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(54) Title: COMPOSITIONS AND METHODS FOR DIAGNOSIS AND TREATMENT OF CENTRAL NERVOUS SYSTEM METASTASES

(57) Abstract: A method of treating or preventing central nervous system (CNS) metastases in a subject afflicted with a CNS metastasizing cancer is provided. The method comprising administering to the subject an agent that down-regulates activity or expression of a polypeptide selected from the group of polypeptides listed in any one of Tables 1-7 herein, thereby treating or preventing CNS metastases. Methods of sensitizing treatment to a CNS metastasizing cancer, methods of detecting CNS metastases and methods of monitoring treatment are also provided. Furthermore, an immunocompetent non-human animal model of CNS metastasizing melanoma, lacking a primary tumor, and methods of generating same are provided.



COMPOSITIONS AND METHODS FOR DIAGNOSIS AND TREATMENT OF CENTRAL NERVOUS SYSTEM METASTASES

FIELD AND BACKGROUND OF THE INVENTION

5 The present invention, in some embodiments thereof, relates to compositions and methods for diagnosis and treatment of central nervous system metastases.

 Brain metastases are cancer cells that have spread to the brain from primary tumors in other organs in the body. Metastatic tumors are among the most common mass lesions in the brain. An estimated 24-45 % of all cancer patients in the United States
10 have brain metastases.

 As primary cancer treatments such as surgery, radiation therapy and chemotherapy have become more effective in the past few decades, people with cancer are living longer after initial treatment than ever before. However, brain metastases still occur in many patients months or even years after their original cancer treatment.

15 The most common sources of brain metastases in a case series of 2,700 patients undergoing treatment at the Memorial Sloan-Kettering Cancer Center were Lung cancer, 48 %, Breast cancer, 15 %, Genitourinary tract cancers, 11 %, Osteosarcoma, 10 %, Melanoma, 9 %, Head and neck cancer, 6%, Neuroblastoma, 5 %, Gastrointestinal cancers, especially colorectal and pancreatic carcinoma, 3 %, Lymphoma, 1 %.

20 Poor prognosis in brain metastasis is mainly attributed to tumor heterogeneity, invasiveness, and drug resistance. Clinically available treatments suffer from deprived pharmacokinetic properties, which lead to severe side effects and low efficacy partly due to inaccessibility through the blood brain barrier (BBB).

 Brain metastases are heterogeneous and harbor multiple cell types. Tumor-associated stroma cells, such as astrocytes, endothelial cells, microglia, peripheral
25 immune cells, and neural precursor cells play a vital role in controlling the course of tumor establishment and progression in the brain. For many tumors, including melanoma, there is a temporal gap between infiltration to distant organs and the capacity to colonize and form macrometastases, implying that disseminated tumor cells
30 need to acquire the ability to “educate” stromal cells in their new microenvironment. Indeed, recent studies have shown that changes in the metastatic microenvironment precede clinically-relevant metastases⁸⁻¹¹.

Astrocytes perform many functions in maintaining central nervous system (i.e. brain and spinal cord) homeostasis. Dysregulation of these cells contributes to the pathogenesis of several diseases, including brain cancers¹². Activated astrocytes were previously shown to surround and infiltrate brain metastases¹³.

5 Neuroinflammation, the immune reaction in the central nervous system (CNS) to tissue damage or pathogen invasion, is characterized by activation of astrocytes and microglia, release of pro-inflammatory cytokines, angiogenesis, increased blood-brain-barrier permeability, and leukocyte infiltration. Sustained inflammation is present in both acute CNS injury and chronic neurodegenerative disorders, while astrocytes are
10 major players in neuroinflammation. Angiogenesis and inflammation are now accepted as hallmarks of cancer. Much data has accumulated on the active role of astrocytes and microglia in producing inflammatory mediators in neurodegenerative and autoimmune diseases in the CNS¹⁴.

One additional important component that determines the metastatic faith of a
15 tumor cell is its angiogenic potential. Endothelial cells that line the blood vessels also respond to growth factors and secrete others that regulate the tumor microenvironment. After a tumor cell extravasates, it can grow along the blood vessels or recruit new blood vessels for angiogenesis. This new source of access to the bloodstream provides oxygen and other nutrients to feed the growth of the distant metastasis.

20 SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of treating or preventing central nervous system (CNS) metastases in a subject afflicted with a CNS metastasizing cancer, the method comprising administering to the subject an agent that down-regulates activity or expression of a
25 polypeptide selected from the group of polypeptides listed in any one of Tables 1-7 herein, thereby treating or preventing CNS metastases.

According to an aspect of some embodiments of the present invention there is provided a n agent that down-regulates activity or expression of a polypeptide selected from the group of polypeptides listed in any one of Tables 1-7 herein, for treating or
30 preventing central nervous system (CNS) metastases in a subject afflicted with a CNS metastasizing cancer.

According to an aspect of some embodiments of the present invention there is provided a method of sensitizing treatment to a CNS metastasizing cancer, the method comprising administering to the subject an agent that down-regulates activity or expression of a polypeptide selected from the group of polypeptides listed in any one of
5 Tables 1-7 herein, and treating the subject with an anti-cancer treatment.

According to an aspect of some embodiments of the present invention there is provided a method of detecting CNS metastases in a subject afflicted with a CNS metastasizing cancer, the method comprising determining the level of at least one polypeptide selected from the group of polypeptides listed in any one of Tables 1-7
10 herein, wherein an upregulation in the level as compared to same in a control sample is indicative of CNS metastases.

According to an aspect of some embodiments of the present invention there is provided a method of monitoring treatment of a CNS metastasizing cancer, the method comprising treating a subject with an anti-cancer treatment and detecting occurrence of
15 CNS metastases according to some embodiments of the invention, thereby monitoring treatment of the CNS metastasizing cancer.

According to an aspect of some embodiments of the present invention there is provided a composition of matter selected from the group consisting of a PGA-NH₂-siMCP-1, PGA-NH₂-siIL-8, PGA-NH₂-siCXCL10, PGA-NH₂-siGRO α , and PGA-NH₂-
20 siSERPINE1, PGA-NH₂-siCXCR1, PGA-NH₂-siCCR4, PGA-NH₂-siCCR2, PGA-NH₂-siCXCR3, PGA-NH₂-siCXCR2 and PGA-NH₂-siuPAR.

According to an aspect of some embodiments of the present invention there is provided a method of generating a non-human animal with a CNS metastasizing melanoma, the method comprising: (a) systemically administering melanoma cells to a
25 non-human animal; (b) allowing growth of a primary melanoma tumor in the non-human animal; (c) surgically removing the primary melanoma tumor; (d) monitoring formation of CNS metastasis; and (e) selecting the non-human animal with a CNS metastasizing melanoma.

According to an aspect of some embodiments of the present invention there is provided an immunocompetent non-human animal comprising a CNS metastasizing
30 melanoma, wherein the non-human animal does not comprise a primary melanoma tumor.

According to some embodiments of the invention, the polypeptide is a secreted polypeptide.

According to some embodiments of the invention, the polypeptide is a cell surface receptor.

5 According to some embodiments of the invention, the polypeptide comprises two or more polypeptides.

According to some embodiments of the invention, the polypeptide is expressed or secreted from a cancer cell.

10 According to some embodiments of the invention, the polypeptide is CXCL10 and/or CXCR3.

According to some embodiments of the invention, the polypeptide is MCP-1, CCR4 and/or CCR2.

According to some embodiments of the invention, the polypeptide is IL-8 and/or CXCR1.

15 According to some embodiments of the invention, the polypeptide is GRO α and/or CXCR2.

According to some embodiments of the invention, the polypeptide is SERPINE1 and/or uPAR.

20 According to some embodiments of the invention, the polypeptide is selected from the group consisting of MCP-1, IL-8, CXCL10, GRO α , SERPINE1, CXCR1, CXCR2, CXCR3, CCR2, CCR4, uPAR, Lipocalin-2 (LCN2), sICAM, MIF, RANTES, IL-6, IL-1 β and CCL17.

According to some embodiments of the invention, the polypeptide is expressed or secreted from a CNS cell.

25 According to some embodiments of the invention, the CNS cell is a glia cell.

According to some embodiments of the invention, the glia cell is an astrocyte.

According to some embodiments of the invention, the polypeptide is CXCL10 or LCN2.

30 According to some embodiments of the invention, determining the level of at least one polypeptide is effected *ex vivo*.

According to some embodiments of the invention, determining the level of at least one polypeptide is effected in a biological sample selected from the group

consisting of a blood sample, a serum sample, urine, cerebro-spinal fluid (CSF), sweat, tears, a saliva and tumor cell sample.

According to some embodiments of the invention, the CNS metastasizing cancer is selected from the group consisting of non-small cell lung cancer (NSCLC), small cell
5 lung cancer (SCLC), breast cancer, mesotheliomas, melanoma, ovarian carcinoma, bladder cancer, renal cancer and colon cancer.

According to some embodiments of the invention, the CNS metastasizing cancer is melanoma.

According to some embodiments of the invention, the agent is a polypeptide
10 agent.

According to some embodiments of the invention, the polypeptide agent is an antibody.

According to some embodiments of the invention, the antibody is a neutralizing antibody.

15 According to some embodiments of the invention, the polypeptide agent is a soluble receptor.

According to some embodiments of the invention, the agent is or comprises a nucleic acid agent.

According to some embodiments of the invention, the nucleic acid agent is an
20 RNA oligonucleotide or a targeted nuclease.

According to some embodiments of the invention, the targeted nuclease is selected from the group CRISPR/Cas-9, zinc finger nuclease, TALEN and a meganuclease.

According to some embodiments of the invention, the RNA oligonucleotide is
25 selected from a mRNA, a micro RNA (miRNA), a small interfering RNA (siRNA) and a tiny noncoding RNA (tnRNA).

According to some embodiments of the invention, the agent is a conjugate which comprises a polymer and a nucleic acid agent associated with the polymer.

According to some embodiments of the invention, the polymer comprises a
30 polyglutamic acid (PGA).

According to some embodiments of the invention, the agent is a siRNA selected from the group consisting of a PGA-NH₂-siMCP-1, PGA-NH₂-siIL-8, PGA-NH₂-siCXCL10, PGA-NH₂-siGRO α , and PGA-NH₂-siSERPINE1.

5 According to some embodiments of the invention, the agent is a siRNA selected from the group consisting of a PGA-NH₂-siCXCR1, PGA-NH₂-siCCR4, PGA-NH₂-siCCR2, PGA-NH₂-siCXCR3, PGA-NH₂-siCXCR2 and PGA-NH₂-siuPAR.

According to some embodiments of the invention, the agent is a small molecule.

According to some embodiments of the invention, the agent is targeted to a melanoma expressed marker.

10 According to some embodiments of the invention, the marker is NCAM or P-selectin.

According to some embodiments of the invention, the agent comprises an NCAM-targeting moiety or a P-selectin-targeting moiety.

15 According to some embodiments of the invention, the agent is formulated for CNS administration.

According to some embodiments of the invention, the agent is formulated for systemic administration.

According to some embodiments of the invention, the agent is formulated for local administration.

20 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
25 examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

30 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 and images 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 and images makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIGs. 1A-E depict mouse models of melanoma brain metastases. Figure 1A, Ret/D4M.3A mCherry, 131/4-5B1 mCherry and patient brain metastases models. Figure 1B, V600E BRAF mutation found in D4M.3A and A375 cell lines; V600G BRAF mutation found in Ret cell line; Normal BRAF sequence found in patient-derived cell lines. Figure 1C, non-invasive imaging of mCherry-labeled melanoma metastases. Figure 1D, Evans Blue extravasates through the BBB in mice with melanoma metastasis. The graph depicts quantification of Evans Blue in normal (grey) and metastatic (red) brains. Figure 1E, melanoma brain metastases proliferate (Ki67), express the melanoma marker MART-1, have activated astrocytes adjacent to the tumor tissue (GFAP) and are highly vascularized (CD31).

FIGs. 2A-L depict detection and molecular quantification of brain micrometastases. Figure 2A, schematic diagram of *ex vivo* micrometastases quantification. Figures 2B-D, quantitative calibration of metastatic load detection. qPCR results for mCherry (Figure 2B), Trp-1 (Figure 2C), and Trp-2 (Figure 2D) in known numbers of cells, as shown in Figure 2A. Representative results of three independent experiments. In all qPCR analyses, results were normalized to Hprt and to the signal of 102 cells. Error bars represent RQ min and max of technical repeats. #, undetected expression. Figure 2E, qPCR analysis of mCherry expression and metastatic load one month after primary tumor excision in injected mice (n = 40) or control mice (n = 8). Figure 2F, metastatic load in a cohort of injected mice (n = 10). Figure 2G, FACS analysis of micrometastases-bearing mouse. Representative plot from four independent cohorts analyzed (n = 21). Figure 2H, summary of injected mice analyzed by FACS for metastatic load, as in Figure 2G. Left y-axis, fold change in number of detected mCherry+ cells per 10,000 events, normalized to control mice (n = 7). Right y-axis, approximate number of cells derived from the calibration curve. Figure 2I, qPCR of mCherry expression at different time points following tumor cell injection, as indicated. Each time point represents the average expression in separate cohorts; n = 4, 3, 4, respectively. Error bars, SEM. *, P < 0.05 (Student t test). Figures 2J-K, fluorescent (Figure 2J) and IHC (Figure 2K) staining in brain tissue sections. Representative images

from multiple fields analyzed in 6 mice. In Figure 2J, scale bar, 25 μm ; Figure 2K, 100 μm . Figure 2L, correlation of qPCR with histology analyses of injected mouse brains; $n = 13$ (Fisher exact test).

FIGs. 3A-H depict that spontaneous formation of brain micrometastases is associated with increased vascular hyperpermeability. Figure 3A, modified Miles assay quantification in normal brains ($n = 7$) or in brains with spontaneous micrometastases ($n = 15$), 1.5 months after tumor excision. Results shown are average of two independent experiments. Error bars, SEM. *, $P < 0.05$ (Student t test). Figure 3B, quantification of FITC-Dextran-labelled blood vessels in brain sections of injected mice ($n = 3$, 92 fields) versus control mice ($n = 3$, 95 fields), 1.5 months after tumor excision. Error bars represent SEM of fields analyzed from each group. **, $P < 0.01$ (Student t test). Figures 3C-E, representative images from multiple fields analyzed from control or injected mice as in Figure 3B. Scale bars, 100 μm . Figures 3F-H, Mart-1 (Magenta) staining depicting melanoma cells near FITC-Dextran-labelled leaky blood vessels. Representative of multiple fields analyzed from control or injected mice as above. In Figures 3F-G, scale bars, 25 μm ; Figure 3H, scale bar, 10 μm .

FIGs. 4A-S depict that astrogliosis is induced in spontaneous melanoma brain metastasis. Figure 4A, macrometastatic lesion in the ventricle. Immunostaining of astrocytes (GFAP, green) in tissue sections of spontaneous brain macrometastases (mCherry, red). Tiling of multiple images. Scale bar, 750 μm . Figure 4B, representative image of tissue sections from human melanoma brain metastases; $n = 4$. Scale bar, 100 μm . Met, macrometastases. Figure 4C, immunostaining, as in Figure 4A, of tissue sections of spontaneous brain micrometastases. Scale bar, 75 μm . Images in Figure 4A and Figure 4C are representative of multiple fields analyzed from 3 mice in each cohort. Figure 4D, qPCR analysis of GFAP expression in normal brains or in brains of injected mice, one month after tumor removal. **, $P < 0.01$ (Student's t -test). Figures 4E-I, qPCR analyses of multiple chemokines and cytokines in total brains of injected mice. $n = 4-6$ mice in each group. Error bars, SEM. *, $P < 0.05$ (Student t test). Con, control mice; micro, injected mice bearing micrometastases. Figure 4J, organotypic co-culture brain slice model. Representative images were taken from the plug-brain slice interface (dashed rectangle). Figure 4K, astrocytes (GFAP, green) and microglia (ILB4, violet) surround, infiltrate, and interact with invasive melanoma cells (mCherry). Scale bar, 50

µm. Figure 4L, astrocyte protrusions penetrate the tumor plug and interact with melanoma cells. Scale bar, 50 µm. Figure 4M, high power magnifications of fields from Figure 4L. Scale bars, 20 µm. Figure 4N, quantification of melanoma cell invasion into brain slices. Data represent percentage of cell invasion after 96-hour co-culture (n = 11).

Figures 4O–S, qPCR analysis of the gliosis-related wound-healing gene signature in astrocytes following incubation with RMS-CM SFM, serum-free medium. Representative results of three independent experiments. Error bars represent SD of technical repeats. **, P < 0.01; *, P < 0.05 (Student t test).

FIGs. 5A–R depict that astrocytes functionally facilitate melanoma brain metastasis. Figure 5A and Figure 5B, representative images from 3D co-cultures in day 1 and day 5. Red, melanoma cells; green, GFP-expressing astrocytes. ii, magnification of image shown in i. Scale bars in Figure 5A, i, 750 µm; scale bar in Figure 5A, ii, 100 µm; in Figure 5B, ii, 250 µm. Figure 5C, quantification of melanoma cell (RMS) number after 3D co-culture with adult primary astrocytes. Representative results of two independent experiments. Experiments were run in duplicate and total of 7 fields/group were analyzed. Error bars, SEM. **, P < 0.01 (Student t test). Figure 5D, representative CT images of brain tumors 5 and 9 days after intracranial inoculation of melanoma cells alone (RMS) or with astrocytes (RMS+AST); lateral (left), superior (middle), and frontal (right) views. Figure 5E, gross anatomical view of the melanotic tumor lesions in RMS (top) or RMS co-injected with astrocytes (bottom). Scale bar, 5 mm. Figure 5F, quantification of tumor volume described in Figure 5D. Results were normalized to control. Representative results of two different cohorts analyzed; n = 16 in each. Error bars, SEM. **, P < 0.01. Figure 5G and Figure 5H, representative immunostaining pictures of astrocytes (GFAP, green) around tumor lesion (mCherry, red) in brains injected with RMS only (left) or in co-injected brains (right). Scale bars, 100 µm. Figure 5I, quantification of total brain GFAP immunostaining in RMS only or in co-injected mice, compared with normal mice injected with SFM as control (CON). n = 3 in each group. Fifteen serial sections per mouse in CON, RMS, and RMS+AST from different brain regions were analyzed. A total of 37, 47, and 27 fields per group were quantified, respectively. Error bars, SEM. **, P < 0.01; *, P < 0.05. Figures 5J–M, the MAPK signaling pathway is activated in brain-tropic melanoma cells (BT-RMS) cells. Figure 5J, RMS or BT-RMS cells were incubated in SFM for 48 hours. pERK activation was

analyzed by Western blot analysis. Figure 5K, quantification of band intensity shown in Figure 5J. Results were normalized to total ERK (tERK) and to RMS. Figure 5L, the MAPK signaling pathway is activated in melanoma cells by astrocytes. Western blot analysis of RMS cells at different time points following incubation with activated astrocytes CM. Figure 5M, quantification of band intensity shown in Figure 5L; results were normalized to tERK and to SFM. Figures 5J–M are representative of two independent experiments. Figures 5N–R, qPCR of gliosis genes in total brain. Results were normalized to Hprt. $n = 3$ mice in each group. Error bars, SEM. *, $P < 0.05$.

FIGs. 6A-B depict that normal astrocytes are activated by melanoma cells to express and secrete pro-inflammatory cytokines. Figure 6A, Normal astrocytes were incubated for 24 hours with CM of Ret-melanoma cells. Astrocytes were lysed, and a total of 200 μ g from each sample was hybridized with the mouse cytokine array panel (R&D). Results were quantified by the ImageJ software. Box = CXCL10. $N = 2$. Figure 6B, astrocytes were incubated for 12 hours with RMS-CM, then washed with PBS, and incubated with SFM for 24 hours. ELISA assay was performed to determine CXCL10 secretion levels. Error bars represent SD of two independent experiments.

FIGs. 7A-D depict that metastases-associated astrocytes express CXCL10. Figures 7A-C, confocal images (Leica sp8) of tissue sections co-stained with GFAP and CXCL10 from Figures 7A-B, spontaneous brain metastases bearing mice, or Figure 7C, macrometastases via intra-cranial injections. Images are representative of multiple fields analyzed from two different mice from each treatment group. Scale bar $s = 5, 25$, and 75μ m respectively. Inset shows larger magnification. Figure 7D, qPCR analysis of Cxcl10 on MAA sorted from intracranial macrometastases bearing mice (pool of 3 mice). Results are representative of two independent experiments. Error bars represent SD of technical repeats. ** $P < 0.01$.

FIGs. 8A-B depict that CXCL10 is highly expressed in serum of brain metastases bearing mice. Figure 8A, a cohort of injected mice was analyzed 2 months after tumor removal, to identify micrometastases bearing mice. FACS analysis of fresh, demyelinated, brain single cell suspensions was utilized to determine the presence of mCherry+ cells in each sample. 3/6 injected mice were found positive, as their % of mCherry cells was more than 1.5 % (approximately 103 cells). Error bars, SEM,

*P<0.05. Figure 8B, representative ELISA quantification of CXCL10 serum levels in the corresponding brains from Figure 8A. Error bars, SEM, *P<0.05.

FIGs. 9A-B depict that Astrocytes facilitate melanoma cell migration at least partially via CXCL10. Figure 9A, transwell migration assay: 50,000 melanoma cells were seeded in the upper chamber and 100,000 astrocytes in the lower chamber. Migration was analyzed after for 48 hours. n=2, Error bars represents SD of two independent experiments Figure 9B, representative images of the quantification shown in Figure 9A.

FIGs. 10A-B depict that CXCR3 is differentially expressed in melanoma cells and correlates with brain metastatic potential. Figure 10A, qPCR analysis of the different metastatic variants, *in vitro*. Representative results from two independent experiments, error bars represent RQ min and max of technical repeats. Figure 10B, Geo data set GSE46517 was analyzed for the expression of CXCR3 in melanoma primary tumors.

FIGs. 11A-C depict that BT-RMS tumors show enriched CXCR3+ expression. FACS analysis of 500,000 cells from single cell suspensions of RMS (Figure 11A), BT-RMS (Figure 11B) or lung tropic variant was lower (LT-RMS) (Figure 11C) primary melanoma tumors, respectively. The gated CD45-CXCR3+ cells are circled in each panel, and the percentages of CXCR3+ cells calculated are shown. Results are representative from three tumors analyzed in each group.

FIGs. 12A-B depict that the BT-RMS variant shows enriched CXCR3+ sub-population *in vitro*. FACS analysis of melanoma cells from culture using CXCR3-APC, n=3. Of note, cells were collected from plates using EDTA rather than trypsin to prevent epitope degradation.

FIGs. 13A-E depict that knockdown of CXCR3 in RMS cells suppresses their migration toward astrocytes *in vitro* and attenuates micrometastasis formation *in vivo*. Figure 13A, qPCR analysis of RMS transduced cells. Representative results of two independent experiments, error bars represent RQ min and max of technical repeats. Figure 13B, migration assay with the KD variants was performed to functionally validate CXCR3 silencing (data shown for sh-2 variant as representative). SC-RMS, scramble controls. Scale bars = 100µm. Figure 13C, 500,000 cells of SC-RMS, Sh-1 or sh-2 lines were subdermally injected and primary tumor growth kinetics was documented. n=15 in each group, error bars represent SEM. Figures 13D-E, primary

tumors were removed when reached 1.5 cm in any diameter. After 2 months, surviving mice were euthanized and heart perfused, their brains harvested and analyzed for the presence of micrometastases by qPCR for TRP-2, a known marker of melanoma.

FIGs. 14A-D depict that LCN2 is expressed and secreted by brain-tropic melanoma cells at early disease stage. Figure 14A, melanoma cells were grown as monolayers in 10 cm plates. When confluent, the cells were lysed and RNA extraction was performed followed by qPCR analysis for Lcn2. Results were normalized to Hprt and to the Ret parental cells basal expression. Result from two independent experiments. Error bars represent RQ min and max of technical repeats. Figure 14B, the different melanoma variants were incubated for 48 hours in SFM and subjected to ELISA assay for LCN2. Results are relative secretion normalized to the RMS. Melanoma cells express Lcn2 at an early disease stage. Figure 14C, publically available GEO data set #GSE46517 of primary vs. metastatic melanoma was analyzed for Lcn2 expression. One-way ANOVA was performed to statistically test the results. Error bar represents SD from the median expression, * $p < 0.05$, *** $p < 0.01$. Figure 14D, ELISA assay for LCN2 serum levels, in injected mice 2 months after tumor removal. $n=3$ in each group.

FIGs. 15A-B depict that LCN2 is expressed in human melanoma primary tumor cells and in the microenvironment. Publically available stainings of melanoma patients ($n=11$) from the Human Protein Atlas were quantified for positive tumor or stromal LCN2 (Figure 15A) or IFN γ (Figure 15B) expressing cells and compared to normal skin as control ($n=3$). Right panels, representative brown stainings from the cohort quantified.

FIGs. 16A-C depict that CXCL10 inducers are mainly stromal derived. Figures 16A-B, schematic diagrams of the sorting experiment accompanied by dot plots and density plot of the different cell populations analyzed. Figure 16A, sorted cells from the normal ear skin (pool of 12 ears) was used as control. Figure 16B, immune cells, fibroblast and tumor cells were isolated from melanoma tumors (pool of 6 tumors). Figure 16C, qPCR analysis for the known CXCL10 inducers LCN2, IL-1 β and IFN γ . Representative of two independent experiments. NDF = Normal dermal fibroblasts, CAFs = cancer associated fibroblasts, N = normal, TA = tumor associated.

FIGs. 17A-B depict that LCN2 and IL-1 β instigate Cxcl10 expression in astrocytes. Figures 17A-B, 105 astrocytes were seeded in 12 well plates, and either incubated in SFM, CM, or with the indicated recombinant or neutralizing antibodies. Representative of two independent experiments, error bars represent SD of biological repeats.

FIGs. 18A-I depict crosstalk between astrocytes and melanoma cells. Figure 18A, cytokine change profile in melanoma cells during starvation; Figure 18B-C, astrocytes grown in primary (Figure 18B) and metastating (Figure 18C) melanoma conditioned media secret elevated levels of cytokines and chemokines; Figure 18D, melanoma cells migrate towards astrocytes conditioned media; Figure 18E, melanoma cell migration towards astrocyte conditioned media supplemented with cytokine neutralizing antibodies for 24 hours. Of note, melanoma cells migration was decreased when neutralizing GRO α , IL-8, MCP-1 and SERPINE1. Figure 18F-G, validation of Cytokine Proteome Array by qRT-PCR. Figure 18H, CCR4, and Figure 18I, CXCR1 expression in stressed or normal astrocytes, 131/4-5B1 and A375 cells. Data represent average $\pm$ SD.

FIG. 19 depicts that normal astrocytes are activated by melanoma cells to express pro-inflammatory cytokines. Normal astrocytes were incubated for 24 hours with CM of ret-melanoma cells. Astrocytes were lysed, and a total of 200 μ g from each sample was hybridized with the mouse cytokine array panel (R&D). Results were quantified by the ImageJ software.

FIGs. 20A-D depict that treating astrocytes with PGA-NH₂-IL-8/MCP-1 siRNA polyplex downregulates the cytokine expression. Figure 20A, chemical structures of PGA-amine, Figure 20B, electrophoretic mobility shift assay (EMSA) evaluation of the nanocarrier capacity to bind siRNA and the optimal ratio PGA/siRNA, Figure 20C and Figure 20D, qRT-PCR analysis verifying IL-8 and MCP-1 knockdown in human astrocytes by the polyplex PGA-siRNA (50 nM siRNA equivalent dose). Data represent means $\pm$ S.D.

FIGs. 21A-B depict MCP-1 levels in human astrocytes following siRNA treatment. Figure 21A, astrocytes were cultured either with or without melanoma cells and treated with MCP-1 siRNA. Of note, MCP-1 levels were significantly decreased

after treatment. Figure 21B, MCP-1 secreted protein levels post treatment were measured. Of note, 500 nM MCP-1 siRNA decreased significantly the protein levels.

FIGs. 22A-F depict intra-cardiac injection of mCherry-labeled 131/4-5B1 cells to mice left ventricle. Brain metastases formation was followed by intra-vital imaging.

5 When a fluorescence signal was found, mice were euthanized and the brain was stained for several markers: Figure 22A, H&E staining showed micrometastasis. Figure 22B, the micrometastasis were highly proliferative as seen by Ki67 immunohistochemistry. Figures 22C-D, the brain microenvironment surrounding the micrometastasis was activated as seen by the positive GFAP (Figure 22C) and IBA-1 (Figure 22D) stainings. 10 Figure 22E, P-Selectin was positively stained in the micrometastasis. Figure 22F, MCP-1 secretion was elevated around the micrometastases.

FIGs. 23A-B depict the permeability in melanoma brain metastasis. Figure 23A, quantitation of Dextran-FITC extravasation through endothelial monolayer following incubation with CM (Excitation=480 nm, Emission=520 nm). Figure 23B, quantitation 15 of Evans Blue extravasation in mice brains. Dye contents in the brain were analyzed by measuring fluorescence signals (Excitation=620 nm, Emission=680 nm).

FIGs. 24A-B depict that P-selectin and NCAM are expressed in melanoma. Figure 24A, FACS analysis of A375, B16-F10, D4M.3A and Ret cell lines expressing P-selectin on cell membranes. Figure 24B, NCAM is highly expressed in primary 20 melanoma cell line WM115, in the metastases cell lines WM239 and in 131/4-5B1 cells.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to compositions and methods for diagnosis and treatment of central nervous system metastases.

25 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.

The major cause of melanoma mortality is metastasis to distant organs, 30 frequently to the brain, despite aggressive treatment, including surgical resection and post-operative radiation therapy. Systemic metastases, including those to the brain are usually associated with limited survival of under a year.

Brain metastases are heterogeneous and harbor multiple cell types. Tumor-associated stroma cells, such as astrocytes, endothelial cells, microglia, peripheral immune cells, and neural precursor cells play a vital role in controlling the course of tumor establishment and progression in the brain. Dysregulation of astrocytes contributes to the pathogenesis of several diseases, including brain cancers and activated astrocytes were previously shown to surround and infiltrate brain metastases. Furthermore, astrocytes and microglia play an active role in producing inflammatory mediators in neurodegenerative and autoimmune diseases in the CNS.

While reducing the present invention to practice, the present inventors have designed novel methods for early prognosis of brain metastases as well as effective therapies for brain metastasis which can be used to overcome ineffectiveness and drug resistance of current therapies. These are effected by (1) targeting multiple biological pathways in order to prevent activation of compensating mechanisms, and (2) interrupting the interactions between the tumor cells and the brain microenvironment.

To unravel the targets, the present inventors have developed a novel model for melanoma brain metastases. This model enabled performing molecular and functional characterization of brain metastases including evaluation of mutations, expression of specific markers, crosstalk between brain metastasis cells and their stroma, invasiveness and passage through the blood brain barrier (BBB). This model led to the identification and validation of brain stroma cells (astrocyte, brain endothelium and microglia)-secreted factors, including IL-8, MCP-1, CXCL10, GRO α , SERPINE1, which facilitate brain colonization and invasiveness of melanoma cells. Furthermore, according to present teachings, these factors and their respective receptors can be targeted, e.g. by nucleic acid agents, by small molecule inhibitors, by antibodies or by soluble receptors (dominant negatives), for prevention or treatment of CNS metastases.

The present inventors have further utilized several polymeric nanocarriers for RNA interference [RNAi, e.g. small interfering RNA (siRNA) and microRNA (miRNA)], including polyaminated polyglycerol-based hyperbranched polymer (PG-NH₂)¹⁶ and polyaminated polyglutamic acid (PGA-NH₂)^{17,18}. These polymer-based nanocarriers improve the pharmacokinetic profile of RNAi prolonging its circulation and selective accumulation at the brain metastatic site. Specifically, the present inventors developed PGA-NH₂-cytokine siRNA polyplexes (i.e. of IL-8/MCP-

1/CXCL10/GRO α /SERPINE1) and/or siRNA polyplexes of the respective receptors (CXCR1/CCR4/CXCR3/CXCR2/uPAR). Target validation of these nanocarriers was evaluated *in vivo* using the selected inhibitors on murine and human mouse models of melanoma brain metastasis.

5 Thus, according to one aspect of the present invention there is provided a method of treating or preventing central nervous system (CNS) metastases in a subject afflicted with a CNS metastasizing cancer, the method comprising administering to the subject an agent that down-regulates activity or expression of a polypeptide selected from the group of polypeptides listed in any one of Tables 1-7 herein, thereby treating or preventing
10 CNS metastases.

 According to another aspect of the invention, there is provided an agent that down-regulates activity or expression of a polypeptide selected from the group of polypeptides listed in any one of Tables 1-7 herein, for treating or preventing central nervous system (CNS) metastases in a subject afflicted with a CNS metastasizing
15 cancer.

 According to another aspect of the invention, there is provided a method of sensitizing treatment to a CNS metastasizing cancer, the method comprising administering to the subject an agent that down-regulates activity or expression of a polypeptide selected from the group of polypeptides listed in any one of Tables 1-8
20 herein, and treating the subject with an anti-cancer treatment.

 As used herein, the term “treating” includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition (i.e. CNS metastasizing cancer), substantially ameliorating clinical or aesthetical symptoms of a condition (i.e. CNS metastasizing cancer) or substantially preventing the appearance of clinical or aesthetical
25 symptoms of a condition (i.e. CNS metastasizing cancer).

 As used herein, the term “preventing” refers to a postponement of development of a condition (i.e. CNS metastasizing cancer) and/or a reduction in the number of metastases which are expected to develop in a CNS of a subject (as compared to a subject not being treated with the agents of the invention).

30 As used herein, the phrase “sensitizing treatment” refers to increasing the sensitivity to treatment (e.g. to an anti-cancer treatment, as discussed in detail below) by

at least about 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % or 100 % as compared to a subject not treated by the agents of the invention.

As used herein, the phrase "central nervous system (CNS) metastases" or "CNS metastasizing cancer" refers to the spread of a metastasizable tumor (e.g. primary tumor) from one part of the body (e.g. skin, lung, breast, liver, pancreas, etc) to the central nervous system (i.e. brain or spinal cord).

The CNS metastasizing tumor is also referred to as a non-neural cancer.

The phrase "non-neural cancer" as used herein, refers to a cancer of cells from a non-neural origin.

Cancer cells may be associated with phenotypes such uncontrolled proliferation, loss of specialized functions, immortality, significant metastatic potential, significant increase in anti-apoptotic activity, rapid growth and proliferation rate, and certain characteristic morphology and cellular markers.

In some circumstances, cancer cells are in the form of a tumor, such cells may exist locally within an animal (e.g. solid tumor), alternatively, cancer cells may circulate in the blood stream as independent cells, for example, leukemic cells (non-solid tumor), or may be dispersed throughout the body (e.g. metastasis). It will be appreciated that the term cancer as used herein encompasses all types of non-CNS e.g., non-neural cancers, at any stage and in any form.

According to one embodiment, the cancer is an adenocarcinoma or a squamous cell carcinoma.

According to a one embodiment, the non-neural cancer includes, but is not limited to, lung cancer e.g., non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), breast cancer, mesotheliomas, melanoma, ovarian carcinoma, bladder cancer, renal cancer and colon cancer.

According to a specific embodiment, the non-neural cancer (e.g. CNS metastasizing cancer) is melanoma. Exemplary types of melanoma include, but are not limited to, Lentigo maligna, Lentigo maligna melanoma, Superficial spreading melanoma, Acral lentiginous melanoma, Mucosal melanoma, Nodular melanoma, Polypoid melanoma, Desmoplastic melanoma, Amelanotic melanoma, Soft-tissue melanoma, Melanoma with small nevus-like cells, Melanoma with features of a Spitz nevus and Uveal melanoma.

Additionally, exemplary cancerous diseases which can be treated using the methods of the present invention include, but are not limited to, tumors of the gastrointestinal tract (colon carcinoma, rectal carcinoma, colorectal carcinoma, colorectal cancer, colorectal adenoma, hereditary nonpolyposis type 1, hereditary
 5 nonpolyposis type 2, hereditary nonpolyposis type 3, hereditary nonpolyposis type 6; colorectal cancer, hereditary nonpolyposis type 7, small and/or large bowel carcinoma, esophageal carcinoma, tylosis with esophageal cancer, stomach carcinoma, pancreatic carcinoma, pancreatic endocrine tumors), endometrial carcinoma, dermatofibrosarcoma protuberans, gallbladder carcinoma, Biliary tract tumors, prostate cancer, prostate
 10 adenocarcinoma, renal cancer (e.g., Wilms' tumor type 2 or type 1), liver cancer (e.g., hepatoblastoma, hepatocellular carcinoma, hepatocellular cancer), bladder cancer, embryonal rhabdomyosarcoma, germ cell tumor, trophoblastic tumor, testicular germ cells tumor, immature teratoma of ovary, uterine, epithelial ovarian, sacrococcygeal tumor, choriocarcinoma, placental site trophoblastic tumor, epithelial adult tumor,
 15 ovarian carcinoma, serous ovarian cancer, ovarian sex cord tumors, cervical carcinoma, uterine cervix carcinoma, small-cell and non-small cell lung carcinoma, nasopharyngeal, breast carcinoma (e.g., ductal breast cancer, invasive intraductal breast cancer, sporadic; breast cancer, susceptibility to breast cancer, type 4 breast cancer, breast cancer-1, breast cancer-3; breast-ovarian cancer), leukemia (e.g., acute lymphatic, acute lymphoblastic,
 20 acute lymphoblastic pre-B cell, acute lymphoblastic T cell leukemia, acute - megakaryoblastic, monocytic, acute myelogenous, acute myeloid, acute myeloid with eosinophilia, B cell, basophilic, chronic myeloid, chronic, B cell, eosinophilic, Friend leukemia, granulocytic or myelocytic, hairy cell, lymphocytic, megakaryoblastic, monocytic, monocytic-macrophage, myeloblastic, myeloid, myelomonocytic, plasma
 25 cell, pre-B cell, promyelocytic, subacute, T cell, lymphoid neoplasm, predisposition to myeloid malignancy, acute nonlymphocytic leukemia), lymphosarcoma, lymphomas (e.g., Hodgkin's disease, non-Hodgkin's lymphoma, B cell, Burkitt, cutaneous T cell, histiocytic, lymphoblastic, T cell, thymic), melanoma, mammary tumor, multiple myeloma, sarcoma (e.g., Ewing's, histiocytic cell, Jensen, osteogenic, reticulum cell),
 30 testicular tumor, thymoma and trichoepithelioma,

As used herein, the term "subject" or "subject in need thereof" refers to a animal, e.g. mammal, e.g., human subject, male or female at any age, who has been

diagnosed with a cancer (e.g. non-neuronal cancer including CNS metastasizing cancer).

The methods of some embodiments of the invention are effected by administering to a subject an agent that down-regulates an activity or expression of a polypeptide listed in any one of Tables 1-7.

