US 20200407421A1

(19) **United States**

(12) **Patent Application Publication**

HERMISTON et al.

(10) **Pub. No.: US 2020/0407421 A1**

(43) **Pub. Date: Dec. 31, 2020**

(54) **DELIVERY OF PAYLOADS TO STEM CELLS**

(71) Applicant: **GLADIATOR BIOSCIENCES, INC.**, Mill Valley, CA (US)

(72) Inventors: **Terry HERMISTON**, Mill Valley, CA (US); **Maxine BAUZON**, Hercules, CA (US); **Christopher H. CONTAG**, San Jose, CA (US); **Jonathan HARDY**, East Lansing, MI (US); **Francis Gerard BLANKENBERG**, Portola Valley, CA (US)

(73) Assignee: **GLADIATOR BIOSCIENCES, INC.**, Mill Valley, CA (US)

(21) Appl. No.: **16/643,942**

(22) PCT Filed: **Sep. 5, 2018**

(86) PCT No.: **PCT/US2018/049618**

§ 371 (c)(1),
(2) Date: **Mar. 3, 2020**

Related U.S. Application Data

(60) Provisional application No. 62/593,014, filed on Nov. 30, 2017, provisional application No. 62/584,565, filed on Nov. 10, 2017, provisional application No. 62/569,411, filed on Oct. 6, 2017, provisional application No. 62/569,403, filed on Oct. 6, 2017, provisional application No. 62/554,533, filed on Sep. 5, 2017, provisional application No. 62/554,530, filed on Sep. 5, 2017.

Publication Classification

(51) **Int. Cl.**

C07K 14/745	(2006.01)
C12N 5/0789	(2006.01)
C12N 5/095	(2006.01)

(52) **U.S. Cl.**

CPC ***C07K 14/745*** (2013.01); ***C12N 5/0695*** (2013.01); ***C12N 5/0647*** (2013.01)

(57) **ABSTRACT**

The present disclosure relates to a method of targeting stems cells, in particular non-apoptotic stem cells, employing a GLA domain, capable of binding surface exposed phosphatidyl serine.

Specification includes a Sequence Listing.

