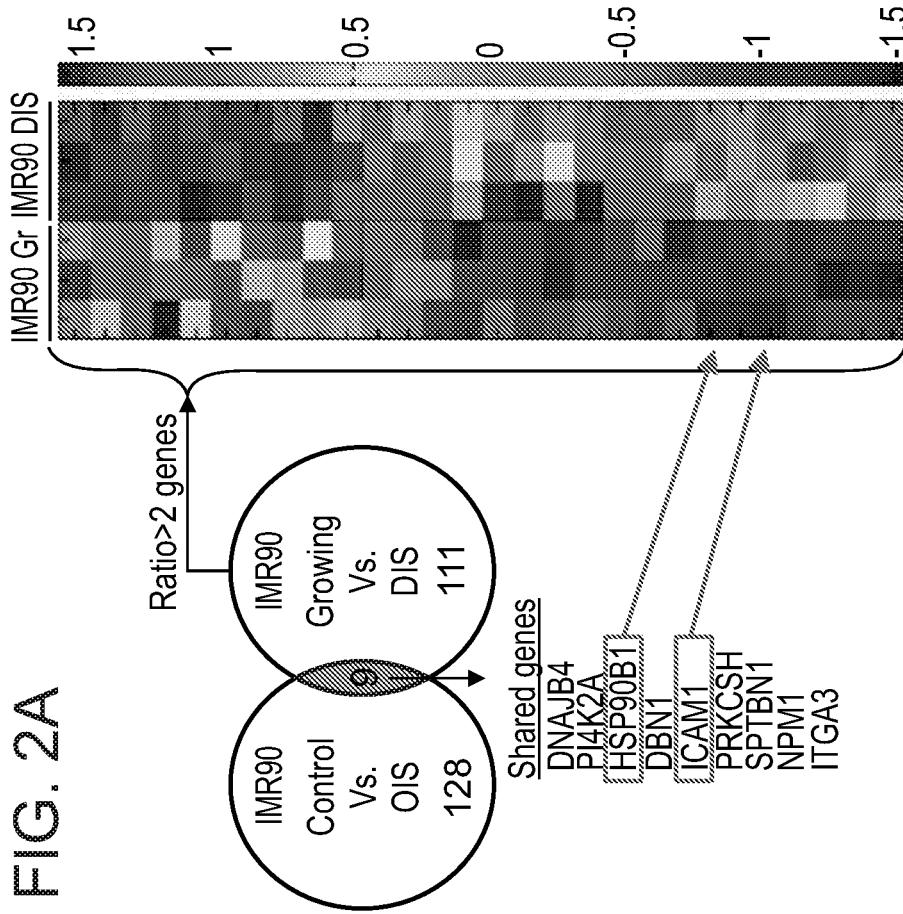
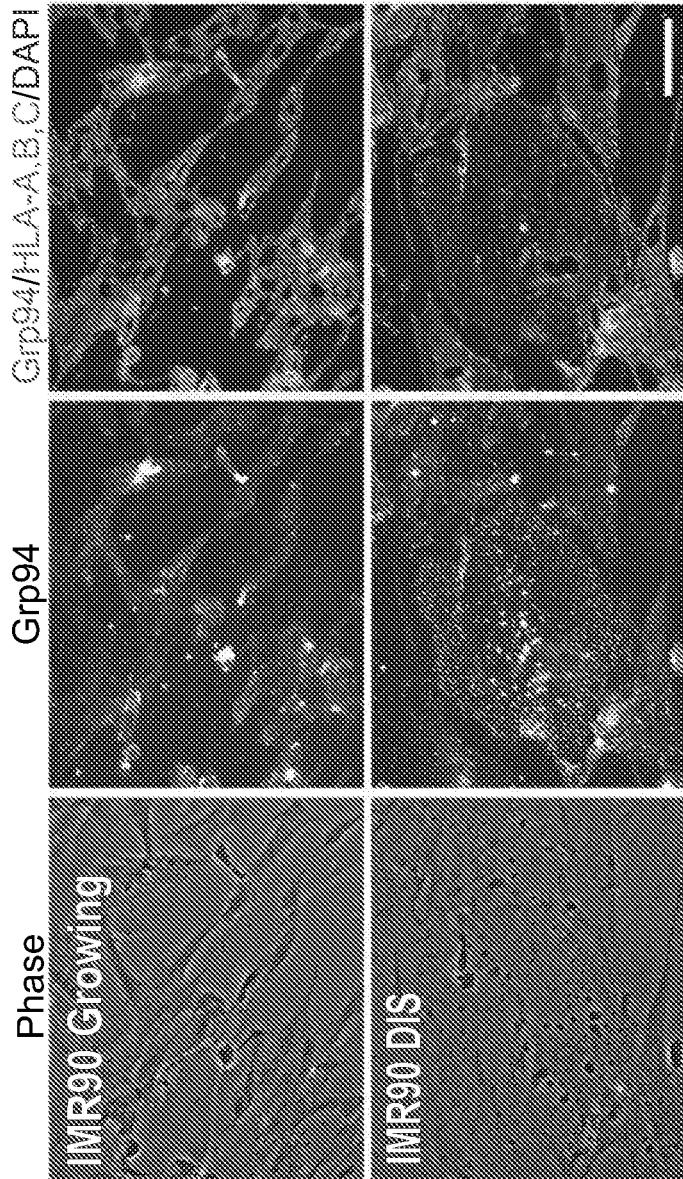