Tables 1-7: polypeptide targets

Table 1: Cytokines secreted from primary Melanoma cells

Target	Full term
CCL2/MCP-1	C-C Motif Chemokine Ligand 2 (Monocyte Chemotactic Protein 1)
IL-8	Interleukin 8
CXCL10	C-X-C Motif Chemokine Ligand 10
CXCL1/GRO α	C-X-C Motif Chemokine Ligand 1
SerpinE1/PAI-1	Serpin Family E Member 1
MIF	Macrophage Migration Inhibitory Factor
Lcn2	Lipocalin 2
Timp-1	TIMP Metalloproteinase Inhibitor 1
IL-6	Interleukin 6
CXCL12/SDF1	C-X-C Motif Chemokine Ligand 12 (Stromal Cell-Derived Factor 1)
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
ICAM-1/CD54	Intercellular Adhesion Molecule 1
RANTES	CC Motif Chemokine Ligand 5 (Small Inducible Cytokine A5)

Table 2: Cytokines secreted from primary melanoma cells elevated in response to stress (starvation medium)

Target	Full term
CCL2/MCP-1	C-C Motif Chemokine Ligand 2 (Monocyte Chemotactic Protein 1)
CXCL1/GRO α	C-X-C Motif Chemokine Ligand 1
MIF	Macrophage Migration Inhibitory Factor
ICAM-CD54	Intercellular Adhesion Molecule 1

Table 3: Cytokines secreted from melanoma brain metastases cells

Target	Full term
CCL2/MCP-1	C-C Motif Chemokine Ligand 2 (Monocyte Chemotactic Protein 1)
IL-8	Interleukin 8
CXCL10	C-X-C Motif Chemokine Ligand 10

CXCL1/GRO α	C-X-C Motif Chemokine Ligand 1
SerpinE1/PAI-1	Serpin Family E Member 1
CXCL12/SDF1	C-X-C Motif Chemokine Ligand 12 (Stromal Cell-Derived Factor 1)
Lcn2	Lipocalin 2
Timp-1	TIMP Metalloproteinase Inhibitor 1
ICAM-1/CD54	Intercellular Adhesion Molecule 1
MIP-1 α /MIP-1 β	C-C Motif Chemokine Ligand 3/C-C Motif Chemokine Ligand 4
MIF	Macrophage Migration Inhibitory Factor

Table 4: Cytokines secreted from Astrocytes

Target	Full term
MCP-1	Monocyte Chemotactic Protein 1
IL-8	Interleukin 8
CXCL10	C-X-C Motif Chemokine Ligand 10
CXCL1/GRO α	C-X-C Motif Chemokine Ligand 1
SerpinE1/PAI-1	Serpin Family E Member 1
MIF	Macrophage Migration Inhibitory Factor
IL-6	Interleukin 6
CXCL12/SDF1	C-X-C Motif Chemokine Ligand 12 (Stromal Cell-Derived Factor 1)
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
ICAM-1/CD54	Intercellular Adhesion Molecule 1
CCL2/MCP-1	C-C Motif Chemokine Ligand 2 (Monocyte Chemotactic Protein 1)
MIP-1 α /MIP-1 β	C-C Motif Chemokine Ligand 3/C-C Motif Chemokine Ligand 4
C5/C5a	Complement C5
IL-32a	Interleukin 32
RANTES	CC Motif Chemokine Ligand 5 (Small Inducible Cytokine A5)
IL-17	Interleukin 17
IL-1 β	Interleukin 1 beta

Table 5: Receptors expression elevated on melanoma brain metastases cells (validated)

Target	Full term
CXCR1	C-X-C Motif Chemokine Receptor 1
CCR2	C-C Motif Chemokine Receptor 2
CCR4	C-C Motif Chemokine Receptor 4

Table 6: Receptors expression elevated on primary melanoma cells (validated)

Target	Full term
CXCR1	C-X-C Motif Chemokine Receptor 1
CCR4	C-C Motif Chemokine Receptor 4

Table 7: Receptors expression elevated on astrocytes (validated)

Target	Full term
CXCR1	C-X-C Motif Chemokine Receptor 1
CCR2	C-C Motif Chemokine Receptor 2
CCR4	C-C Motif Chemokine Receptor 4
CXCR2	C-X-C Motif Chemokine Receptor 2
CXCR3	C-X-C Motif Chemokine Receptor 3
uPAR	Urokinase Plasminogen Activator Surface Receptor

5 According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 1.

 According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 2.

10 According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 3.

 According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 4.

 According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 5.

15 According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 6.

 According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 7.

 According to one embodiment, the polypeptide is a human polypeptide.

20 According to one embodiment, the polypeptide is a secreted polypeptide.

 According to one embodiment, the polypeptide is one that is involved in cell growth, cell proliferation, cell proliferation or cell maturation, such as but not limited to, a cytokine, a chemokine or a growth factor.

25 According to one embodiment, the polypeptide is a cell surface receptor (e.g. transmembrane receptor).

According to one embodiment, the cell surface receptor is one that binds to and mediates signaling of a cytokine, a chemokine or a growth factor.

According to one embodiment, the polypeptide is expressed or secreted from a cancer cell (e.g. melanoma cell).

5 According to one embodiment, the target comprises two or more targets (e.g. polypeptides or polynucleotides encoding same), e.g. 2-3 polypeptides, 3-4 polypeptides, 4-5 polypeptides, e.g. 2, 3, 4, 5 polypeptides.

According to a specific embodiment, the polypeptide (or polynucleotide encoding same) comprises no more than 3, 4 or 5 polypeptides.

10 According to one embodiment, the two or more polypeptide (or polynucleotides encoding same) are of the same pathway.

According to one embodiment, the two or more polypeptides (or polynucleotides encoding same) are of different pathways.

15 According to one embodiment, the two or more polypeptides (or polynucleotides encoding same) comprise different secreted polypeptides (e.g. cytokine, chemokine, growth factor).

According to one embodiment, the two or more polypeptides (or polynucleotides encoding same) comprise different cell surface receptors.

20 According to one embodiment, the two or more polypeptides (or polynucleotides encoding same) comprise a combination of a secreted polypeptide (e.g. cytokine, chemokine, growth factor) and a cell surface receptor.

According to one embodiment, targeting the two or more polypeptides (or polynucleotides encoding same) results in synergy of the downregulation effect.

According to a specific embodiment, the polypeptide is CXCL10 and/or CXCR3.

25 According to a specific embodiment, the polypeptide is MCP-1, CCR4 and/or CCR2.

According to a specific embodiment, the polypeptide is IL-8 and/or CXCR1.

According to a specific embodiment, the polypeptide is GRO α and/or CXCR2.

30 According to a specific embodiment, the polypeptide is SERPINE1 and/or uPAR.

According to a specific embodiment, the polypeptide is selected from the group consisting of MCP-1, IL-8, CXCL10, GRO α , SERPINE1, CXCR1, CXCR2, CXCR3, CCR2, CCR4, uPAR, LCN2, sICAM, MIF, RANTES, IL-6, IL-1 β and CCL17.

According to one embodiment, the polypeptide is expressed or secreted from a
5 CNS cell, e.g. from a glia cell such as an astrocyte.

According to a specific embodiment, the polypeptide is CXCL10 or LCN2.

Downregulation of any one of the above polypeptide targets (e.g. one or more e.g., 1-2, 2-3) can be effected on the genomic and/or the transcript level using a variety of molecules which interfere with transcription and/or translation [e.g., RNA silencing
10 agents (e.g., antisense, siRNA, shRNA, micro-RNA), Ribozyme, DNazyme and a guided nuclease e.g., CRISPR system (e.g. CRISPR/Cas)], or on the protein level using e.g., antibodies, dominant negative polypeptides and the like.

Down regulation of expression may be transient, stable or induced.

According to specific embodiments, downregulating expression refers to a
15 decrease in gene expression, in the level of mRNA and/or in the level of a protein, as detected e.g. by RT-PCR or Western blot, respectively. The reduction may be by at least a 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60 %, at least 70 %, at least 80 %, at least 90 %, at least 95 % or at least 99 % reduction as compared to the expression in the absence of the downregulating agent.

According to specific embodiments, downregulating activity refers to the protein
20 activity, as detected for example by Western blot or Cell migration assay. The reduction may be by at least a 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60 %, at least 70 %, at least 80 %, at least 90 %, at least 95 % or at least 99 % reduction as compared to the protein activity in the absence of the downregulating
25 agent.

Following is a list of agents capable of downregulating expression level and/or activity of the polypeptide targets listed in the above tables 1-7.

One example, of an agent capable of downregulating the polypeptide targets is an antibody or antibody fragment capable of specifically binding the polypeptide target
30 and preferably block its activity or interaction with another polypeptide e.g., neutralizing antibody. Preferably, the antibody specifically binds at least one epitope of

the polypeptide. As used herein, the term "epitope" refers to any antigenic determinant on an antigen to which the paratope of an antibody binds.

Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or carbohydrate side chains and usually have specific
5 three-dimensional structural characteristics, as well as specific charge characteristics.

The term "antibody" as used in this invention includes intact molecules as well as functional fragments thereof (such as Fab, F(ab')₂, Fv, scFv, dsFv, or single domain molecules such as VH and VL) that are capable of binding to an epitope of an antigen.

Suitable antibody fragments for practicing some embodiments of the invention
10 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
15 light and heavy chains such as an Fv, a single chain Fv Fv (scFv), a disulfide-stabilized Fv (dsFv), an Fab, an Fab', and an F(ab')₂.

As used herein, the terms "complementarity-determining region" or "CDR" are used interchangeably to refer to the antigen binding regions found within the variable region of the heavy and light chain polypeptides. Generally, antibodies comprise three
20 CDRs in each of the VH (CDR HI or HI; CDR H2 or H2; and CDR H3 or H3) and three in each of the VL (CDR LI or LI; CDR L2 or L2; and CDR L3 or L3).

The identity of the amino acid residues in a particular antibody that make up a variable region or a CDR can be determined using methods well known in the art and include methods such as sequence variability as defined by Kabat et al. (See, e.g., Kabat
25 et al., 1992, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, NIH, Washington D.C.), location of the structural loop regions as defined by Chothia et al. (see, e.g., Chothia et al., Nature 342:877-883, 1989.), a compromise between Kabat and Chothia using Oxford Molecular's AbM antibody modeling software (now Accelrys®, see, Martin et al., 1989, Proc. Natl Acad Sci USA. 86:9268; and world
30 wide web site [www\(dot\)bioinf-org\(dot\)uk/abs](http://www.bioinf-uk.org/abs)), available complex crystal structures as defined by the contact definition (see MacCallum et al., J. Mol. Biol. 262:732-745,

1996) and the "conformational definition" (see, e.g., Makabe et al., Journal of Biological Chemistry, 283:1156-1166, 2008).

As used herein, the "variable regions" and "CDRs" may refer to variable regions and CDRs defined by any approach known in the art, including combinations of approaches.

Functional antibody fragments comprising whole or essentially whole variable regions of both light and heavy chains such as provided in Fv, scFv, dsFv, Fab, Fab', F(ab')₂, Single domain antibodies or nanobodies.

Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art (See for example, Harlow and Lane, 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 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 sulfhydryl groups resulting 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. Natl. 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. 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.

5 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*
10 332:323-327 (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,
15 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
20 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991)].

According to one embodiment, the antibody is a neutralizing antibody.

Downregulation of the polypeptide targets can be also achieved by RNA silencing. As used herein, the phrase "RNA silencing" refers to a group of regulatory mechanisms [e.g. RNA interference (RNAi), transcriptional gene silencing (TGS), post-
25 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
30 capable of specifically 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.

According to an embodiment of the invention, the RNA silencing agent is specific to the target RNA (e.g., any one of the targets in the above tables e.g., CXCL10 or CXCR3) and does not cross inhibit or silence a gene or a splice variant which exhibits 99 % or less global homology to the target gene, e.g., less than 98 %, 97 %, 96 %, 95 %, 94 %, 93 %, 92 %, 91 %, 90 %, 89 %, 88 %, 87 %, 86 %, 85 %, 84 %, 83 %, 82 %, 81 % global homology to the target gene.

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, some embodiments of the invention contemplates use of dsRNA to downregulate protein expression from mRNA.

5 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
10 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.

 In particular, the invention according to some embodiments thereof contemplates introduction of long dsRNA (over 30 base transcripts) for gene silencing
15 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 invention according to some embodiments thereof also contemplates introduction of long dsRNA specifically designed not to induce the interferon and PKR
20 pathways for down-regulating gene expression.

 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
25 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
30 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.

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 some embodiments of the 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: 13, Brummelkamp, T. R. et al. (2002) Science 296: 550) and 5'-UUUGUGUAG-3' (SEQ ID NO: 14, 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.

Synthesis of RNA silencing agents suitable for use with some embodiments of the invention can be effected as follows. First, the mRNA sequence is scanned downstream of the AUG start codon for AA dinucleotide sequences. Occurrence of each AA and the 3' adjacent 19 nucleotides is recorded as potential siRNA target sites. Preferably, siRNA target sites are selected from the open reading frame, as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex [Tuschl ChemBiochem. 2:239-245]. It will be appreciated though, that siRNAs directed at untranslated regions may also be effective, as

demonstrated for GAPDH wherein siRNA directed at the 5' UTR mediated about 90 % decrease in cellular GAPDH mRNA and completely abolished protein level ([www\(·\)ambion\(·\)com/techlib/tn/91/912\(·\)html](http://www.ambion.com/techlib/tn/91/912.html)).

Second, potential target sites are compared to an appropriate genomic database (e.g., human, mouse, rat etc.) using any sequence alignment software, such as the BLAST software available from the NCBI server ([www\(·\)ncbi\(·\)nlm\(·\)nih\(·\)gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)). Putative target sites which exhibit significant homology to other coding sequences are filtered out.

Qualifying target sequences are selected as template for siRNA synthesis. Preferred sequences are those including low G/C content as these have proven to be more effective in mediating gene silencing as compared to those with G/C content higher than 55 %. Several target sites are preferably selected along the length of the target gene for evaluation. For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative control siRNA preferably include the same nucleotide composition as the siRNAs but lack significant homology to the genome. Thus, a scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene.

For example, a suitable siRNA can be obtained from Ambion Inc., Austin, TX.

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

In some embodiments, the RNA silencing agent provided herein can be functionally associated with a cell-penetrating peptide."

According to another embodiment the RNA silencing agent may be a miRNA.

According to another embodiment the RNA silencing agent may be a tiny noncoding RNA (tnRNA).

Another agent capable of downregulating expression of the polypeptide target is a ribozyme molecule capable of specifically cleaving an mRNA transcript encoding the polypeptide target. Ribozymes are being increasingly used for the sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding proteins of interest [Welch et al., Curr Opin Biotechnol. 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both

basic research and therapeutic applications. In the therapeutics area, ribozymes have been exploited to target viral RNAs in infectious diseases, dominant oncogenes in cancers and specific somatic mutations in genetic disorders [Welch et al., Clin Diagn Virol. 10:163-71 (1998)]. Most notably, several ribozyme gene therapy protocols for HIV patients are already in Phase 1 trials. More recently, ribozymes have been used for transgenic animal research, gene target validation and pathway elucidation. Several ribozymes are in various stages of clinical trials. ANGIOZYME was the first chemically synthesized ribozyme to be studied in human clinical trials. ANGIOZYME specifically inhibits formation of the VEGF-r (Vascular Endothelial Growth Factor receptor), a key component in the angiogenesis pathway. Ribozyme Pharmaceuticals, Inc., as well as other firms have demonstrated the importance of anti-angiogenesis therapeutics in animal models. HEPTAZYME, a ribozyme designed to selectively destroy Hepatitis C Virus (HCV) RNA, was found effective in decreasing Hepatitis C viral RNA in cell culture assays (Ribozyme Pharmaceuticals, Incorporated - WEB home page).

An additional method of regulating the expression of the polypeptide target in cells is via triplex forming oligonucleotides (TFOs). Recent studies have shown that TFOs can be designed which can recognize and bind to polypurine/polypyrimidine regions in double-stranded helical DNA in a sequence-specific manner. These recognition rules are outlined by Maher III, L. J., et al., Science, 1989; 245:725-730; Moser, H. E., et al., Science, 1987; 238:645-630; Beal, P. A., et al., Science, 1992; 251:1360-1363; Cooney, M., et al., Science, 1988; 241:456-459; and Hogan, M. E., et al., EP Publication 375408. Modification of the oligonucleotides, such as the introduction of intercalators and backbone substitutions, and optimization of binding conditions (pH and cation concentration) have aided in overcoming inherent obstacles to TFO activity such as charge repulsion and instability, and it was recently shown that synthetic oligonucleotides can be targeted to specific sequences (for a recent review see Seidman and Glazer, J Clin Invest 2003; 112:487-94).

Downregulation of the polypeptide target can be also achieved using nucleic acid agents operating at the DNA level as summarized infra.

Downregulation of a polypeptide target can also be achieved by inactivating the gene (e.g., encoding a polypeptide) via introducing targeted mutations involving loss-of function alterations (e.g. point mutations, deletions and insertions) in the gene structure.

As used herein, the phrase “loss-of-function alterations” refers to any mutation
5 in the DNA sequence of a gene (e.g., encoding a polypeptide) which results in downregulation of the expression level and/or activity of the expressed product, i.e., the mRNA transcript and/or the translated protein. Non-limiting examples of such loss-of-function alterations include a missense mutation, *i.e.*, a mutation which changes an amino acid residue in the protein with another amino acid residue and thereby abolishes
10 the enzymatic activity of the protein; a nonsense mutation, *i.e.*, a mutation which introduces a stop codon in a protein, e.g., an early stop codon which results in a shorter protein devoid of the enzymatic activity; a frame-shift mutation, *i.e.*, a mutation, usually, deletion or insertion of nucleic acid(s) which changes the reading frame of the protein, and may result in an early termination by introducing a stop codon into a
15 reading frame (e.g., a truncated protein, devoid of the enzymatic activity), or in a longer amino acid sequence (e.g., a readthrough protein) which affects the secondary or tertiary structure of the protein and results in a non-functional protein, devoid of the enzymatic activity of the non-mutated polypeptide; a readthrough mutation due to a frame-shift mutation or a modified stop codon mutation (*i.e.*, when the stop codon is mutated into
20 an amino acid codon), with an abolished enzymatic activity; a promoter mutation, *i.e.*, a mutation in a promoter sequence, usually 5' to the transcription start site of a gene, which results in down-regulation of a specific gene product; a regulatory mutation, *i.e.*, a mutation in a region upstream or downstream, or within a gene, which affects the expression of the gene product; a deletion mutation, *i.e.*, a mutation which deletes
25 coding nucleic acids in a gene sequence and which may result in a frame-shift mutation or an in-frame mutation (within the coding sequence, deletion of one or more amino acid codons); an insertion mutation, *i.e.*, a mutation which inserts coding or non-coding nucleic acids into a gene sequence, and which may result in a frame-shift mutation or an in-frame insertion of one or more amino acid codons; an inversion, *i.e.*, a mutation
30 which results in an inverted coding or non-coding sequence; a splice mutation *i.e.*, a mutation which results in abnormal splicing or poor splicing; and a duplication

mutation, *i.e.*, a mutation which results in a duplicated coding or non-coding sequence, which can be in-frame or can cause a frame-shift.

According to specific embodiments loss-of-function alteration of a gene may comprise at least one allele of the gene.

5 The term "allele" as used herein, refers to any of one or more alternative forms of a gene locus, all of which alleles relate to a trait or characteristic. In a diploid cell or organism, the two alleles of a given gene occupy corresponding loci on a pair of homologous chromosomes.

According to other specific embodiments, loss-of-function alteration of a gene
10 comprises both alleles of the gene. In such instances the gene may be in a homozygous form or in a heterozygous form. According to this embodiment, homozygosity is a condition where both alleles are characterized by the same nucleotide sequence. Heterozygosity refers to different conditions of the gene at each locus.

Methods of introducing nucleic acid alterations to a gene of interest are well
15 known in the art [see for example Menke D. Genesis (2013) 51: - 618; Capecchi, Science (1989) 244:1288-1292; Santiago et al. Proc Natl Acad Sci USA (2008) 105:5809-5814; International Patent Application Nos. WO 2014085593, WO 2009071334 and WO 2011146121; US Patent Nos. 8771945, 8586526, 6774279 and UP Patent Application Publication Nos. 20030232410, 20050026157, US20060014264;
20 the contents of which are incorporated by reference in their entireties] and include targeted homologous recombination, site specific recombinases, PB transposases and genome editing by engineered nucleases. Agents for introducing nucleic acid alterations to a gene of interest can be designed publically available sources or obtained commercially from Transposagen, Addgene and Sangamo Biosciences.

25 Following is a description of various exemplary methods used to introduce nucleic acid alterations to a gene of interest and agents for implementing same that can be used according to specific embodiments of the present invention.

Genome editing using engineered endonucleases - this approach refers to a reverse genetics method using artificially engineered nucleases to cut and create specific
30 double-stranded breaks at a desired location(s) in the genome, which are then repaired by cellular endogenous processes such as, homology directed repair (HDR) and non-homologous end-joining (NHEJ). NHEJ directly joins the DNA ends in a double-

stranded break, while HDR utilizes a homologous sequence as a template for regenerating the missing DNA sequence at the break point. In order to introduce specific nucleotide modifications to the genomic DNA, a DNA repair template containing the desired sequence must be present during HDR. Genome editing cannot be performed using traditional restriction endonucleases since most restriction enzymes recognize a few base pairs on the DNA as their target and the probability is very high that the recognized base pair combination will be found in many locations across the genome resulting in multiple cuts not limited to a desired location. To overcome this challenge and create site-specific single- or double-stranded breaks, several distinct classes of nucleases have been discovered and bioengineered to date. These include the meganucleases, Zinc finger nucleases (ZFNs), transcription-activator like effector nucleases (TALENs) and CRISPR/Cas system.

For example, meganucleases can be designed using the methods described in e.g., Certo, MT et al. *Nature Methods* (2012) 9:073-975; U.S. Patent Nos. 8,304,222; 8,021,867; 8, 119,381; 8, 124,369; 8, 129,134; 8,133,697; 8,143,015; 8,143,016; 8, 148,098; or 8, 163,514, the contents of each are incorporated herein by reference in their entirety. Alternatively, meganucleases with site specific cutting characteristics can be obtained using commercially available technologies e.g., Precision Biosciences' Directed Nuclease Editor™ genome editing technology. Alternatively, DNA interacting amino acids of the meganuclease can be altered to design sequence specific meganucleases (see e.g., US Patent 8,021,867, incorporated herein by reference).

Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), have both proven to be effective at producing targeted double-stranded breaks (Christian *et al.*, 2010; Kim *et al.*, 1996; Li *et al.*, 2011; Mahfouz *et al.*, 2011; Miller *et al.*, 2010).

Method for designing and obtaining ZFNs are described in e.g. Carlson *et al.*, 2012; Lee *et al.*, 2010, Li *et al.*, 2011; Miller *et al.*, 2010; Urnov *et al.*, 2005, the contents of each are incorporated herein by reference in their entirety. ZFNs can be designed and obtained commercially from e.g., Sangamo Biosciences™ (Richmond, CA).

Method for designing and obtaining TALENs are described in e.g. Reyon et al. *Nature Biotechnology* 2012 May;30(5):460-5; Miller et al. *Nat Biotechnol.* (2011) 29:

143-148; Cermak et al. Nucleic Acids Research (2011) 39 (12): e82 and Zhang et al. Nature Biotechnology (2011) 29 (2): 149–53, the contents of each are incorporated herein by reference in their entirety. A recently developed web-based program named Mojo Hand was introduced by Mayo Clinic for designing TAL and TALEN constructs for genome editing applications (can be accessed through www.talendesign.org). TALEN can also be designed and obtained commercially from e.g., Sangamo Biosciences™ (Richmond, CA).

Another agent capable of downregulating expression of the polypeptide is a RNA-guided endonuclease technology e.g. CRISPR system.

As used herein, the term "CRISPR system" also known as Clustered Regularly Interspaced Short Palindromic Repeats refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated genes, including sequences encoding a Cas gene (e.g. CRISPR-associated endonuclease 9), a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat) or a guide sequence (also referred to as a "spacer") including but not limited to a crRNA sequence (i.e. an endogenous bacterial RNA that confers target specificity yet requires tracrRNA to bind to Cas) or a sgRNA sequence (i.e. single guide RNA).

In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence (i.e. guide RNA e.g. sgRNA or crRNA) is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. Thus, the CRISPR system comprises two distinct components, a guide RNA (gRNA) that hybridizes with the target sequence, and a nuclease (e.g. Type-II Cas9 protein), wherein the gRNA targets the target sequence and the nuclease (e.g. Cas9 protein) cleaves the target sequence. Introducing CRISPR/Cas into a cell may be effected using one or more vectors driving expression of one or more elements of a CRISPR system such that expression of the elements of the CRISPR system direct formation of a CRISPR complex at one or more target sites.

A "Hit and run" or "in-out", a two-step recombination procedure (as described in Wu et al. Proc Natl Acad Sci U S A. 1994 Mar 29; 91(7): 2819–2823, incorporated

herein by reference) can be utilized for downregulating expression of the polypeptide. Also, the “double-replacement” or “tag and exchange” strategy which - involves a two-step selection procedure similar to the hit and run approach, but requires the use of two different targeting constructs can be utilized.

5 Site-Specific Recombinases (as described in Dymecki, Proc. Natl. Acad. Sci. USA (1996) 93: 6191-6196, incorporated herein by reference) can be utilized for downregulating expression of the polypeptide. In this system, the Cre recombinase derived from the P1 bacteriophage and Flp recombinase derived from the yeast *Saccharomyces cerevisiae* are site-specific DNA recombinases each recognizing a
10 unique 34 base pair DNA sequence (termed “Lox” and “FRT”, respectively) and sequences that are flanked with either Lox sites or FRT sites can be readily removed via site-specific recombination upon expression of Cre or Flp recombinase, respectively.

A number of transposon systems that are able to transpose in cells e.g. vertebrates have been isolated or designed and can be used in accordance with the
15 present teachings to downregulate protein expression, such as Sleeping Beauty [Izsvák and Ivics Molecular Therapy (2004) 9, 147–156, incorporated herein by reference], piggyBac [Wilson et al. Molecular Therapy (2007) 15, 139–145, incorporated herein by reference], Tol2 [Kawakami et al. PNAS (2000) 97 (21): 11403–11408, incorporated herein by reference] or Frog Prince [Miskey et al. Nucleic Acids Res. Dec 1, (2003)
20 31(23): 6873–6881, incorporated herein by reference].

Genome editing using recombinant adeno-associated virus (rAAV) platform can be used to downregulate protein expression. rAAV genome editing technology is commercially available, for example, the rAAV GENESIS™ system from Horizon™ (Cambridge, UK).

25 It will be appreciated that the agent can be a mutagen that causes random mutations and the cells exhibiting downregulation of the expression level and/or activity may be selected.

The mutagens may be, but are not limited to, genetic, chemical or radiation agents. For example, the mutagen may be ionizing radiation, such as, but not limited to,
30 ultraviolet light, gamma rays or alpha particles. Other mutagens may include, but not be limited to, base analogs, which can cause copying errors; deaminating agents, such as nitrous acid; intercalating agents, such as ethidium bromide; alkylating agents, such

as bromouracil; transposons; natural and synthetic alkaloids; bromine and derivatives thereof; sodium azide; psoralen (for example, combined with ultraviolet radiation). The mutagen may be a chemical mutagen such as, but not limited to, ICR191, 1,2,7,8-diepoxy-octane (DEO), 5-azaC, N-methyl-N-nitrosoguanidine (MNNG) or ethyl methane sulfonate (EMS).

Methods for qualifying efficacy and detecting sequence alteration are well known in the art and include, but not limited to, DNA sequencing, electrophoresis, an enzyme-based mismatch detection assay and a hybridization assay such as PCR, RT-PCR, RNase protection, in-situ hybridization, primer extension, Southern blot, Northern Blot and dot blot analysis.

Sequence alterations in a specific gene can also be determined at the protein level using e.g. chromatography, electrophoretic methods, immunodetection assays such as ELISA and western blot analysis and immunohistochemistry.

In addition, one ordinarily skilled in the art can readily design a knock-in/knock-out construct including positive and/or negative selection markers for efficiently selecting transformed cells that underwent a homologous recombination event with the construct. Positive selection provides a means to enrich the population of clones that have taken up foreign DNA. Non-limiting examples of such positive markers include glutamine synthetase, dihydrofolate reductase (DHFR), markers that confer antibiotic resistance, such as neomycin, hygromycin, puromycin, and blasticidin S resistance cassettes. Negative selection markers are necessary to select against random integrations and/or elimination of a marker sequence (e.g. positive marker). Non-limiting examples of such negative markers include the herpes simplex-thymidine kinase (HSV-TK) which converts ganciclovir (GCV) into a cytotoxic nucleoside analog, hypoxanthine phosphoribosyltransferase (HPRT) and adenine phosphoribosyltransferase (ARPT).

It will be appreciated that a non-functional analogue of at least a catalytic or binding portion of the polypeptide target e.g., soluble cytokine or chemokine receptor e.g., soluble CXCR3 devoid of the TM domain can be also used as the agent.

Another agent which can be used along with some embodiments of the invention to downregulate the activity of the polypeptide is a small molecule chemical.

Small molecules suitable for use in the context of these embodiments can be retrieved by screening libraries of compounds for an agent that downregulates the

activity of the polypeptide. Alternatively, or in addition, new chemical entities are designed and synthesized, optionally based on the structural features of the compounds retrieved in such a screening.

A method for screening for compounds (small molecules) usable in treating
5 CNS metastases, by screening for compounds that inhibit a target polypeptide as described herein is also contemplated.

According to one embodiment, the agent is a conjugate which comprises a polymer and a nucleic acid agent associated with the polymer.

Polymeric Conjugates:

As used herein throughout, the term "polymer" describes an organic substance composed of a plurality of repeating structural units (backbone units) covalently
10 connected to one another. The term "polymer" as used herein encompasses organic and inorganic polymers and further encompasses one or more of a homopolymer, a copolymer or a mixture thereof (a blend). The term "homopolymer" as used herein describes a polymer that is made up of one type of monomeric units and hence is composed of homogenic backbone units. The term "copolymer" as used herein
15 describes a polymer that is made up of more than one type of monomeric units and hence is composed of heterogenic backbone units. The heterogenic backbone units can differ from one another by the pendant groups thereof.

Polymers which are suitable for use in the context of the present embodiments are biocompatible, non-immunogenic and non-toxic. The polymers serve as carriers
20 that enable specific delivery into tumor tissue. As described hereinabove, the specific delivery is due to the enhanced permeability and retention (EPR) effect discussed hereinabove. Furthermore, conjugation to polymers may restrict the passage through the blood brain barrier and may prolong the circulating half-life of the drugs, hence inhibiting the growth of tumor endothelial and epithelial cells by exposing the cells to
25 the conjugated drugs in the circulation for a longer time compared to the free drugs. Additionally, polymer-drug conjugates may act as drug depots for sustained release, producing prolonged drug exposure to tumor cells. Water soluble polymers may be used to stabilize drugs, as well as to solubilize otherwise insoluble compounds.

A polymer may be a biostable polymer, a biodegradable polymer or a
30 combination thereof.

The term "biostable", as used in this context of embodiments of the invention, describes a compound or a polymer that remains intact under physiological conditions (e.g., is not degraded *in vivo*).

5 The term "biodegradable" describes a substance which can decompose under physiological and/or environmental conditions into breakdown products. Such physiological and/or environmental conditions include, for example, hydrolysis (decomposition via hydrolytic cleavage), enzymatic catalysis (enzymatic degradation), and mechanical interactions. This term typically refers to substances that decompose under these conditions such that 50 weight percents of the substance decompose within
10 a time period shorter than one year.

The term "biodegradable" as used in the context of embodiments of the invention, also encompasses the term "bioresorbable", which describes a substance that decomposes under physiological conditions to break down products that undergo bioresorption into the host-organism, namely, become metabolites of the biochemical
15 systems of the host-organism.

The polymers can be water-soluble or water-insoluble. In some embodiments, the polymers are water soluble at room temperature.

The polymers can further be charged polymers or non-charged polymers. Charged polymers can be cationic polymers, having positively charged groups and a
20 positive net charge at a physiological pH; or anionic polymers, having negatively charged groups and a negative net charge at a physiological pH. Non-charged polymers can have positively charged and negatively charged group with a neutral net charge at physiological pH, or can be non-charged.

In some embodiments, the polymer has an average molecular weight in the
25 range of 100 Da to 800 kDa. In some embodiments, the polymer has an average molecular weight lower than 60 kDa. In some embodiments, the polymer's average molecular weight range is 15 to 60 kDa.

Polymeric substances that have a molecular weight higher than 10 kDa typically exhibit an EPR effect, as described herein, while polymeric substances that have a
30 molecular weight of 100 kDa and higher have relatively long half-lives in plasma and an inefficient renal clearance. Accordingly, a molecular weight of a polymeric

conjugate can be determined while considering the half-life in plasma, the renal clearance, and the accumulation in the tumor of the conjugate.

The molecular weight of the polymer can be controlled, at least to some extent, by the degree of polymerization (or co-polymerization).

5 The polymer used in the context of embodiments of the invention can be a synthetic polymer or a naturally-occurring polymer. In some embodiments, the polymer is a synthetic polymer.

Exemplary polymers which are suitable for use in the context of the present embodiments include polymers such as, but are not limited to, polyglutamic acid
10 (PGA), a poly(hydroxyalkylmethacrylamide) (HPMA), a polylactic acid (PLA), a poly(lactic-co-glycolic acid) (PLGA), a poly(D,L-lactide-co-glycolide) (PLA/PLGA), a polyamidoamine (PAMAM), a polyethylenimine (PEI), dextran, pullulan, a water soluble polyamino acid, a polyethyleneglycol (PEG) and a polyaminoglycerol.

These polymers can be of any molecular weight, as described herein, and
15 preferably have a molecular weight within the range of 10 to 60 kDa, or of 10 to 40 kDa.

The polymer in the conjugate can be a polymer per se, or a polymeric backbone derived from, or corresponding to, the polymer.

Herein, the term “conjugate” describes a chemical entity in which two or more
20 moieties (e.g., the polymer and the oligonucleotide or nucleic acid agent) are associated to one another.

In some embodiments, the association is via electrostatic interactions. In some embodiments, the electrostatic interactions are between phosphate groups of the oligonucleotide and terminal amine groups of the polymer.

25 A conjugate as described herein in which association is via electrostatic interactions is also referred to herein throughout as a “polyplex”.

In some embodiments the association is via covalent bonds.

In some embodiments, the association is a physical association, and the nucleic acid agent can be embedded, entrapped or encapsulated in a polymeric matrix (e.g.,
30 polymeric particles such as nanoparticles) or absorbed to a polymeric matrix.

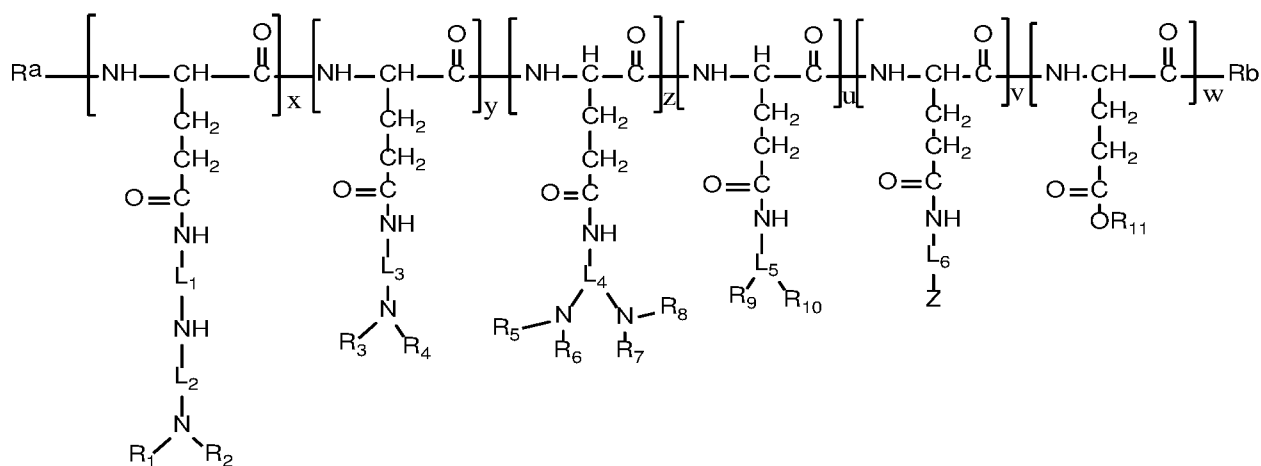
In some of these embodiments, the conjugate comprises polymeric nanoparticles, for example, PLGA nanoparticles, entrapping therein a nucleic acid agent as described herein.

The terms “oligonucleotide” and “nucleic acid agent” agent are used herein interchangeably.

The Polymer in a polyplex conjugate as described herein is, in some embodiments, a PGAamine polymer.

Herein throughout, the terms “PGAamine polymer” and “aminated PGA polymer”, “PGA-based polymer” and simply “polymer” are used interchangeably.

Polymers according to embodiments of the present invention can be collectively represented by Formula I:



Formula I

wherein:

x, y, z, u, v and w each independently represents the mol % of the respective backbone unit, such that $x+y+z+u+v+w = 100$ mol %, wherein, in some embodiments, $x+y+z+u+v \geq 40$ mol %;

Ra is selected from hydrogen and alkyl, preferably an alkyl (linear or branched) of at least 4 carbon atoms in length;

Rb is selected from hydroxyl, alkoxy, amine and pyrrolidinone;

L₁, L₂, L₃ and L₆ is each independently a linear linking moiety;

L₄ and L₅ are each independently a branched linking moiety;

R₁-R₁₁ are each independently selected from H, alkyl and cycloalkyl; and

Z is a nitrogen-containing heterocyclic moiety,
provided that at least one of x, y and z is other than 0.

In some of any of the embodiments described herein, each of x, y, a, u and v,
when other than 0, independently ranges from 10 to 80 %, including subranges and
5 intermediate values therebetween.

In some of any of the embodiments described herein, y ranges from 50 to 100
mol %, or from 60 to 100 mol %, or from 70 to 100 mol %. In some embodiments, y is
about 100 mol %. See, for example, Polymers A and B.

In some of any of the embodiments in which y is other than 0, R₃ and R₄ are each
10 H.

In some of any of the embodiments in which y is other than 0, L₃ is an
unsubstituted alkylene being 2-10, or 2-8, or 2-6, carbon atoms in length.

In some of any of the embodiments described herein, x ranges from 50 to 100
mol %, or from 60 to 100 mol %, or from 70 to 100 mol %.

15 In some of any of the embodiments in which x is other than 0, one or more of,
and preferably each of, R₁ and R₂ is alkyl, for example, a C1-4 alkyl such as methyl.
See, for example, Polymers F and I.

In some of these embodiments, at least 50 %, or 60 % or 70 % of y are units in
which each of R₁ and R₂ is alkyl, for example, methyl, and in the remaining units one or
20 both of R₁ and R₂ is H. See, for example, Polymer I.