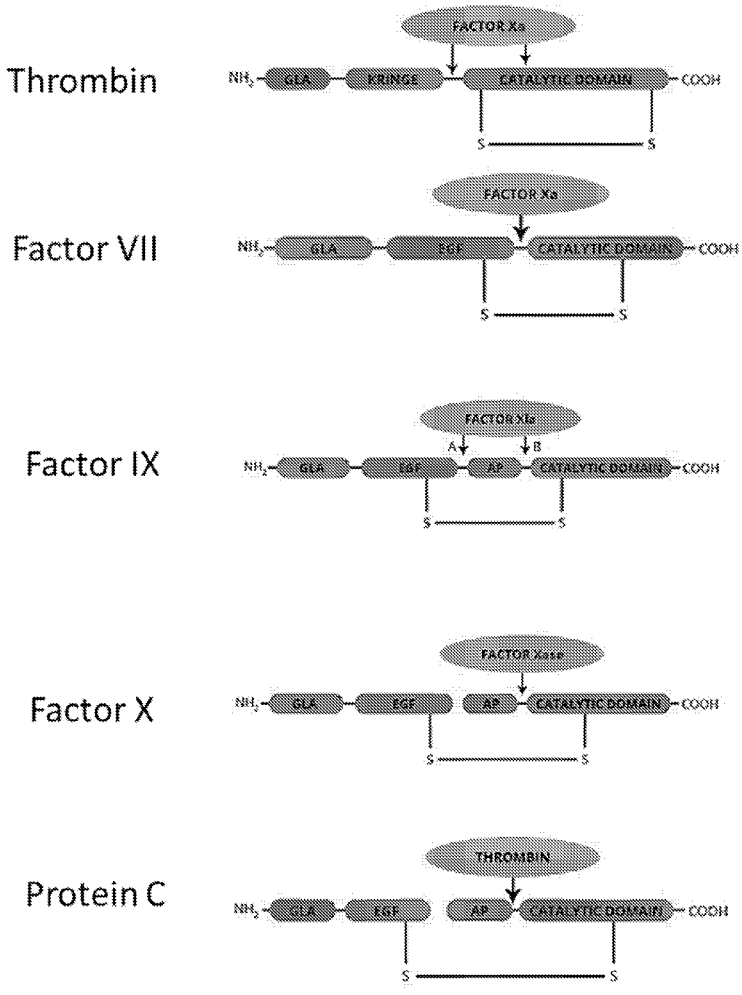


Figure 1A

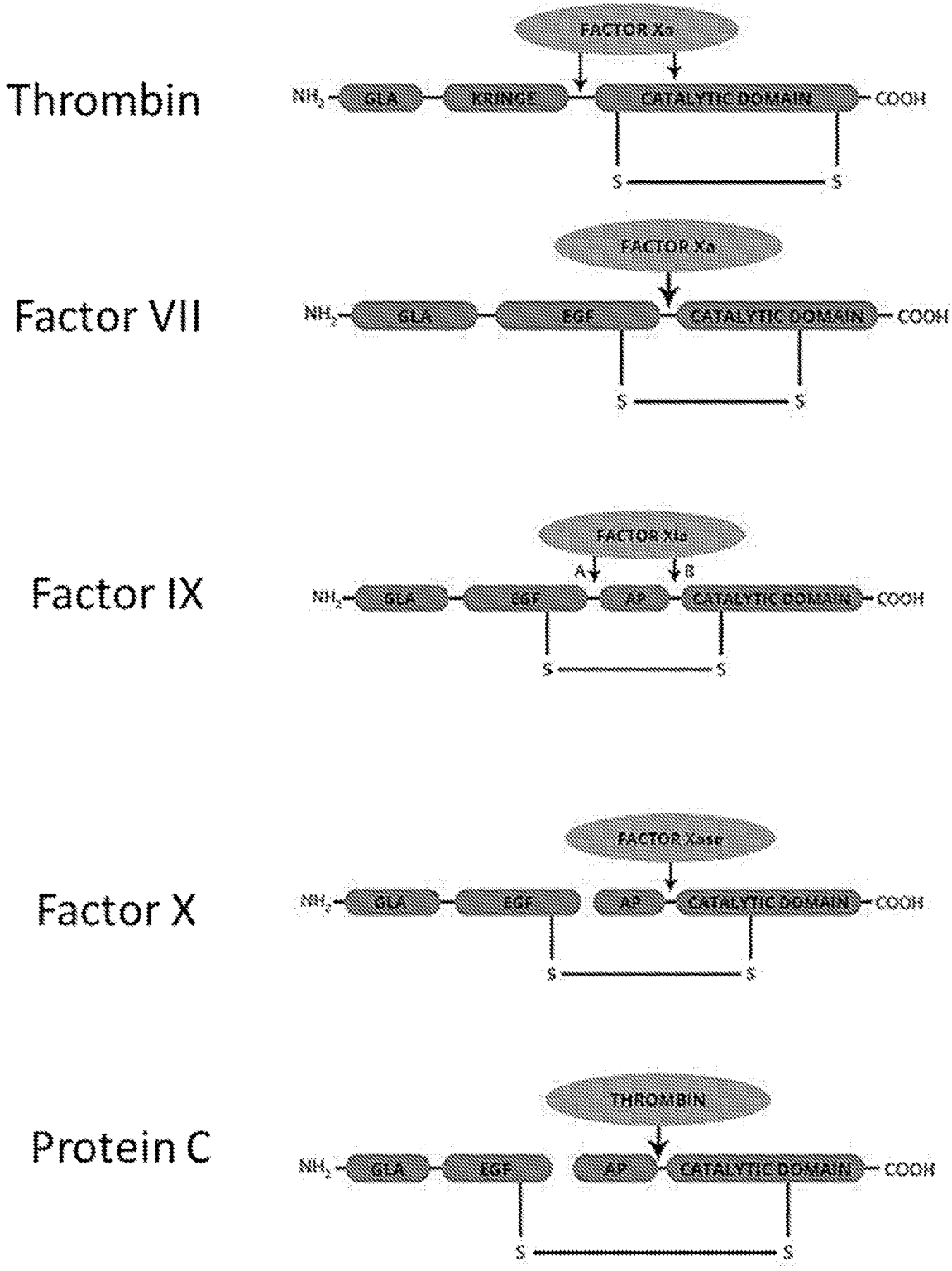

Thrombin
FACTOR Xa
NH2
GLA
KRINGE
CATALYTIC DOMAIN
COOH
S
S
Factor VII
FACTOR Xa
NH2
GLA
EGF
CATALYTIC DOMAIN
COOH
S
S
Factor IX
FACTOR XIa
A
B
NH2
GLA
EGF
AP
CATALYTIC DOMAIN
COOH
S
S
Factor X
FACTOR Xase
NH2
GLA
EGF
AP
CATALYTIC DOMAIN
COOH
S
S
Protein C
THROMBIN
NH2
GLA
EGF
AP
CATALYTIC DOMAIN
COOH
S
S

Figure 1B

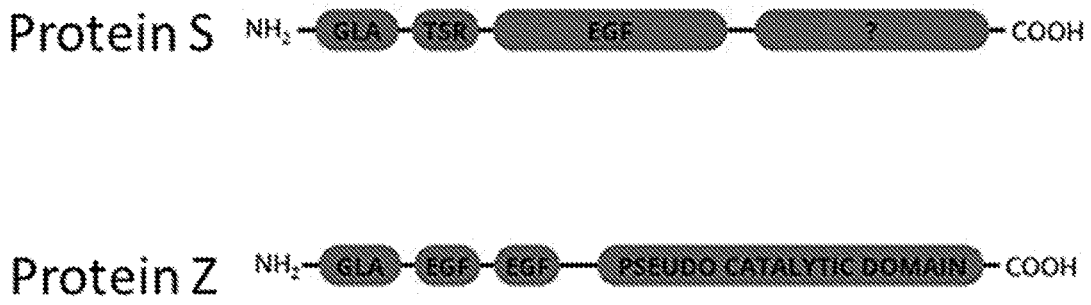
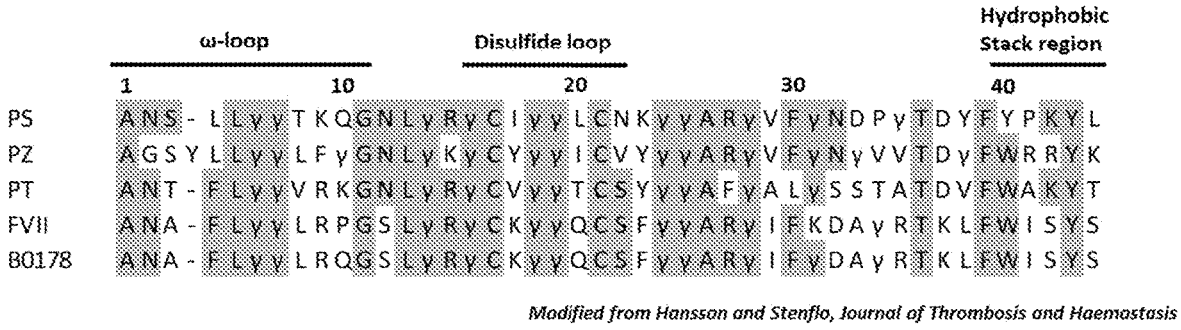