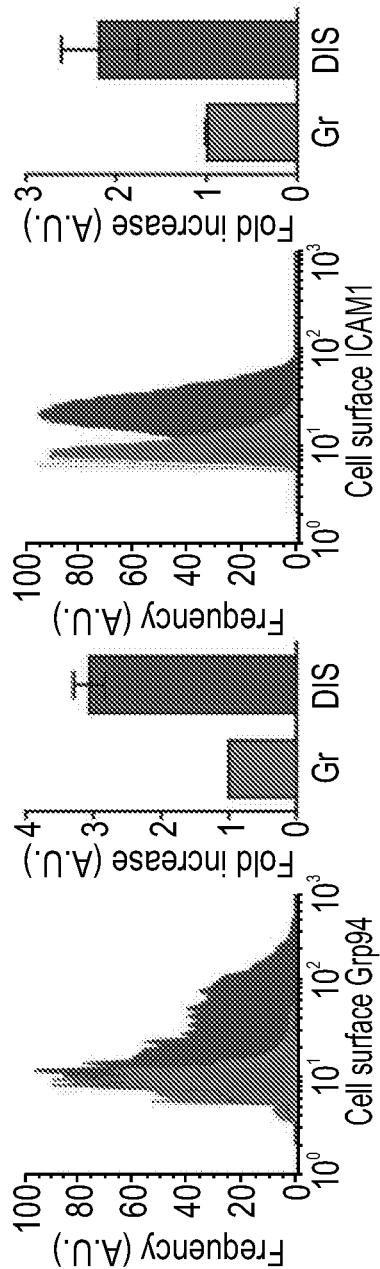