In some of any of the embodiments described herein, x is at least 40 mol %, and
at least one of R₁ and R₂, preferably each, is other than H. In some of these
embodiments, y is lower than 40 mol %, and can also be 0.

In some of any of the embodiments described herein, u is other than 0, such that
25 the polymer comprises alkyl pendant groups. In some of these embodiments, at least
one of x, y, z and v is other than 0.

In some of any of the embodiments described herein, u is at least 40 mol %.

In some of these embodiments, u ranges from 40 to 50 mol %.

In some of any of the embodiments described herein, u is at least 40 mol % and y
30 is other than 0. See, for example, Polymers K, M, O and P, and Polymers V and W.

In some of these embodiments, y ranges from 60 to 50 mol %, respectively. That is, for example, u and y together are 100 mol %, and, for example, when u is 40 mol %, y is 60 mol %, when u is 45 mol %, y is 55 mol %, etc.

In some of any of the embodiments described herein, u is at least 40 mol % and x is other than 0. In some of these embodiments, x is at least 40 mol %. See, for example, Polymer X.

In some of any of the embodiments described herein, u is at least 40 mol % and both x and y are other than 0. In some of these embodiments, x is at least 40 mol %. See, for example, Polymer Y.

In some of any of the embodiments described herein for u other than 0, R₉ is H and R₁₀ is alkyl.

In some of any of the embodiments described herein for u other than 0, each of R₉ and R₁₀ is alkyl.

The alkyl in these embodiments can be 3 to 10, or 5 to 10, or 5 to 8, or 6 to 8, carbon atoms in length.

L₅ is a branched linking moiety as defined herein.

The branched linking moiety, according to any of the embodiments described herein is R_c-CR_d-R_f, wherein R_d is H or alkyl; and R_c and R_f are each independently an alkylene or absent.

In some embodiments, in L₅, R_c and R_f are absent. In some embodiments of L₅, R_d is H. In some of these embodiments, when R₉ is H, L₅ and R₁₀ can be regarded as forming together a linear alkyl, and, in some embodiments, this linear alkyl is at least 5 carbon atoms in length.

In some embodiments, in L₅, R_c and R_f are absent. In some embodiments of L₅, R_d is H, and each of R₉ and R₁₀ is alkyl, such that L₅, R₉ and R₁₀ can be regarded as forming together a branched alkyl. In some of these embodiments, each branch of the branched alkyl is at least 3 carbon atoms in length.

In some of any of embodiments described herein, when u is other than 0, at least one of R₉ and R₁₀ is an alkyl being 3 or more, preferably 4 or more, carbon atoms in length, and at least one of x, y, z and v is other than 0.

In some of any of the embodiments described herein, v is other than 0.

In some of these embodiments, v is at least 20, or at least 30 mol %.

In some of any of the embodiments where v is other than 0, u is at least 20, or at least 30 mol %.

In some of any of the embodiments where v is other than 0, at least one of x , y and z is other than 0. In some of these embodiments, y is other than 0. In some of these
5 embodiments, u is at least 20, or at least 30 mol %. In some of any of these
embodiments, y ranges from 40 to 80 mol %, preferably from 40 to 60 mol %.

In some of any of the embodiments described herein, Z is a nitrogen-containing heteroaryl, for example, imidazole.

In some of any of the embodiments described herein L_1 , L_2 and L_3 are each
10 independently, if present, a linear linking moiety. In some embodiments, the linear
linking moiety is a substituted or unsubstituted alkylene, for example, ethylene.

In some embodiments, the linear linking moiety is an unsubstituted ethylene ($-\text{CH}_2-\text{CH}_2-$) or an unsubstituted propylene ($-\text{CH}_2-\text{CH}_2-\text{CH}_2-$).

In some of any of the embodiments described herein, the polymers described
15 herein are collectively represented by Formula I*, in which all variables are as defined in
any of the embodiments described herein for Formula I, yet, one of the following exists:

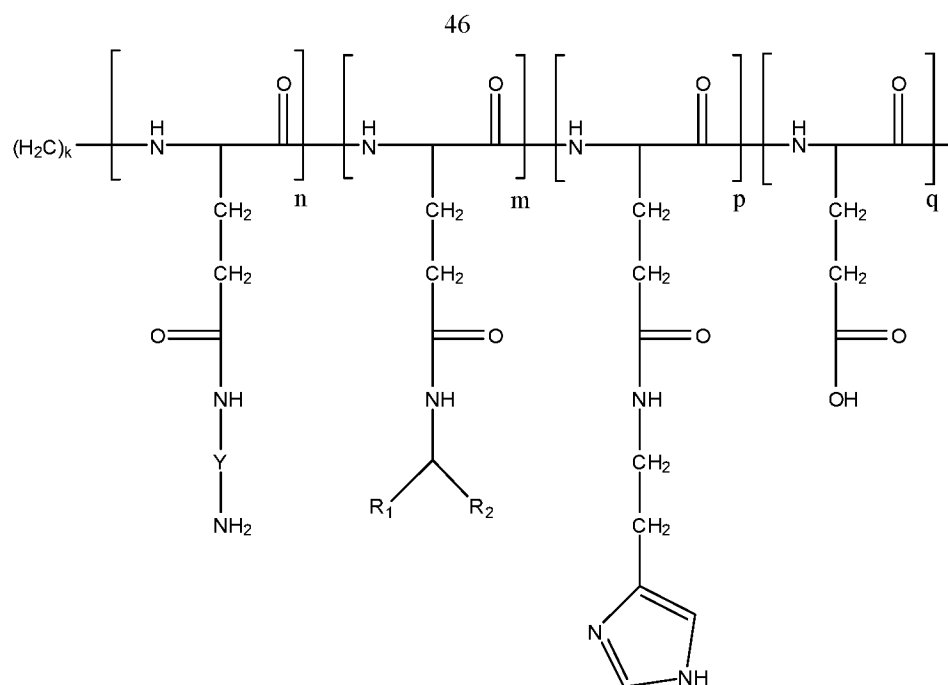
(i) x is at least 40 mol %, y is lower than 40 mol %, and at least one of R_1 and R_2
is other than H; or

(ii) when u is other than 0, at least one of R_9 and R_{10} is an alkyl being more than
20 3 carbon atoms in length, and at least one of x , y , z and v is other than 0; or

(iii) when v is other than 0, u is other 0; or

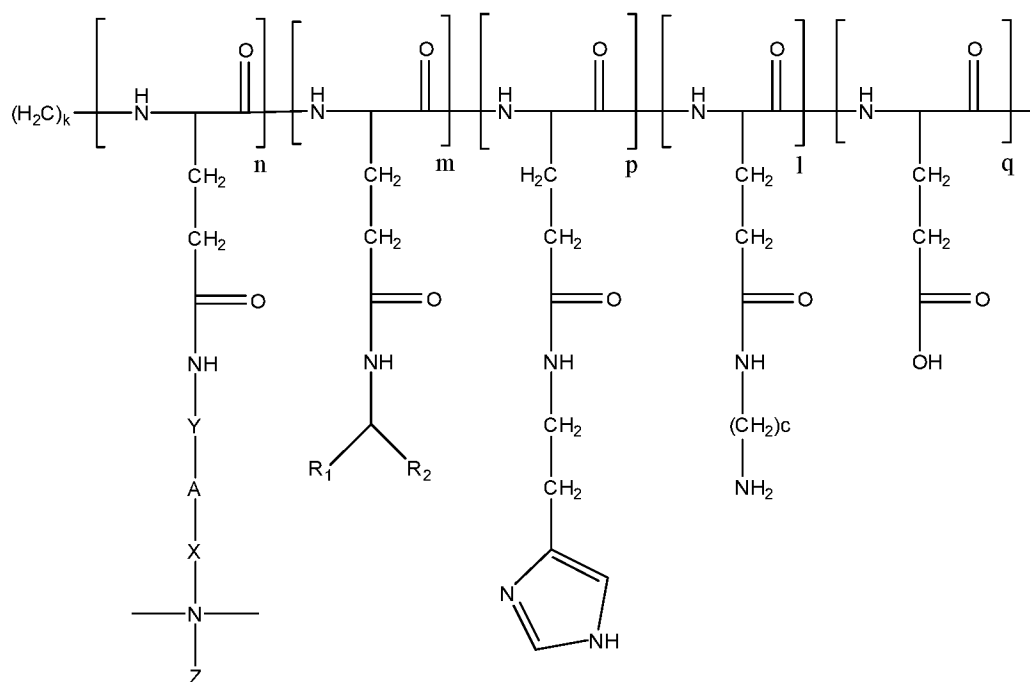
(iv) z is greater than 40 mol %.

Exemplary polymers according to some embodiments of the present invention
include Polymers of Groups I-III, as presented below:



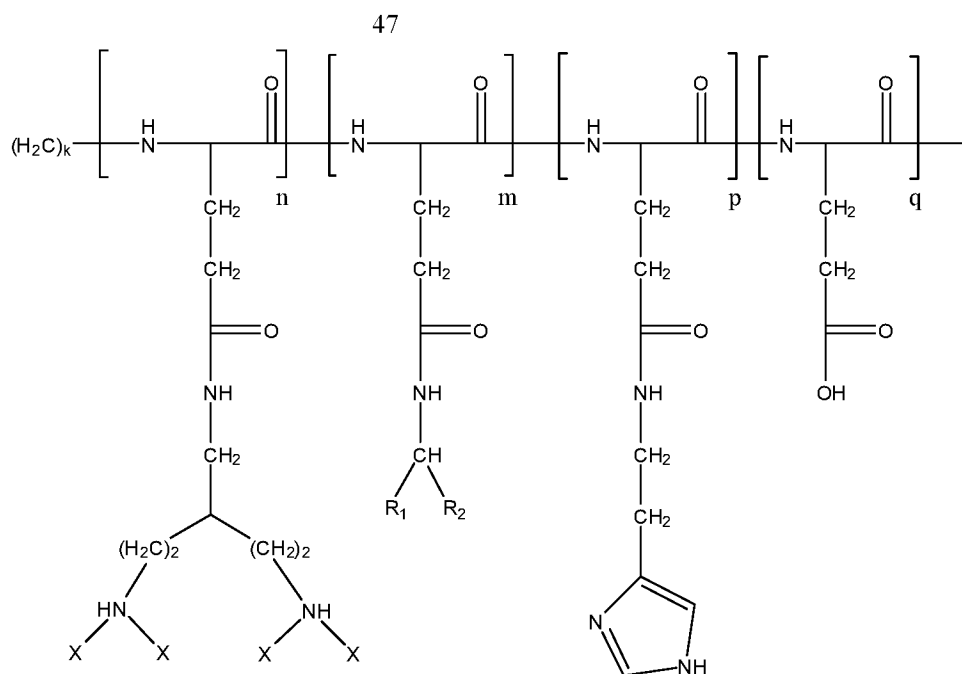
3) $Y = (CH_2)_a$ $a = 2-6$, $R_1 = H$, $R_2 = (CH_2)_bCH_3$ $b = 4-8$, $n = 40-100\%$, $m = 0-45\%$, $p = 0-30\%$, $q = 0-60\%$, $k = 4-18$

4) $Y = (CH_2)_a$ $a = 2-6$, $R_1 = R_2 = (CH_2)_bCH_3$ $b = 2-5$, $n = 40-100\%$, $m = 0-45\%$, $p = 0-30\%$, $q = 0-60\%$, $k = 4-18$



1) $Y = (CH_2)_a$ $a = 2-10$, $X = (CH_2)_{a'}$ $a' = 2-10$, $Z = H, CH_3$, $R_1 = (CH_2)_bCH_3$ $b = 4-8$ $R_2 = H$, $c = 2-6$, $n = 40-100\%$, $m = 0-45\%$, $p = 0-30\%$, $l = 0-50\%$, $q = 0-60\%$, $k = 4-18$, $A = N, CH_2$

2) $Y = (CH_2)_a$, $X = (CH_2)_a$ $a = 2, 3$, $Z = H, CH_3$, $R_1 = R_2 = (CH_2)_bCH_3$ $b = 2-5$, $n = 40-100\%$, $m = 0-45\%$, $p = 0-30\%$, $l = 0-50\%$, $q = 0-60\%$, $k = 4-18$

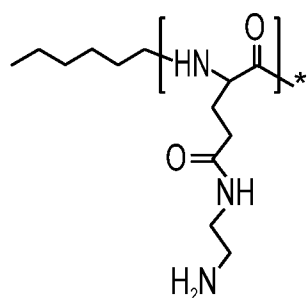


- 5) X=H, CH₃, R₁=(CH₂)_bCH₃ b=4-8, R₂=H, n=40-100%, m=0-45%, p=0-30%, q=0-60%, k=4-18
 6) X=H, CH₃, R₁=R₂=(CH₂)_bCH₃ b=2-5, n=40-100%, m=0-45%, p=0-30%, q=0-60%, k=4-18
 7) X=H, CH₃, R₁=(CH₂)₂NH(CH₂)₅CH₃, R₂=H, n=40-100%, m=0-45%, p=0-30%, q=0-60%, k=4-18

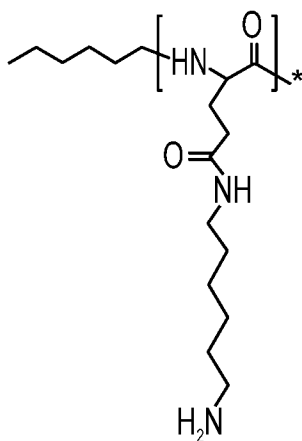
Exemplary polymers according to some embodiments of the present invention include Polymers A-Y.

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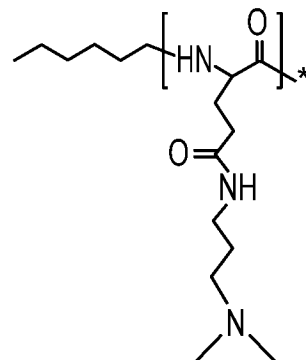
A



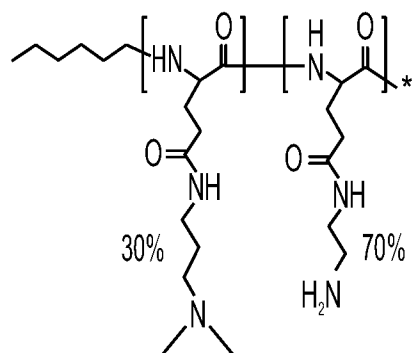
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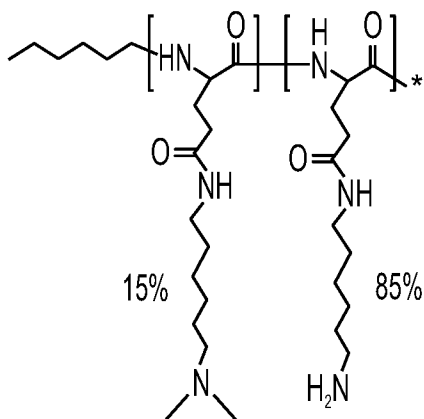
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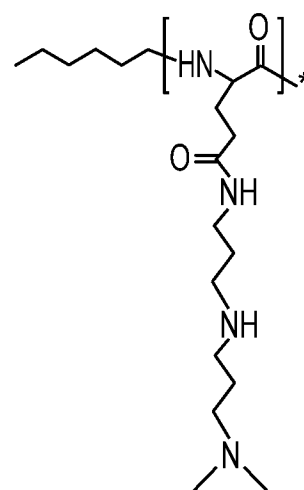
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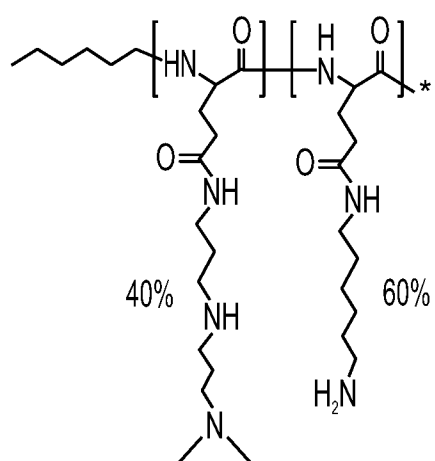
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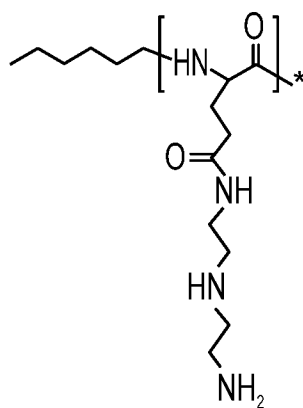
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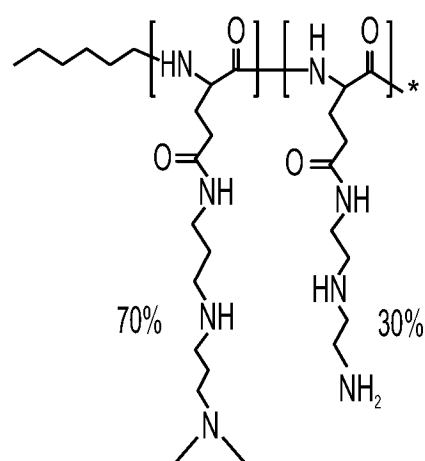
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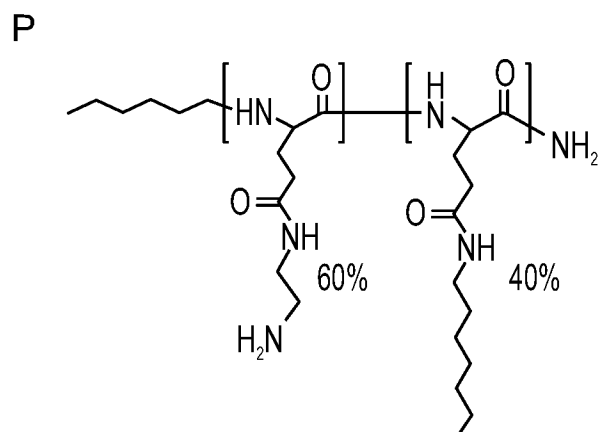
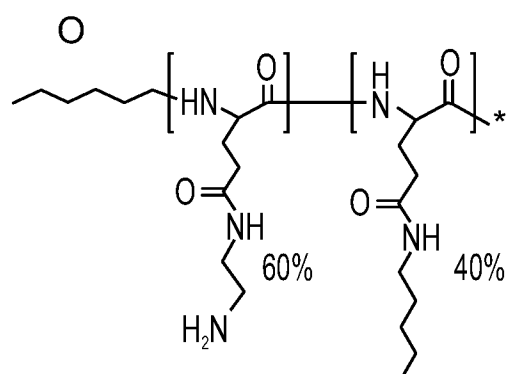
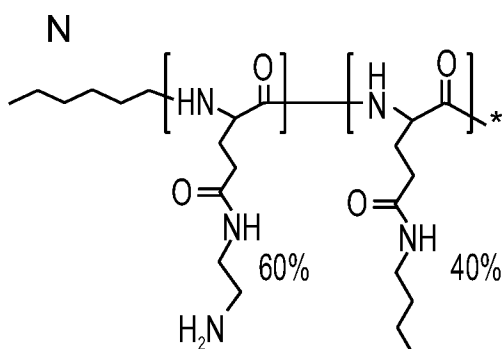
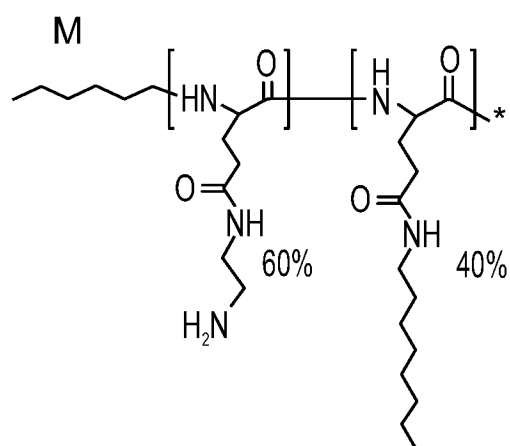
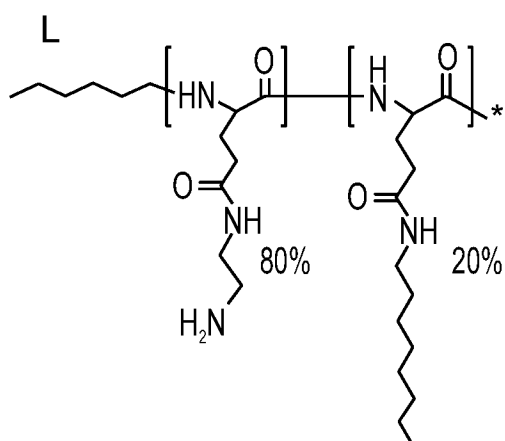
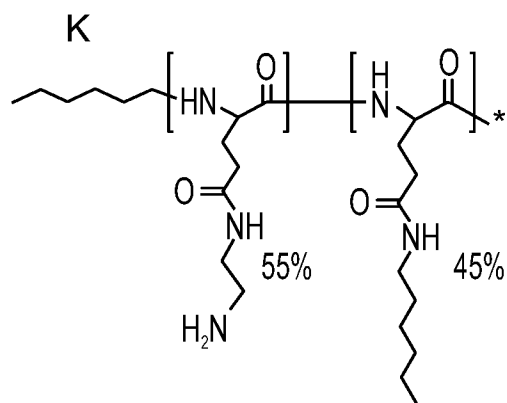
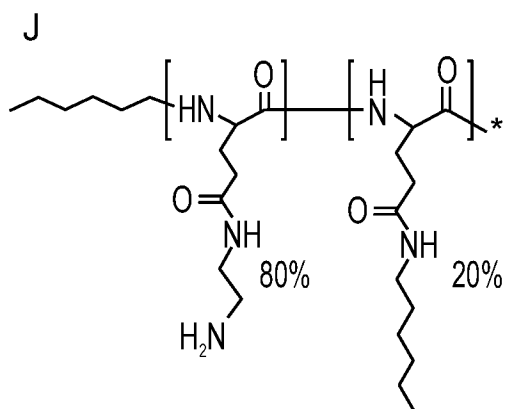
H



I

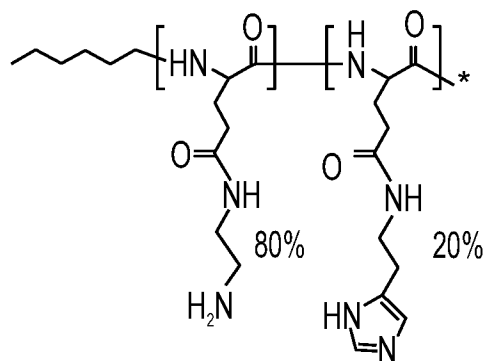


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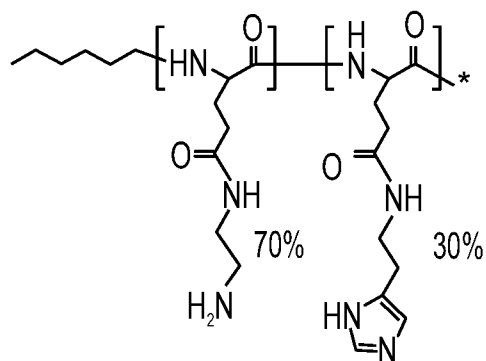


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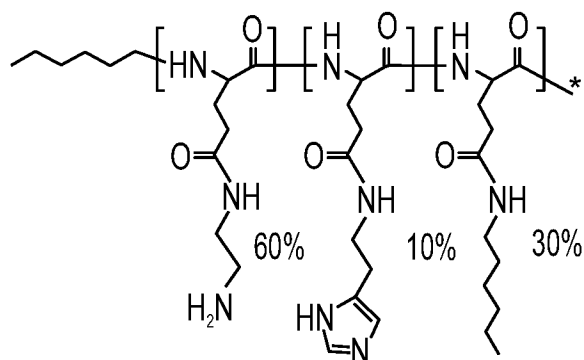
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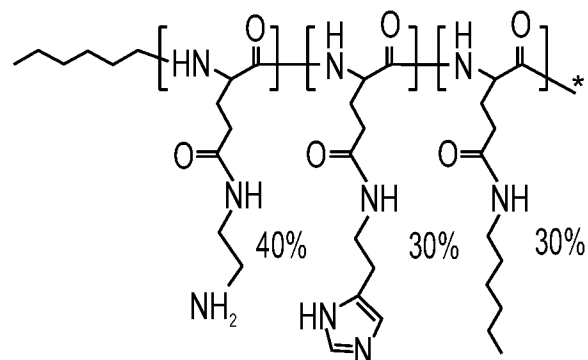
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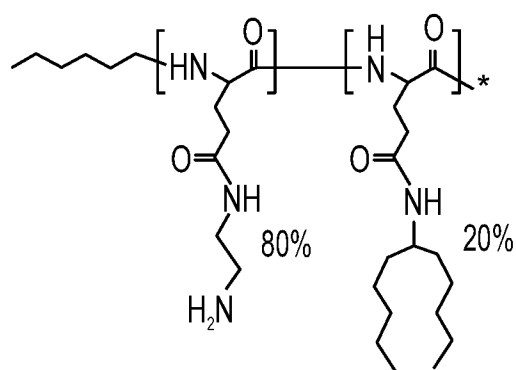
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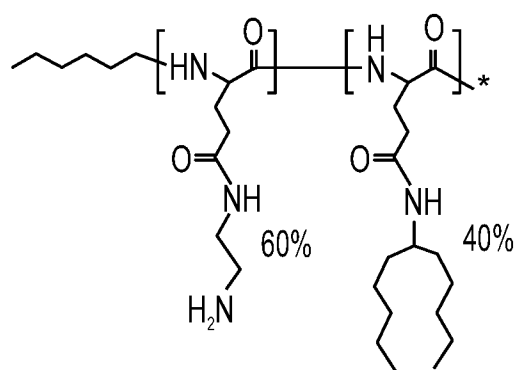
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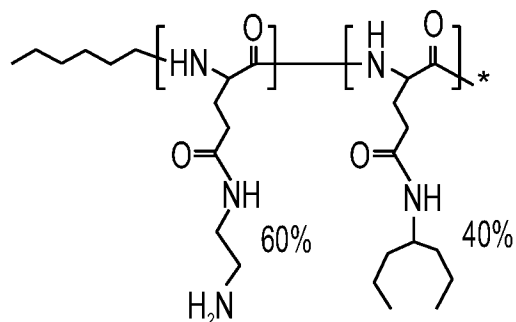
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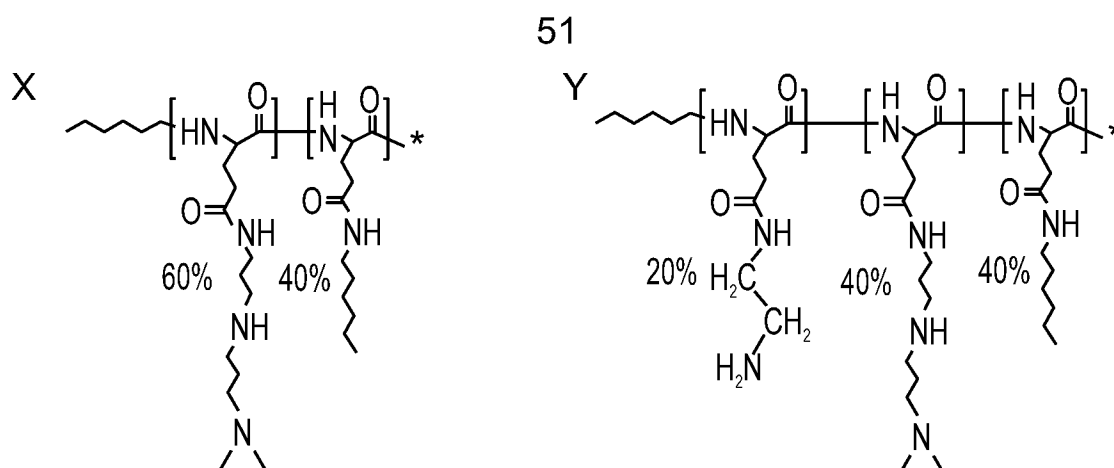


V



W





Exemplary polymers according to some embodiments of the present invention include Polymer A, Polymer B, Polymer F, Polymer I, Polymer K, Polymer M, Polymer O, Polymer P, and Polymer T.

5 In some embodiments, exemplary polymers according to some embodiments of the present invention include Polymer F, Polymer I, Polymer K, Polymer M, Polymer O, Polymer P, and Polymer T, as described herein.

The polymers described herein are generally prepared by coupling PGA to a respective amine-containing moiety, as described herein.

10 Any coupling agent useful in forming peptide bonds is contemplated. One or more types of coupling agents can be used, depending on the type(s) of the amine to be conjugated.

Exemplary coupling agents include, without limitation, CDI and DIC.

15 A conjugate as described herein comprises a polymer as described herein in any one of the respective embodiments and any combination in association with an oligonucleotide or a nucleic acid agent, as described herein.

In some embodiments, the oligonucleotide is complexed to the polymer via electrostatic interactions, and the conjugate is therefore referred to herein interchangeably as a polyplex.

20 In some embodiments, the association is by electrostatic interactions, or bonds, formed between the amine moieties and optionally other nitrogen-containing moieties (e.g., imidazole) in the polymer and the negatively charged groups of the oligonucleotide.

In some embodiments, the electrostatic interactions are between terminal amine groups or other terminal nitrogen-containing moieties (e.g., imidazole) of the polymer and phosphate groups of the oligonucleotide.

N/P ratio is the ratio between phosphate groups of the oligonucleotide and terminal amines of the pendant groups of the PGA backbone. For example: 5 N/P means 5 terminal nitrogen groups for each phosphate group (can be also written as 5:1 ratio).

In some embodiments, this N/P ratio between a number of the terminal amine groups and a number of the phosphate groups ranges from 15:1 to 1:1, or from 10:1 to 1:1, or from 5:1 to 1:1.

A polyplex as described herein can alternatively be as described in WO 2009/141170, which is incorporated by reference as if fully set forth herein, wherein the nanocarrier system described therein is associated with a nucleic acid agent as described herein.

According to some embodiments of the present invention, there are provided polymeric conjugates comprising a polymer and a nucleic acid agent as described herein associated with the polymer.

The conjugates described herein can be utilized *per se* or as a pharmaceutical composition comprising same, as discussed in further detail hereinbelow.

According to some of any of the embodiments described herein there is provided a pharmaceutical composition comprising a conjugate as described herein in any of the respective embodiments, and a pharmaceutically acceptable carrier.

In some embodiments, the pharmaceutical composition is for use in any of the methods and uses described herein.

According to some embodiments of the present invention a pharmaceutical composition comprising the conjugate as described herein comprises an aqueous carrier.

A pharmaceutical composition as described herein is also referred to as a formulation.

According to some embodiments, the conjugate is in a form of a plurality of particles (e.g., nanoparticles) dispersed in the carrier.

According to some embodiments, the carrier further comprises a dispersing agent.

According to some embodiments, the carrier further comprises glucose.

According to some embodiments, the dispersing agent is selected so as to prevent aggregation of the nanoparticles and/or to maintain the discrete particles of the conjugate in the composition.

5 According to some embodiments, the dispersing agent is selected so as to obtain and maintain nanoparticles featuring an average particle size (diameter) of said particles is lower than 1 micron, or lower than 500 nm or lower than 300 nm, or lower than 200 nm and/or PDI lower than 1, or lower than 0.5, or lower than 0.3.

10 In some embodiments, for conjugates in which the polymer features amine-containing groups, the dispersing agent is a surfactant, such as Tween®. Other surfactants are also contemplated.

In some embodiments, a concentration of the surfactant ranges from 0.1 % to 40 % by volume, of the total volume of the composition.

15 In some embodiments, a concentration of the surfactant ranges from 0.1 to 10, or from 0.1 to 40 mol %, relative to the conjugate.

In some embodiments, for conjugates in which the polymer features amine-containing pendant groups and alkyl-containing pendant groups, the dispersing agent can be a polyethylene glycol and/or a glucose (for isotonicity) as described herein.

20 In some embodiments, a concentration of the PEG ranges from 1 to 20 %, or from 5 to 15 %, or is about 10 %, by volume, of the total volume of the composition.

In some embodiments, a MW of the PEG is at least 400 grams/mol.

In some embodiments, for conjugates in which the polymer features amine-containing pendant groups and alkyl-containing pendant groups, the composition is prepared by means of a microfluidic system.

25 According to some embodiments of the present invention the conjugate is in a form of particles dispersed in said carrier, wherein an average particle size (in diameter) of said particles is lower than 1 micron, or lower than 500 nm or lower than 300 nm, or lower than 200 nm; and/or a PDI of said particles is lower than 1, or lower than 0.5, or lower than 0.3.

30 According to a specific embodiment, the agent is a siRNA polyplex selected from the group consisting of a PGA-NH₂-siMCP-1, PGA-NH₂-siIL-8, PGA-NH₂-siCXCL10, PGA-NH₂-siGRO α , and PGA-NH₂-siSERPINE1.

According to another specific embodiment, the agent is a siRNA polyplex selected from the group consisting of a PGA-NH₂-siCXCR1, PGA-NH₂-siCCR4, PGA-NH₂-siCCR2, PGA-NH₂-siCXCR3, PGA-NH₂-siCXCR2 and PGA-NH₂-siuPAR.

According to one embodiment of the invention, there is provided a composition of matter selected from the group consisting of a PGA-NH₂-siMCP-1, PGA-NH₂-siIL-8, PGA-NH₂-siCXCL10, PGA-NH₂-siGRO α , and PGA-NH₂-siSERPINE1, PGA-NH₂-siCXCR1, PGA-NH₂-siCCR4, PGA-NH₂-siCCR2, PGA-NH₂-siCXCR3, PGA-NH₂-siCXCR2 and PGA-NH₂-siuPAR.

According to one embodiment, the agent of some embodiments of the invention is targeted to a metastasizing cell expressed marker.

According to one embodiment, the agent of some embodiments of the invention is targeted to a melanoma expressed marker.

According to one embodiment, the marker is NCAM, P-Selectin, or $\alpha_v\beta_3$ integrin.

NCAM or P-Selectin targeting:

In some embodiments, an agent as described herein is targeted to the cell-surface receptor NCAM (neural cell adhesion molecule).

In some embodiments, the agent further comprises NCAM targeting moiety.

In some embodiments, the NCAM targeting moiety is a ligand of the NCAM receptor, and, in some embodiments, it is a ligand that is specific to NCAM receptor.

In some embodiments, the ligand of NCAM is a peptide. Such peptides are referred to herein and in the art as NCAM-targeting peptides or abbreviated as NTP.

In some embodiments, the peptide comprises the amino acid sequence DDSDEEN (SEQ ID NO: 3), which is known to target NCAM. In some of these embodiments, the NTP may further comprise one or more amino acid residues, at each terminus thereof, which may serve, for example, to facilitate attachment to the agent and/or to improve its targeting by improved exposure (reduced steric hindrance). In some embodiments, the NCAM targeting peptide is of the 8-amino acid sequence GDDSDEEN (SEQ ID NO: 4).

In some embodiments, the NCAM targeting peptide comprises an amino acid sequence of the C3 peptide, ASKKPKRNIKA (SEQ ID NO: 5). In some of these embodiments, the peptide may further comprise one or more amino acid residues, at

each terminus thereof, which may serve, for example, to facilitate attachment to the agent and/or to improve its targeting by improved exposure (reduced steric hindrance). In some embodiments, the NCAM targeting peptide is of the sequence GASKKPKRNIKA (SEQ ID NO: 15), with added glycine spacer at the N-terminal.

5 Additional NCAM targeting moieties, for example antibodies, are also contemplated.

According to some embodiments of the present invention there is provided a polymeric conjugate as described herein which further comprises an NCAM targeting moiety attached to the polymeric backbone. The NCAM targeting moiety can be a
10 ligand of NCAM as described herein.

In some embodiments, an agent as described herein is targeted to the cell-surface receptor P-Selectin.

In some embodiments, the agent further comprises P-Selectin targeting moiety.

In some embodiments, the P-Selectin targeting moiety is a ligand of the P-
15 Selectin receptor, and, in some embodiments, it is a ligand that is specific to P-Selectin receptor.

In some embodiments, the ligand of P-Selectin is a peptide. Such peptides are referred to herein and in the art as P-Selectin-targeting peptides.

In some embodiments, the targeting moiety is such that exhibits high
20 affinity to P-selectin, which is expressed in melanoma, glioblastoma and other cancer cells. An exemplary targeting moiety is e.g. a monosaccharide such as mannose.

In some embodiments, the ligand of P-Selectin is a selective antibody.

Additional P-Selectin targeting moieties, include for example antibodies, such as those commercially available from R&D Systems or from Biolegend.

25 Each of the downregulating agents described hereinabove can be administered to the individual *per se* or as part of a pharmaceutical composition which also includes a physiologically acceptable carrier. The purpose of a pharmaceutical composition is to facilitate administration of the active ingredient to an organism.

Pharmaceutical Compositions:

30 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 agent or conjugate (polyplex) accountable for the biological effect, as described herein.

5 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.

10 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.

15 Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

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

According to one embodiment, the agent is formulated for local administration
25 e.g. to the central nervous system (i.e. CNS administration).

According to one embodiment, the agent is formulated for systemic administration.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition
30 directly into a tissue region of a patient (e.g. CNS).

The term "tissue" refers to part of an organism consisting of cells designed to perform a function or functions. Examples include, but are not limited to, brain tissue,

retina, skin tissue, hepatic tissue, pancreatic tissue, bone, cartilage, connective tissue, blood tissue, muscle tissue, cardiac tissue brain tissue, vascular tissue, renal tissue, pulmonary tissue, gonadal tissue, hematopoietic tissue.

Pharmaceutical compositions of some embodiments of the 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 some embodiments of the 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. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the 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, and the like, for oral ingestion by a patient.

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.

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 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 suspensions.

Pharmaceutical compositions suitable for use in context of some embodiments of the invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredient (an agent or a conjugate as described herein) effective to prevent, alleviate or ameliorate symptoms of a disorder (e.g., as described herein) or prolong the survival of the subject being treated.

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

For example, the effect of the active ingredients (e.g., down-regulating agent of some embodiments of the invention) on the pathology (e.g. cancer) can be evaluated by monitoring the size and number of metastases (e.g. CNS metastases) in a treated subject using well known methods e.g. CT, pet-CT, MRI, ultrasound, X-ray, or by measuring the expression of cancer markers or inflammatory markers in a biological sample of the treated subject using well known methods (e.g. standard blood tests, ELISA, FACS, PCR, etc).

According to one embodiment, the therapeutically effective amount of the down-regulating agent is an amount capable of inhibiting or preventing formation of CNS metastases.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in vitro* and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Animal models for CNS metastases, which can be used to evaluate the down-regulating agents, are described in detail below.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and 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 e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

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 some embodiments of the invention may, if desired, be presented in a pack or 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 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 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 is further detailed above.