Figure 1C



Modified from Hansson and Stenflo, Journal of Thrombosis and Haemostasis

Figure 1D

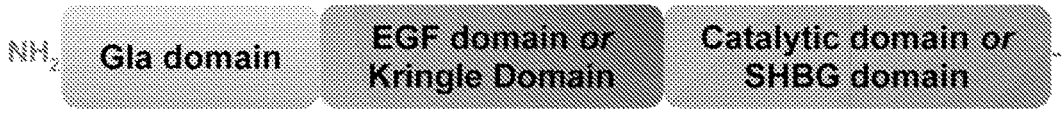


Figure 1E An embodiment of a GLA-component

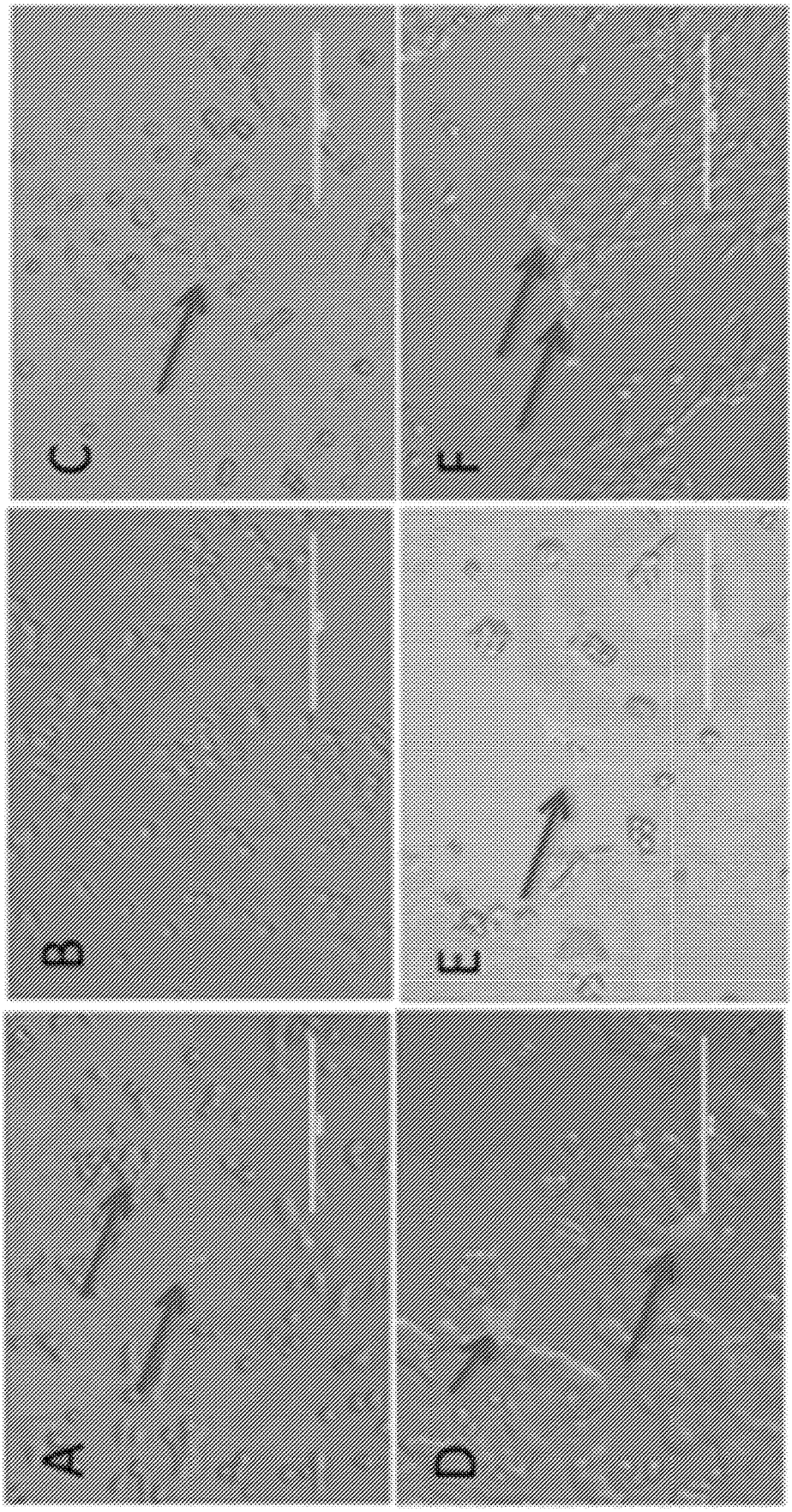
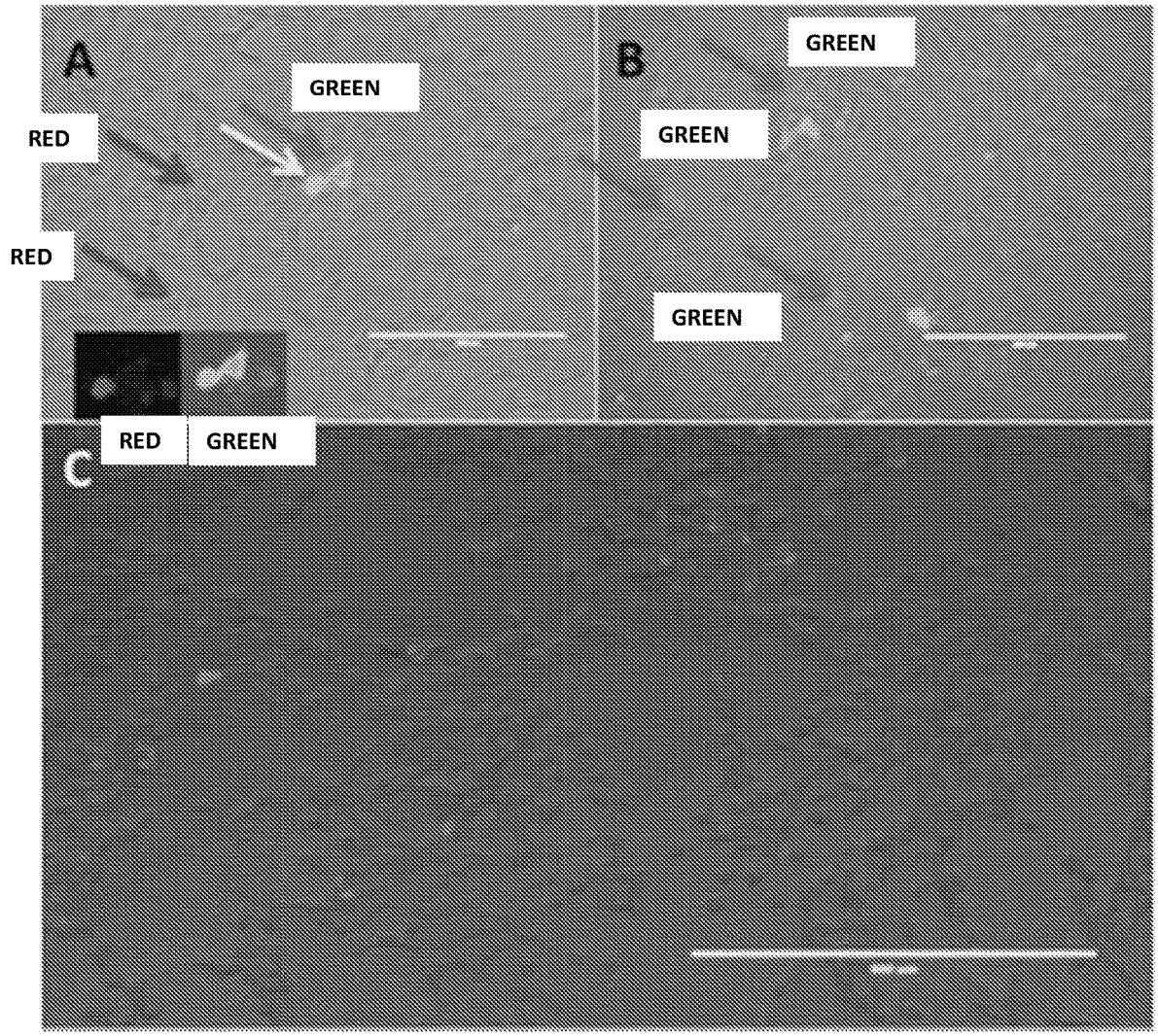