FIG. 1E

**FIG. 2D**



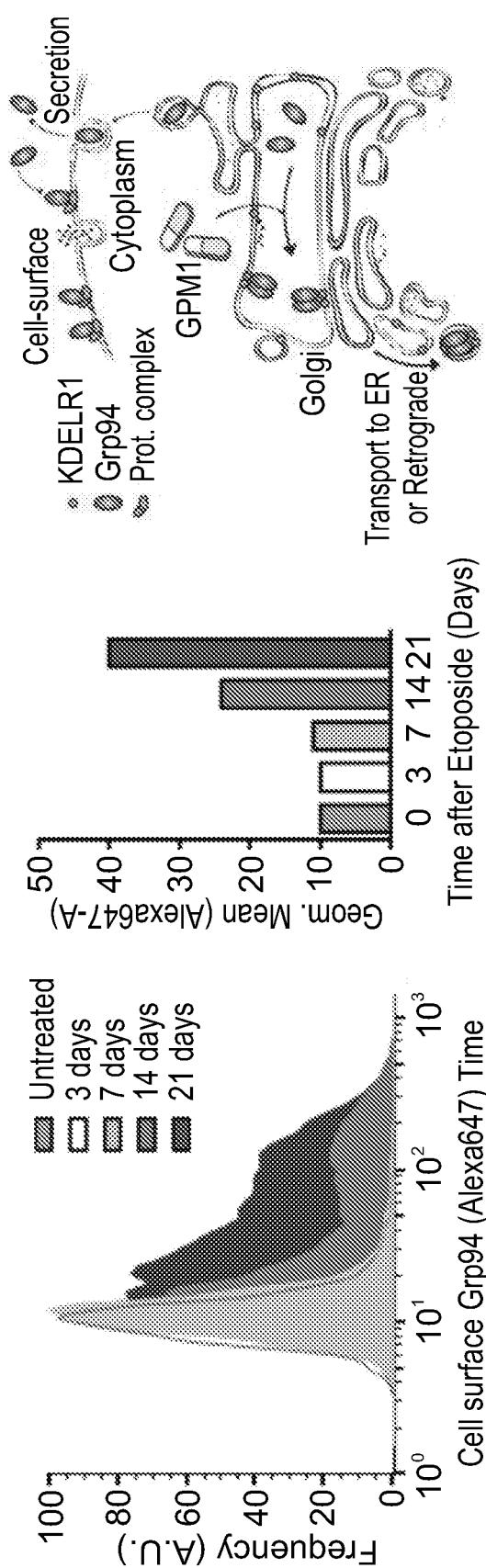


FIG. 3C

FIG. 3B

FIG. 3E