In order to enhance treatment of the CNS metastasizing cancer, the present invention further envisions administering to the subject an additional anti-cancer therapy such as radiotherapy, chemotherapy, biological therapy e.g., immunotherapy (e.g. antibody immunotherapy), phototherapy and photodynamic therapy, surgery, nutritional therapy, ablative therapy, combined radiotherapy and chemotherapy, brachiotherapy, proton beam therapy, cellular therapy and photon beam radiosurgical therapy, or combinations thereof. Analgesic agents and other treatment regimens are also contemplated. Examples of chemotherapeutic agents are described in detail below.

As used herein, the terms "chemotherapy" or "chemotherapeutic" refer to an agent that reduces, prevents, mitigates, limits, and/or delays the growth of neoplasms or metastases, or kills neoplastic cells directly by necrosis or apoptosis of neoplasms or any other mechanism, or that can be otherwise used, in a pharmaceutically-effective amount, to reduce, prevent, mitigate, limit, and/or delay the growth of neoplasms or metastases in a subject with neoplastic disease (e.g. cancer).

Chemotherapeutic agents include, but are not limited to, fluoropyrimidines; pyrimidine nucleosides; purine nucleosides; anti-folates, platinum agents; anthracyclines/anthracenediones; epipodophyllotoxins; camptothecins (e.g., Karenitecin); hormones; hormonal complexes; antihormonals; enzymes, proteins, peptides and polyclonal and/or monoclonal antibodies; immunological agents; vinca alkaloids; taxanes; epothilones; antimicrotubule agents; alkylating agents; antimetabolites; topoisomerase inhibitors; antivirals; and various other cytotoxic and cytostatic agents.

According to a specific embodiment, the chemotherapeutic agent includes, but is not limited to, abarelix, aldesleukin, aldesleukin, alemtuzumab, alitretinoin, allopurinol, altretamine, amifostine, anastrozole, arsenic trioxide, asparaginase, azacitidine, bevacuzimab, bexarotene, bleomycin, bortezomib, busulfan, calusterone, capecitabine, carboplatin, carmustine, celecoxib, cetuximab, cisplatin, cladribine, clofarabine, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, actinomycin D, Darbepoetin alfa, Darbepoetin alfa, daunorubicin liposomal, daunorubicin, decitabine, Denileukindiftitox, dexrazoxane, dexrazoxane, docetaxel, doxorubicin, dromostanolone

propionate, Elliott's B Solution, epirubicin, Epoetin alfa, erlotinib, estramustine, etoposide, exemestane, Filgrastim, floxuridine, fludarabine, fluorouracil 5-FU, fulvestrant, gefitinib, gemcitabine, gemtuzumabozogamicin, goserelin acetate, histrelin acetate, hydroxyurea, IbritumomabTiuxetan, idarubicin, ifosfamide, imatinibmesylate, interferon alfa 2a, Interferon alfa-2b, irinotecan, lenalidomide, letrozole, leucovorin, Leuprolide Acetate, levamisole, lomustine, CCNU, meclorethamine, nitrogen mustard, megestrol acetate, melphalan, L-PAM, mercaptopurine 6-MP, mesna, methotrexate, mitomycin C, mitotane, mitoxantrone, nandrolonephenpropionate, nelarabine, Nofetumomab, Oprelvekin, Oprelvekin, oxaliplatin, paclitaxel, palifermin, pamidronate, pegademase, pegasparase, Pegfilgrastim, pemetrexed disodium, pentostatin, pipobroman, plicamycinmithramycin, porfimer sodium, procarbazine, quinacrine, Rasburicase, Rituximab, sargramostim, sorafenib, streptozocin, sunitinib maleate, tamoxifen, temozolomide, teniposide VM-26, testolactone, thioguanine 6-TG, thiotepa, thiotepa, topotecan, toremifene, Tositumomab, Trastuzumab, tretinoin ATRA, Uracil Mustard, valrubicin, vinblastine, vinorelbine, zoledronate and zoledronic acid.

Additionally or alternatively, an anti-inflammatory therapy may be used to enhance treatment of the CNS metastasizing cancer. An exemplary anti-inflammatory therapy includes, without being limited to, NSAIDs (Non-Steroidal Anti-inflammatory Drugs), corticosteroids (such as prednisone) and anti-histamines.

Applicant asserts that one of skill in the art is capable of determining which anti-cancer and/or anti-inflammatory treatment should be used for therapy. Specifically, such a determination is based on the type of primary cancer (e.g. melanoma), staging, the number of metastasis, location of the metastasis, etc.

According to another aspect of the invention, there is provided a method of detecting CNS metastases in a subject afflicted with a CNS metastasizing cancer, the method comprising determining the level of at least one polypeptide selected from the group of polypeptides listed in any one of Tables 1-7 herein, wherein an upregulation in the level as compared to same in a control sample is indicative of CNS metastases.

According to another aspect of the invention, there is provided a method of monitoring treatment of a CNS metastasizing cancer, the method comprising treating a subject with an anti-cancer treatment (as discussed in detail hereinabove) and detecting

occurrence of CNS metastases according to some embodiments of the invention, thereby monitoring treatment of the CNS metastasizing cancer.

The terms “detecting” or “diagnosing” as used herein refer to determining the presence of a CNS metastasis, classifying a CNS metastasis, determining a severity of
5 CNS metastasis, monitoring CNS metastasis progression, forecasting an outcome of the CNS metastasis and/or prospects of recovery.

According to one embodiment, the polypeptide is a human polypeptide.

According to one embodiment, the at least one polypeptide comprises two or more polypeptides (or polynucleotides encoding same), e.g. 2-3 polypeptides, 3-4
10 polypeptides, 4-5 polypeptides, e.g. 2, 3, 4, 5 polypeptides.

According to a specific embodiment, the polypeptides (or polynucleotide encoding same) comprise no more than 3, 4 or 5 polypeptides.

According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 1.

15 According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 2.

According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 3.

20 According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 4.

According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 5.

According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 6.

25 According to one embodiment, the polypeptide is selected from the group of polypeptides listed in Table 7.

According to one embodiment, determining the level of at least one polypeptide is effected *ex vivo*.

30 According to one embodiment, determining the level of at least one polypeptide is effected in a biological sample.

As used herein “a biological sample” refers to a sample of fluid or tissue sample derived from a subject. Examples of fluid samples include, but are not limited to,

blood, plasma, serum, cerebro-spinal fluid (CSF), lymph fluid, urine, sweat, tears, saliva, sputum, milk and tumor cell sample. An example of a tissue sample includes a brain tissue sample.

Methods of obtaining such biological samples are known in the art including but not limited to standard blood retrieval procedures, lumbar puncture (LP), and biopsy (e.g. needle puncture and aspiration).

Control samples to which the subject's samples are compared may be obtained from cancer patients with the same cancer type, preferably at the same stage, wherein the clinical outcome of the cancer is known not to comprise brain metastasis. It is preferable that the non-metastatic cancerous control sample come from a subject of the same species, age and from the same sub-population (e.g. smoker/non-smoker). Alternatively, control data may be taken from databases and literature. It will be appreciated that the control sample may also be taken from the diseased subject at a particular time-point, prior to metastasis in order to analyze the progression of the disease.

According to some embodiments of the invention, upregulation in the level of at least one polypeptide (as compared to same in a control sample) is indicative of CNS metastases. According to some embodiments of the invention, upregulation by at least about 5 %, 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 %, 100 % or more is indicative of CNS metastases.

Determining the level of at least one polypeptide can be carried out using any method known in the art, as discussed infra.

Methods of detecting expression of the target on the RNA level

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 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 radioisotopes or enzyme linked nucleotides. Detection may be using autoradiography, 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 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 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.

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
5 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
10 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
15 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
20 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
25 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
30 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 target on the protein level

5 Determining expression of the polypeptide is typically effected using an antibody capable of specifically interacting with the polypeptide. Methods of detecting the polypeptide 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"
10 immunoassays, and immunoprecipitation assays and immunohistochemical assays as detailed herein below.

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
15 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
20 the amount of color produced. A substrate standard is generally employed to improve quantitative accuracy.

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
25 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 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
30 relative position on the membrane which is indicative of a migration distance in the acrylamide gel during electrophoresis.

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 I^{125}) immobilized on a precipitable carrier such as agarose beads. The number of counts in the precipitated pellet is proportional to the amount of substrate.

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.

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.

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.

The present inventors further provide novel animal models of melanoma brain metastases.

Thus, according to another aspect of the invention, there is provided a non-human animal comprising a CNS metastasizing melanoma, wherein the non-human animal does not comprise a primary melanoma tumor.

According to one embodiment, the non-human animal is immunocompetent.

As used herein, the term “immunocompetent” refers to an animal that is not immunodeficient (e.g. an animal that is capable of developing an immune response against an antigen).

According to some embodiments of the invention, the non-human animal is a
5 mammal.

According to some embodiments of the invention, the mammal is a mouse, a rat, a rabbit, a hamster, a sheep, a goat, a dog or a pig.

According to one embodiment, the melanoma cells are of an organism different from the non-human animal (e.g. allogeneic or xenogeneic with respect to the recipient
10 organism).

According to another embodiment, when xenogeneic (e.g. human) melanoma cells are implanted, the non-human animal is an immunodeficient non-human animal (e.g. SCID mouse).

According to one embodiment, the melanoma cells comprise human melanoma
15 cells.

According to one embodiment, the non-human animal does not comprise a primary melanoma tumor as detected by e.g. ultrasound, x-ray or using a caliper. Thus, according to one embodiment, the primary melanoma tumor is removed from the non-human animal prior to formation of the CNS metastasis. Accordingly, the primary
20 melanoma tumor may be removed (e.g. surgically) from the animal several days, a week, two weeks, one month, two months, three months, four months, six months or more, prior to formation of the CNS metastasis.

The non-human animal comprising the CNS metastasizing melanoma may be generated using any method known in the art.

According to one aspect of the invention, the non-human animal with the CNS metastasizing melanoma is generated by a method comprising: (a) systemically administering melanoma cells to a non-human animal; (b) allowing growth of a primary melanoma tumor in the non-human animal; (c) surgically removing the primary melanoma tumor; (d) monitoring formation of CNS metastasis; and (e) selecting the
30 non-human animal with a CNS metastasizing melanoma.

According to one embodiment, the non-human animal is immunocompetent.

According to one embodiment, the non-human animal is immunodeficient (i.e. immunocompromised).

According to one embodiment, when xenogeneic melanoma cells are administered (e.g. in situations in which donor and recipient are of different species),
5 the non-human animal is an immunodeficient non-human animal (e.g. SCID mouse).

According to a specific embodiment, when allogeneic melanoma cells are administered (e.g. in situations in which both donor and recipient are of the same species), the non-human animal is an immunocompetent or an immunodeficient non-human animal.

10 According to one embodiment, systemically administering melanoma cells to a non-human animal is effected by injection of the melanoma cells into a peripheral location (e.g. not to a CNS tissue). Any peripheral location of the animal can be selected, as for example, over the shoulders, into the loose skin over the neck, over the flank, etc.

15 According to one embodiment, the systemic administration comprises an intradermal administration. According to one embodiment, the systemic administration comprises a subcutaneous administration.

Other modes of administration of melanoma cells include, but are not limited to, intra-cardially, intra-carotid artery, or intra-cranially injection.

20 According to one embodiment, administration is effected in single dose.

According to another embodiment, administration is effected in two or more doses. When repeated doses are used, varying the site of injection can be used to reduce the likelihood of local skin reactions.

When several doses are used, these can be administered on the same day, on
25 consecutive days, or over several days, weeks or months. One of skill in the art is capable of making such a determination.

According to one embodiment, the melanoma cells are allogeneic or xenogeneic with respect to the recipient non-human animal.

According to a specific embodiment, the melanoma cells comprise human
30 melanoma cells.

According to a specific embodiment, the melanoma cells are derived from a patient (i.e. from a melanoma tumor removed from a patient).

According to a specific embodiment, the melanoma cells are melanoma cell lines. Exemplary cell lines which can be used to generate the animals of the invention include, but are not limited to, WM115, WM239, 131/4-5B1, A375, MEL-526, Ret, D4M.3A, B16 cell lines.

5 According to a specific embodiment, the melanoma cells comprise a V600E BRAF mutation. Accordingly, the melanoma cells may be D4M.3A or A375 cell lines.

According to another specific embodiment, the melanoma cells comprise a V600G BRAF mutation. Accordingly, the melanoma cells may be a Ret cell line.

10 According to another specific embodiment, the melanoma cells comprise a V600D BRAF mutation. Accordingly, the melanoma cells may be WM115, WM239 or 131/4-5B1 cell lines.

Using melanoma cells comprising specific mutations can be beneficially utilized to assess the efficiency of treatment of different anti-cancer agents (e.g. BRAF inhibitors) towards the CNS metastases.

15 According to one embodiment, the melanoma cells express a reporter gene. For example, a reporter gene can encode for a fluorescent protein, such as mCherry. Exemplary reporter genes which can be used in accordance with the present invention include, but are not limited to, *lacZ* (encoding β -galactosidase), *cat* (Chloramphenicol acetyltransferase), *luciferase*, *gfp* (encoding Green fluorescent protein), or *rfp* (encoding
20 Red fluorescent protein).

According to one embodiment, the melanoma cells are transduced to express the reporter gene (e.g. mCherry) by viral infection using, for example, a retrovirus or a lentivirus.

Following administration of the melanoma cells to an animal, the melanoma cells
25 are allowed to grow to form a primary melanoma tumor. Once the primary melanoma tumor reaches a size of approximately 250-1000 mm³ (e.g. 500-700 mm³), as can be measured by e.g. ultrasound, x-ray or using a caliper, the primary tumor is surgically removed from the animal.

CNS metastatic development is then monitored for expression of melanoma cells
30 e.g. by monitoring melanoma cells expressing the reporter gene. According to one embodiment, the formation of CNS metastases is monitored by non-invasive *in vivo* imaging. Exemplary non-invasive *in vivo* imaging includes, but is not limited to, CRI™

Maestro non-invasive intravital imaging system. According to another embodiment, the formation of CNS metastases is monitored every day, every other day, every week or every month, or until identification of CNS metastases is achieved.

According to another embodiment, metastasis tumor size is monitored for appearance, size and location. In order to confirm the diagnosis, the animal may be subjected to a biopsy (e.g. in which a sample of the tumor metastasis is removed and evaluated *ex vivo* for cancer specific markers, e.g. MART-1 (MELAN-A), Tyrosinase, MITF, S100 protein family, TRP-1, TRP-2).

Once a non-human animal has been determined as having a CNS metastasizing melanoma, the non-human animal is selected.

In order to further enhance the number of non-human animals exhibiting CNS metastasizing melanoma, the CNS metastases may be removed from the non-human animal (described above), minced into single cell suspensions (e.g. cell lines) and re-administered to new non-human animals (as described in detail above), also referred to as passaging. According to one embodiment, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more passages may be effected in order to enhance the number of non-human animals exhibiting CNS metastasizing melanoma.

According to one embodiment, the above described non-human animal with the CNS metastasizing melanoma may be used as a model animal for CNS metastases. Furthermore, this animal model may be used for evaluation of therapeutic agents for the treatment of CNS metastasizing cancer, such as the down-regulating agents described above.

As used herein the term "about" refers to $\pm 10\%$.

The terms "comprises", "comprising", "includes", "including", "having" and 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.

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.

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

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.

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.

When reference is made to particular sequence listings, such reference is to be understood to also encompass sequences that substantially correspond to its complementary sequence as including minor sequence variations, resulting from, e.g., sequencing errors, cloning errors, or other alterations resulting in base substitution, base deletion or base addition, provided that the frequency of such variations is less than 1 in 50 nucleotides, alternatively, less than 1 in 100 nucleotides, alternatively, less than 1 in 200 nucleotides, alternatively, less than 1 in 500 nucleotides, alternatively, less than 1 in 1000 nucleotides, alternatively, less than 1 in 5,000 nucleotides, alternatively, less than 1 in 10,000 nucleotides.

As used herein, the term "alkyl" describes an aliphatic hydrocarbon including straight chain and branched chain groups. Preferably, the alkyl group has 1 to 20 carbon atoms, and more preferably 1-10 carbon atoms. Whenever a numerical range; e.g., "1-10", is stated herein, it implies that the group, in this case the alkyl group, may contain 1 carbon atom, 2 carbon atoms, 3 carbon atoms, etc., up to and including 10 carbon atoms. In the context of the present invention, a "long alkyl" is an alkyl having at least 5 carbon atoms in its main chain (the longest path of continuous covalently attached atoms). A short alkyl therefore has 4 or less main-chain carbons. The alkyl can be substituted or unsubstituted. When substituted, the substituent can be, for example, an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an aryl, a heteroaryl, a halide, an amine, a hydroxyl, a thiol, an alkoxy and a thioalkoxy, as these terms are defined herein.

The term "alkyl", as used herein, also encompasses saturated or unsaturated hydrocarbon, hence this term further encompasses alkenyl and alkynyl.

The term "alkenyl" describes an unsaturated alkyl, as defined herein, having at least two carbon atoms and at least one carbon-carbon double bond. The alkenyl may be substituted or unsubstituted by one or more substituents, as described hereinabove.

The term "alkynyl", as defined herein, is an unsaturated alkyl having at least two carbon atoms and at least one carbon-carbon triple bond. The alkynyl may be substituted or unsubstituted by one or more substituents, as described hereinabove.

The term "heteroalicyclic" describes a monocyclic or fused ring group having in the ring(s) one or more atoms such as nitrogen, oxygen and sulfur. The rings may also have one or more double bonds. However, the rings do not have a completely conjugated pi-electron system. The heteroalicyclic may be substituted or unsubstituted. Substituted heteroalicyclic may have one or more substituents, whereby each substituent group can independently be, for example, hydroxyalkyl, trihaloalkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, heteroalicyclic, amine, halide, hydroxy, alkoxy and thioalkoxy. Representative examples are piperidine, piperazine, tetrahydrofurane, tetrahydropyrane, morpholino and the like.

Piperidine and piperazine are exemplary nitrogen-containing heterocyclic.

The term "hydroxy", as used herein, refers to an -OH group.

The term "alkoxy" refers to a $-OR'$ group, where R' is alkyl, aryl, heteroalicyclic or heteroaryl.

As used herein, the term "amine" describes a $-NR'R''$ group where each of R' and R'' is independently hydrogen, alkyl, cycloalkyl, heteroalicyclic, aryl or heteroaryl,
5 as these terms are defined herein.

The term "aryl" describes an all-carbon monocyclic or fused-ring polycyclic (i.e., rings which share adjacent pairs of carbon atoms) groups having a completely conjugated pi-electron system. The aryl group may be substituted or unsubstituted by one or more substituents, as described hereinabove.

10 The term "heteroaryl" describes a monocyclic or fused ring (i.e., rings which share an adjacent pair of atoms) group having in the ring(s) one or more atoms, such as, for example, nitrogen, oxygen and sulfur and, in addition, having a completely conjugated pi-electron system. Examples, without limitation, of heteroaryl groups include pyrrole, furane, thiophene, imidazole, oxazole, thiazole, pyrazole, pyridine,
15 pyrimidine, quinoline, isoquinoline and purine. The heteroaryl group may be substituted or unsubstituted by one or more substituents, as described hereinabove. Representative examples of nitrogen-containing heterocyclics include imidazole, thiadiazole, pyridine, pyrrole, oxazole, indole, purine and the like.

As used herein, the terms "halo" and "halide", which are referred to herein
20 interchangeably, describe an atom of a halogen, that is fluorine, chlorine, bromine or iodine, also referred to herein as fluoride, chloride, bromide and iodide.

The term "haloalkyl" describes an alkyl group as defined above, further substituted by one or more halide(s).

The term "alkylene" as used herein describes a $-(CR'R'')_f-$, wherein R' and R''
25 are as described herein, and f is an integer from 1 to 20, or from 1 to 10.

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
30 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

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.

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); "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
5 (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 **GENERAL MATERIALS AND EXPERIMENTAL PROCEDURES**

Cell Culture

mCherry-labeled cell lines were obtained as previously described ²¹. Cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS), 100 µg/ml Penicillin, 100 U/ml Streptomycin, 12.5 U/ml Nystatin, 2 mM L-glutamine
15 (Biological Industries, Israel). D4M.3A cell line was cultured in DMEM/F12 medium supplemented with 5 % FBS, Glutamax supplement, 100 U/ml penicillin and 100 µg/ml streptomycin (ThermoFisher Scientific). WM115 cell line was cultured in MEM medium supplemented with 10 % FBS, 100 µg/ml Penicillin, 100 U/ml Streptomycin, 12.5 U/ml Nystatin, 2 mM L-glutamine (Biological Industries, Israel).

20 Human Umbilical Vein Endothelial Cells or human microvascular endothelial cells (HUVEC/HMVEC from ATCC) were cultured in M199 medium, supplemented with 20 % fetal bovine serum (FBS), 100 mg/ml Penicillin, 100 U/ml Streptomycin, 12.5 U/ml Nystatin, 2 mM L-glutamine, 100 mg/ml Heparin and 50 mg/ml of Endothelial Cell Growth Supplement (ECGS).

25 Murine and human astrocytes were purchased from ScienCell Research Laboratories (CA, USA) and grown in custom made medium purchased from the same company.

Murine microglial C8-B4 cell line (ATCC, CRL-2540, Manassas, USA) ²⁶ are maintained in DMEM/F12 medium supplemented with 10 % FBS, 100 units/ml
30 penicillin and 100 µg/ml streptomycin.

All cells are grown at 37 °C in 5 % CO₂ humidified incubator.

Primary cells isolation from brain stroma (murine and human patient-derived brain metastasis samples)

Murine microglia and astrocytes are isolated from healthy and melanoma metastasis-bearing mouse brains, using CD11b (Microglia) and Anti-GLAST (Astrocytes) MicroBeads Kits (Miltenyi Biotec, Germany), according to manufacturer's instructions. The same kits are adapted for isolation of human microglia and astrocytes from human patient-derived samples of melanoma brain metastasis.

Mouse models of melanoma brain metastasis

Mice (Harlan laboratories, Israel) were maintained at Tel-Aviv University imaging facility. All experiments were approved by the Tel-Aviv University Institutional Animal Care and Use Committee (IACUC).

Orthotropic inoculation and tumor excision

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (12 mg/kg). 1 x 10⁶/100 µl mCherry labeled melanoma cells (WM115, WM239, 131/4-5B1, A375, Ret-mCherry, D4M.3A or patient-derived cell lines) were inoculated intra-dermally or subcutaneously to the mouse flank. Primary tumor progression was monitored by caliper measurement (width x length² x 0.52) and resected when reached the size of 500 to 700 mm³. Brain metastatic development was monitored by CRITM Maestro non-invasive intravital imaging system. Multispectral image-cubes were acquired through 450-720 nm spectral range in 10 nm steps using excitation (465 nm longpass) and emission (515 nm longpass) filter set. Mice autofluorescence and undesired background signals were eliminated by spectral analysis and linear unmixing algorithm.

Intra-cardiac injection

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (12 mg/kg). 0.5 x 10⁶/50 µl mCherry labeled melanoma cells (WM115, WM239, 131/4-5B1, A375, Ret-mCherry, D4M.3A or patient-derived cell lines) were injected to the left heart ventricle, guided by live ultrasound imaging (Vevo 770 High-Resolution System; Visual-Sonics Inc.). Brain metastatic development was monitored by CRITM Maestro non-invasive intravital imaging system.

Patient-derived melanoma brain metastases

Tissue samples are used for the following purposes: (1) snap freeze by liquid nitrogen for RNA isolation and genetic characterization by qRT-PCR; (2) formalin-

fixed for analysis by IHC for melanoma markers (MART-1), morphology (H&E), proliferation (PCNA or Ki67), apoptosis (caspase) activated astrocytes (GFAP) microglia (IBA-1) and microvessel density (CD31); (3) rinsed into small pieces and cultured into 35 mm plate in Dulbecco's Modified Eagle Medium (supplemented by 10 % fetal bovine serum (FBS), 100 mg/ml penicillin, 100 U/ml streptomycin and 2 mM L-glutamine). Cell line are propagated; (4) Fragments (5 mm³ in size) are implanted subcutaneously into the flank of 5-7 weeks old male SCID mice. Once the tumor reaches a size of approximately 1,000 mm³, it is excised and passaged to a new mouse.

Magnetic Resonance Imaging (MRI)

MRI is performed using 7T MRI scanner (Bruker, Germany) with a 30-cm bore and a gradient strength of up to 400 mT/m with a mouse head quadrature coil serving as a receiver coil. The MRI protocol includes T1 weighted image pre- and post GD-DTPA injection. During MRI, mice are anesthetized using isoflurane (1.5 %-2 %) and their breathing are monitored with a breathing sensor. T1 weighted scan of mouse brain are performed using RARE sequence (rapid acquisition with relaxation enhancement). Sequence parameters: TR = 728 ms, TE = 7.2 ms, RARE factor 4, 14 averages, in-plane resolution 0.07 x 0.07 mm, slice thickness 0.8 mm. The T1 weighted scan are repeated after i.p injection of 0.25 mmol/kg GD-DTPA (Omniscan). Image analysis is performed by WiseImage Advanced Imaging Services, using in-house software (Matlab). Pre- and post-Gd images are compared, enhanced regions of interest (ROIs) are outlined manually and the signal intensity is used to calculate the tumor volumes. For comparison, a region in the contralateral hemisphere is outlined manually in a normal appearing region. The signal intensity values is extracted in the enhanced and normal signal regions, and tumor volume is calculated. Regions with high signal intensity compared to the surrounding tissue are considered as lesions, and outlined manually in each slice. The volume is calculated as number of outlined voxels multiplied by voxel size. Mean signal intensity is extracted in each lesion. The suspected pathological brain regions are analyzed by a neuro-radiologist.

Gene sequencing

Melanoma cell lines and brain metastatic melanoma cells were analyzed for the presence of the V600 BRAF mutation in exon 15. DNA was extracted from the cells using QIAamp DNA Mini Kit (Qiagen), following the manufacturer's protocol. PCR

reaction was performed using BRAF primers: for human cell lines: forward primer – TGCTTGCTCTGATAGGAAAATG (SEQ ID NO: 1), reverse primer – TCAGGGCCAAAAATTTAATCA (SEQ ID NO: 2); for murine cell lines: forward primer - TCCCAGCAGAGTTAAAGTGTATC (SEQ ID NO: 6), reverse primer – GTGAGTAGTGGGAACTGTGAAA (SEQ ID NO: 7). Following DNA amplification, samples were loaded on an agarose gel. The appropriate band was cut and cleaned using Wizard SV Gel and PCR Clean-Up System (Promega). Purified DNA was sequenced using ABI 3500xl Genetic Analyzer.

Proliferation assay

Cells are seeded on 24-well plates and allowed to attach for 24 hours. Cells are then incubated for 72 hours with conditioned media (CM) or media with selective inhibitor treatments (from 0.01 nM to 10,000 nM drug equivalents). Following incubation, cells are washed, detached by trypsin EDTA and counted by Coulter Counter® (Beckman Coulter). IC₅₀ is presented as the concentration at which 50 % of inhibition rate is reached.

Conditioned Media (CM) Preparation

To generate conditioned media, 1 x 10⁶ cells were plated on 10 cm² tissue culture plate in its appropriate medium supplemented with 10 % FBS, 100 ug/ml Penicillin, 100 U/ml Streptomycin, 12.5 U/ml Nystatin and 2 mM L-glutamine. Following a 48 hour incubation at 37 °C in 5 % CO₂, cells' media was harvested and filtered through 0.45 µm syringe filter to remove cells and debris.

Quantitative Real-Time PCR

Total RNA was isolated from cultured cells or tumor tissues using EZ-RNA II total RNA isolation kit (Biological industries). One microgram of RNA was reverse transcribed using EZ-first strand cDNA synthesis kit for RT-PCR (Biological industries).

SYBR green based real-time PCR assays (QIAGEN) was used to analyze gene expression levels. Specific primers were designed using Primer Express software (Applied Biosystems). The relative amount of RNA was normalized to the expression of GAPDH or Beta actin.

FACS analysis

Cells were washed twice with PBS, then harvested and centrifuged for 5 minutes at 1200 rpm. Then cells were resuspended and washed three times in FACS buffer (0.5 % BSA in PBS). After the last cycle, the supernatant were discarded and cells were resuspended in 100 µl of FACS buffer. Cells were then incubated in gentle rocking with the relevant antibodies (e.g. NCAM), according to the manufacturer's instructions, washed in FACS buffer and resuspended in 500 µl of FACS buffer. Results were read in FACS Aria.

Cytokine array analysis

Astrocytes or melanoma cells were grown in serum free medium (SFM) for 24 hours. The conditioned medium (CM) was collected and filtered through 0.45 micron filter. Astrocytes or melanoma cells were then grown for 24 hours in the counter-CM. The medium was collected and the secreted cytokines were concentrated using centricons. The concentrated medium was then mixed with an antibody cocktail and placed on a membrane for an overnight incubation at 2-8 °C on a rocking platform. After a serial of washes and incubations with detectors, the membranes were exposed to X-ray film for 1-10 minutes. The intensity of the detected cytokines was measured using the ImageJ software.

ELISA

Astrocytes were grown to 80 % confluence and then treated with a polyplex of siRNA of selected cytokines and a polymer. 24 hours post-treatment, medium was aspirated and replaced with SFM. 24 hours post medium-replacement, the medium was collected, concentrated using centricons and distributed to ELISA plates. After 2 hours incubation, and a serial of washes and incubation with detection antibodies, a substrate solution was distributed to the wells. Once a color development was seen, the plates were placed in an ELISA reader and monitored at 405 nm with a wavelength correction of 650 nm. The amount of secreted protein was calculated using a standard curve.

Migration of melanoma cells towards CM from brain stroma cells

Cell migration assays were performed using 8 micron Transwell inserts (Costar Corp., Corning, NY, USA). Melanoma cells (1×10^5 cells/200 µl) were seeded in the upper chamber. Astrocytes/microglia/endothelial cells or their CM were placed in the lower chamber with or without neutralizing antibodies blocking the following

cytokines: RANTES (1 µg/µl), IL-8 (0.1 µg/µl), IL-6 (0.1 µg/µl), SERPINE1 (0.1 µg/µl), GRO α (0.1 µg/µl), IP-10 (0.1 µg/µl), MCP-1 (1 µg/µl) (Peprotech Asia). Cells were allowed to migrate to the lower chamber for an additional 5 - 24 hours (according to preliminary migration assays), then fixed with ice cold methanol and stained (Hema 3 Stain System). The stained migrated cells were imaged using Nikon TE2000E inverted microscope integrated with Nikon DS5 cooled CCD camera by 10⁶ objective, brightfield illumination. Total area of migrated cells from the captured images per membrane were analyzed using NIH imageJ software.

Electrophoretic Mobility Shift Assay (EMSA)

The optimal ratio between PGA-amine and siRNA was determined by electrophoretic mobility shift assay (EMSA). Fifty pmole siRNA was incubated with the nanocarrier at a ratio of 1:0, 1:1, 1:2, 1:3, 1:5 for 20 minutes. Following incubation, polyplexes mobility shift was analyzed by agarose gel electrophoresis.

Dynamic light scattering (DLS) and zeta potential determination

The mean hydrodynamic diameter of the PGA-NH₂-siRNA polyplex and the zeta-potential measurements are performed using a ZetaSizer Nano ZS instrument with an integrated 4 mW He-Ne laser (λ =633 nm; Malvern Instruments Ltd., Malvern, Worcestershire, UK). PGA-NH₂-siRNA polyplexes are prepared by mixing polymer and siRNA at the indicated molar ratio in 1 mL DDW. Samples are incubated for 20-30 minutes at room temperature, then PBS is added from X10 stock to a final buffer concentration of 15 mM, pH=7.4 to the zeta potential sample. All measurements are performed at 25 °C using polystyrol/polystyrene (10×4×45 mm) cell for DLS analysis and folded capillary cell (DTS 1070) for zeta-potential measurements.

siRNA knockdown in vitro

siRNA sequences (TriFECTa® RNAi Kit, IDT) were used to knockdown IL-8 and MCP-1. siRNA at different concentrations (1 nM-100 nM) was incubated either with lipofectamine (Invitrogen) as a control or with PGA-NH₂ for 20 minutes at room temperature. The formed polyplexes were then added to astrocytes/microglia/endothelial cells, seeded the previous day. 48 hours following treatment, RNA was isolated and qRT-PCR was performed to analyze the expression of the knocked-down factors.

Knockdown of CXCR3 expression

To prepare stable melanoma cell lines that are knocked down for the CXCR3 receptor (NM_009910.2), lentiviral LVRU6GH plasmids containing shRNA for CXCR3 or scramble constructs (purchased from GeneCopoeia) were used to prepare
 5 lentiviral particles. The following sequences were used: SC-RMS gcttcgcgcgcgtagtctta (SEQ ID NO: 8), Sh-1 ggtagtgaacgtcaagtgt (SEQ ID NO: 9), Sh-2 tcagcctgaactttgacagaa (SEQ ID NO: 10), Sh-3 gccctactaaattagcaagta (SEQ ID NO: 11), Sh-4 gcgccttggtcaacatcaact (SEQ ID NO: 12). 1.5×10^6 293T packaging cells were plated and grown in RPMI, transfected with the plasmids utilizing 2M CaCl_2 . 2×10^5
 10 ret-melanoma cells were plated in 10 cm and were 50 % confluent on day of infection. 24 hours post-transfection 293T cell media were collected, filtered (0.45 μm filter, Millipore) and supplemented with 8 $\mu\text{g/ml}$ Polybrene (H-9268, sigma). Melanoma cells were infected with the virus-containing supernatant for 3 hours. Infectants were selected with 200 $\mu\text{g/ml}$ hygromycin (H-270-5-1, ENCO).

Immunohistochemistry

Immunohistochemistry of tumor nodules was performed using 5 mm thick formalin-fixed, paraffin-embedded tissue sections. Paraffin sections were deparaffinized, rehydrated, and stained by hematoxylin and eosin (H&E). Sections were further stained for proliferating cells using anti-Ki67 antibody (1:100, Abcam), for
 20 apoptotic cells using cleaved caspase 3 antibody (1:100 dilution; Epitomics) and for microvessels using anti-CD31 antibody (1:10 dilution; Dianova). For Ki67 and caspase 3 stainings slides were subjected to heat induced antigen retrieval procedure using 10 mM citric buffer (pH 6.0). Rabbit monoclonal anti-ki67 and rabbit monoclonal anti-caspase-3 were applied in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1 % Tween-20,
 25 pH 7.5) for 1 hour. Slides were then embedded in anti-rabbit immunoperoxidase polymer (N-histofine Simple Stain Mouse Max PO; Nichirei) for 30 minutes, followed by DAB/ H_2O_2 for 3 minutes. For CD31 staining slides were subjected to heat induced antigen retrieval using TE buffer (Sigma-Aldrich). Rat monoclonal anti-murine CD31 antibody was applied in TBST for 2 hours. Slides were then embedded in anti-rat
 30 immunoperoxidase polymer for 30 minutes, followed by DAB/ H_2O_2 for 3 minutes. Following DAB all slides were weakly stained with hematoxylin, dehydrated through

ascending ethanol concentrations and mounted in Eukitt (Sigma Aldrich). Microvessel density (MVD) was calculated as previously described ²⁷.

Modified-Miles assay

Evans blue 1 % (100 µl) was injected to the mouse orbital vein. Twenty minutes later, heart perfusion was performed with 10 ml PBS. Brains were extracted, minced and incubated in 300 µl formamide. Five days later, fluorescence of the extracted Evans blue dye was evaluated. Extracted Evans Blue in formamide (100 µl) from each mouse was divided into triplicates and read in a 96 well plate reader (Excitation= 620 nm, Emission= 680 nm).

Statistical Analysis

In vitro data from proliferation and migration assays on melanoma cells, astrocytes, endothelia cells and microglia was expressed as mean $\pm$ 95 % confidence interval (CI). *In vivo* data of Miles assay and evaluation of antitumor activity of the polymeric conjugates was expressed as mean $\pm$ 95 % CI (n=15mice/group). The Student's t test was used to compare treated versus untreated melanoma cell lines with respect to continuous data including *in vitro* tumor cell doubling times, migration assays, vascular permeability, and ELISA. Two-tailed P values less than .05 was considered statistically significant. Statistical analyses was performed with the SPSS package. For each of the tumor types, times to reach 700 mm³ at the primary site or time to reach macrometastases in the brain was compared using the Kaplan – Meier product limit method (Kaplan EL, Meier P. J Am Stat Assoc 1958; 53: 457 – 81).

EXAMPLE 1

Establishment and characterization of in vivo melanoma brain metastases model

The present inventors worked with three mouse models for melanoma brain metastases. The Ret-mCherry model is based on a transgenic mouse model of spontaneous malignant melanoma, the Ret-Melanoma ¹⁹. This cell line was derived from a spontaneously occurring local tumor ²⁰. The cells were engineered to express the fluorescent reporter gene mCherry and selected for highly fluorescent cells by FACS ²¹. An additional murine melanoma used is the cell line D4M.3A previously described ³³. 131/4-5B1 cells are also engineered to express mCherry. These two cell lines, along with ATCC-purchased melanoma cell lines (WM115, MEL526 and A375 human

primary melanoma and WM239 human metastatic melanoma) are either inoculated intra-dermally to mouse flank or injected to mice left heart ventricle. Once a primary tumor (in the orthotopic model) reaches a size of 500 mm³ it is surgically removed. In both models, brain metastases development is monitored by an intravital imaging system. These tissues are used for the following purposes: (1) snap freeze by liquid nitrogen to be genetically characterized by qRT-PCR; (2) formalin-fixed for analysis by IHC for melanoma markers (MART-1), morphology (H&E), proliferation (PCNA or Ki67), apoptosis (caspase) activated astrocytes (GFAP) and microvessel density (CD31); (3) rinsed into small pieces and cultured into 35 mm plate for the establishment of a new cell line, which is then propagated; (4) fragments (*circa* 5 x 5 x 5 mm³ in size) are implanted subcutaneously into the flank of 5-7 weeks old SCID mice. Once the tumor reaches a size of approximately 1,000 mm³, it is excised and passaged (Figure 1A).

The present inventors sequenced all of the cell lines and found that A375 and MEL526 as well as the murine D4M.3A bear V600E BRAF mutation), while the WM115, WM239 and 131/4-5B1 have V600D mutation). The Ret-mCherry cell line was found to express the more scarce mutation, V600G, and the patient derived cell lines that do not bear a BRAF mutation (Figure 1B).

In order to determine the ability of the present nanocarrier to extravasate through the compromised BBB at the neuroinflammatory pre-metastatic site, and later at the tumor site and accumulate in melanoma brain metastases due to the EPR effect, the present inventors performed a modified-Miles assay²⁴. Human melanoma cells were injected to the left ventricle of the heart of immunodeficient mice, guided by live ultrasound imaging. Three weeks post injection, the present inventors observed brain metastases by non-invasive imaging (Figure1C). At that point, Evans blue dye, which binds to albumin and the complex extravasates at location of vascular hyperpermeability, was injected to mice orbital vein, brains were extracted, minced and incubated in formamide for 5 days. Dye accumulation was quantified and an increase in the dye signal of metastases-bearing brains was observed compared to healthy brains (Figure 1D). This result suggests that metastatic formation disrupts the integrity of the blood brain barrier. Histology stainings of the metastatic brains show that the metastases

are highly proliferative (Ki67) and angiogenic (CD31) and that the microenvironment is activated (as seen by the GFAP positive staining) (Figure 1E).