Figure 2

Figure 3



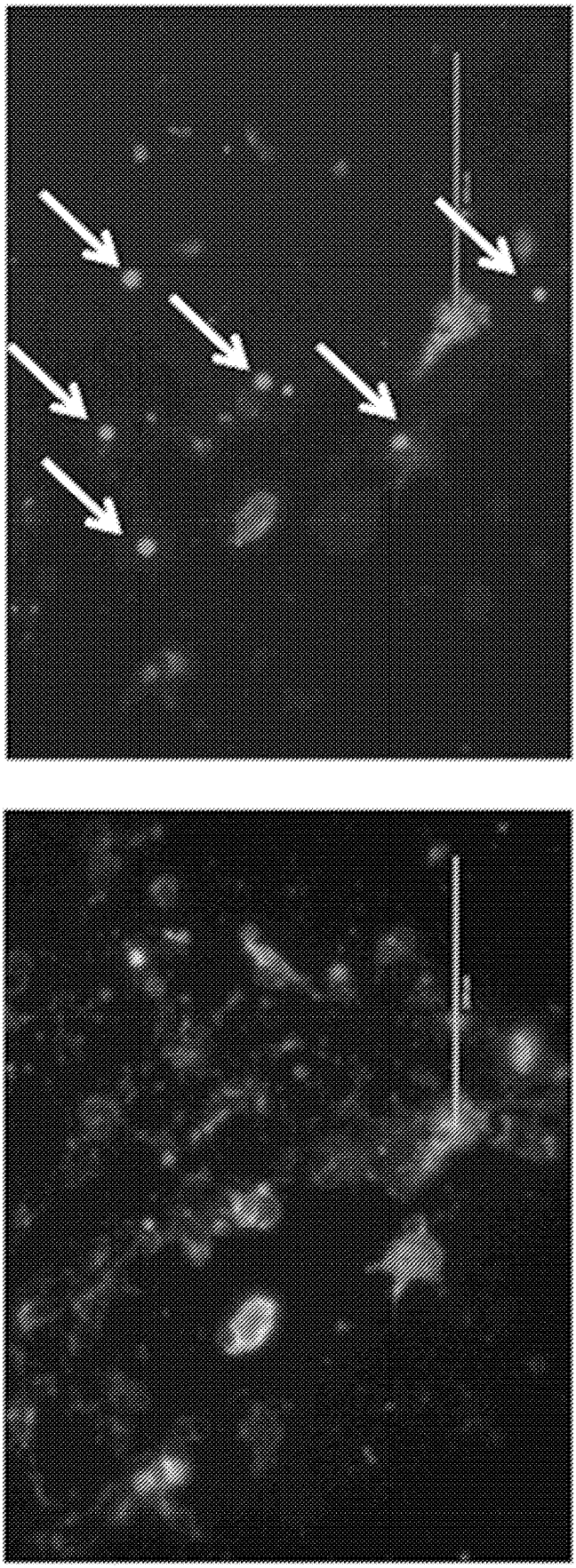
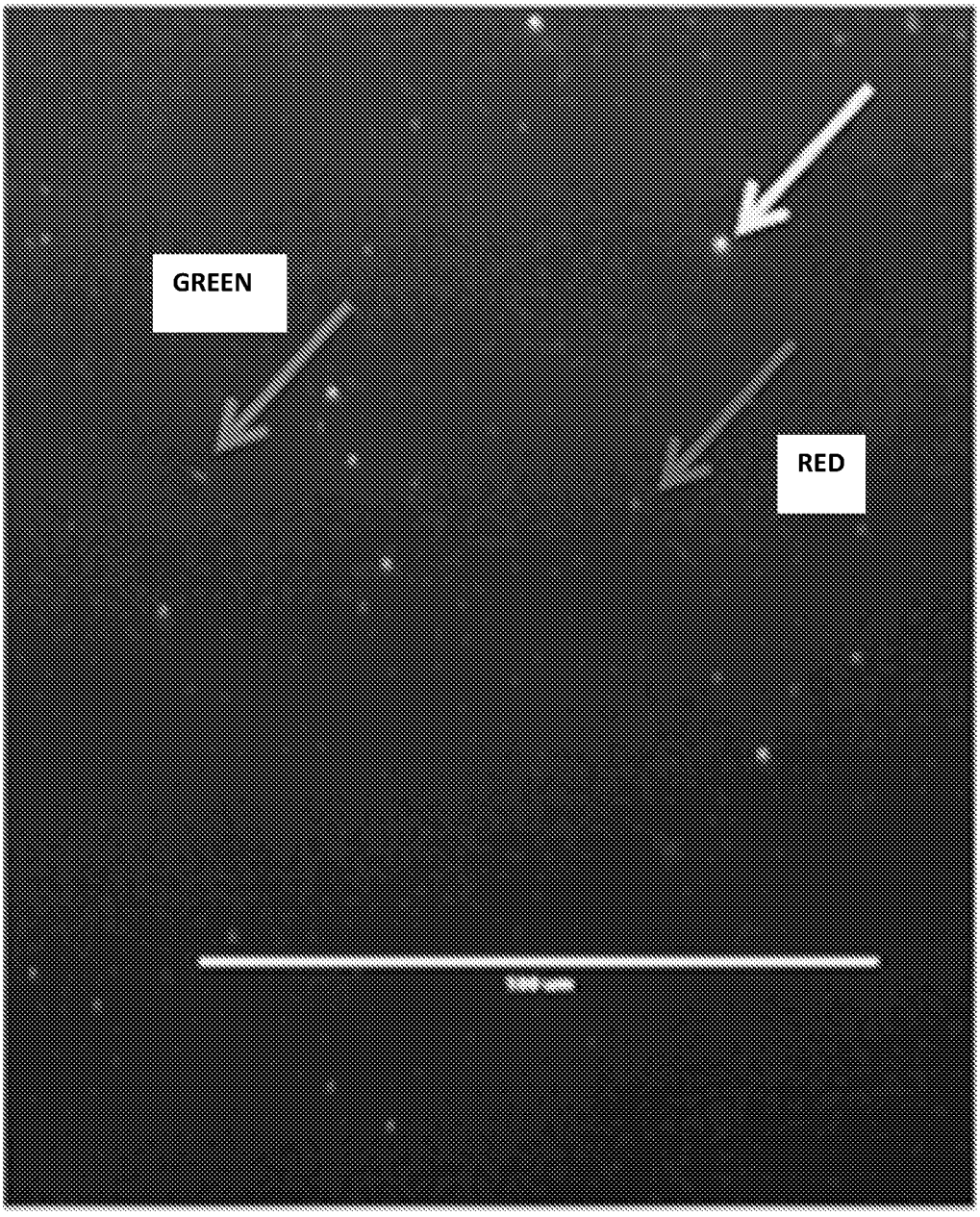