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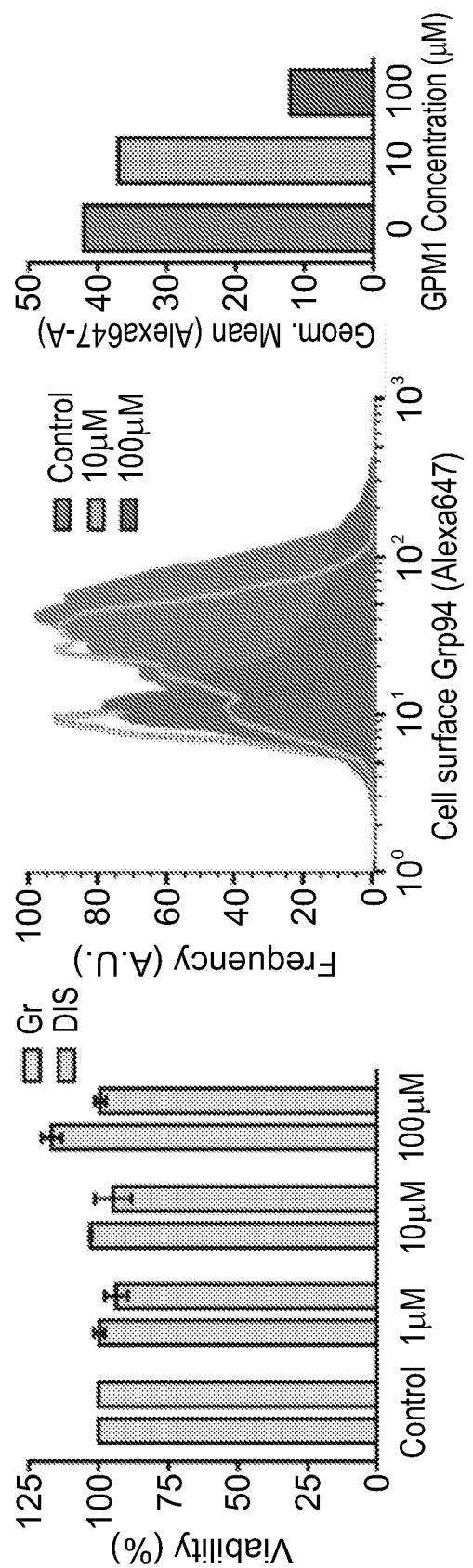
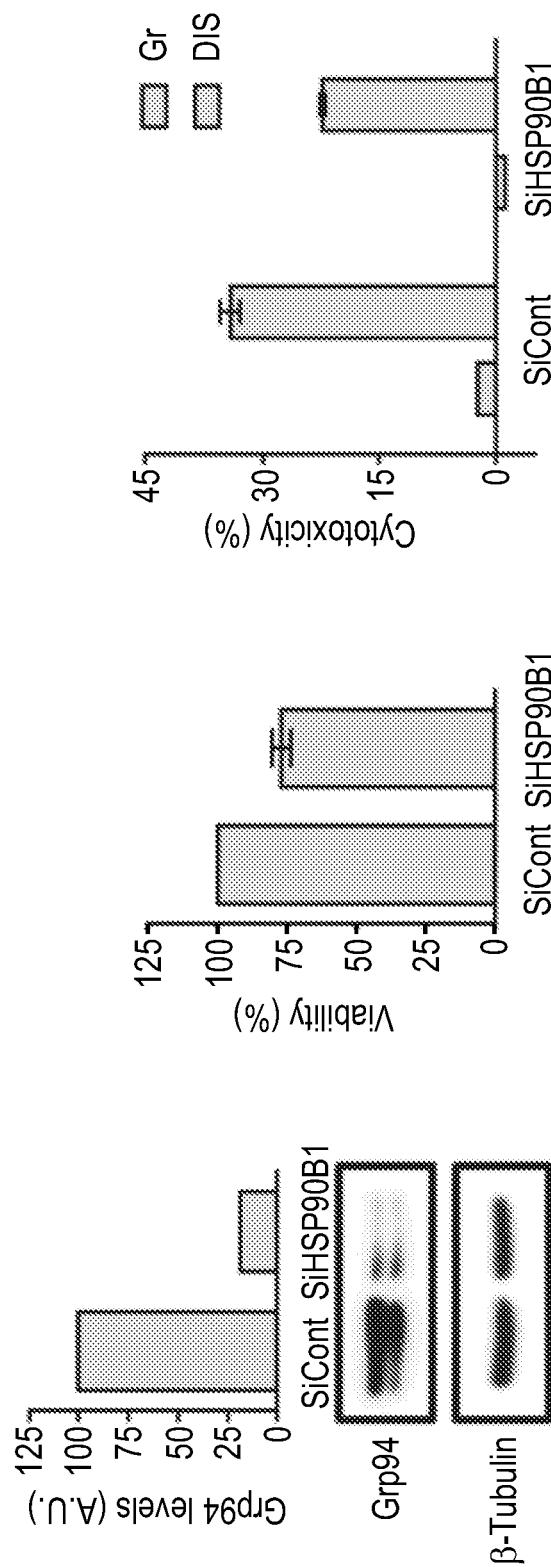