EXAMPLE 2

Quantitative molecular detection of micrometastases in the murine model

Macrometastases are the final stage of a long complex process. In order to gain insight on the initial steps of metastasis, the present inventors analyzed the formation of brain micrometastases in the immunocompetent murine model. First, an evaluation was performed to determine whether the expression of mCherry could be quantitatively assessed as a reporter for the presence of micrometastases, and whether quantitative detection of mCherry could be utilized to determine brain metastatic load. To that end, an *ex vivo* modeling system of micrometastases was established by mixing known numbers of RMS cells with normal brains followed by combined homogenization and RNA extraction (Figure 2A). qPCR analysis of mCherry revealed a linear correlation between melanoma cell numbers and mCherry expression ($r^2=0.98$, Figure 2B). The same linearity was obtained for the known melanoma markers *Trp-1*, *Trp-2* ($r^2=0.99$, Figures 2C, 2D), *Mart-1* and *Mitf-v2* (not shown), confirming that this *ex vivo* calibration system could be used to quantify the number of melanoma cells. Strikingly, quantification of melanoma cells in brains of mice with no detectable macrometastases revealed mCherry positive signal equivalent to as few as 100 cells (Figures 2E, 2F). Notably, mice were heart perfused to ascertain that the mCherry signal did not originate in circulating melanoma cells, but rather from parenchymal metastases. Thus, this molecular quantification system provides a reliable tool to identify incipient metastatic lesions and to study the earliest stages of metastatic disease.

Utilizing this molecular detection system, the present inventors next quantified the percentage of micrometastases. Analysis revealed that one month following primary tumor removal, 40-50 % of the mice had micrometastases composed of less than 10^4 cells (Figures 2E, 2F). In addition, micrometastases were detected and quantified by FACS analysis. Micrometastases consisting of less than 10^4 cells corresponded to a population of approximately 1-1.5 % mCherry positive cells of total brain cells (Figures 2G, 2H).

Next, the present inventors asked whether metastases formation in this model is a continuous process. To that end, the relative expression signal from mCherry positive brains was averaged at different micrometastases end-points: 7, 14, and 56 days post-subdermal inoculation. Analysis indicated that the mCherry signal increased exponentially with time in three independent cohorts analyzed (Figure 2I). These results indicate that micrometastases are proliferative; or alternatively, that additional micrometastatic lesions form with disease progression. In order to obtain spatial insight of micrometastases, mCherry detection was utilized to analyze the histopathology of brain micrometastases. Analysis of staining revealed that disseminated cells were located in the choroid plexus and in the brain parenchyma (Figures 2J, 2K). To test whether micrometastases detected by qPCR correlate with the histological findings, the corresponding hemispheres were examined by qPCR, as described above. A significant correlation was found between the presence of melanoma cells in brain sections and their detection by qPCR in the contralateral hemisphere, thus enabling efficient and accurate screening for micrometastases-bearing brains (Figure 2L).

EXAMPLE 3

Brain micrometastases are associated with vascular hyperpermeability

Tumor vasculatures are known to be pathologically hyperpermeable in advanced tumor lesions. However, very little is known about changes in the permeability of blood vessels in micrometastases. To test whether vessel hyperpermeability is a feature of brain micrometastases, a modified Miles assay was performed. Brains of injected mice analyzed were free of macrometastases, as confirmed by *ex vivo* fluorescent imaging and qPCR analysis of mCherry expression. Analysis of Evans blue extravasation into brain tissue revealed that spontaneous formation of brain micrometastases was associated with increased vascular hyperpermeability (Figure 3A). To further quantify the leakiness of brain blood vessels and the integrity of the blood-brain barrier (BBB), mice were injected with FITC-Dextran (70 kDa). Quantification of staining indicated that mice with micrometastases had significantly more leaky vessels than control mice (Figures 3B-E). Furthermore, staining of melanoma cells in mice injected with FITC-Dextran indicated that vessel permeability is correlated with the presence of disseminated melanoma cells around leaky blood vessels (Figures 3C-H). These

findings suggest that spontaneous brain micrometastases instigate breakdown of the blood-brain barrier.

EXAMPLE 4

5 *Astrogliosis and neuroinflammation are instigated in incipient melanoma brain metastases*

The present inventors next wanted to test the applicability of the model to characterize the formation of a hospitable metastatic niche. It was previously demonstrated that astrocytes are recruited to experimental melanoma macrometastases
10 in brain ²¹. Therefore, the present inventors set out to characterize the role of astrocytes in spontaneously occurring brain metastases. To that end, astrocyte recruitment and activation was analyzed by immunostaining with GFAP, a marker of activated astrocytes, in brain sections from mice injected subdermally with RMS cells. Analysis of the results confirmed that activated astrocytes surrounded macrometastatic lesions
15 (Figure 4A). Moreover, the expression of GFAP in brain sections was analyzed from patients and validated that astrogliosis is evident also in human melanoma brain metastasis (Figure 4B). Importantly, analysis of staining in brains bearing micrometastases indicated that astrocyte recruitment and activation is an early event (Figure 4C). Therefore, the present inventors next sought to determine when astrogliosis
20 is first instigated during brain metastases formation. Analysis of brains bearing micrometastases (as determined by mCherry expression) indicated that the expression of GFAP is upregulated as early as 2-4 weeks after primary tumor removal (Figure 4D), suggesting that astrogliosis precedes metastases formation.

Neuroinflammation, manifested by upregulation of pro-inflammatory cytokines
25 and chemokines is a feature of astrogliosis. To test whether this physiologic characteristic of astrogliosis is operative also in the metastatic niche, the expression of multiple pro-inflammatory mediators in brains of mice bearing micrometastases was analyzed, as above. Analysis of the expression results revealed upregulation in known pro-inflammatory cytokines and chemokines, including CCL17, CCL2, CXCL10, IL-6
30 and IL-1 β (Figures 4E-I), suggesting that instigation of neuroinflammation, associated with astrogliosis, is an early event in the metastatic cascade.

It was previously demonstrated that astrocytes facilitate the invasiveness of brain-tropic human melanoma cells *in vitro*²¹. The functional role of astrocytes in facilitating the invasiveness of melanoma cells was further supported in an organotypic 3D intact brain slices co-cultured with a tumor plug containing melanoma cells (Figure 4J). Analysis of brain slices by immunostaining indicated that activated astrocytes and microglia are recruited to the tumor-brain interface (Figures 4K-N). Seeking to obtain functional evidence that astrogliosis could be induced by paracrine signaling from disseminated melanoma cells, the present inventors next tested whether conditioned media (CM) from RMS cells could activate primary adult astrocytes that had been isolated from brains of normal mice. In addition to auditing the expression of *Gfap*, the expression of an astrogliosis-related wound-healing gene signature was analyzed, including *Cxcl10*, *Lcn2*, *Timp-1*, *Serpine1*, and *Serpina3n* (Figures 4O-S). This gene signature was demonstrated to be operative during stroke and LPS-induced astrogliosis. Expression analysis of astrocytes incubated with melanoma cells CM indicated that secreted factors from melanoma cells could upregulate the astrogliosis-related wound-healing gene signature in normal astrocytes. These results suggest that the same molecular pathways that are induced during brain tissue damage and in neuroinflammation are operative also during brain metastases formation.

EXAMPLE 5

Astrocytes facilitate the initial growth of metastatic melanoma cells

In addition to invasiveness, persistence of disseminated tumor cells at a hostile microenvironment is a critical limiting step of metastasis. The present inventors therefore asked whether astrocytes affect the initial growth of disseminated melanoma cells. To that end, a 3D co-culture system to model incipient micrometastases was established. When dispersed melanoma cells (as few as 25 cells) were seeded in the presence of primary astrocytes they were more proliferative than control melanoma cells (Figures 5A-C), suggesting that astrocytes are functionally necessary for the initial growth of melanoma cells.

To further assess the functional importance of astrocytes to the initial growth of melanoma cells *in vivo*, a limiting number of melanoma cells admixed with adult primary astrocytes were co-injected to mice brains. Strikingly, melanoma cells co-

injected with astrocytes gave rise to significantly (9-fold) larger brain lesions than RMS-only controls (Figures 5D-F), implying that astrocytes play a central role in supporting the growth of melanoma cells in the brain. Moreover, quantification of reactive astrocytes *in vivo* by immunostaining demonstrated that larger brain lesions are characterized by increased GFAP expression in the brain, reflecting recruitment and activation of host astrocytes (Figures 5G-I). This astrogliosis was specifically instigated by tumor cells rather than by the tissue damage caused by intracranial injection, since intracranial injection of serum-free medium (SFM) to control mice did not induce astrogliosis at the indicated time-point (Figure 5I). To get molecular insight on the effect of the brain microenvironment on brain-metastasizing melanoma cells, a variant of brain-tropic melanoma cells (BT-RMS) was established by isolating tumor cells from brain macrometastases and re-injecting them to mice. Analysis of the ensuing brain metastasis after two rounds of selection revealed that the brain-tropic variants exhibited an increased incidence of brain metastasis and a shortened timeline from subdermal injection to the formation of spontaneous brain metastasis. Moreover, analysis of known signaling pathways indicated that the MAPK pathway was activated in brain-tropic melanoma cells as compared with the parental tumor cells (Figures 5J-K). These results indicate that the MAPK pathway is activated by the brain microenvironment. Importantly, *in vitro* experiments in which melanoma cells were incubated with secreted factors of activated astrocytes revealed that astrocytes induce MAPK activation in RMS melanoma cells (Figures 5L-M). These results imply that the *in vivo* activation of this pathway in brain-tropic melanoma cells is mediated, at least partially, by astrocytes. Thus, astrocytes support the growth of melanoma cells and activate signaling pathways associated with enhanced proliferation.

Finally, the present inventors analyzed the expression of the gliosis wound-healing gene signature in brains of mice co-injected with astrocytes as compared with brains of mice injected with melanoma cells only (Figures 5N-R). Expression results revealed upregulation of genes known to be associated with astrogliosis. Notably, the upregulation of most genes was significant even when analyzed in total brain, containing multiple other cells in addition to activated astrocytes. These results functionally implicate the induction of astrogliosis and a wound-healing program in facilitating metastatic colonization and the initial growth of melanoma cells.

EXAMPLE 6***Metastases-associated astrocytes express and secrete the chemokine CXCL10***

To further study the role of astrocytes, the present inventors set out to identify possible candidates that could point out key interactions between astrocytes and melanoma cells. Since RMS cells were isolated from a cutaneous melanoma tumor, studying the effect of RMS-derived soluble factors on normal adult primary astrocytes is an *in vitro* system to model possible interactions between systemic tumor cell secreted factors and the distant brain niche.

Since it was found (see above) that astrocytes become pro-inflammatory when reprogrammed by melanoma cells, and upregulate *Cxcl10* mRNA levels, the present inventors set out to further characterize the effect of melanoma-secreted factors on pro-inflammatory gene expression in astrocytes. A cytokine array analysis was performed on melanoma-activated astrocytes. Astrocytes were incubated with melanoma cell conditioned medium, lysed, and analyzed for their protein-level expression of various cytokines and chemokines. It was found that CXCL10, IL-17 and IL-1 β were highly upregulated in activated astrocytes (Figure 6A). Moreover, the control non-activated astrocytes had almost no basal CXCL10 expression, strengthening the previous results at the mRNA level. In order to test whether CXCL10 is secreted from activated astrocytes, normal primary adult astrocytes were incubated in RMS-CM for 12 hours. CM was then replaced with fresh media, and collected 24 hours later. ELISA assay revealed that the secretion of CXCL10 was approximately 5 folds elevated in melanoma activated astrocytes compared with SFM treated, non-activated, astrocytes (Figure 6B).

CXCL10 is a known T cell chemoattractant, operating via the CXCR3 receptor. Astrocytes were recently shown to orchestrate T cell recruitment to the brain in Multiple Sclerosis and during viral CNS infections via CXCL10 secretion. The present inventors therefore hypothesized that melanoma cells hijack this physiological pathway, resulting in brain tropism. Specifically, the present inventors hypothesized that astrocyte-derived CXCL10 might have a role in attracting melanoma cells to the brain.

To validate these findings *in vivo*, the present inventors analyzed whether CXCL10 is expressed by metastases-associated astrocytes. To this end, co-stainings of CXCL10 and GFAP were performed in brains bearing spontaneous micro- and

macrometastases as well as from intra-cranial injected mice. The results illustrate that metastases-associated astrocytes express CXCL10 *in vivo* (Figures 7A-C).

To further validate the expression of CXCL10 in astrocytes *in vivo* FACS-sorting of mCherry⁻CD45⁻ACSA2⁺ astrocytes was utilized. Normal astrocytes or metastases-associated astrocytes (MAA) were isolated from brain single cell suspensions of either normal mice, or macrometastases bearing mice (via intracranial injections). qPCR analysis revealed that *Cxcl10* was highly upregulated in MAAs compared to normal astrocytes (Figure 7D).

EXAMPLE 7

CXCL10 serum levels correlate with brain micrometastases

The present inventors hypothesized that CXCL10 may affect brain tropism of melanoma cells. To test this hypothesis, the present inventors next asked whether higher levels of CXCL10 in serum are correlated with brain metastases. To address this, the present inventors analyzed serum from mice with or without metastases as determined by FACS analysis and found that micrometastases-bearing mice had higher CXCL10 serum levels (Figures 8A-B), suggesting that CXCL10 may have systemic effects on melanoma brain metastases formation.

EXAMPLE 8

CXCL10 is functionally necessary for melanoma cell migration toward astrocytes

To further test the hypothesis, the present inventors next studied the functional role of astrocyte-derived CXCL10 in mediating migration of melanoma cells. Transwell assays were utilized in which melanoma cells were seeded in the upper chamber, and astrocytes in the lower chamber. rCXCL10 and neutralizing antibodies were added to CXCL10 in order to test its effect on melanoma cell migration. Notably, while rCXCL10 treatment upregulated the migration of melanoma cells to some extent, the migration of melanoma cells towards astrocytes was significantly better. Since recombinant proteins lack post-translational modifications, this might suggest that such modifications in astrocytes are important for CXCL10 function as a chemoattractant. Importantly, CXCL10 neutralization attenuated melanoma cell migration towards

astrocytes (Figures 9A-B). These results suggest the migration of melanoma cells is mediated, at least partially, by astrocyte-derived CXCL10.

EXAMPLE 9

CXCR3 is highly expressed in brain-tropic melanoma cells

The role of astrocyte-derived CXCL10 in facilitating melanoma brain tropism via the CXCL10-CXCR3 axis is unknown. Therefore, the present inventors next asked whether melanoma cells express CXCR3. To that end, CXCR3 expression was analyzed by qPCR in the melanoma brain-tropic variants which have been isolated. Interestingly, the selected brain-tropic variant (BT-RMS-2 melanoma) expressed higher levels of CXCR3 compared to the local tumor variant. This was specific to brain-tropic cells as the expression of CXCR3 in lung tropic variant was lower (LT-RMS, Figure 10A). Moreover, an available GEO dataset was analyzed and found that CXCR3 is expressed in human melanoma tumors (Figure 10B).

To further validate these observations, the expression of CXCR3 was analyzed at the protein level *in vivo* by FACS analysis of primary tumors. $5 \cdot 10^5$ RMS, BT-RMS and LT-RMS cells were injected subdermally to mice, and the ensuing primary tumors were surgically removed, digested into single cell suspensions, and stained with anti-CXCR3 antibodies. FACS analysis revealed that BT-RMS tumors were highly enriched in CXCR3⁺ melanoma cells, compared to the parental RMS tumors, and compared to the lung tropic variant. Interestingly, the analysis also revealed a prominent CD45⁺CXCR3⁺ population infiltrating the BT-RMS local tumors (Figures 11A-C). These results validate the observations at the mRNA level of higher CXCR3 expression in BT-RMS cells compared with RMS, suggesting that reciprocal interactions with the brain stroma may select for CXCR3⁺ cells, which are more responsive to CXCL10.

Intrigued by these observations, the present inventors next asked if this upregulation of CXCR3 is stable *in vitro*, in order to further manipulate CXCR3 expression in functional experiments. FACS analysis revealed a sub-population of CXCR3⁺ tumor cells within the RMS and BT-RMS variants. Indeed, the results illustrated that the percentage of CXCR3 expressing cells was higher in cultured BT-RMS cells compared to RMS cells (Figures 12A-B). Interestingly, for both RMS and

BT-RMS variants, the proportions of CXCR3⁺ cells found *in vivo* were much higher than those *in vitro*.

EXAMPLE 10

5 *CXCR3 is functionally necessary for brain micrometastases formation*

In order to functionally test the effect of CXCR3 on brain metastasis formation, the present inventors knocked down CXCR3 expression by lentiviral transduction of shRNA targeting CXCR3. qPCR analysis of the resulting shCXCR3 variants (sh-1 to sh-4) confirmed knockdown of the receptor (Figure 13A). Scramble-infected cells were
10 used as controls. Next, the migration of RMS cells toward astrocytes was evaluated and revealed a decreased migration of the Sh-1 (data not shown) and sh-2 clones (Figure 13B). To assess the role of CXCR3 in facilitating brain tropism *in vivo*, mice were injected with shCXCR3-RMS or control cells (SC-RMS) subdermally. Analysis of primary tumor growth indicated no differences in growth kinetics following CXCR3
15 ablation (Figure 13C). The local tumors were resected and the mice were monitored for 2 months. Surviving mice were, heart perfused, euthanized and their brains analyzed by qPCR for TRP-2, an established melanoma marker, to determine brain metastasis (Figure 13D). Normal mice were used as controls and results were compared to a calibration curve, as described above. Strikingly, knockdown of CXCR3 decreased the
20 occurrence of brain metastasis by approximately 50 %: while 66 % of SC-RMS injected mice developed brain micrometastases, as expected in the present model, only 33 % and 25 % in the sh-1 and sh-2 clones, respectively, had micrometastases. Taken together, the present results implicate the CXCR3-CXCL10 axis in brain tropism.

25 EXAMPLE 11

LCN2 is highly expressed and secreted by brain-tropic melanoma cells

Since it was observed that CXCL10 is upregulated in astrocytes prior to detection of metastases, the present inventors hypothesized that systemic signaling from the primary tumor or from circulating melanoma cells may activate astrocytes to form a
30 hospitable brain metastatic niche. Therefore, in order to identify tumor-secreted factors that may activate astrocytes in incipient metastatic niches, and specifically, factors that can induce the expression of CXCL10, the present inventors searched for known

CXCL10 activators. LCN2, a member of the lipocalin ligand-binding protein family, was previously shown to induce CXCL10 expression in astrocytes during neuroinflammation³⁴⁻³⁶. LCN2 was also shown to mediate GFAP upregulation in astrocytes via the Rho/ROCK pathway. Moreover, LCN2 is upregulated in astrocytes during gliosis³⁷. However, the role of tumor-derived LCN2 in inducing CXCL10 expression in astrocytes is unknown. The present inventors therefore hypothesized that melanoma cell-derived LCN2 may activate CXCL10 expression in astrocytes. To test this hypothesis, the expression and secretion of LCN2 was analyzed in the different melanoma variants. Analysis of the results revealed that LCN2 is indeed expressed in and secreted by melanoma cells. Importantly, when comparing the different melanoma variants it was found that LCN2 was more highly expressed and secreted by the brain-tropic cells (Figures 14A-B). Next, the present inventors asked whether LCN2 is expressed in human melanoma. Analysis of expression data in publically available databases indicated that LCN2 is mainly expressed in local tumors, suggesting it plays a role in early disease stage (Figure 14C). The present inventors further validated that LCN2 is systemically secreted utilizing ELISA assay of serum from mice injected with RMS or BT-RMS, collected two months after tumor removal. The analysis revealed that mice injected with BT-RMS had higher levels of LCN2 in their serum compared to mice injected with RMS, supporting the hypothesis of systemic LCN2 secretion (Figure 14D). Notably, even after the long latency from tumor removal, the injected mice showed higher serum LCN2 levels compared to normal mice. This result might suggest circulating tumor cells as an additional source of systemic LCN2 secretion.

EXAMPLE 12

The stromal compartment of primary tumors expresses LCN2

In order to gain further insight on LCN2 expression in the primary tumor, the present inventors analyzed tissue samples of primary melanomas from the Human Protein Atlas for staining with LCN2 (Figure 15A). Interestingly, LCN2 was expressed in both tumor cells and the stromal compartment (fibroblasts and immune cells, see inset in Figure 15A) in primary tumors, suggesting a functional role for LCN2 in the communication between tumors and their microenvironment. IFN γ , another known inducer of CXCL10, showed the same trend (Figure 15B).

The present inventors therefore asked whether LCN2 is expressed in the stromal compartment of RMS tumors. Single cell suspensions from RMS tumors were FACS sorted to dissect the expression of LCN2 by various cells in the tumor microenvironment. Normal skin from mouse ears was used as control. qPCR analysis revealed that cancer associated fibroblasts (CAFs) and immune cells expressed higher levels of the CXCL10 inducers *Lcn2*, *Il-1 β* and *Ifn γ* as compared to tumor cells. These results suggest that in addition to tumor cell-derived signaling, the stromal compartment in the primary tumor might affect distant astrocytes in the brain pre-metastatic niche (Figures 16A-C).

EXAMPLE 13

Tumor-derived LCN2 is necessary to induce CXCL10 expression in astrocytes

Next, the present inventors tested whether RMS-derived LCN2 could upregulate the expression of CXCL10 in astrocytes. To that end, functional *in vitro* experiments were performed in which astrocytes were incubated with SFM supplemented with recombinant proteins, or with RMS CM, or with CM supplemented with neutralizing antibodies to LCN2 or IL-1 β . The results illustrated that tumor cell CM, rLCN2 and rIL-1 β upregulated the expression of *Cxcl10* in astrocytes. Interestingly, anti-LCN2 antibodies attenuated *Cxcl10* upregulation following CM treatment, but anti-IL-1 β antibodies did not affect *Cxcl10* expression. These results suggest that *Cxcl10* expression is instigated, at least partially, by tumor derived-LCN2, but tumor-derived IL-1 β is not required for upregulation of CXCL10 in astrocytes (Figures 17A-B). The findings that sorted stromal cells from primary melanoma tumors express high levels of IL-1 β (data not shown) suggest that the main source of IL-1 β in melanoma tumors may be stromal cells in the microenvironment, rather than tumor cells. Taken together, the present data suggest possible role for LCN2 (and IL-1 β) as an upstream regulator of CXCL10 expression in astrocytes.

EXAMPLE 14***The molecular crosstalk between circulating melanoma cells, astrocytes and brain-metastasizing melanoma cells***

By using a cytokine array, the present inventors have recently identified
5 cytokines changes in melanoma cell's profile while stressing (Figure 18A) and
cytokines that were upregulated in astrocytes following paracrine signaling from
melanoma cells, while circulating in the blood (Figure 18B) or brain metastases (Figure
18C) including: sICAM, GRO, MIF, SERPINE1, CCL2, IL-8, and RANTES. Human
astrocytes were grown in serum-free medium for 24 hours. In a transwell assay,
10 melanoma cells were seeded in an insert and astrocyte-conditioned media (CM) was
placed in the lower well. The present results illustrate that melanoma cells migrate
towards the astrocytes-conditioned media (Figure 18D). Cell migration assays were
performed in triplicates using 8 micron Transwell inserts (Costar). WM115 and 131/4-
5B1 cells (1×10^5 cells/200 μ l) were seeded in the upper chamber. Astrocytes' CM was
15 placed in the lower chamber with or without neutralizing antibodies blocking the
following cytokines: RANTES (1 μ g/ μ l), IL-8 (0.1 μ g/ μ l), IL-6 (0.1 μ g/ μ l), SERPINE1
(0.1 μ g/ μ l), GRO α (0.1 μ g/ μ l), IP-10 (0.1 μ g/ μ l), MCP-1 (1 μ g/ μ l) (Peprotech Asia).
Cells were allowed to migrate to the lower chamber for an additional 5 hours and 24
hours for WM115 cell line or 131/4-5B1 cells, respectively. Cells were fixed with
20 methanol, stained (Hema 3 Stain System) and imaged. Migrated cells from the
documented images were counted using NIH imageJ software. Next, cytokines-
neutralizing antibodies were added to the astrocytes conditioned media, the results
illustrate that by neutralizing IL-8, MCP-1, GRO α and SERPINE1 a decrease in
melanoma cell migration was significantly attained (Figure 18E). Cytokine gene
25 expression in astrocytes activated by melanoma cells was also validated by real-time
PCR. mRNA was isolated from human astrocytes 72 hours post co-incubation with
melanoma cells or astrocyte medium (Figure 18F-G). IL-8 and MCP-1 receptors
expression were evaluated and the results illustrated that brain metastasizing melanoma
cells as well as astrocytes grown in starvation media express higher levels of CXCR1
30 (IL-8 receptor), and CCR4 receptor (MCP-1 receptor) compared to full medium (Figure
18H-I).

EXAMPLE 15***Astrocytes are activated by melanoma cells to express pro-inflammatory factors***

In order to identify astrocyte-derived metastasis promoting factors, we performed a proteomic analysis utilizing a cytokine array platform. Primary astrocytes
5 treated with conditioned medium of melanoma cells exhibited upregulation of the pro-inflammatory genes IL-17, IL-1 β , M-CSF and a prominent upregulation of the chemokine CXCL10 (Figure 19).

EXAMPLE 16***IL-8 and MCP-1 knockdown in human astrocytes by siRNA***

The present inventors have developed a novel PGA-amine nanocarrier which is positively charged and can be complexed with negatively charged nucleotides (siRNA, miRNA) (Figure 20A). The optimal ratio between PGA-amine and IL-8/MCP-1 siRNA was determined by electrophoretic mobility shift assay (EMSA) and was found to be 1:3
15 (Figure 20B). siRNA sequences were used to knockdown IL-8 and MCP-1 genes in human astrocytes. siRNA at different concentrations (1 nM-100 nM) was incubated either with lipofectamine (as control) or with the PGA-amine nanocarrier for 20 minutes at room temperature. The polyplexes were then added to astrocytes which were seeded at a 2.5×10^5 cells-confluence per well in 6 wells plate the previous day. Twenty four
20 hours post treatment, cells were lysed and RNA was isolated. qRT-PCR was performed to analyze cytokine expression (Figures 20C-D).

EXAMPLE 17***Blood brain barrier permeability in melanoma brain metastases***

In order to determine the ability of the presently described nanocarrier to extravasate through the compromised BBB at the neuroinflammatory pre-metastatic site and later at the tumor site and accumulate in melanoma brain metastases due to the EPR effect, the present inventors performed a modified-Miles assay²⁴. Human melanoma cells were injected to the left heart ventricle of immunodeficient mice, guided by live
30 ultrasound imaging. Forty days post injection, brain metastases was observed by non-invasive imaging. At that point, Evans blue dye, which binds to albumin and the complex extravasates at location of vascular hyperpermeability, was injected to mice

orbital vein, brains were extracted, minced and incubated in formamide for 5 days. Dye fluorescence was quantified and an increase in the fluorescence signal of metastases-bearing brains was observed compared to healthy brains (Figures 23A-B). This result suggests that metastatic formation disrupts the integrity of the blood brain barrier

EXAMPLE 18

P-Selectin and Neural Cell Adhesion Molecule (NCAM) as potential targeting moieties.

Selectins

Selectins are carbohydrate-binding proteins that are physiologically expressed on the surface of endothelial cells (P-sel and E-selectin), activated platelets (P-sel) and leukocytes (L-selectin)²⁸. Selectins are also involved in tumor cell adhesion and promote their invasion by supporting a permissive microenvironment and protecting them from recognition by immune cells²⁹. Upon endothelial activation with ionizing radiation, P-sel translocates to the cell membrane³⁰. Elevated P-sel expression has been previously found in the vasculature of lung, breast, colon³¹ and kidney cancers³².

P-selectin expression

The present inventors evaluated by flow cytometry P-sel expression in several melanoma cell lines (Figure 24A). Cells were detached with a rubber spatula and incubated with either mouse anti-human E/P-selectin antibody (R&D Systems, Minneapolis, MN, USA) or mouse anti mouse/rat P-selectin (Biolegend, San Diego, CA, USA) for 60 minutes at 4 °C in the dark. All washing steps were performed with freshly prepared FACS buffer consisting of 0.5 % BSA in PBS. NCAM expression of several cell lines was evaluated by flow cytometry. Cells were detached with a rubber spatula and incubated with mouse anti-human NCAM-APC antibody (Biolegend, San Diego, California, USA) for 45 minutes at 4 °C in the dark. All washing steps were performed with freshly prepared FACS buffer consisting of 0.5% BSA in PBS.

NCAM expression in cancer cells

In order to select candidates for a model tumor cell line, NCAM expression of various melanoma cell lines was evaluated by FACS. The present inventors uncovered that the cell lines WM115, WM239 and 131/4-5B1 express high levels of NCAM (Figure 24B), the cell line A375 expresses about 30 % NCAM (data not shown). Taken

together, NCAM can be used as a targeting moiety for the presently described nanomedicine.

NCAM expression in human umbilical vein endothelial cells (HUVEC)

NCAM expression of human umbilical vein endothelial cells (HUVEC) was examined in cells that were seeded on Matrigel, which promotes cell organization into capillary-like tubes, and in cells grown on uncoated plates. It was found that HUVEC express NCAM during the process of capillary-like tube formation (48 % expression) but not when grown on uncoated plates (data not shown).

EXAMPLE 19

Molecular and functional characterization of established models of melanoma brain metastases

Mouse models of human and murine melanoma previously established are characterized at distinct stages of brain metastasis. Specifically, mCherry-labeled human melanoma cells WM115, WM239, 131/4-5B1, or A375 inoculated orthotopically intra-dermally in SCID mice (n=30 mice/cell line) are characterized. Once a primary tumor reaches a size of 500 mm³ as measured by caliper, it is resected. From previous experience, only 30-50 % of the mice, in which their primary tumors were resected, develop brain metastases. Alternatively, for a faster, reproducible and high "tumor-take" procedure, mCherry-labeled melanoma cells are injected intracardially under ultrasound imaging (as described in the 'general material and experimental procedures' section). Mice are followed-up for tumor recurrence and brain metastases development by intravital non-invasive imaging (Maestro, CRI). These tissues are treated as follows: (1) snap frozen by liquid nitrogen for RNA isolation and q-RT-PCR analysis of specific genes previously found to be overexpressed in melanoma brain metastases. (2) DNA is isolated for BRAF sequencing. (3) formalin-fixed and paraffin-embedded (FFPE) for analysis by immunohistochemistry (IHC) for melanoma markers (MART-1), morphology (H&E), proliferation (PCNA or Ki67), apoptosis (caspase), activated astrocytes (GFAP), NCAM, IL-8 and MCP-1 receptors (CXCR1 and CCR4) and *in situ* hybridization for IL-8 and MCP-1; (4) snap-frozen for OCT frozen sections for CD31 fluorescence staining for microvessel density analysis; (5) resected into small pieces and cultured into 35 mm plates for the establishment of a

new cell line, which is then propagated; (6) fragments (5 x 5 x 5 mm³ in size) are implanted subdermally into the flank of 5-7 weeks old SCID mice (n=4 mice per tumor sample). Once the tumor xenograft reaches a size of approximately 1,000 mm³, it is excised and passaged to additional mice (n = 5 mice per generation from each tumor resected). Mice that underwent surgery removing the primary melanoma, are maintained and followed up by MRI (as described in the 'general material and experimental procedures' section). Once brain metastasis are detected, mice are euthanized at different time points, brains are resected and metastasis progression is analyzed in parallel with astrocyte proliferation and activation markers (Ki67, GFAP), microglia (Iba1), blood vessel density (CD31, Meca32), and vessel maturation (smooth muscle cells (SMC) and pericytes), compared to no metastasis-bearing (healthy) brain. General morphology is assessed and compared to FFPE samples. A similar analysis is performed in immunocompetent mouse model of melanoma brain metastasis (mCherry-labeled Ret-melanoma cells, derived from metallothionein-I (MT)/ret transgenic mice).

EXAMPLE 20

Identification and validation of brain stroma cells (astrocyte, brain endothelium and microglia)-secreted factors in facilitating brain colonization and invasiveness of melanoma cells.

Secretome profiling of brain stroma cells before and after the onset of brain metastases:

Following the findings obtained from the above described experiments, the present inventors are co-culturing several melanoma cell lines (WM115, WM239, 131/4-5B1, A375, Ret-mCherry, patient-derived cell lines) with brain stroma including cells purchased from the ATCC or ScienCell Research Laboratories, or freshly-isolated astrocytes, microglia and brain endothelial cells. The present inventors are identifying secreted factors that play a key role in the interaction between brain metastasis-forming melanoma cells and the brain stroma. Briefly, astrocytes, microglia and brain endothelial cells are incubated with melanoma cells conditioned media (CM). Twenty four hours later, cells supernatant are analyzed by a fully quantitative ELISA-based chemiluminescent assay, allowing the concurrent measurement of multiple biomarkers (circa 100 growth factors, cytokines involved in processes such as angiogenesis and

inflammation) and are analyzed using the human or murine Proteome Profiler Human Cytokine Array Kit (R&D systems, USA).

In order to validate the results obtained from these assays, mCherry-labeled melanoma cells are co-cultured with human astrocytes, microglia or brain endothelial cells for 72 hours. Following incubation, stroma cells are separated from melanoma cells by flow cytometry. Total RNA is isolated from stroma cells, and expression levels of cytokines and/ or growth factors highly secreted in the ELISA arrays and their respective receptors are analyzed by q-RT-PCR.

The present inventors are further isolating astrocytes, microglia and brain endothelial cells from naïve mice *versus* mice bearing melanoma brain metastasis and comparing the factors secreted in each case using the previously described ELISA and q-RT-PCR techniques.

Stroma secreted factors validation in human patient-derived brain metastasis samples:

In order to test whether the changes in cytokines/growth factor secretion in mouse tumor-associated stroma are relevant to human brain metastasis, the present inventors are validating the expression of the candidate genes that were significantly altered in mouse astrocytes, microglia and/or endothelial cells in fresh human tissue samples and FFPEs from tissue banks by q-RT-PCR and IHC.

The ability of mCherry-labeled 131/4-5B1 cells to develop brain metastasis was analyzed by injecting the cells intra-cardiacally to mice left ventricle. Brain metastases formation was followed by intra-vital imaging. As illustrated in Figure 22A, hematoxylin and eosin (H&E) staining showed micrometastasis. These micrometastasis were highly proliferative as seen by Ki-67 immunohistochemistry (Figure 22B), and the brain microenvironment surrounding the micrometastasis was activated as seen by the positive GFAP (Figure 22C) and IBA-1 (Figure 22D) stainings. Moreover, P-Selectin was positively stained in the micrometastasis and MCP-1 secretion was elevated around the micrometastases (Figures 22E-F).

EXAMPLE 21***Assessing the pro-oncogenic potential of brain metastasis-related secreted factors by RNA interference (in vitro and in vivo)***

The present inventors are functionally elucidating the role of the newly discovered factors in facilitating brain metastasis establishment by silencing the relevant genes with specific small interfering RNAs (siRNAs). The present inventors are establishing a library of siRNAs targeting the newly discovered factors in order to validate their potential pro-oncogenic activity. Following molecular knockdown of each of the factors, the present inventors are co-culturing cytokine siRNA-treated brain stroma cells with melanoma cells or alternatively brain stroma cells with receptors siRNA-treated melanoma cells. Then, the effects on proliferation, migration and invasion of melanoma cells are evaluated *in vitro* in real time PCR and ELISA assay (Figures 21A-B). The ability of melanoma cells to establish metastatic brain tumors in mice are compared following culture in two conditions: (i) CM from naive astrocytes/microglia/endothelial cells *versus* (ii) CM from astrocytes/microglia/endothelial cells pre-treated with cytokine siRNA preselected and validated in example 20, above.

Synthesis, characterization and optimization of PGA-NH₂-cytokine (IL-8/MCP-1/CXCL10/GRO α /SERPINE1) siRNA polyplex and/or respective receptors (CXCR1/CCR4/CXCR3/CXCR2/uPAR) siRNA polyplex:

Following the findings obtained from the above described experiments, PGA-NH₂ is first complexed with IL-8, MCP-1, CXCL10, GRO α and SERPINE1 siRNA at several ratios and evaluated in an electrophoresis mobility shift assay (EMSA). Thereafter, it is characterized by dynamic light scattering (DLS) and zeta potential (as described in the 'general material and experimental procedures' section), in order to find the optimal ratio to achieve maximal siRNA activity and minimal toxicity. The effect of PGA-NH₂-siIL-8, PGA-NH₂-siGRO α , PGA-NH₂-siMCP-1, PGA-NH₂-siSERPINE1 and PGA-NH₂-siCXCL10 siRNA polyplexes on astrocytes and the ability of their CM to inhibit melanoma cell proliferation and migration is evaluated.

Moreover, based on the cytokines/growth factors discovered, that are highly secreted from the brain stroma of melanoma brain metastasis-bearing mice or their

respective receptors overexpressed on the melanoma cells, the present inventors are developing additional PGA-NH₂-siRNA polyplexes and characterizing them using the techniques described herein (see 'general material and experimental procedures' section).

EXAMPLE 22

Assessment of synergistic potential of the proposed combination therapy in 3D co-cultures of melanoma cells with astrocytes, microglia and endothelial cells.

PGA-NH₂ siRNA polyplexes are evaluated for their ability to inhibit the proliferation and migration of murine and human melanoma cells.

10 PGA-NH₂-siRNA effect on astrocytes, microglia, endothelial and brain metastasis cells:

Astrocytes are treated with any one of PGA-NH₂ IL-8/MCP-1/GRO α /SERPINE1/CXCL10 siRNA polyplex, and the cytokine expression and secretion levels are measured by qPCR and ELISA, respectively. Melanoma cells grown in CM from PGA-NH₂-siIL-8/MCP-1/GRO α /SERPINE1/CXCL10 (i.e. siRNA
15 polyplexes) - treated astrocytes are followed for the functional effect of the cytokines silencing (proliferation, invasion, transendothelial migration as described above). A similar approach is undertaken, treating microglia and endothelial cells with siRNA targeting a factor that is highly secreted from these cells following co-culture with melanoma cells.

20 PGA-NH₂-siRNA polyplex that significantly inhibits proliferation, invasion and transendothelial migration of melanoma cells *in vitro* at the most pronounced level is selected for further *in vivo* evaluation.