Figure 4

Figure 5



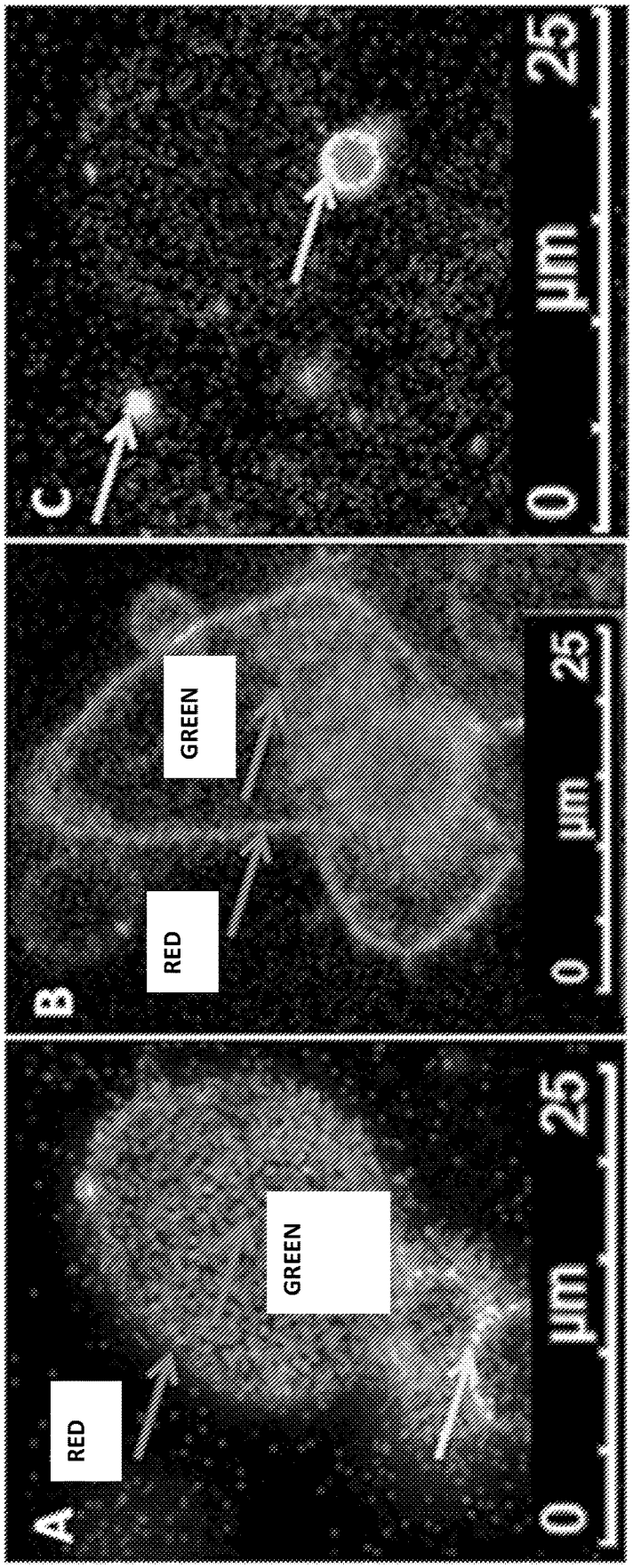


Figure 6

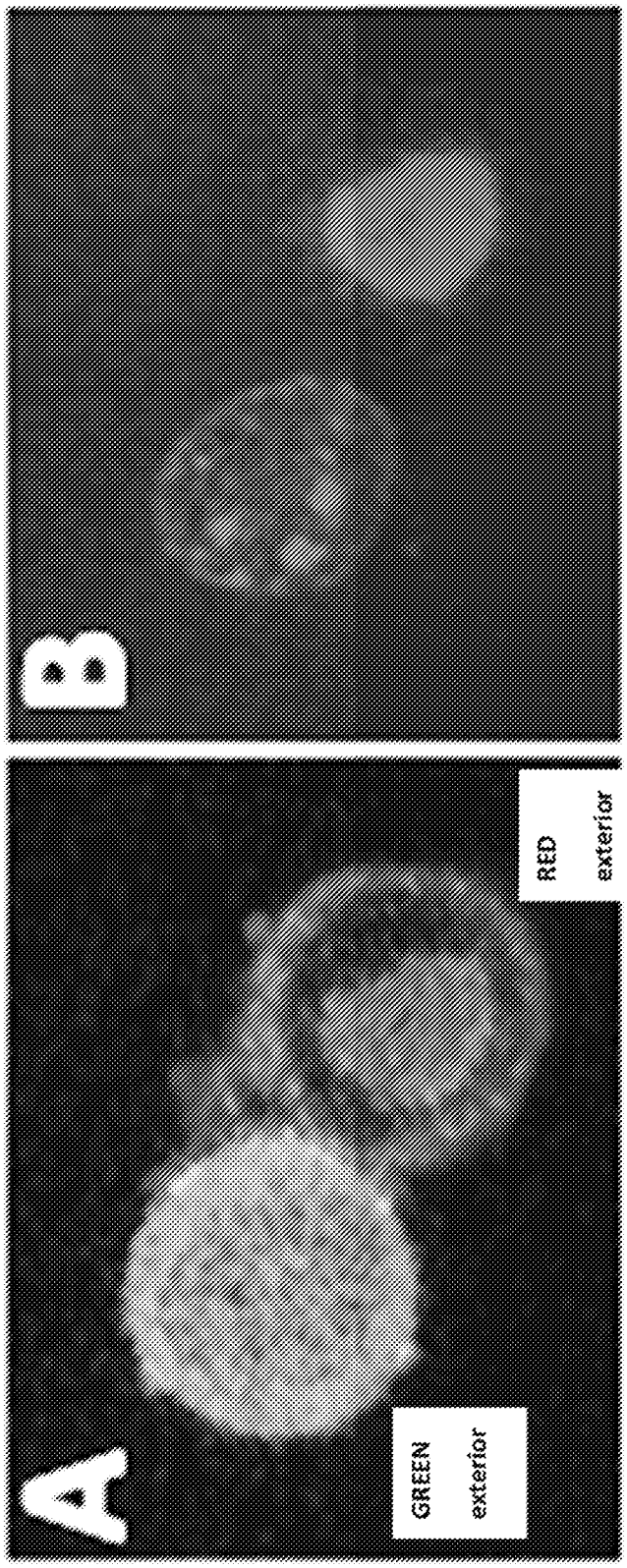


Figure 7

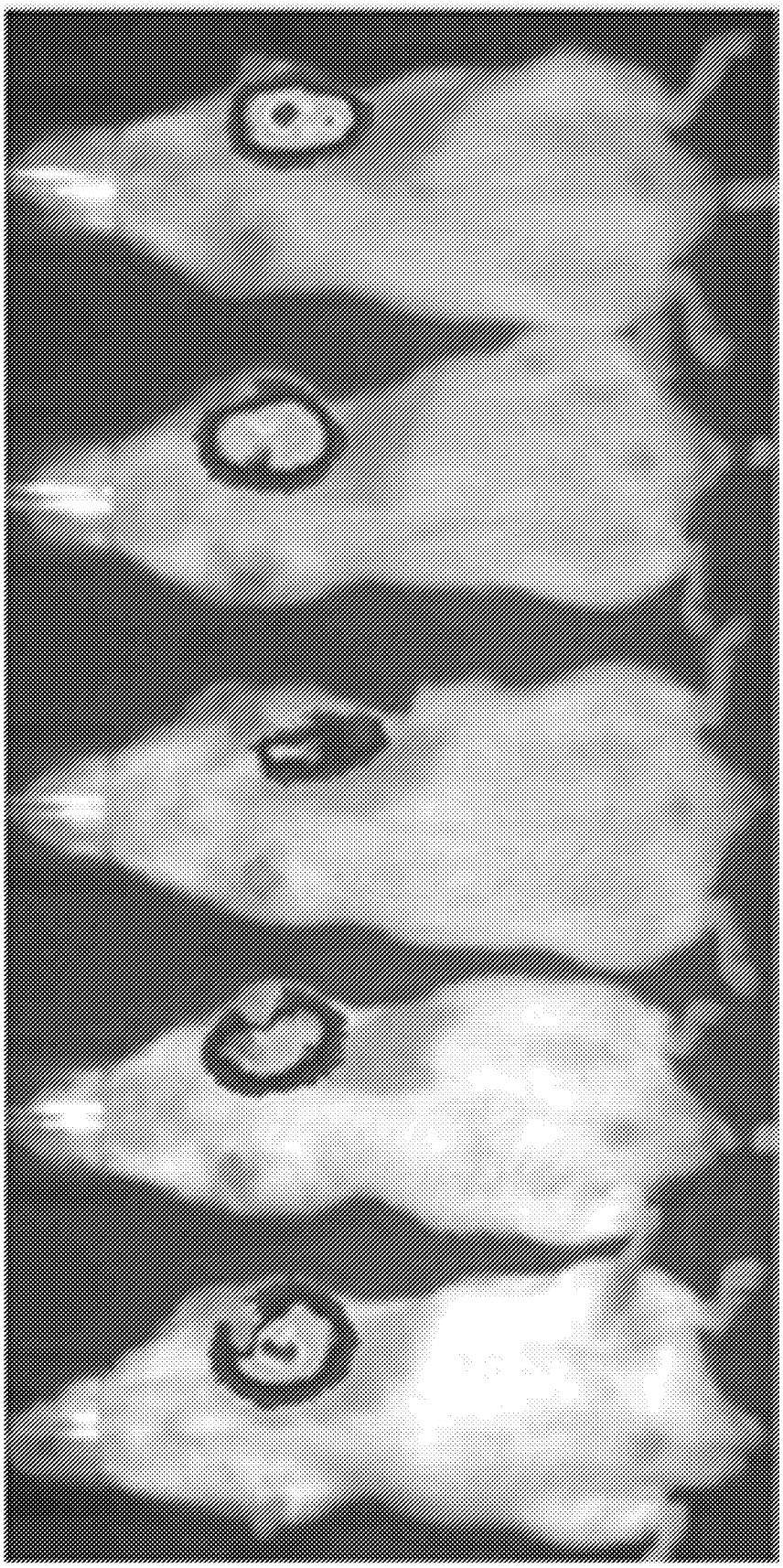