FIG. 3F

FIG. 3E

FIG. 3D



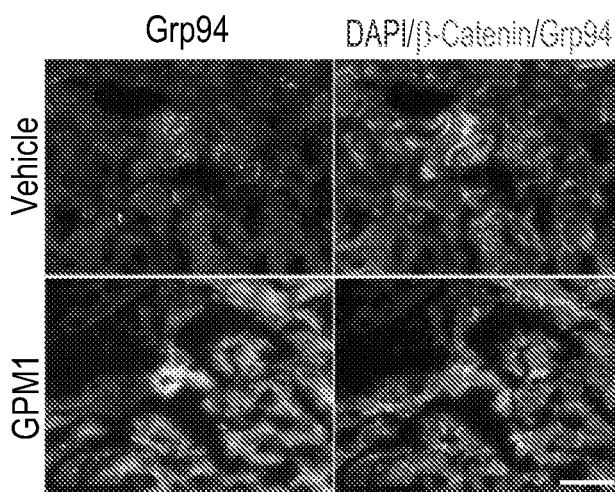


FIG. 5A

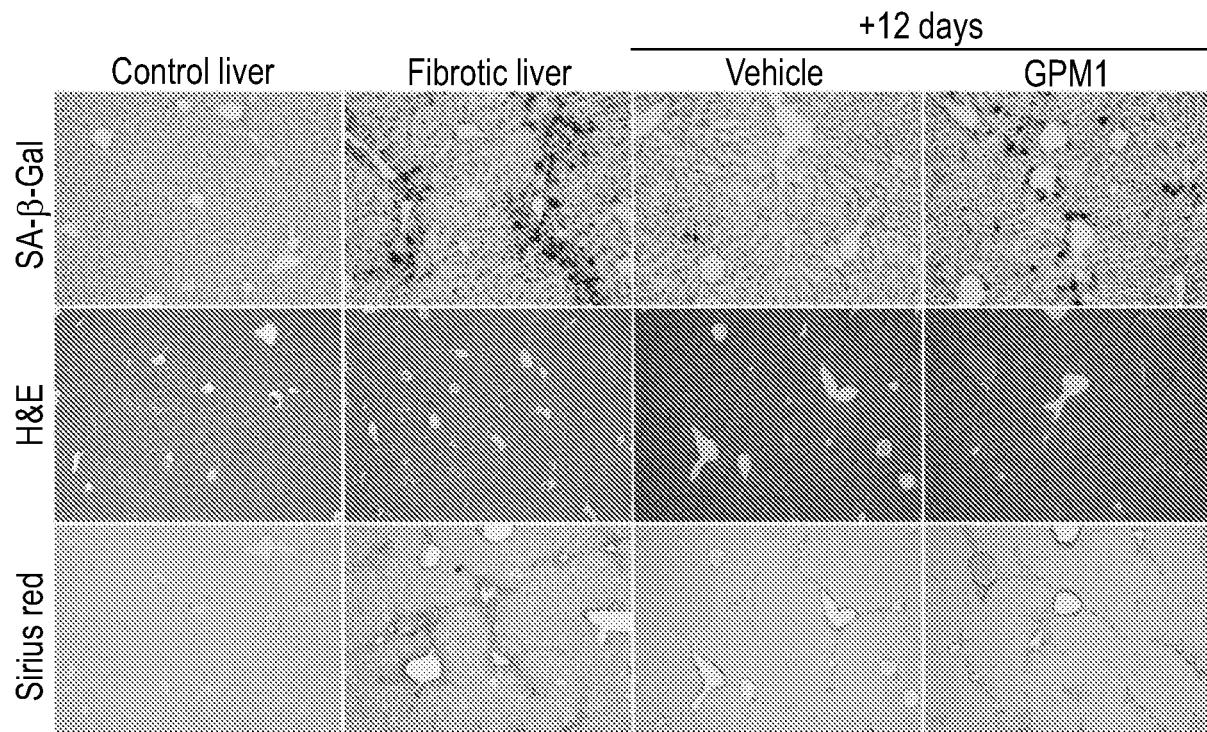


FIG. 5B

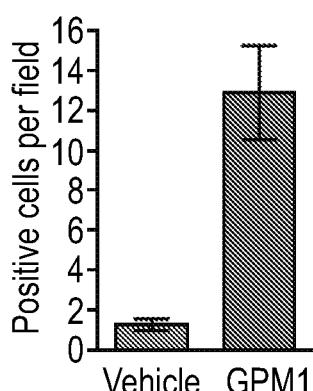


FIG. 5C

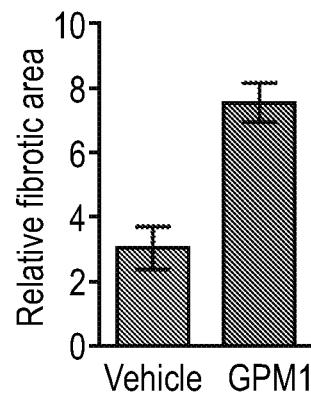


FIG. 5D