EXAMPLE 23

25 *In vivo evaluation of the combined nanomedicine on established murine and human mouse models of melanoma brain metastasis*

Biodistribution:

In order to assess the accumulation of the polyplex elements at the tumor site, mice are injected intravenously with Cy5-labeled PGA-NH₂-polyplex. At different time
30 points after injection (5 min, 30 min, 1, 3, 6, 12, and 24 hours), perfusion is performed

by saline injection to the mouse left ventricle. Organs and tumors are resected, fixed, and imaged as whole mount by confocal microscopy as previously shown ¹⁶.

Tumor microdialysis:

5 Tumor microdialysis is performed to determine siRNAs concentrations in tumor interstitial fluid as previously described ²⁵. Following a 60 minutes period of equilibration, brain extracellular fluid (ECF) microdialysate samples are collected every 30 minutes, starting one hour prior to drug administration. Blood samples are collected 10, 30, 60, 120, 240 and 480 minutes after drug dosing. The brain ECF and plasma
10 samples are assessed for drugs levels via HPLC.

Systemic administration of PGA-combined nanomedicine for the treatment of melanoma brain metastasis:

Growth rate of melanoma tumors and brain metastases in mice treated PGA-NH₂
15 IL-8/MCP-1/GRO α /SERPINE1/CXCL10 siRNA is monitored by non-invasive intravital microscopy. Body weight, general health and tumor size are monitored q.o.d. (every other day).

Immunohistochemistry of tumor tissues:

20 At termination, tumors are resected and analyzed by immunohistochemistry determining morphology, necrosis (H&E), melanoma markers (MART-1 and TRP2), microvessel density (CD-31), proliferation (PCNA or Ki67), apoptosis (TUNEL or caspase), MEK, IL-8, MCP-1, GRO α , SERPINE1, CXCL10, CXCR1, CCR4, CXCR2, uPAR, CXCR3 etc. Specific targets' expression levels are assessed by IHC and qPCR,
25 according to the relevant inhibited pathway.

Improvement and optimization of the PGA-siRNA formulations is performed by testing different flow rates of the drug nanoprecipitation using a syringe pump or a microfluidic system (Nanoassembler). Secondly, different sonication time and intensities are further used to modulate particle size and stability. The present inventors are further
30 exploring small molecular excipients to stabilize the particles (PEG 400, Tween-20, etc.).

The PGA formulation is administered systemically as nanoparticles and is expected to accumulate in the leaky vasculature of the tumors. Tumor accumulation is enhanced by NTP targeting or alternatively by RGD peptidomimetics, transferrin, folic acid and others. Complementary approaches are employed:

- 5 1. Reducing nanoparticles size to further improve penetration, as well as stability in serum.
2. Modulating surface charge with positively charged hydrophobic counter ion for improved BBB penetration.
3. Adding polymeric layers to improve drug release (polyethyleneglycol-
10 (PEGylation) via cleavable biodegradable bonds, for example).

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
15 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
20 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.

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WHAT IS CLAIMED IS:

1. A method of treating or preventing central nervous system (CNS) metastases in a subject afflicted with a CNS metastasizing cancer, the method comprising administering to the subject an agent that down-regulates activity or expression of a polypeptide selected from the group of polypeptides listed in any one of Tables 1-7 herein, thereby treating or preventing CNS metastases.
2. An agent that down-regulates activity or expression of a polypeptide selected from the group of polypeptides listed in any one of Tables 1-7 herein, for treating or preventing central nervous system (CNS) metastases in a subject afflicted with a CNS metastasizing cancer.
3. A method of sensitizing treatment to a CNS metastasizing cancer, the method comprising administering to the subject an agent that down-regulates activity or expression of a polypeptide selected from the group of polypeptides listed in any one of Tables 1-7 herein, and treating the subject with an anti-cancer treatment.
4. A method of detecting CNS metastases in a subject afflicted with a CNS metastasizing cancer, the method comprising determining the level of at least one polypeptide selected from the group of polypeptides listed in any one of Tables 1-7 herein, wherein an upregulation in said level as compared to same in a control sample is indicative of CNS metastases.
5. A method of monitoring treatment of a CNS metastasizing cancer, the method comprising treating a subject with an anti-cancer treatment and detecting occurrence of CNS metastases according to claim 4, thereby monitoring treatment of the CNS metastasizing cancer.
6. The method of any one of claims 1 or 3-5, or agent of claim 2, wherein said polypeptide is a secreted polypeptide.

7. The method of any one of claims 1 or 3-5, or agent of claim 2, wherein said polypeptide is a cell surface receptor.

8. The method of any one of claims 1 or 3-7, or agent of any one of claims claim 2 or 7, wherein said polypeptide comprises two or more polypeptides.

9. The method of any one of claims 1, or 3-8, or agent of any one of claims 2 or 6-8, wherein said polypeptide is expressed or secreted from a cancer cell.

10. The method of any one of claims 1 or 3-9, or agent of any one of claims 2 or 6-9, wherein said polypeptide is CXCL10 and/or CXCR3.

11. The method of any one of claims 1 or 3-9, or agent of any one of claims 2 or 6-9, wherein said polypeptide is MCP-1, CCR4 and/or CCR2.

12. The method of any one of claims 1 or 3-9, or agent of any one of claims 2 or 6-9, wherein said polypeptide is IL-8 and/or CXCR1.

13. The method of any one of claims 1 or 3-9, or agent of any one of claims 2 or 6-9, wherein said polypeptide is GRO α and/or CXCR2.

14. The method of any one of claims 1 or 3-9, or agent of any one of claims 2 or 6-9, wherein said polypeptide is SERPINE1 and/or uPAR.

15. The method of any one of claims 1 or 3-9, or agent of any one of claims 2 or 6-9, wherein said polypeptide is selected from the group consisting of MCP-1, IL-8, CXCL10, GRO α , SERPINE1, CXCR1, CXCR2, CXCR3, CCR2, CCR4, uPAR, Lipocalin-2 (LCN2), sICAM, MIF, RANTES, IL-6, IL-1 β and CCL17.

16. The method of any one of claims 1 or 3-8, or agent of any one of claims 2 or 6-8, wherein said polypeptide is expressed or secreted from a CNS cell.

17. The method or agent of claim 16, wherein said CNS cell is a glia cell.
18. The method or agent of claim 16, wherein said glia cell is an astrocyte.
19. The method of any one of claims 4-9 or 16-18, wherein said polypeptide is CXCL10 or LCN2.
20. The method of any one of claims 4-19, wherein said determining the level of at least one polypeptide is effected *ex vivo*.
21. The method of any one of claims 4-20, wherein said determining the level of at least one polypeptide is effected in a biological sample selected from the group consisting of a blood sample, a serum sample, urine, cerebro-spinal fluid (CSF), sweat, tears, a saliva and tumor cell sample.
22. The method of any one of claims 1 or 3-21, or agent of any one of claims 2 or 6-18, wherein said CNS metastasizing cancer is selected from the group consisting of non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), breast cancer, mesotheliomas, melanoma, ovarian carcinoma, bladder cancer, renal cancer and colon cancer.
23. The method of any one of claims 1 or 3-21, or agent of any one of claims 2 or 6-18, wherein said CNS metastasizing cancer is melanoma.
24. The method of any one of claims 1, 3, 6-18 or 22-23, or agent of any one of claims 2, 6-18 or 22-23, wherein said agent is a polypeptide agent.
25. The method or agent of claim 24, wherein said polypeptide agent is an antibody.
26. The method or agent of claim 25, wherein said antibody is a neutralizing antibody.

27. The method or agent of claim 24, wherein said polypeptide agent is a soluble receptor.

28. The method of any one of claims 1, 3, 6-18 or 22-23, or agent of any one of claims 2, 6-18 or 22-23, wherein said agent is or comprises a nucleic acid agent.

29. The method or agent of claim 28, wherein said nucleic acid agent is an RNA oligonucleotide or a targeted nuclease.

30. The method or agent of claim 29, wherein said targeted nuclease is selected from the group CRISPR/Cas-9, zinc finger nuclease, TALEN and a meganuclease.

31. The method or agent of claim 29, wherein said RNA oligonucleotide is selected from a mRNA, a micro RNA (miRNA), a small interfering RNA (siRNA) and a tiny noncoding RNA (tnRNA).

32. The method or agent of any one of claims 28, 29 or 31, wherein said agent is a conjugate which comprises a polymer and a nucleic acid agent associated with said polymer.

33. The method or agent of claim 32, wherein said polymer comprises a polyglutamic acid (PGA).

34. The method or agent of claim 33, wherein said agent is a siRNA selected from the group consisting of a PGA-NH₂-siMCP-1, PGA-NH₂-siIL-8, PGA-NH₂-siCXCL10, PGA-NH₂-siGRO α , and PGA-NH₂-siSERPINE1.

35. The method or agent of claim 33, wherein said agent is a siRNA selected from the group consisting of a PGA-NH₂-siCXCR1, PGA-NH₂-siCCR4, PGA-NH₂-siCCR2, PGA-NH₂-siCXCR3, PGA-NH₂-siCXCR2 and PGA-NH₂-siuPAR.

36. The method of any one of claims 1, 3, 6-18 or 22-23, or agent of any one of claims 2, 6-18 or 22-23, wherein said agent is a small molecule.

37. The method of any one of claims 1, 3, 6-18 or 22-23, or agent of any one of claims 2, 6-18 or 22-23, wherein said agent is targeted to a melanoma expressed marker.

38. The method or agent of claim 37, wherein said marker is NCAM or P-selectin.

39. The method or agent of claim 37 or 38, wherein said agent comprises an NCAM-targeting moiety or a P-selectin-targeting moiety.

40. The method of any one of claims 1, 3, 6-18 or 22-39, or agent of any one of claims 2, 6-18 or 22-39, wherein said agent is formulated for CNS administration.

41. The method of any one of claims 1, 3, 6-18 or 22-39, or agent of any one of claims 2, 6-18 or 22-39, wherein said agent is formulated for systemic administration.

42. The method of any one of claims 1, 3, 6-18 or 22-39, or agent of any one of claims 2, 6-18 or 22-39, wherein said agent is formulated for local administration.

43. A composition of matter selected from the group consisting of a PGA-NH₂-siMCP-1, PGA-NH₂-siIL-8, PGA-NH₂-siCXCL10, PGA-NH₂-siGRO α , and PGA-NH₂-siSERPINE1, PGA-NH₂-siCXCR1, PGA-NH₂-siCCR4, PGA-NH₂-siCCR2, PGA-NH₂-siCXCR3, PGA-NH₂-siCXCR2 and PGA-NH₂-siuPAR.

44. A method of generating a non-human animal with a CNS metastasizing melanoma, the method comprising:

- (a) systemically administering melanoma cells to a non-human animal;
- (b) allowing growth of a primary melanoma tumor in said non-human animal;

- (c) surgically removing said primary melanoma tumor;
- (d) monitoring formation of CNS metastasis; and
- (e) selecting said non-human animal with a CNS metastasizing melanoma.

45. An immunocompetent non-human animal comprising a CNS metastasizing melanoma, wherein said non-human animal does not comprise a primary melanoma tumor.

FIG. 1A

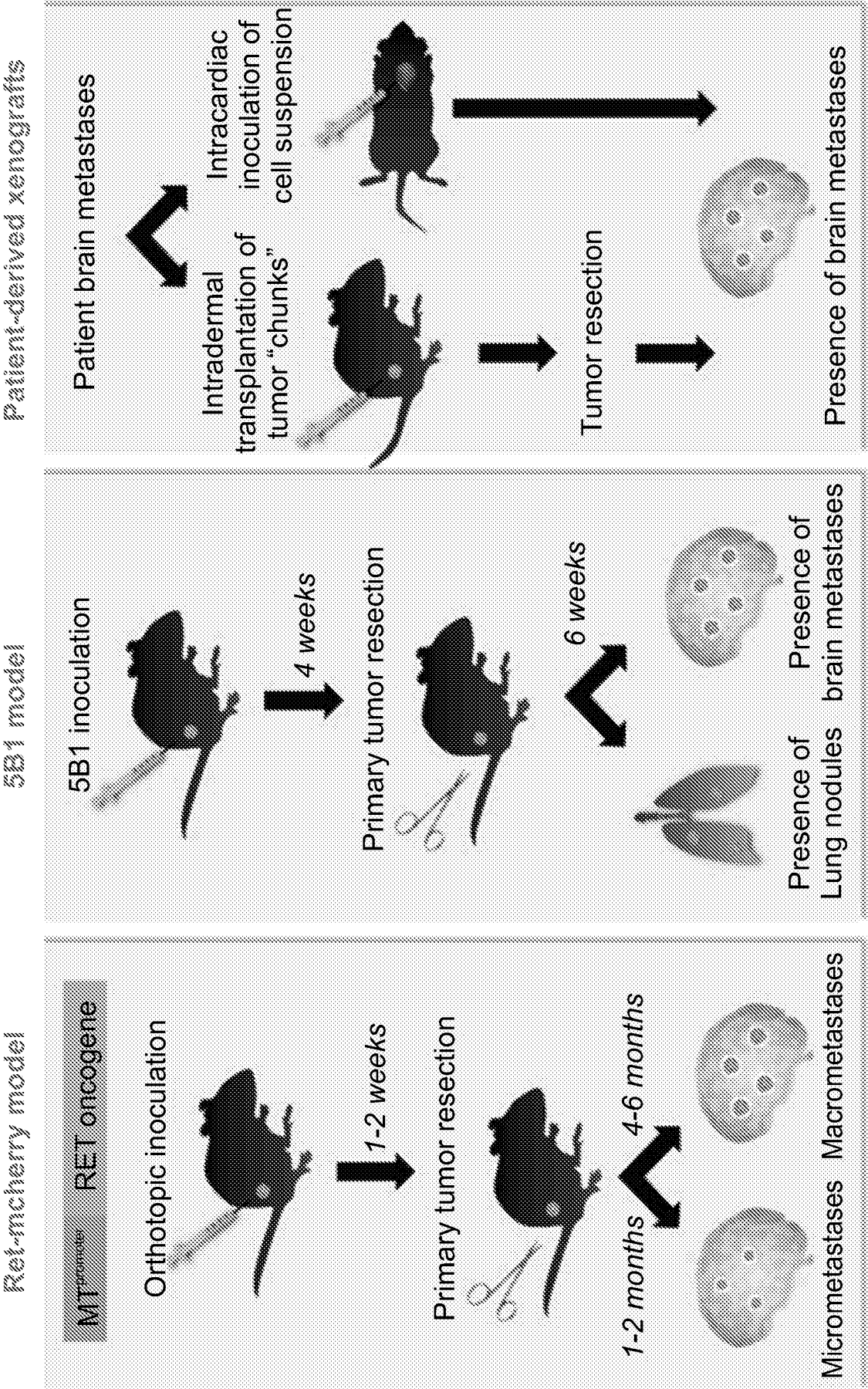
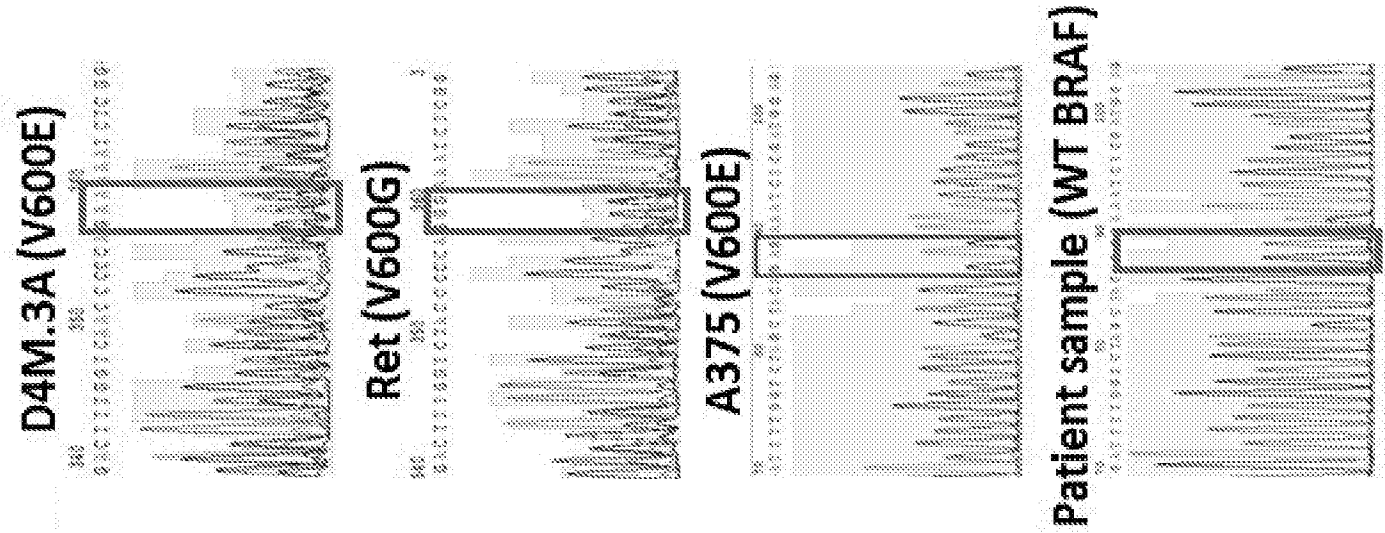


FIG. 1B



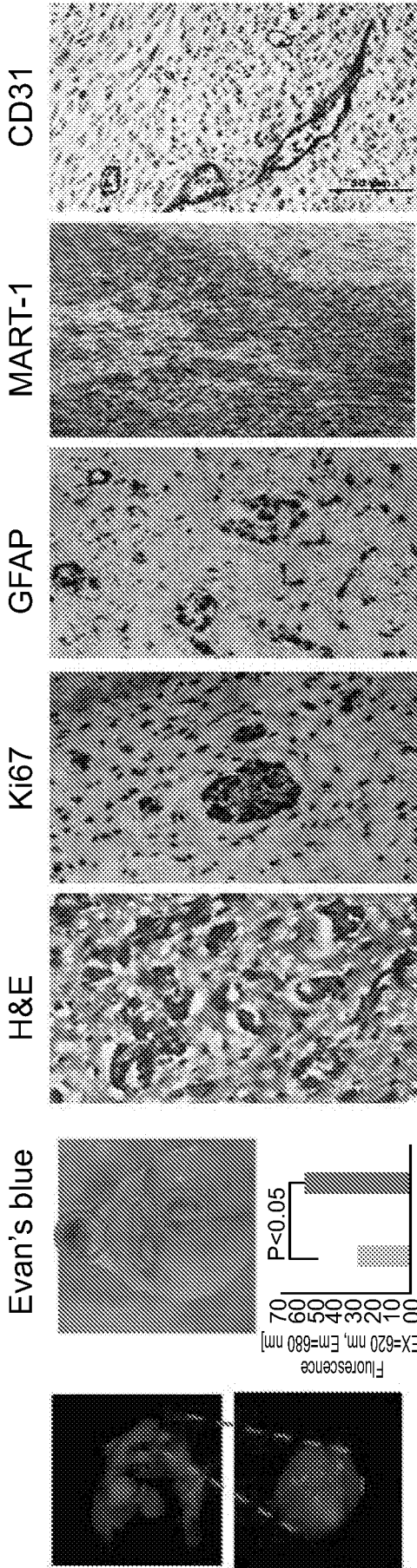


FIG. 1E

FIG. 1C FIG. 1D

FIG. 2A

FIG. 2B

FIG. 2C

FIG. 2D

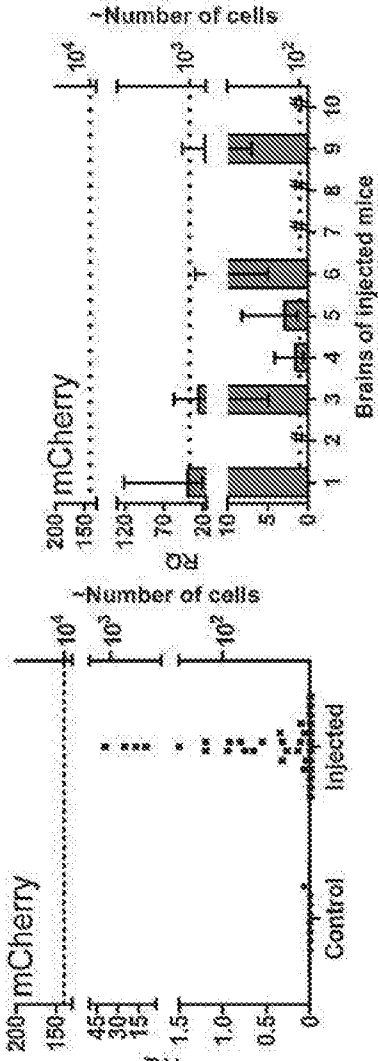
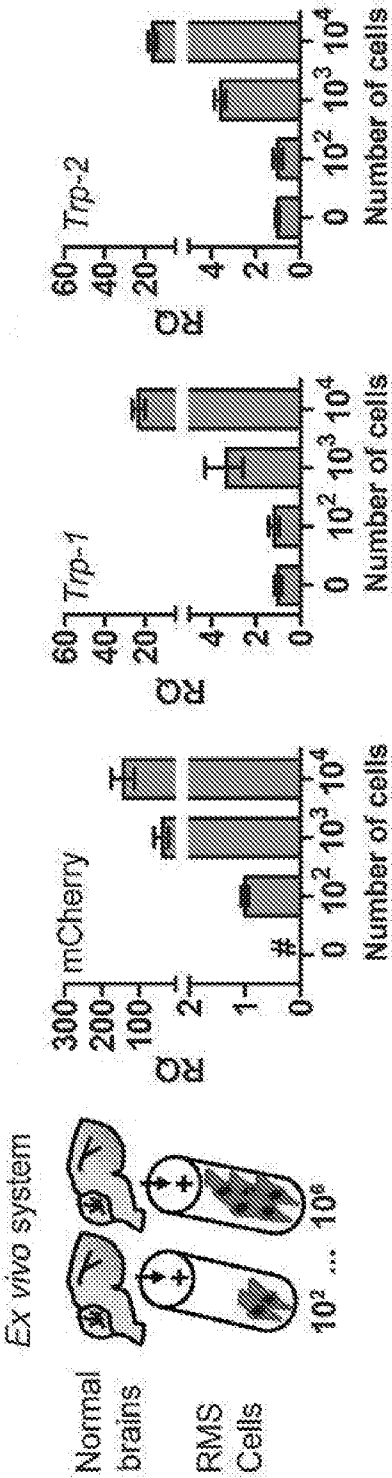


FIG. 2E

FIG. 2F

FIG. 2G

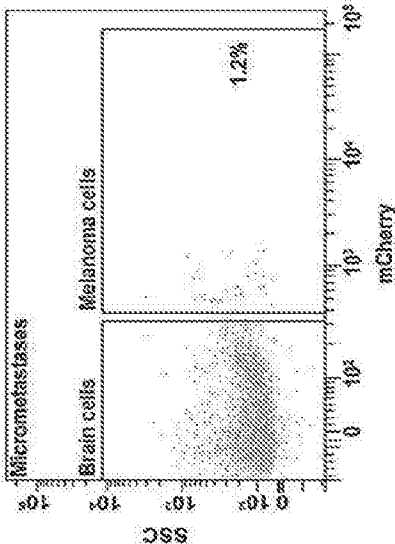


FIG. 2H

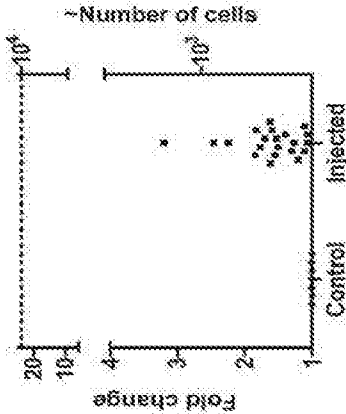


FIG. 2I

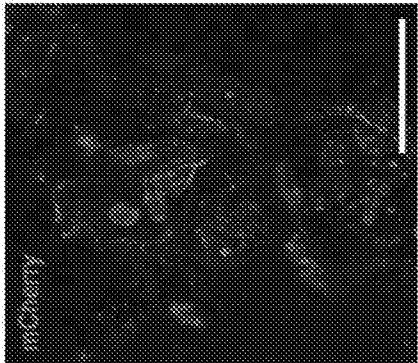
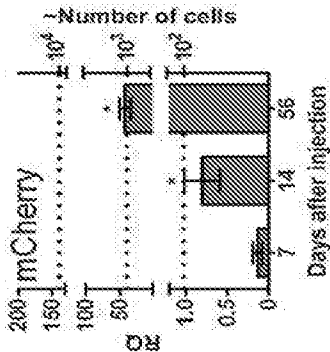


FIG. 2J

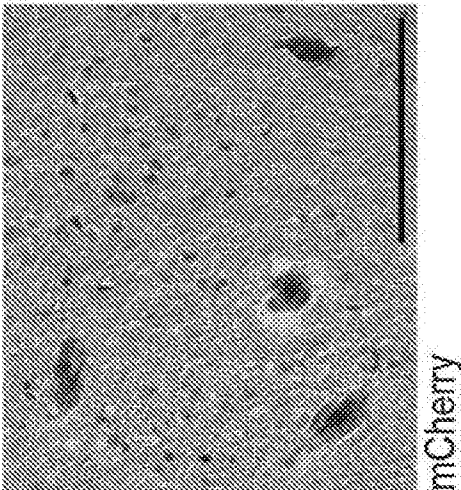


FIG. 2K

$P < 0.05$

	Histology negative	Histology positive
qPCR Positive	1	9
qPCR Negative	3	0

FIG. 2L

FIG. 3A

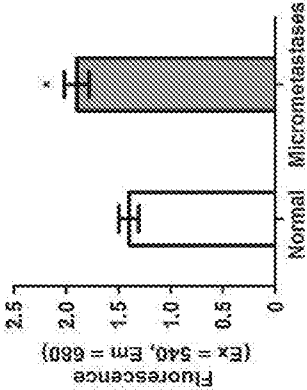


FIG. 3B

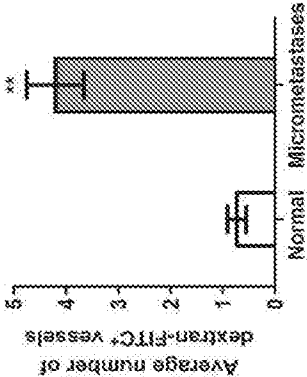


FIG. 3C

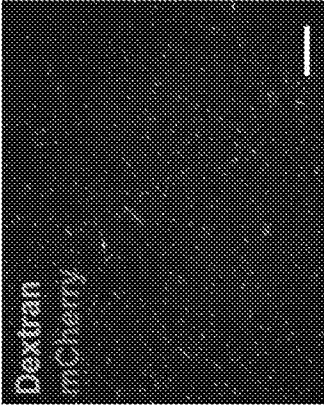


FIG. 3D

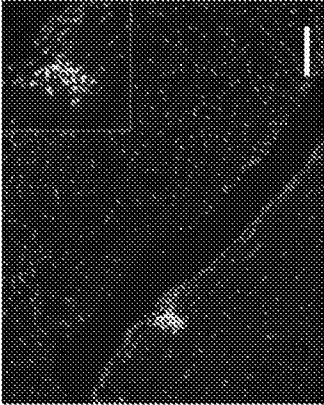


FIG. 3E

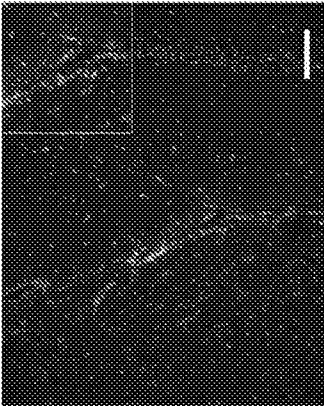


FIG. 3F



FIG. 3G

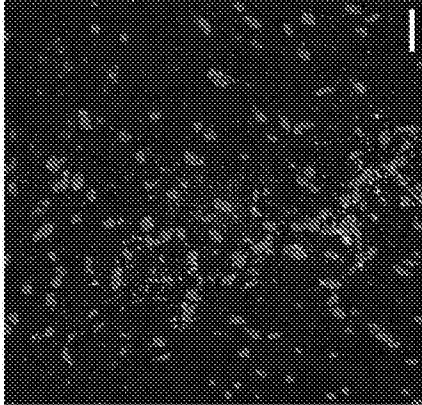


FIG. 3H

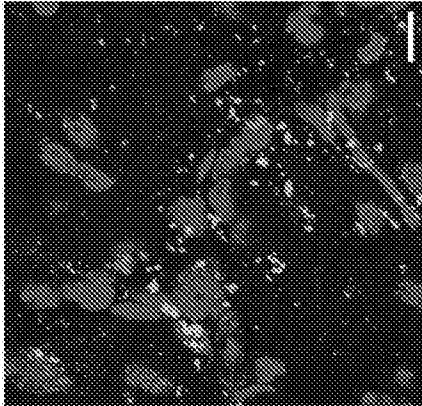


FIG. 4A

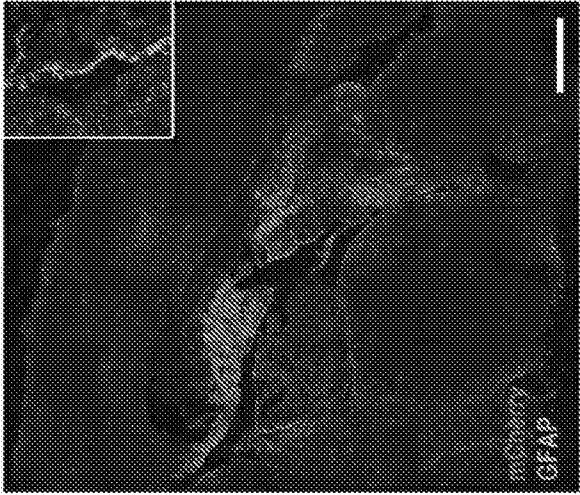


FIG. 4B

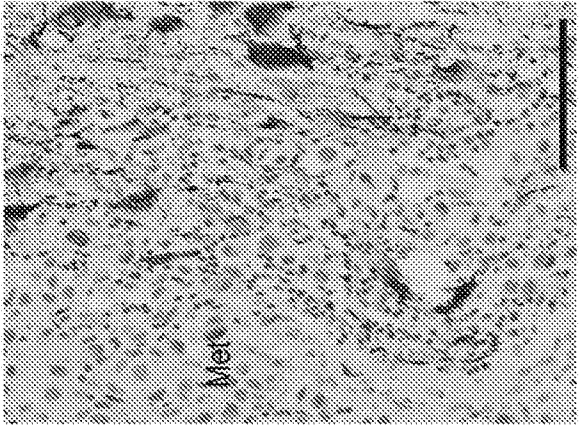
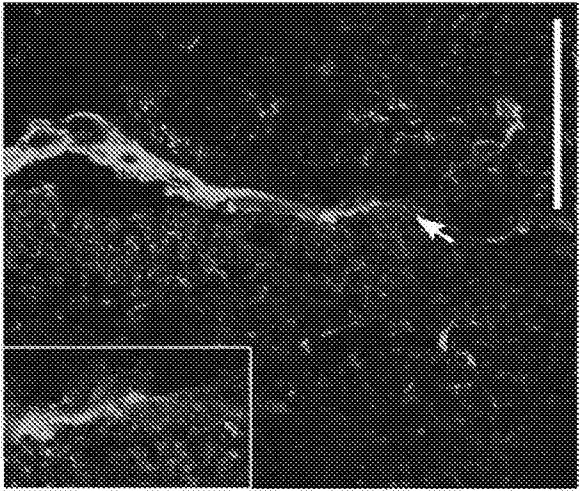


FIG. 4C



GFAP

FIG. 4D

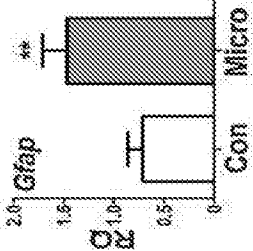


FIG. 4E

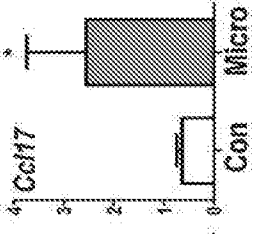


FIG. 4F

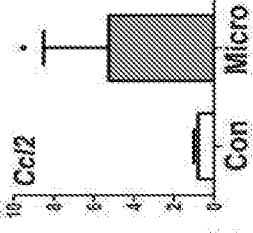


FIG. 4G

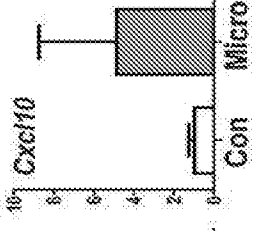


FIG. 4H

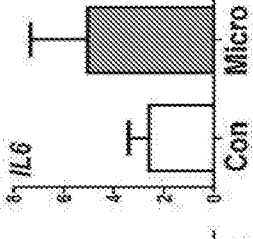


FIG. 4I

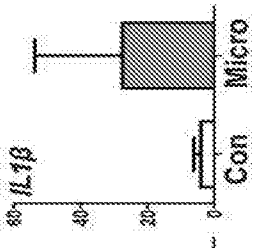


FIG. 4J

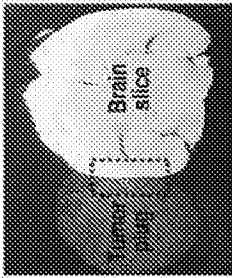


FIG. 4K

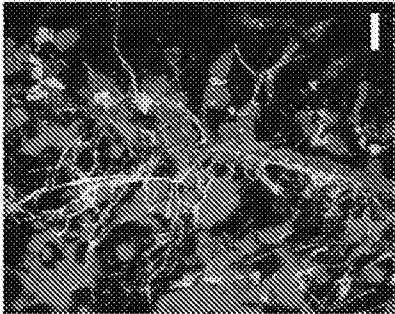


FIG. 4L

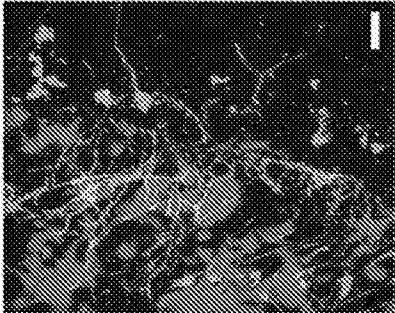


FIG. 4M



FIG. 4N

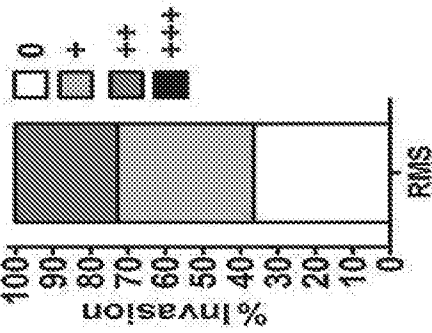


FIG. 4O

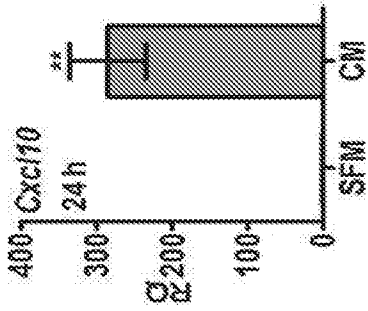


FIG. 4P

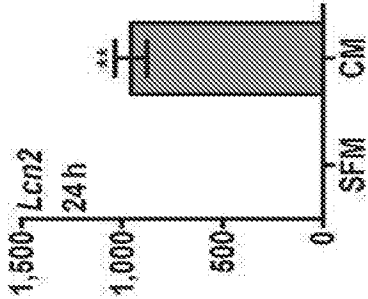


FIG. 4Q

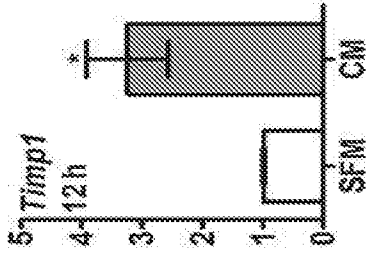


FIG. 4R

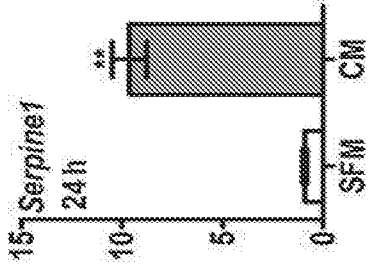


FIG. 4S

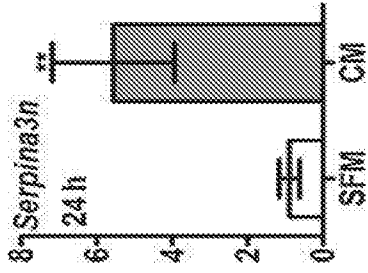


FIG. 5A

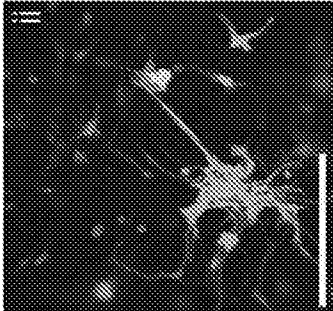
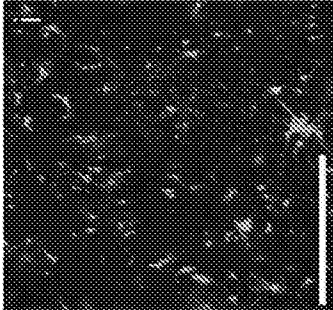
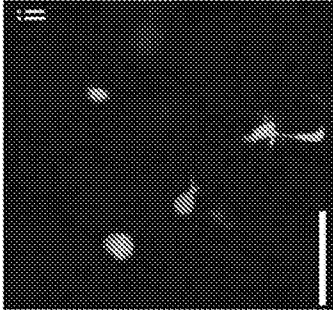
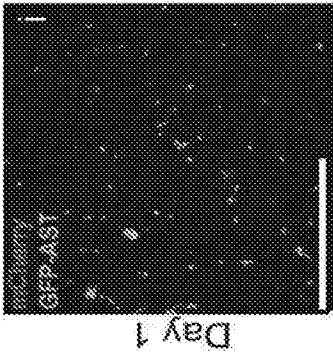