Figure 8

Figure 9

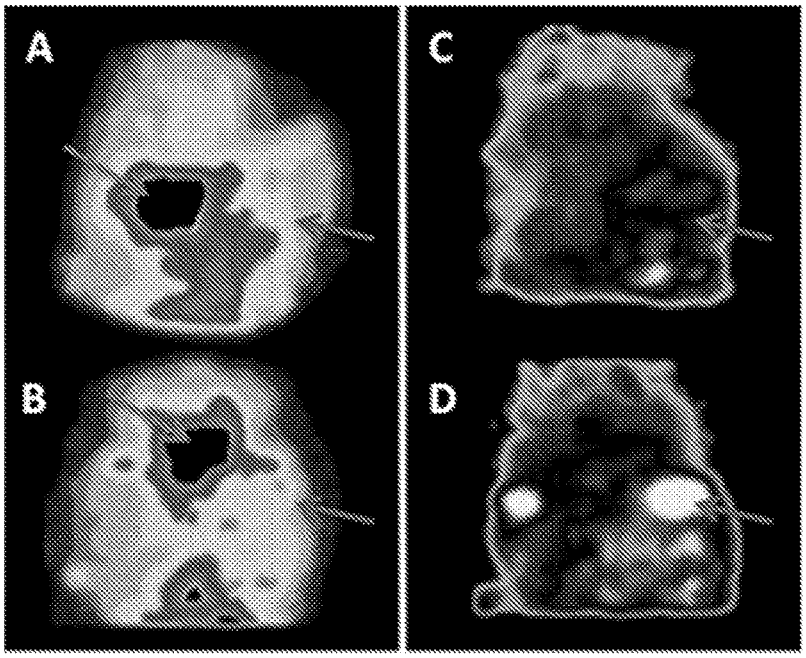


Figure 10

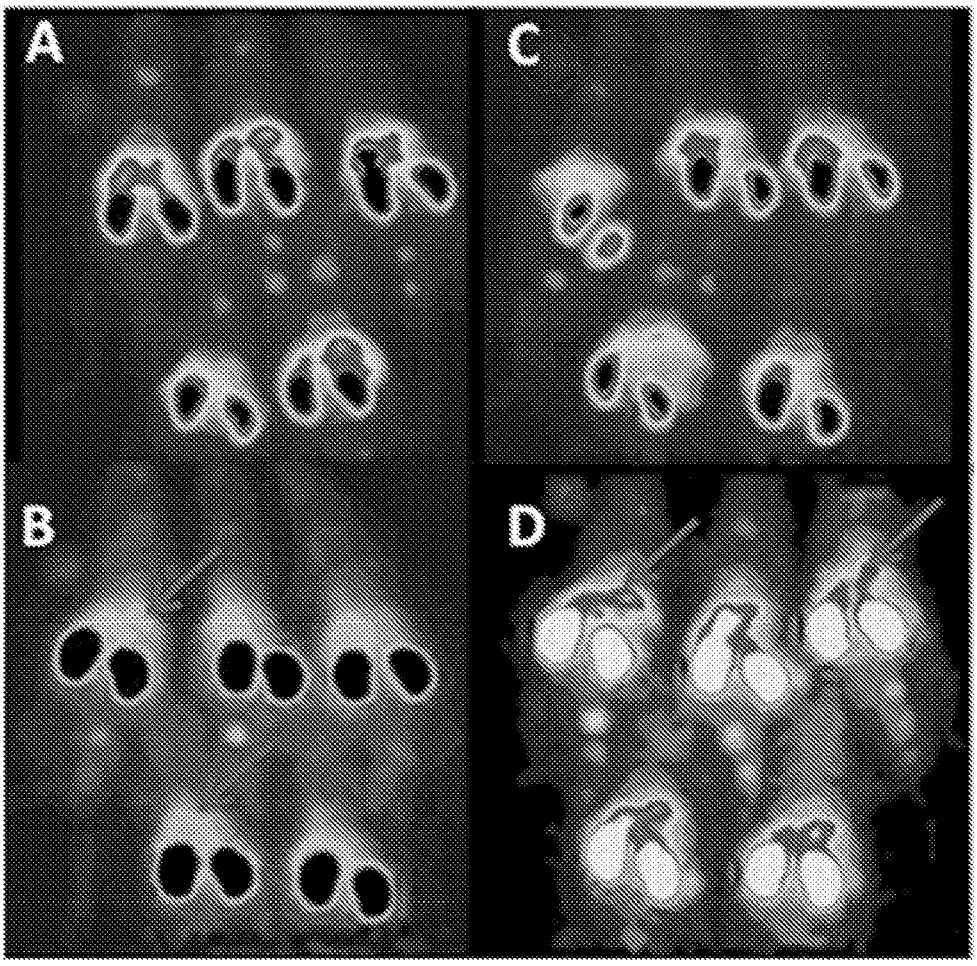
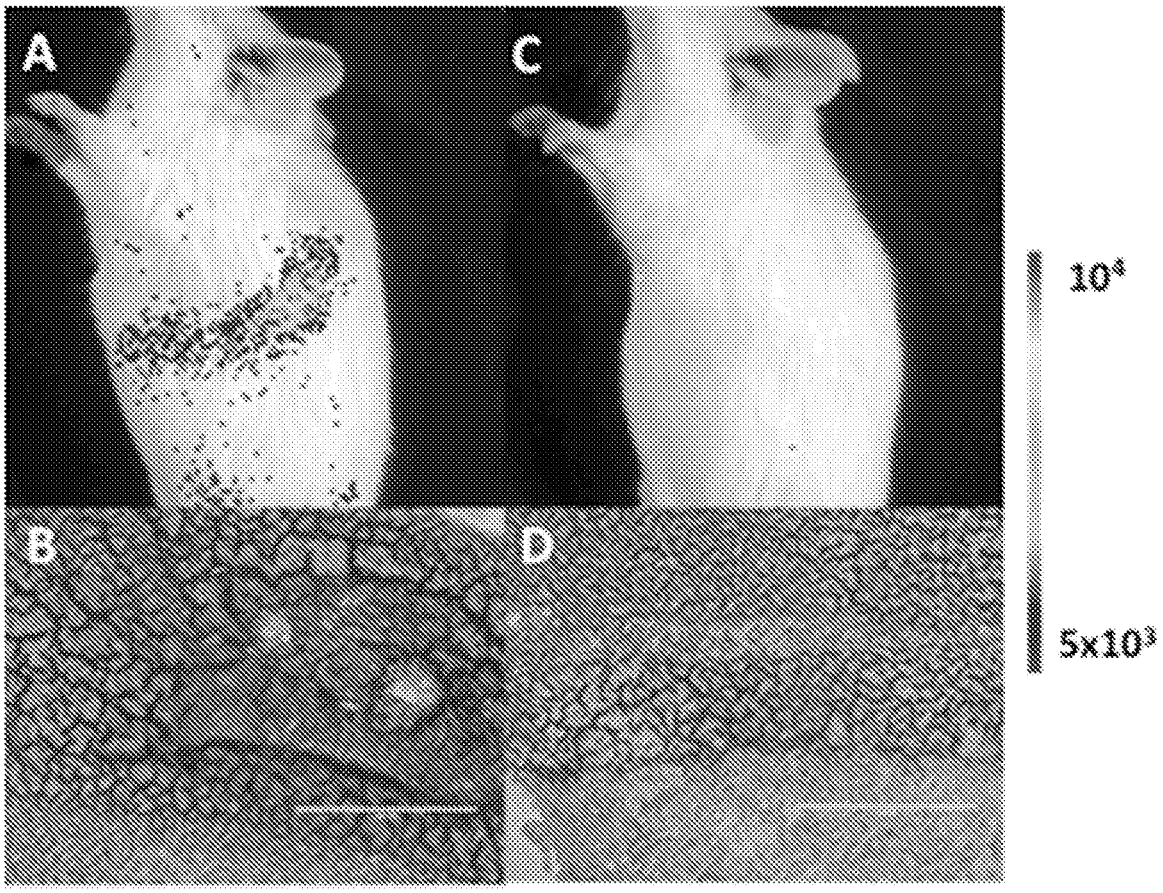