FIG. 5B

FIG. 5D

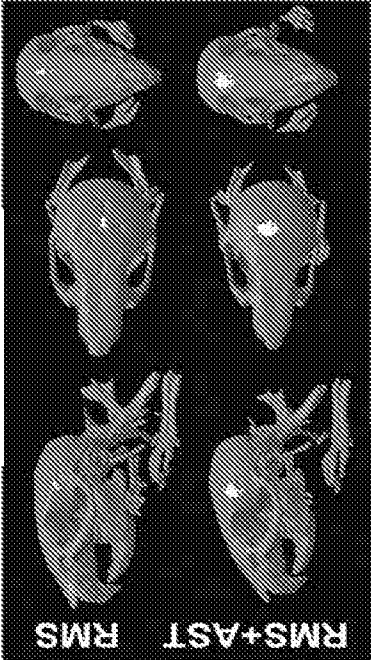


FIG. 5C

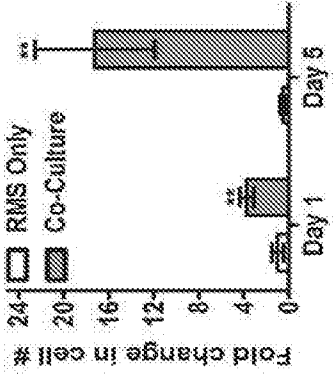


FIG. 5E

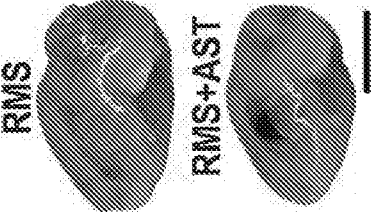


FIG. 5F

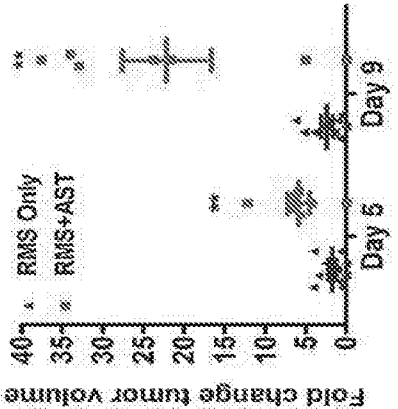


FIG. 5I

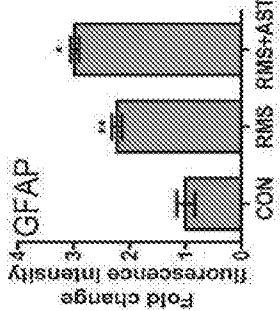
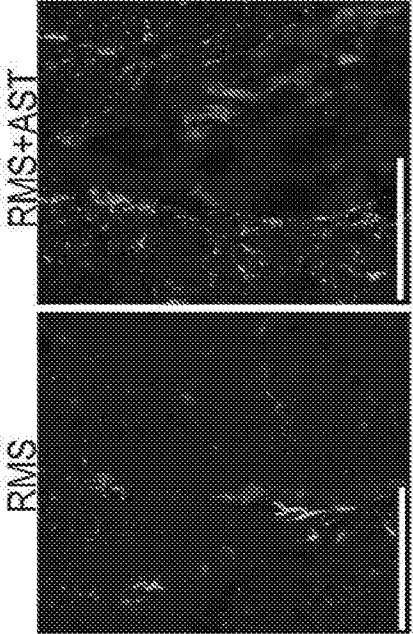


FIG. 5H

FIG. 5G

FIG. 5J

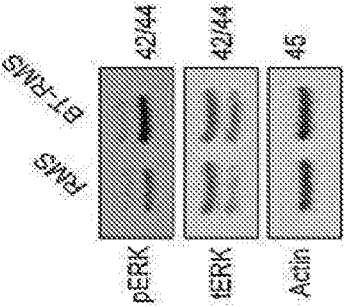


FIG. 5K

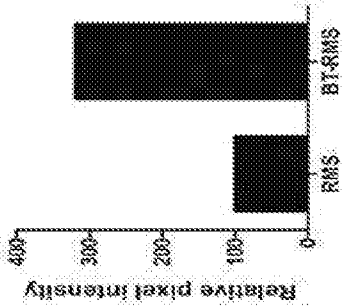


FIG. 5L

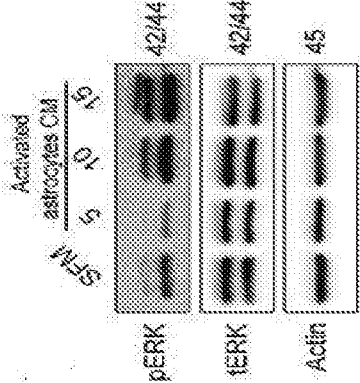


FIG. 5M

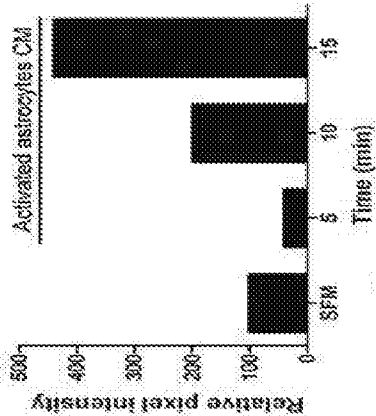


FIG. 5N

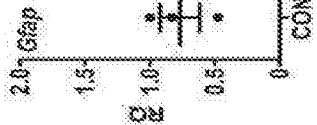


FIG. 5O

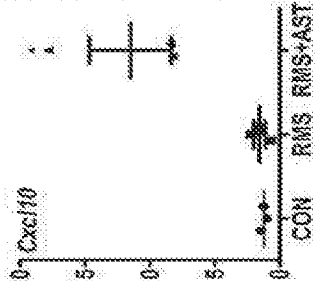


FIG. 5P

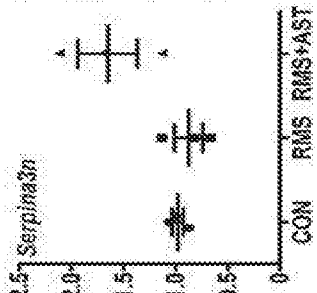


FIG. 5Q

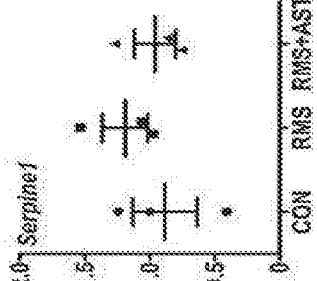


FIG. 5R

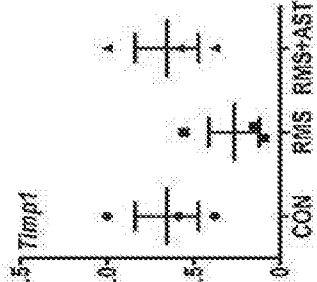


FIG. 6B

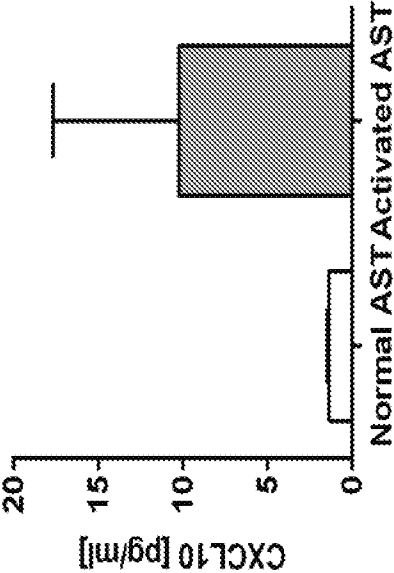


FIG. 6A

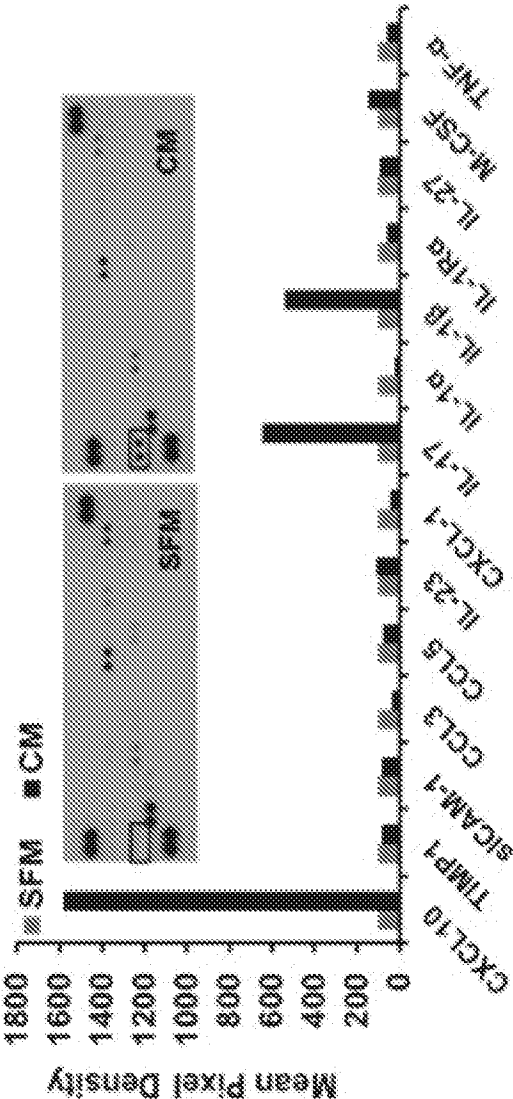


FIG. 7A

Spontaneous Micro

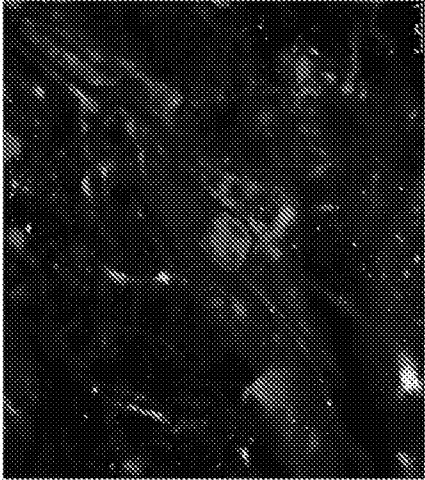


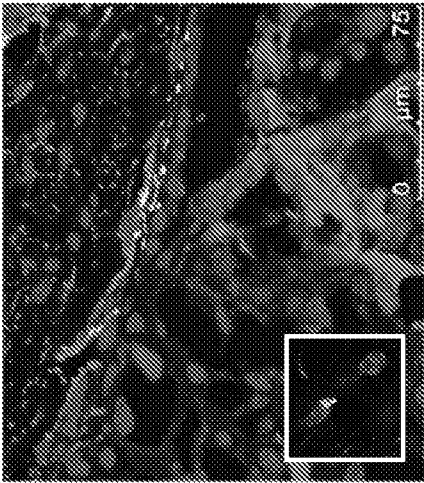
FIG. 7B

Spontaneous Macro



Melanoma cells | Astrocytes GFAP | CXCL10 | DAPI

Intra-Cranial



Sorted astrocytes

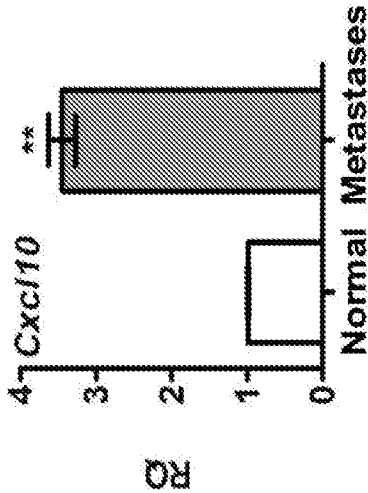


FIG. 7C

FIG. 7D

FIG. 8B

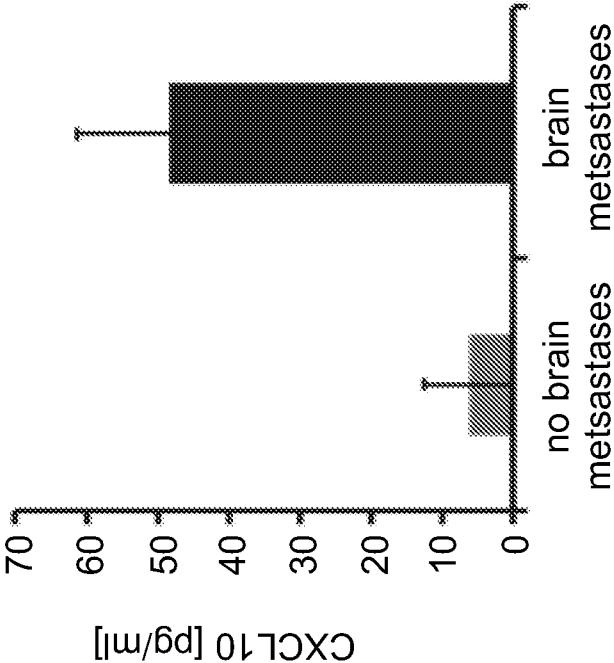


FIG. 8A

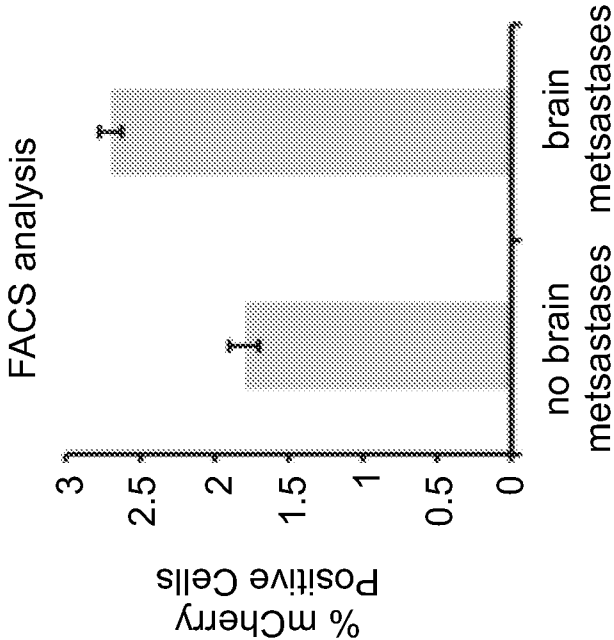


FIG. 9B

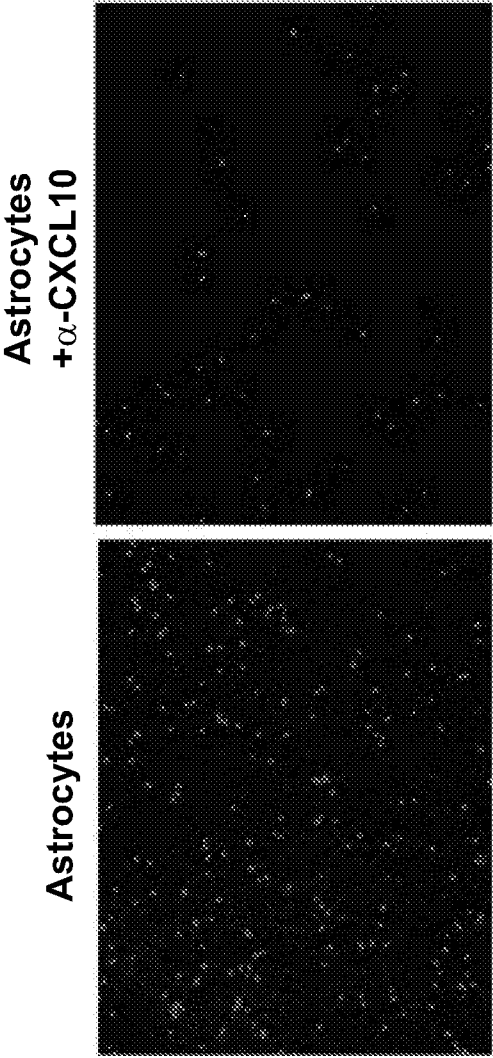


FIG. 9A

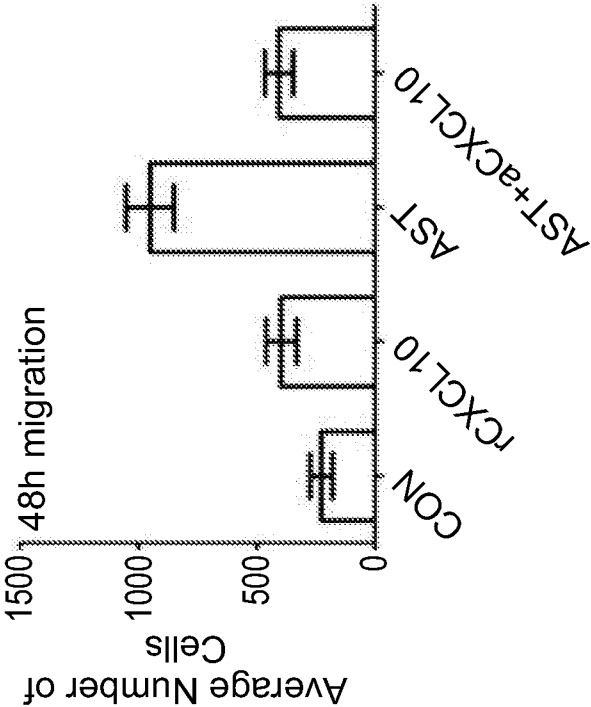


FIG. 10A

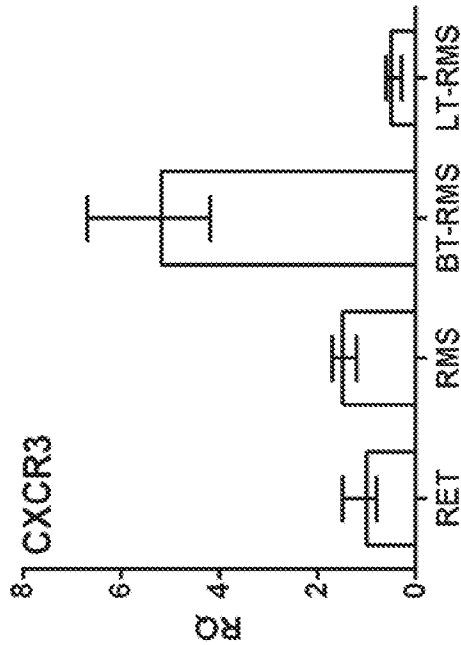


FIG. 10B

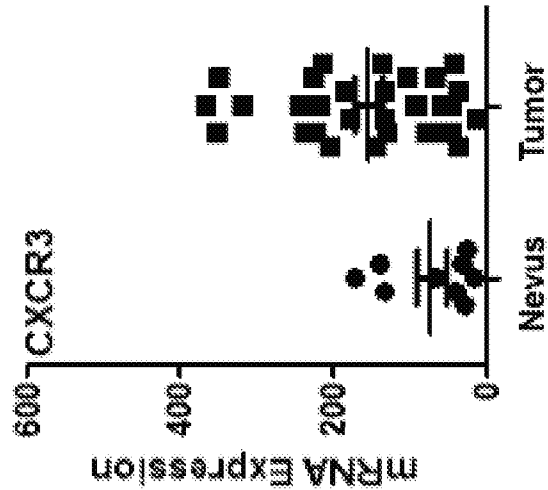


FIG. 11A

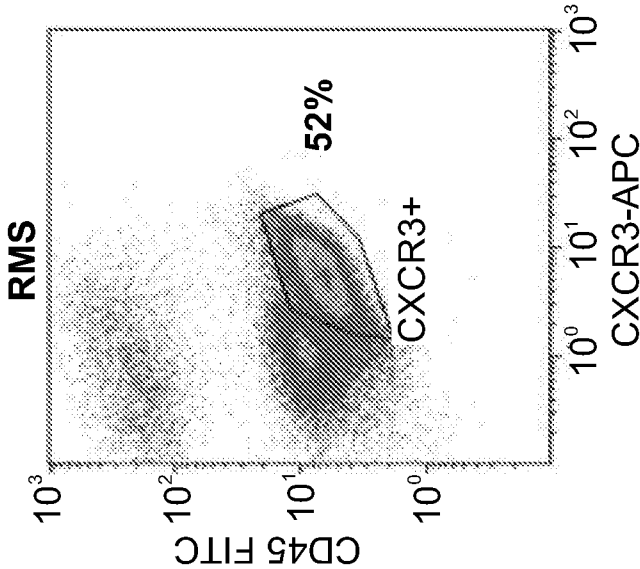


FIG. 11B

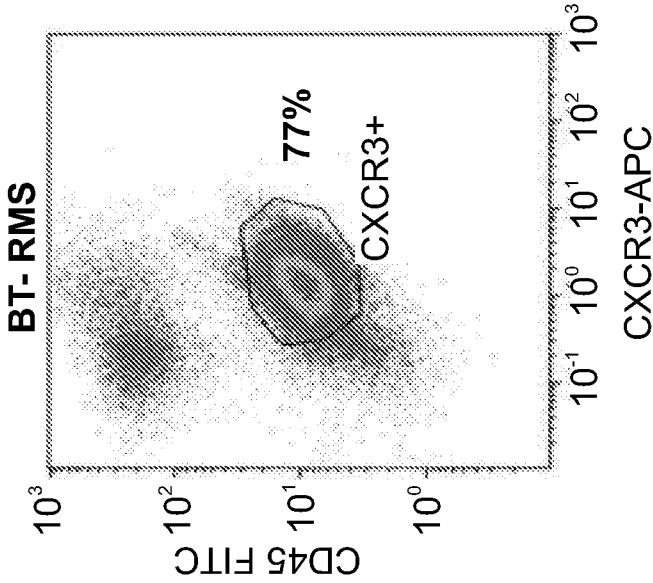


FIG. 11C

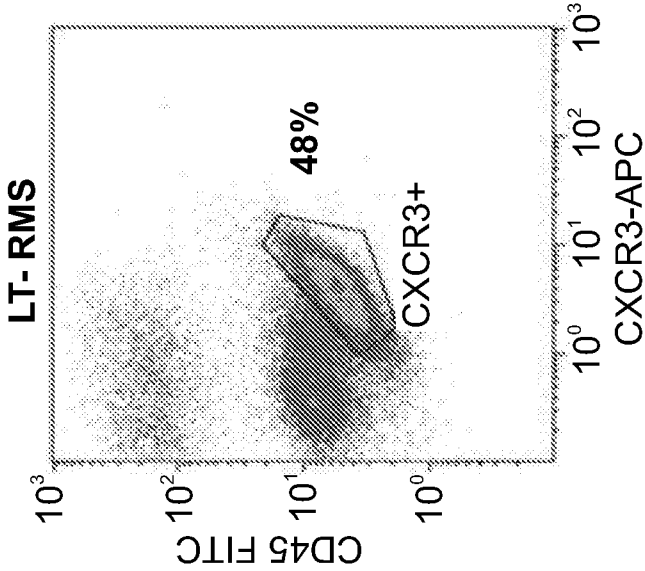


FIG. 12A

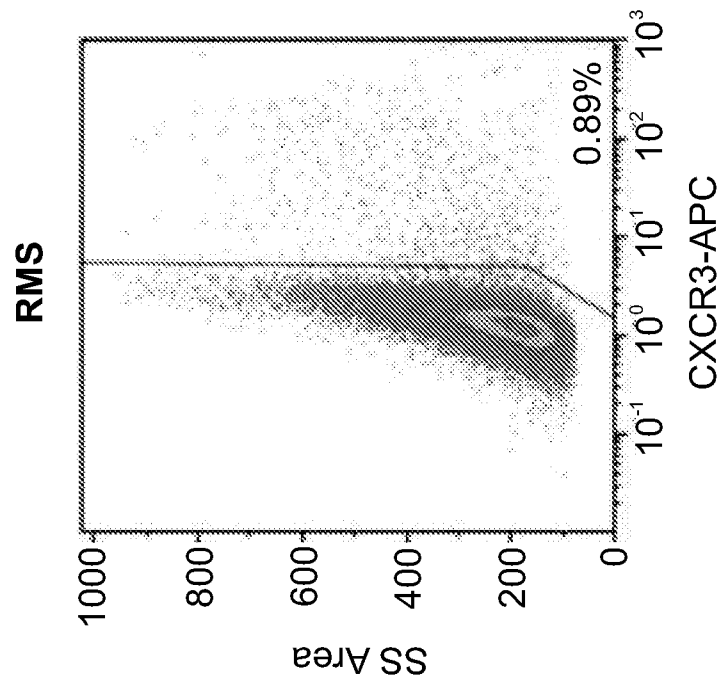


FIG. 12B

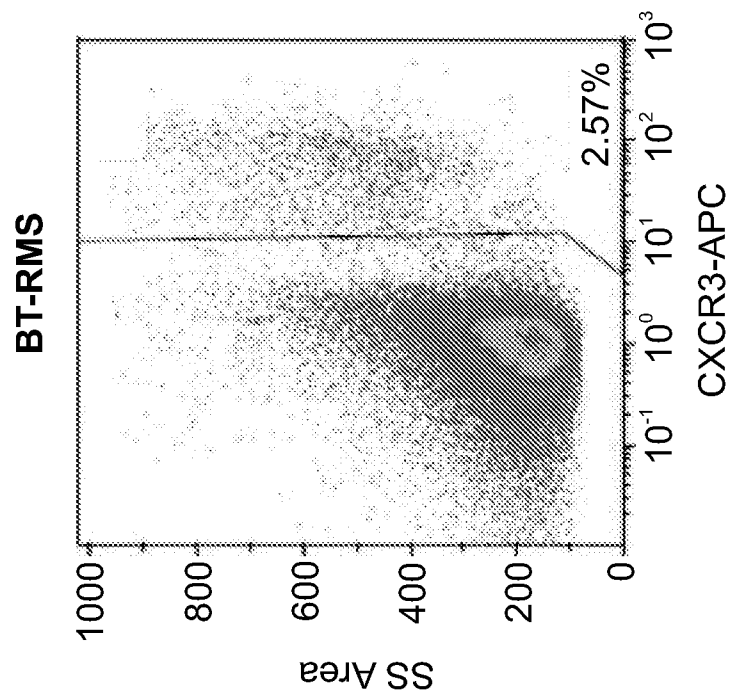


FIG. 13A

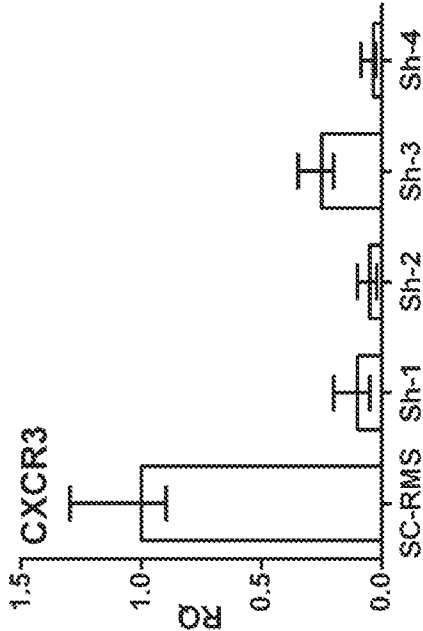


FIG. 13B

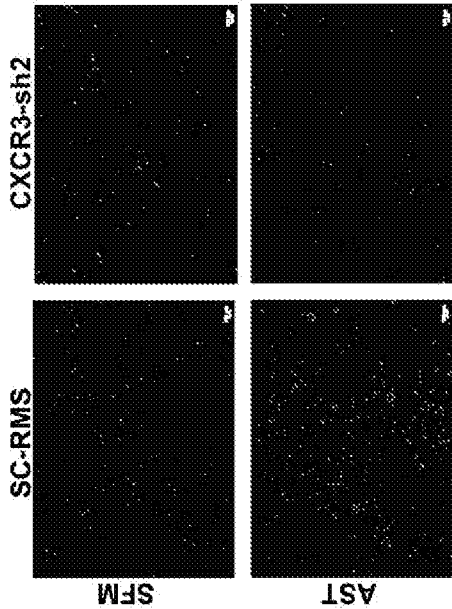


FIG. 13C

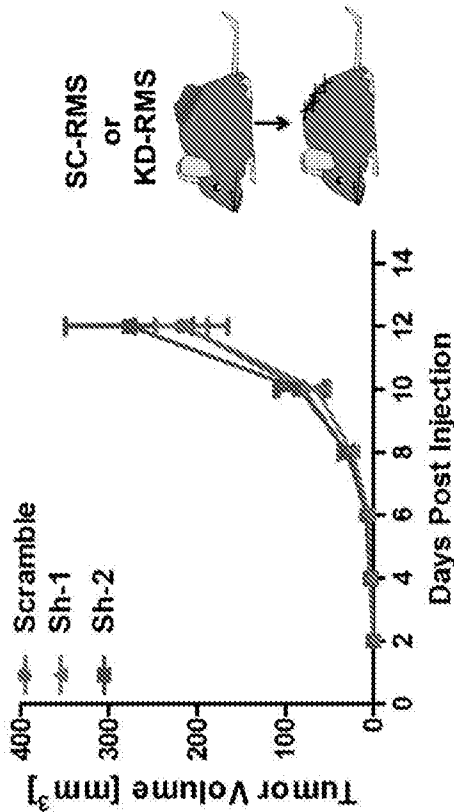


FIG. 13D

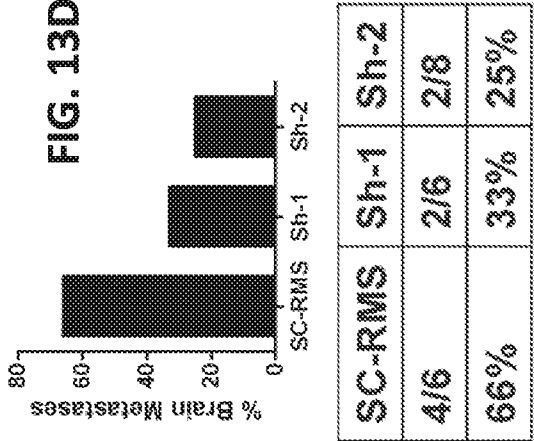


FIG. 13E

FIG. 14A

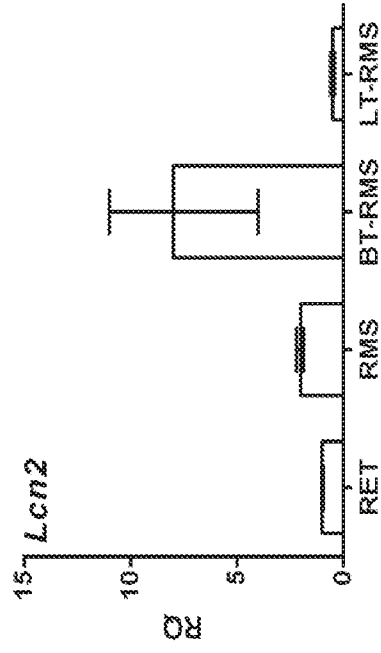


FIG. 14B

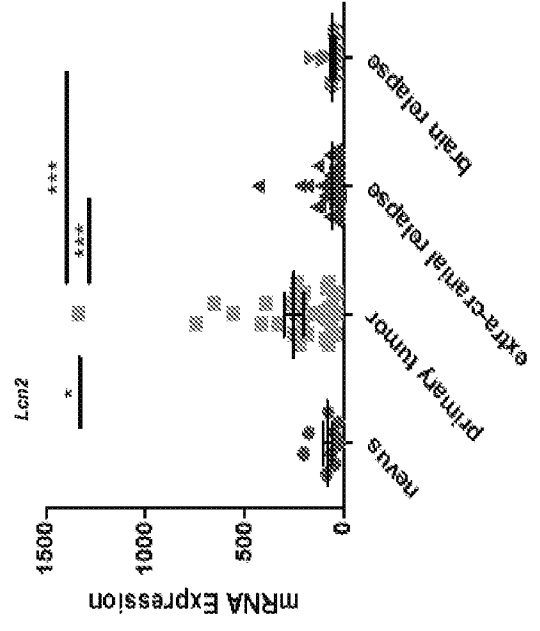
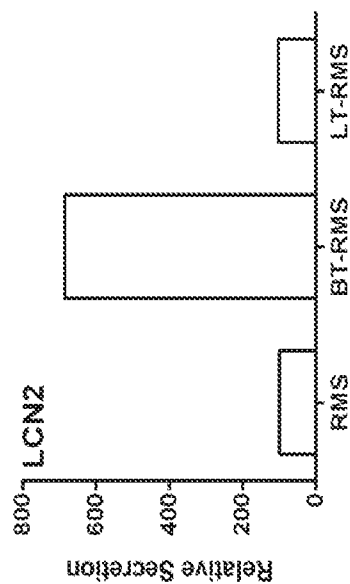


FIG. 14C

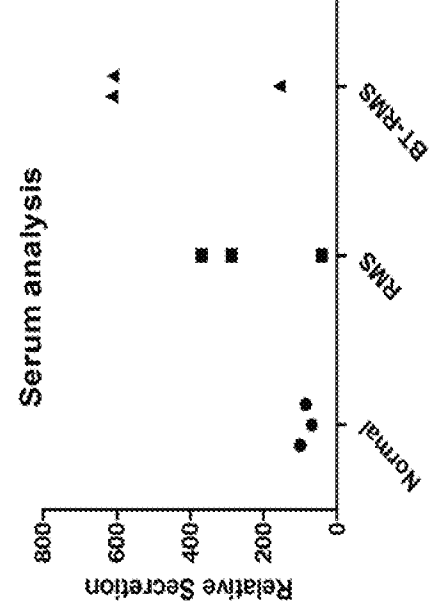


FIG. 14D

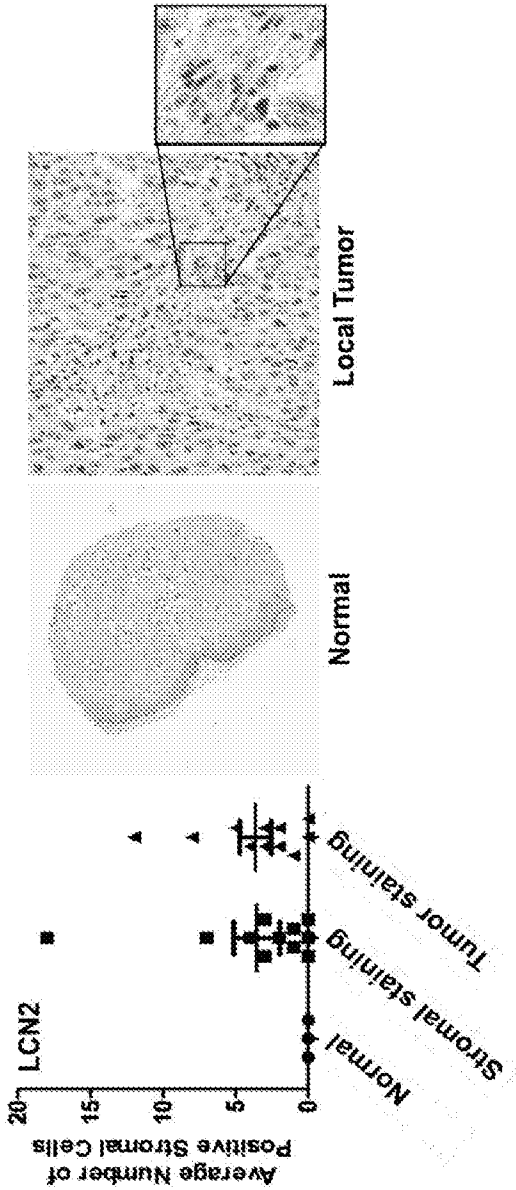


FIG. 15A

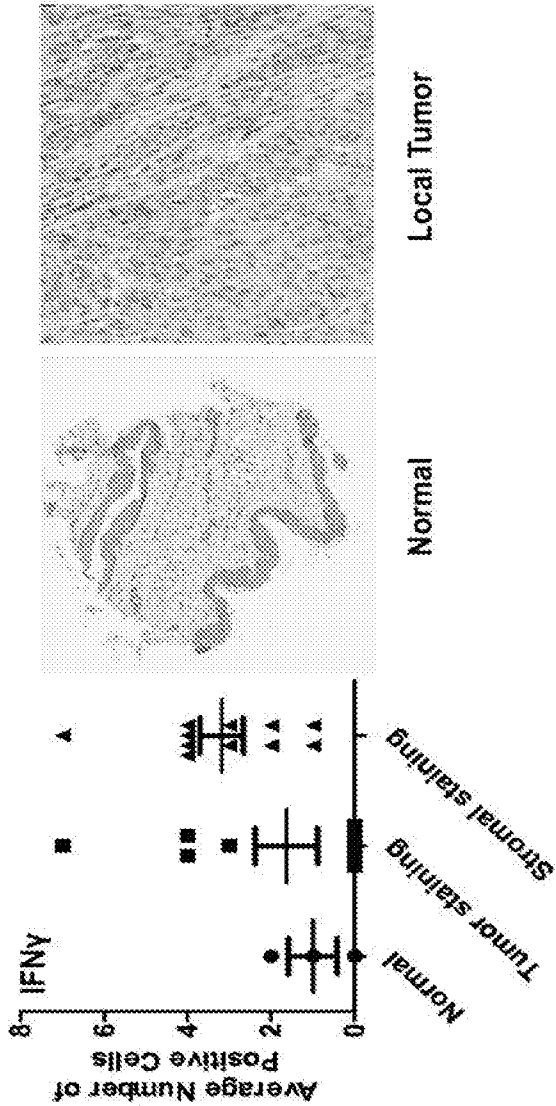


FIG. 15B

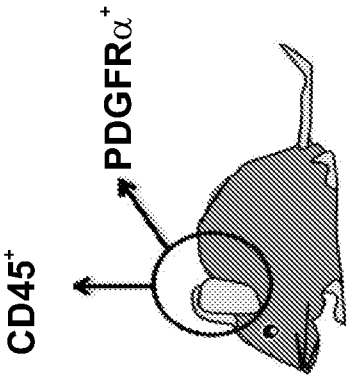
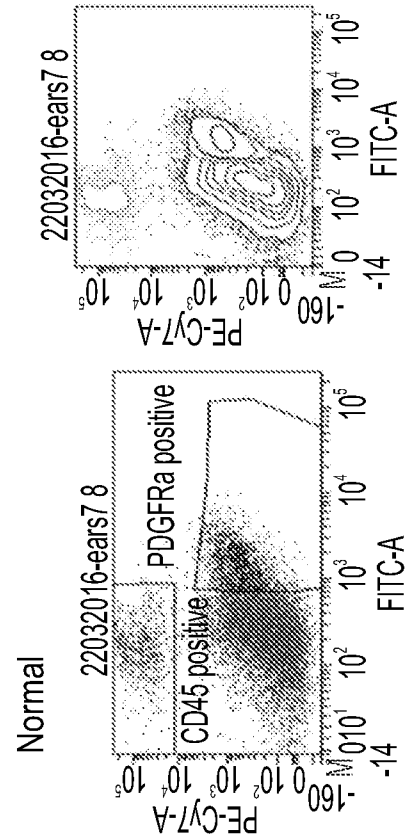


FIG. 16A

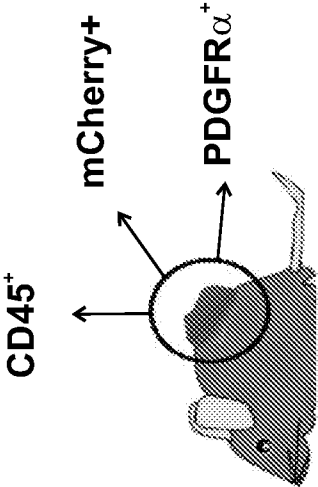
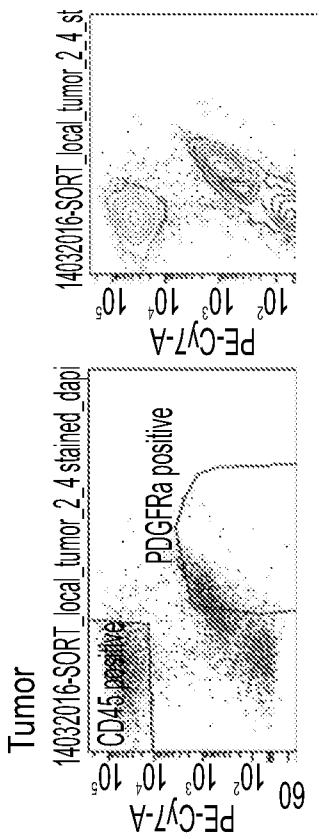


FIG. 16B

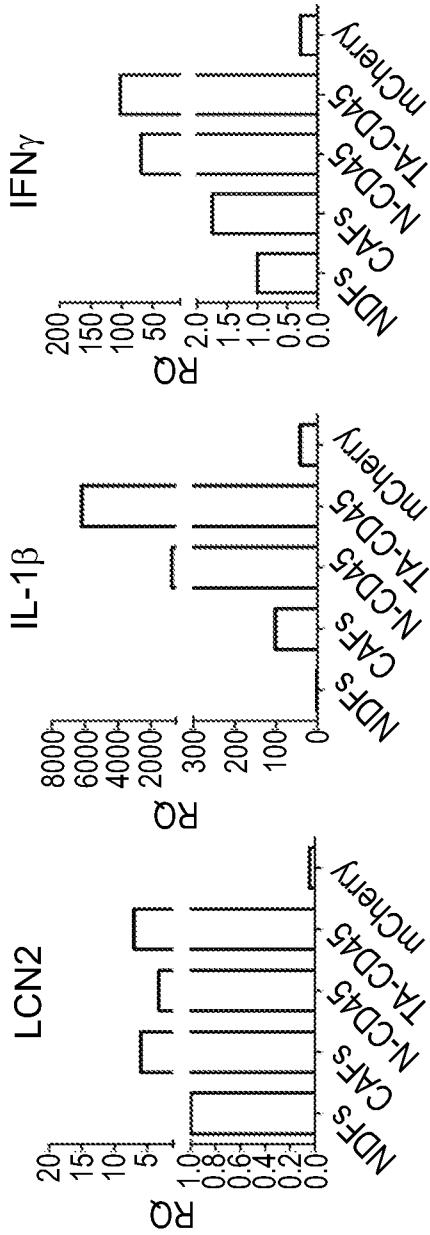


FIG. 16C

FIG. 17B

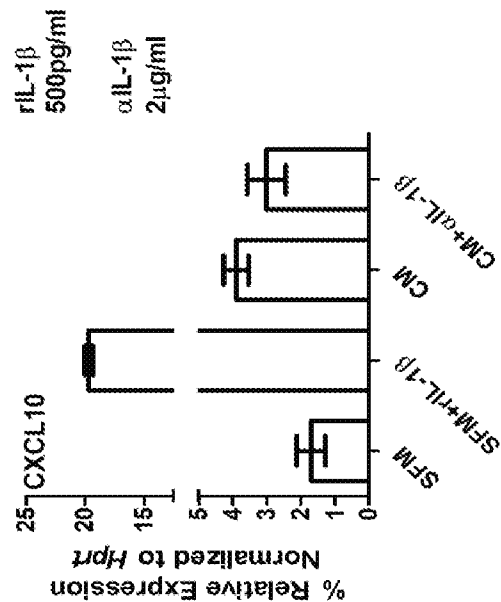


FIG. 17A

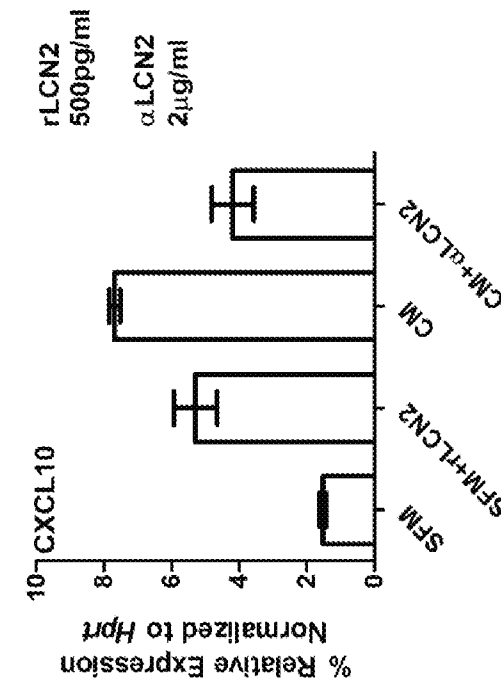


FIG. 18B

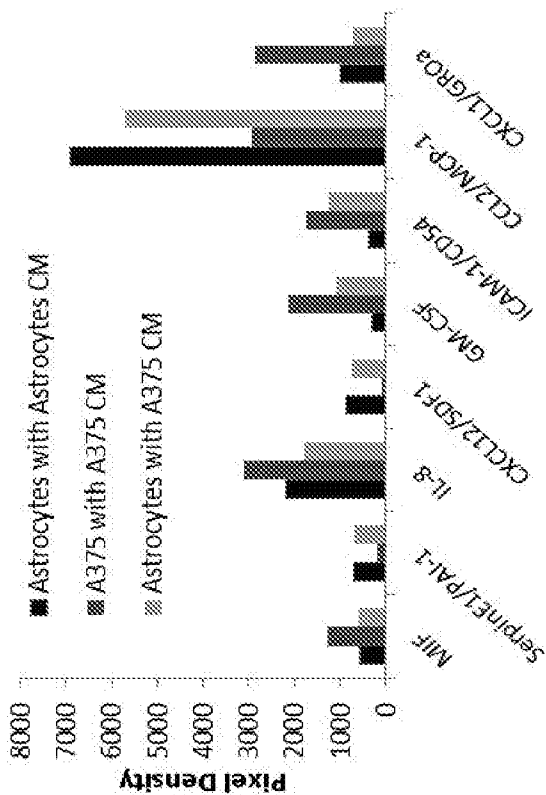


FIG. 18A

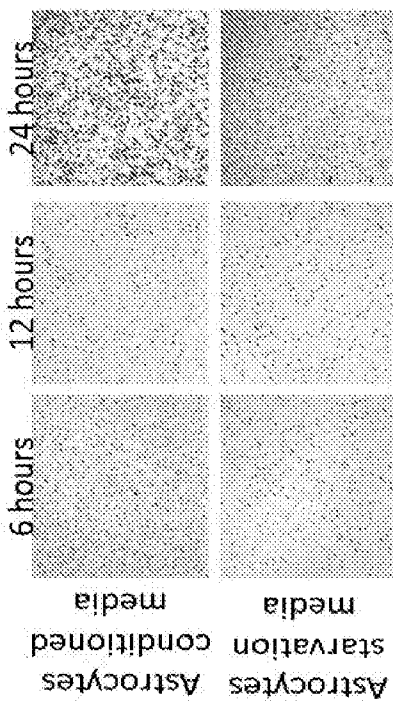
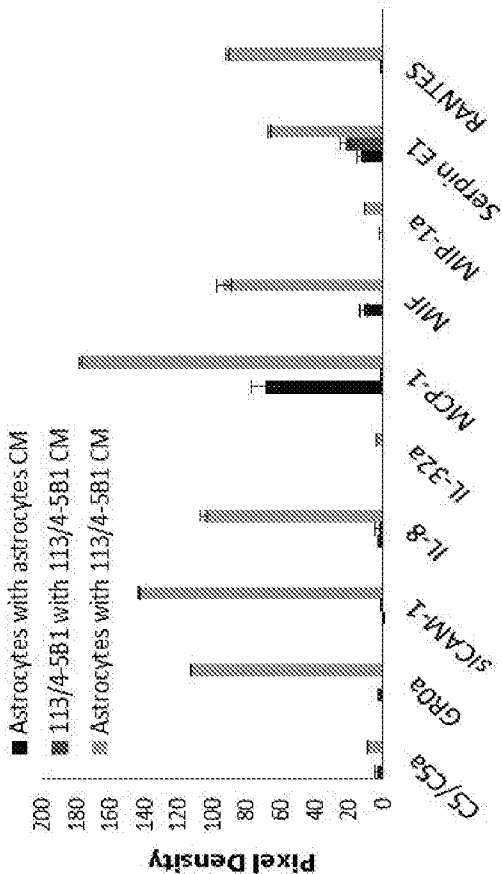
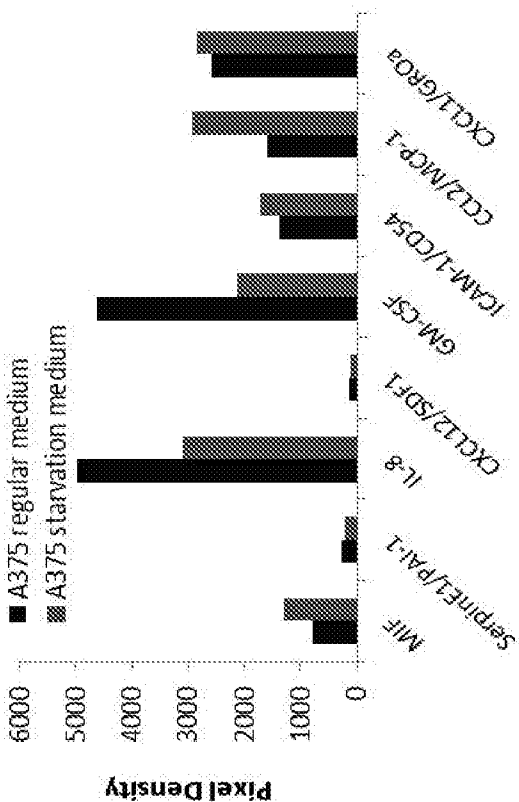


FIG. 18D

FIG. 18C

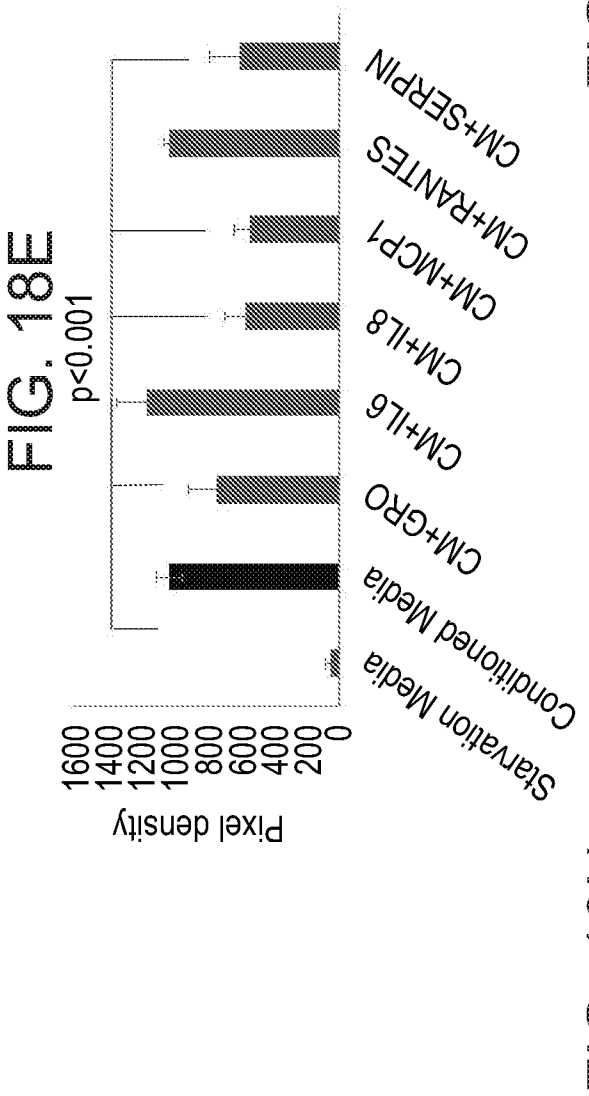
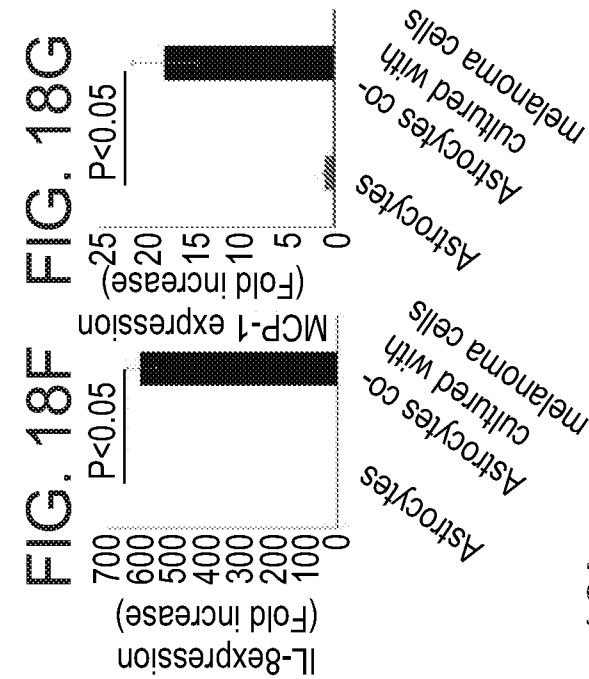
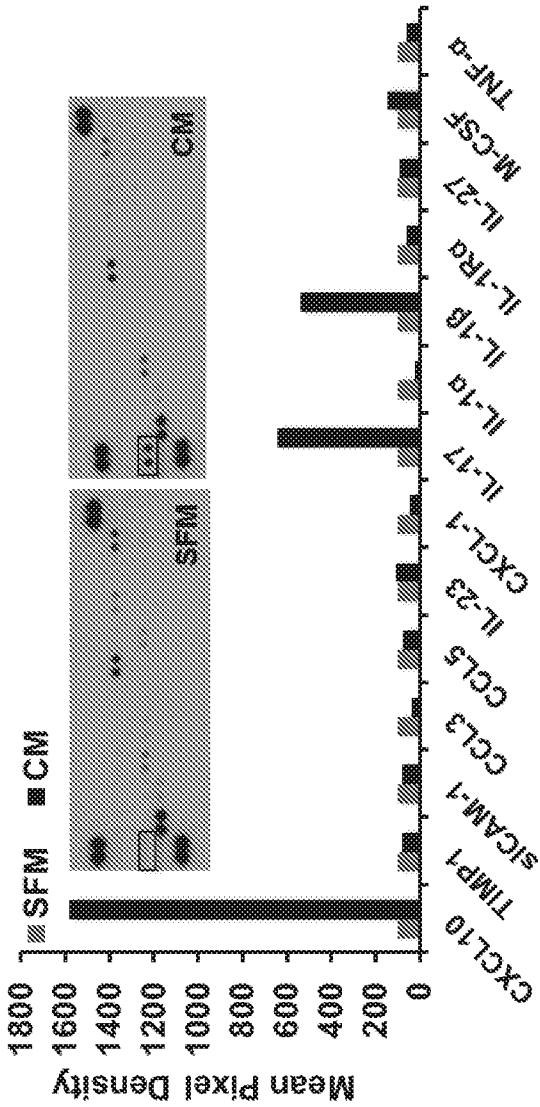


FIG. 18H

FIG. 18I

FIG. 19



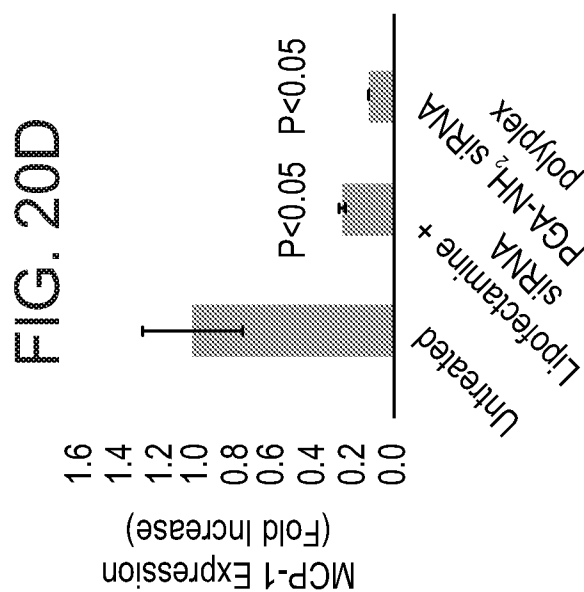
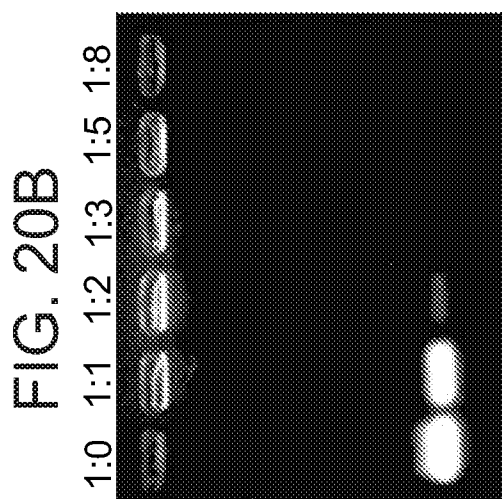
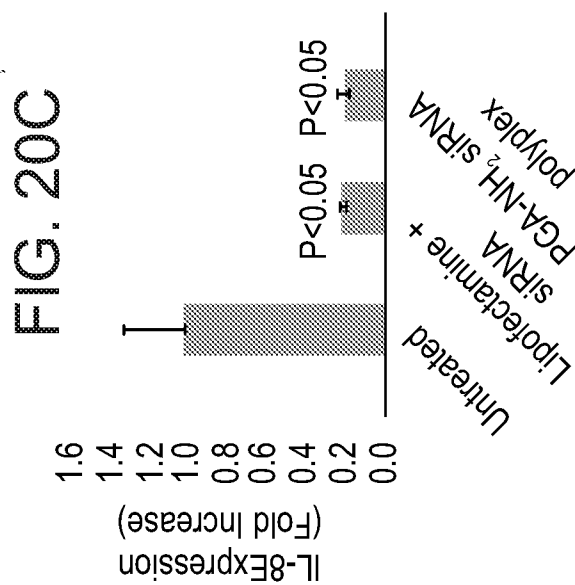
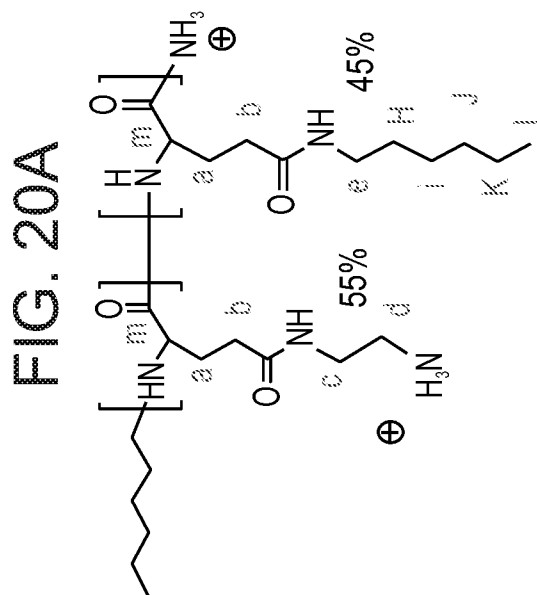


FIG. 21A

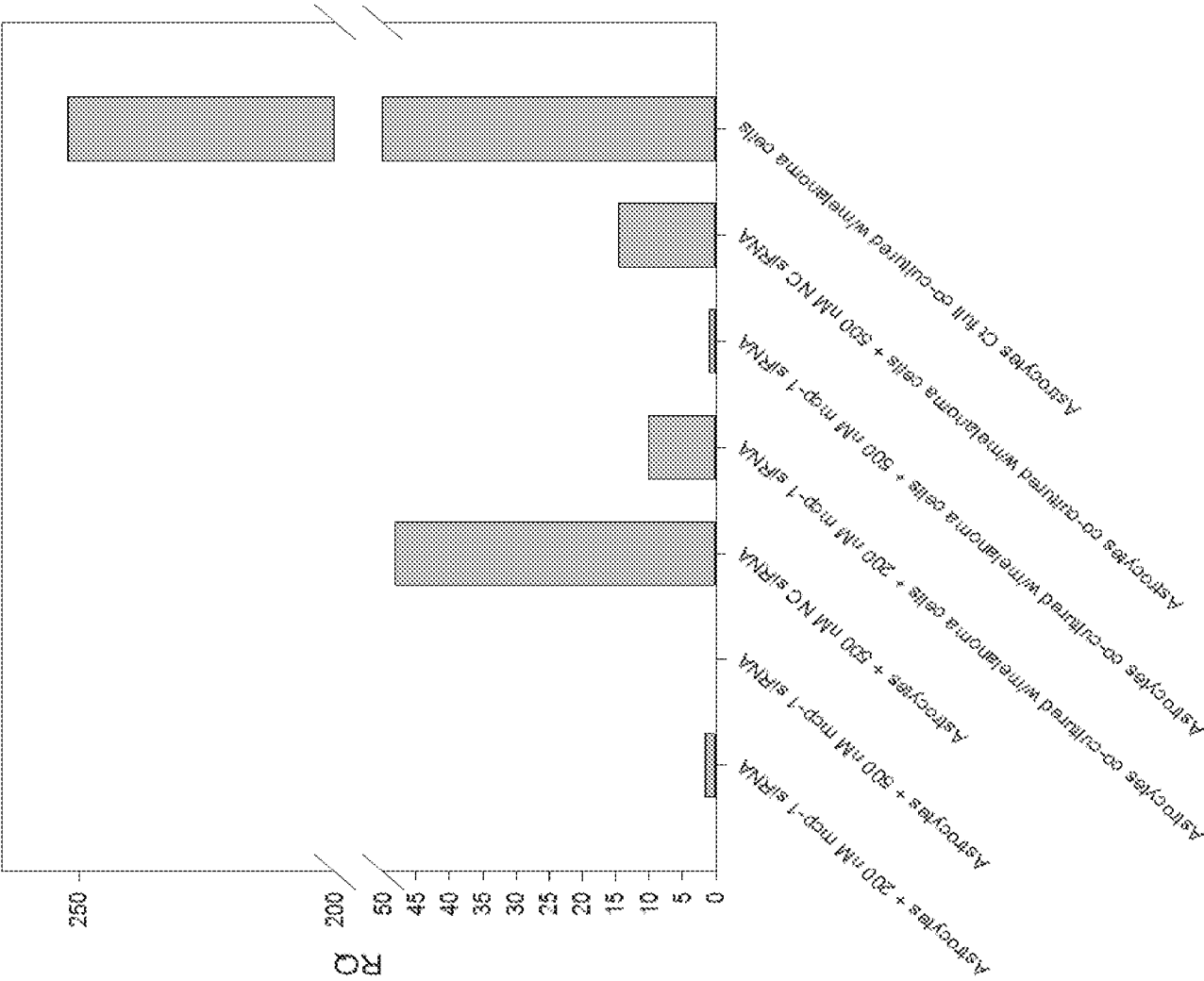


FIG. 21B

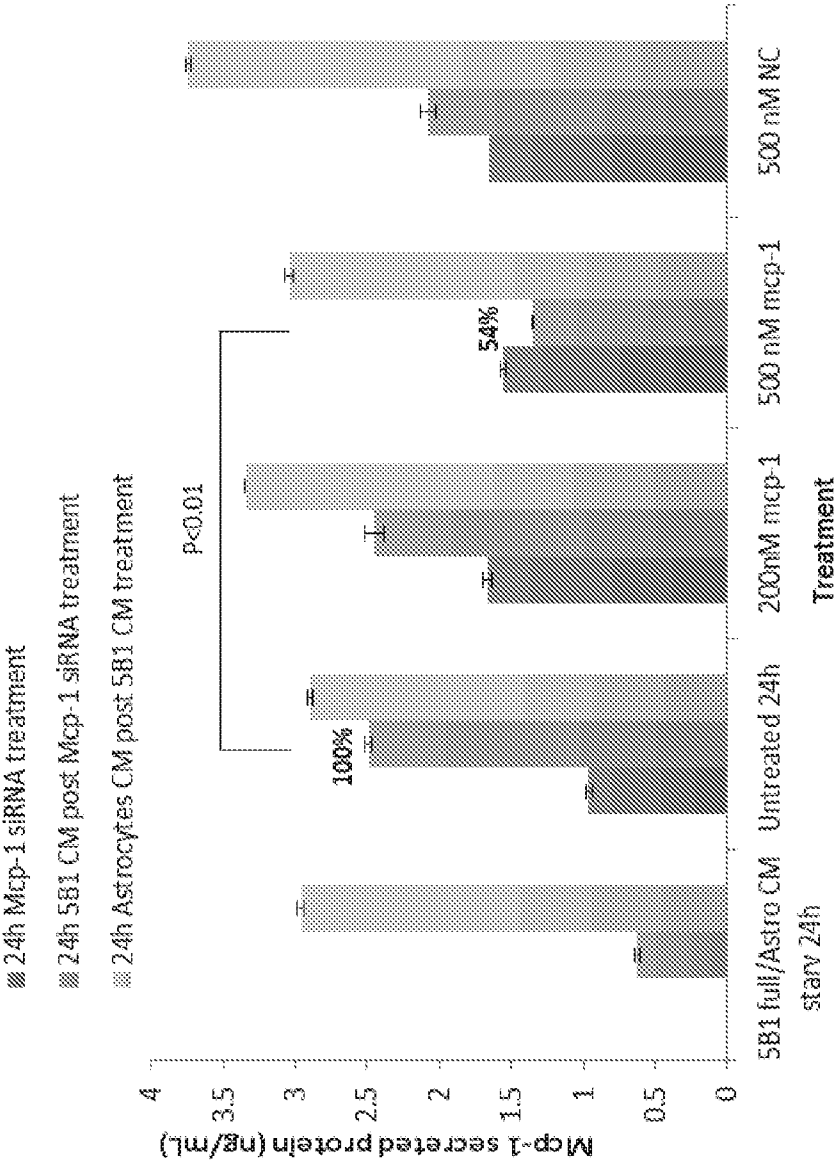


FIG. 22A

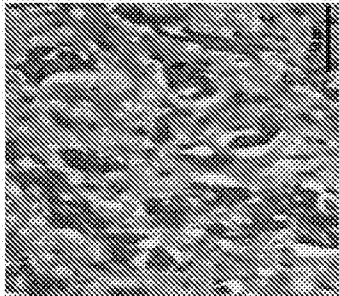


FIG. 22B

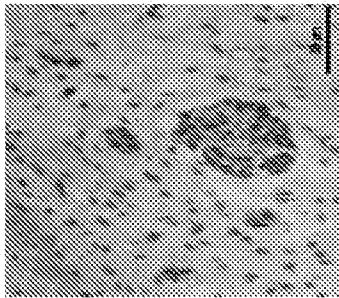


FIG. 22C

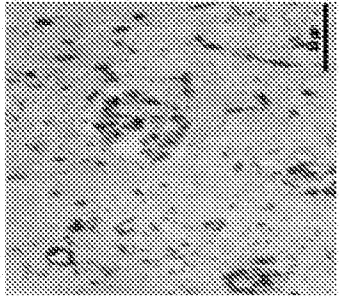


FIG. 22D

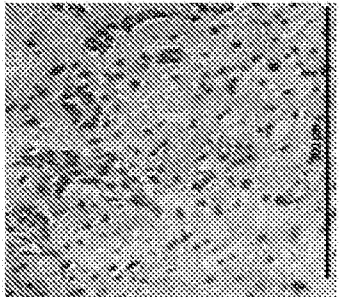


FIG. 22E

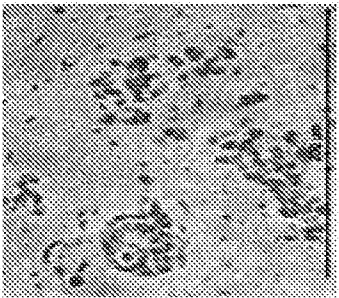


FIG. 22F

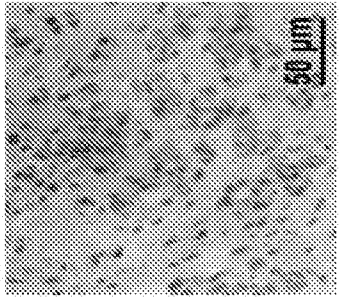


FIG. 23B

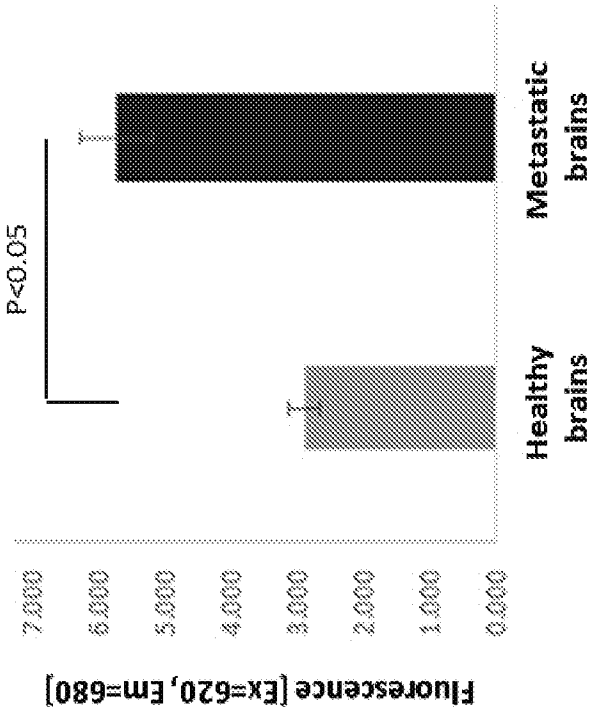
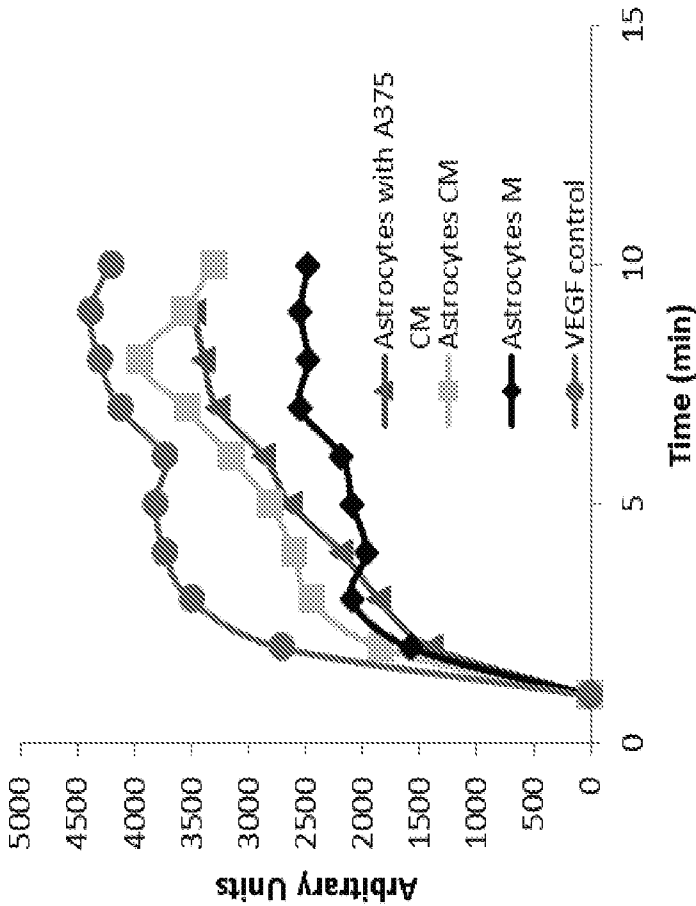


FIG. 23A



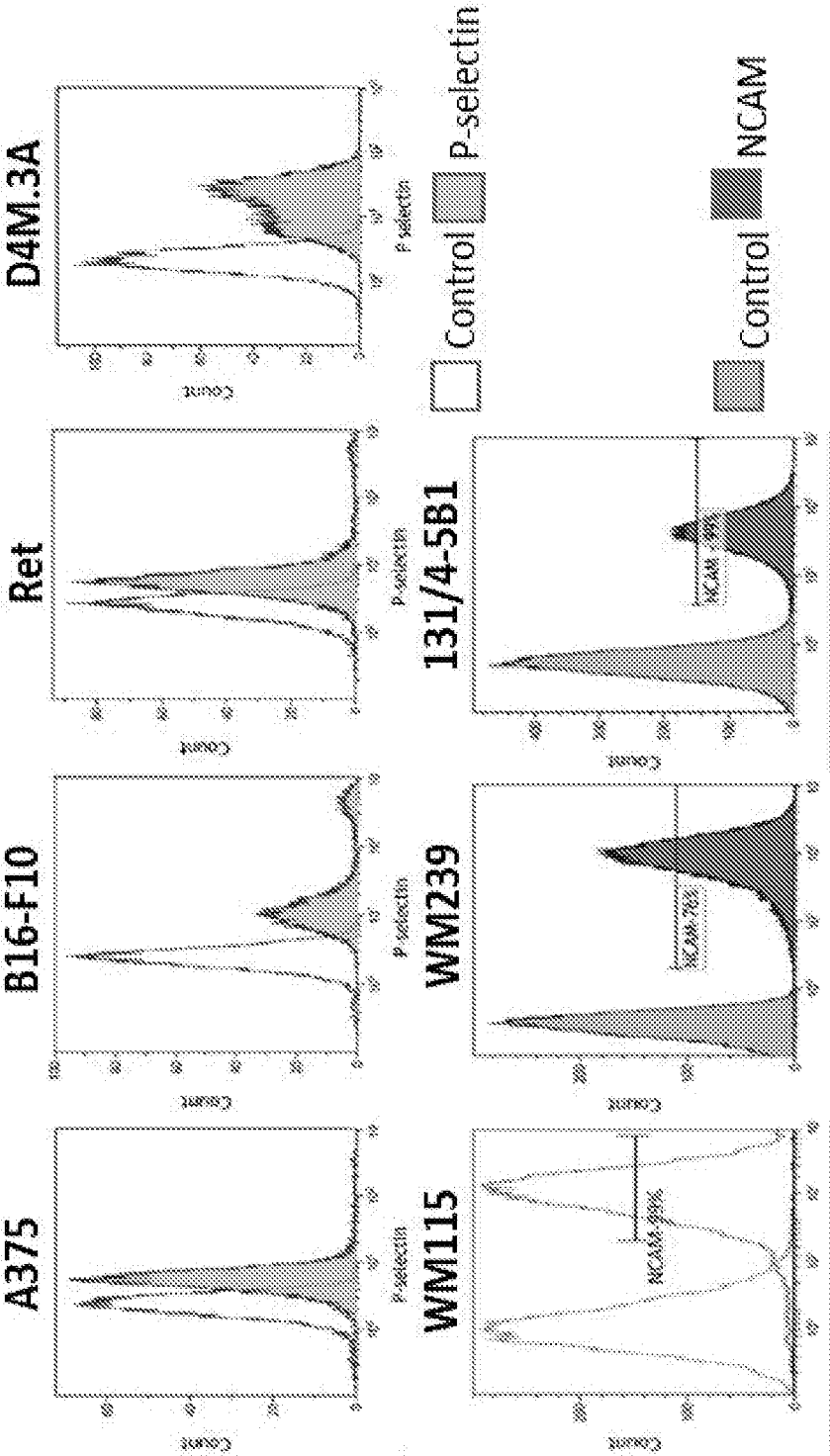


FIG. 24A

FIG. 24B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2017/050237

A. CLASSIFICATION OF SUBJECT MATTER IPC (2017.01) A61K 39/395, A61P 35/04, C07K 16/24, C12N 15/113 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC (2017.01) A61K 39/395, A61P 35/04, C07K 16/24, C12N 15/113 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Databases consulted: Google Patents, CAPLUS, BIOSIS, EMBASE, MEDLINE, Google Scholar Search terms used: Metastasis and (CNS or brain) and treatment and (antibody or siRNA or antisense or TALEN or CRISPR) and (MCP-1 or CCL2 or MCPT1)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Liu, Y., Kosaka, A., Ikeura, M., Kohanbash, G., Fellows-Mayle, W., Snyder, L. A., & Okada, H. (17 April 2013). Premetastatic soil and prevention of breast cancer brain metastasis. <i>Neuro-oncology</i> , Vol. 15, no. 7, pages 891-903. 17 Apr 2013 (2013/04/17) page 901, left column, second paragraph; figures 1d, 5, and 6	1,2,4,6,9,11,15, 20-22,24-26,41,42
X	Zhang, L., Zhang, S., Yao, J., Lowery, F. J., Zhang, Q., Huang, W. C., & Wang, H. (19 October 2015). Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. <i>Nature</i> , Vol. 527, no. 7576, pages 1-34, URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4819404/pdf/nihms-769849.pdf . 19 Oct 2015 (2015/10/19) page 2, first and third paragraphs; figure 4 (c, i, j); extended data figures 6 (f-i) and 7-10	1,2,4,6,9,11,15, 20-22,24-26,28,29,36,41, 42
A	Hattori, Y., Nakamura, A., Arai, S., Nishigaki, M., Ohkura, H., Kawano, K., & Yonemochi, E. (25 January 2014). In vivo siRNA delivery system for targeting to the liver by poly-l-glutamic acid-coated lipoplex. <i>Results in pharma sciences</i> , Vol. 4, pages 1-7. 25 Jan 2014 (2014/01/25) whole document, especially page 4, right column, second paragraph; figure 3	33,34,43
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 06 Jun 2017		Date of mailing of the international search report 14 Jun 2017
Name and mailing address of the ISA: Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel Facsimile No. 972-2-5651616		Authorized officer MAZEL Alexander Telephone No. 972-2-5651716

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2017/050237

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6,8,9,11,15,20-34,36,40-43

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2017/050237

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet):

* This International Searching Authority found multiple inventions in this international application, as follows:

Invention/s 1	An agent and its medical use in a subject afflicted with CNS metastasizing cancer, said agent down-regulates activity or expression of CCL2.	Claim/s 1-6,8,9,11,15,20-34,36,40-43
Invention/s 2 - 25	An agent and its medical use in a subject afflicted with CNS metastasizing cancer, said agent down-regulates activity or expression of a polypeptide selected from the group of polypeptides listed in any one of tables 1-7 (excluding CCL2).	Claim/s 1-43
Invention/s 26	Non-human animal comprising a CNS metastasizing melanoma, wherein the non-human animal does not comprise a primary melanoma tumor and a method for generating said animal.	Claim/s 44,45

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2017/050237

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dwyer, J., Hebda, J. K., Le Guelte, A., Galan-Moya, E. M., Smith, S. S., Azzi, S., & Gavard, J. (2012). Glioblastoma cell-secreted interleukin-8 induces brain endothelial cell permeability via CXCR2. PLoS One, 7(9), e45562. 30 Sep 2012 (2012/09/30) whole document	1-6,8,9,11,15,20-34, 36,40-43
P,Y	Polyak, D., Krivitsky, A., Scomparin, A., Eliyahu, S., Kalinski, H., Avkin-Nachum, S., & Satchi-Fainaro, R. (26 June 2016). Systemic delivery of siRNA by aminated poly (?) glutamate for the treatment of solid tumors. Journal of Controlled Release. 26 Jul 2016 (2016/07/26) whole document	33,34,43