Figure 11



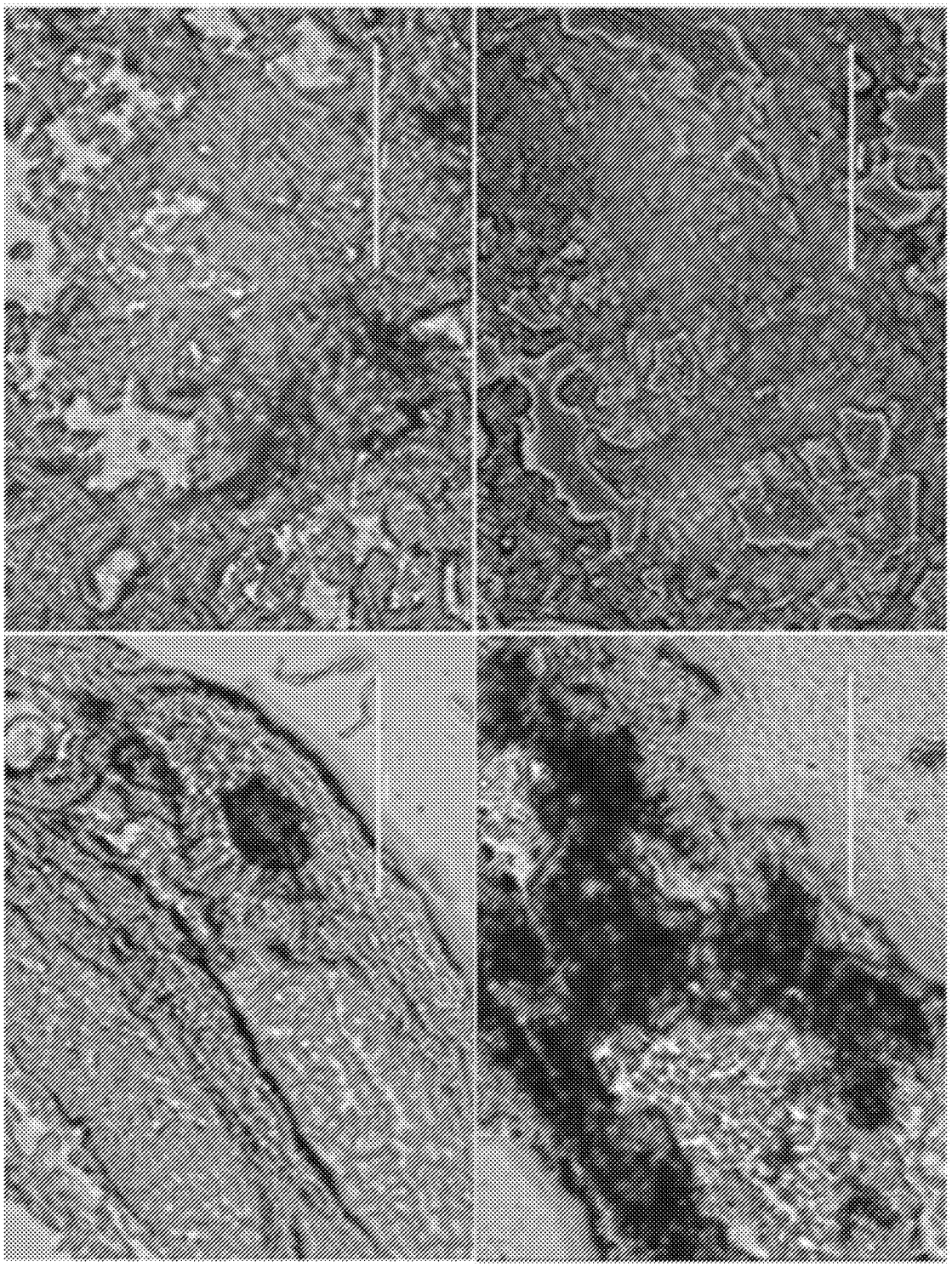


Figure 12

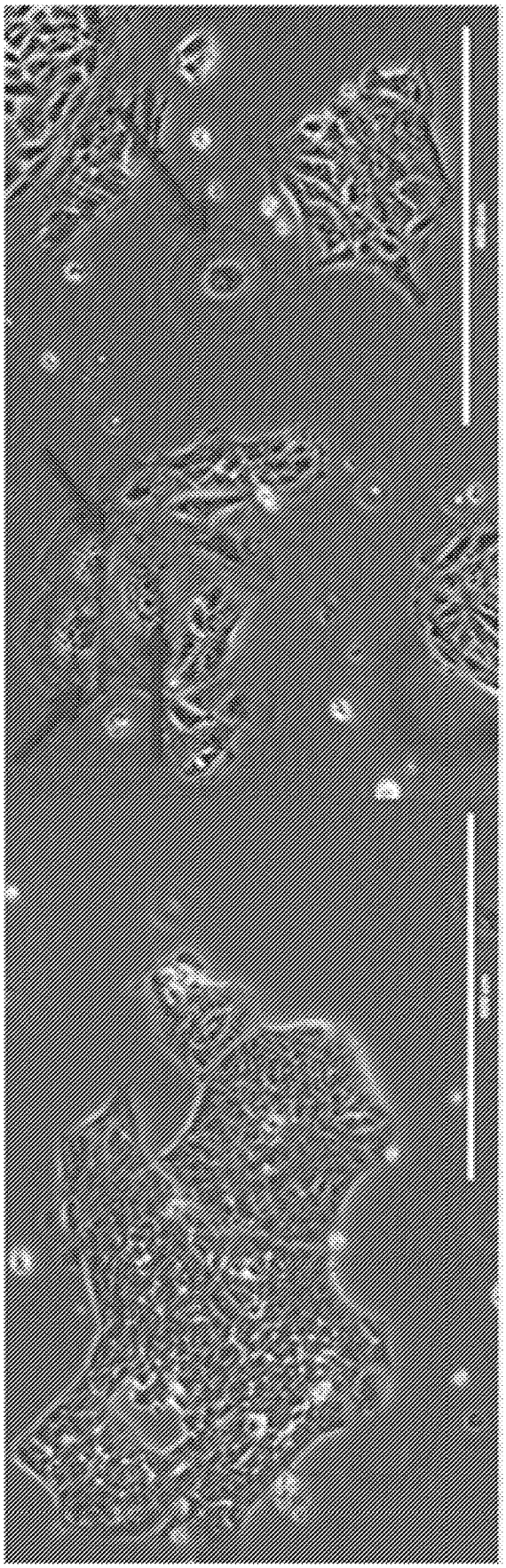


Figure 13

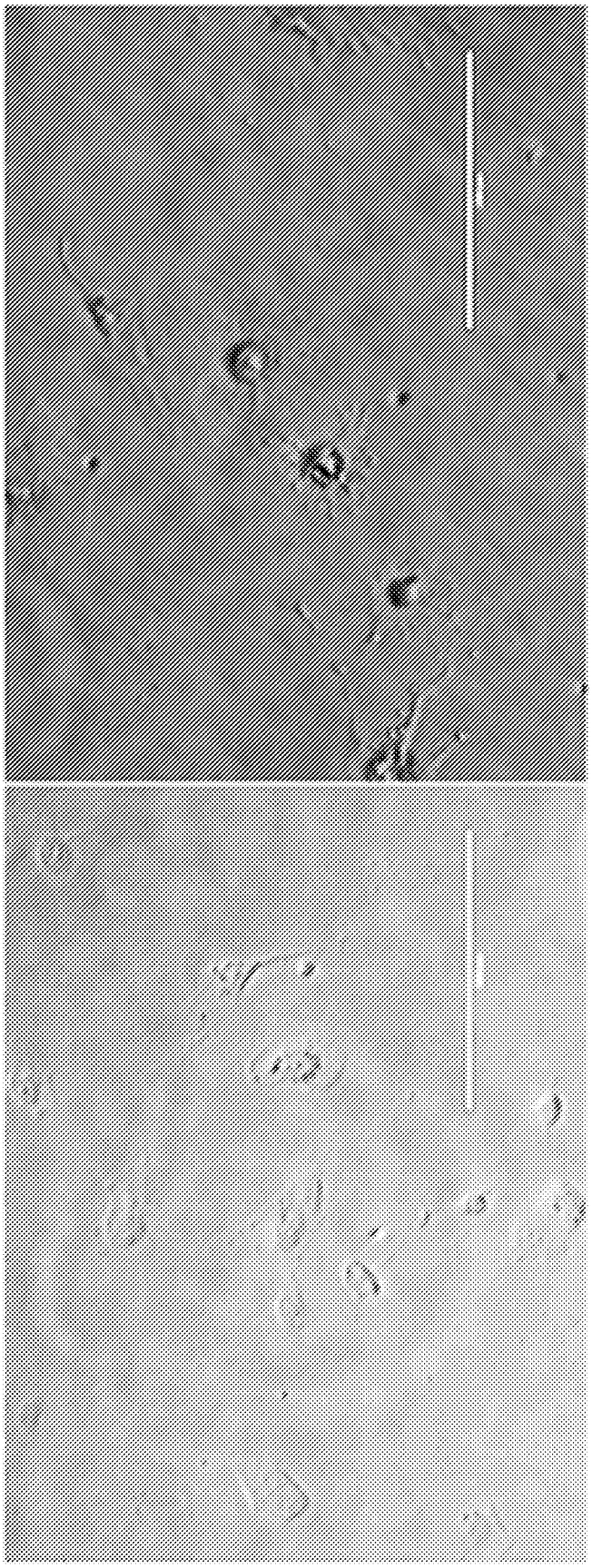


Figure 14

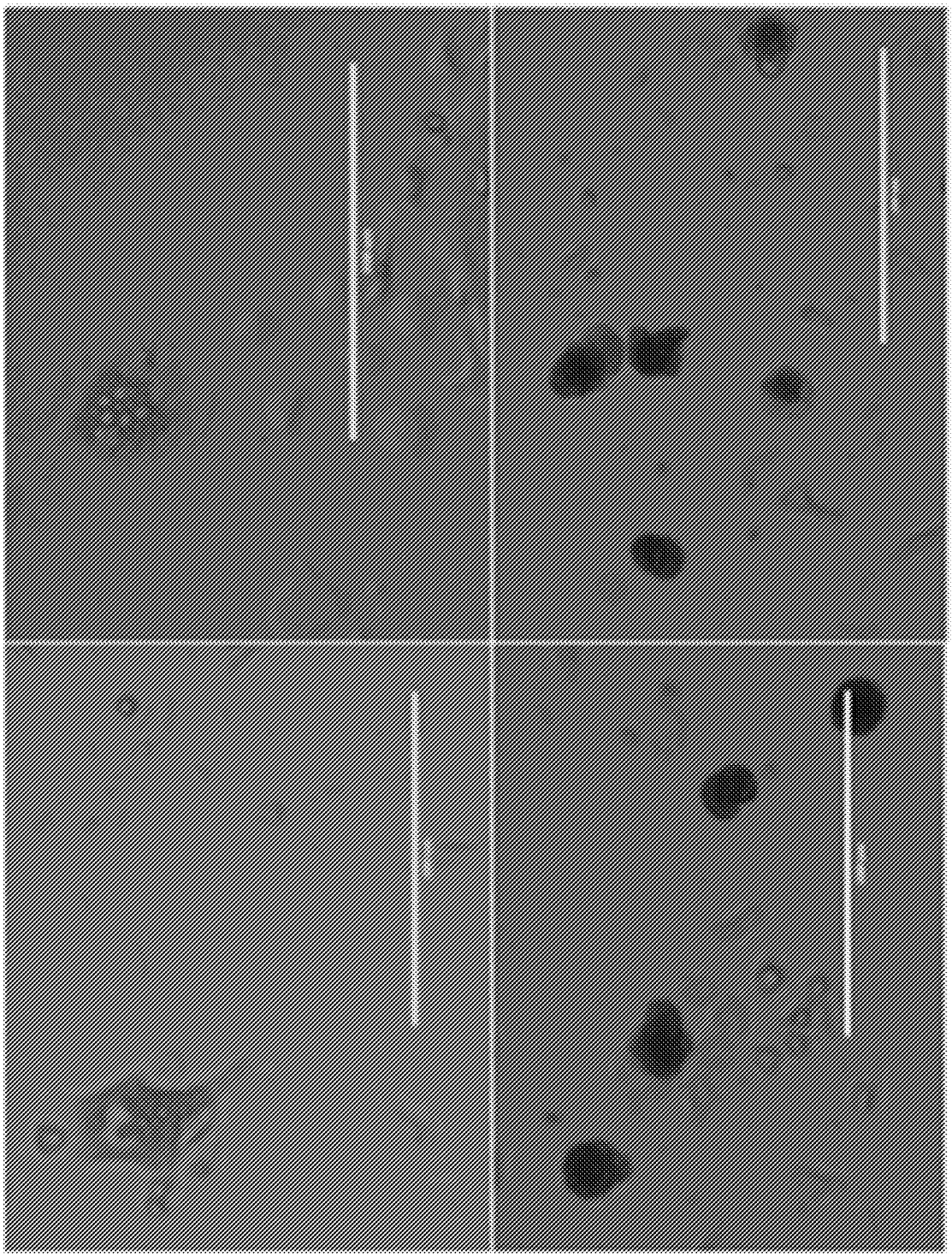


Figure 15

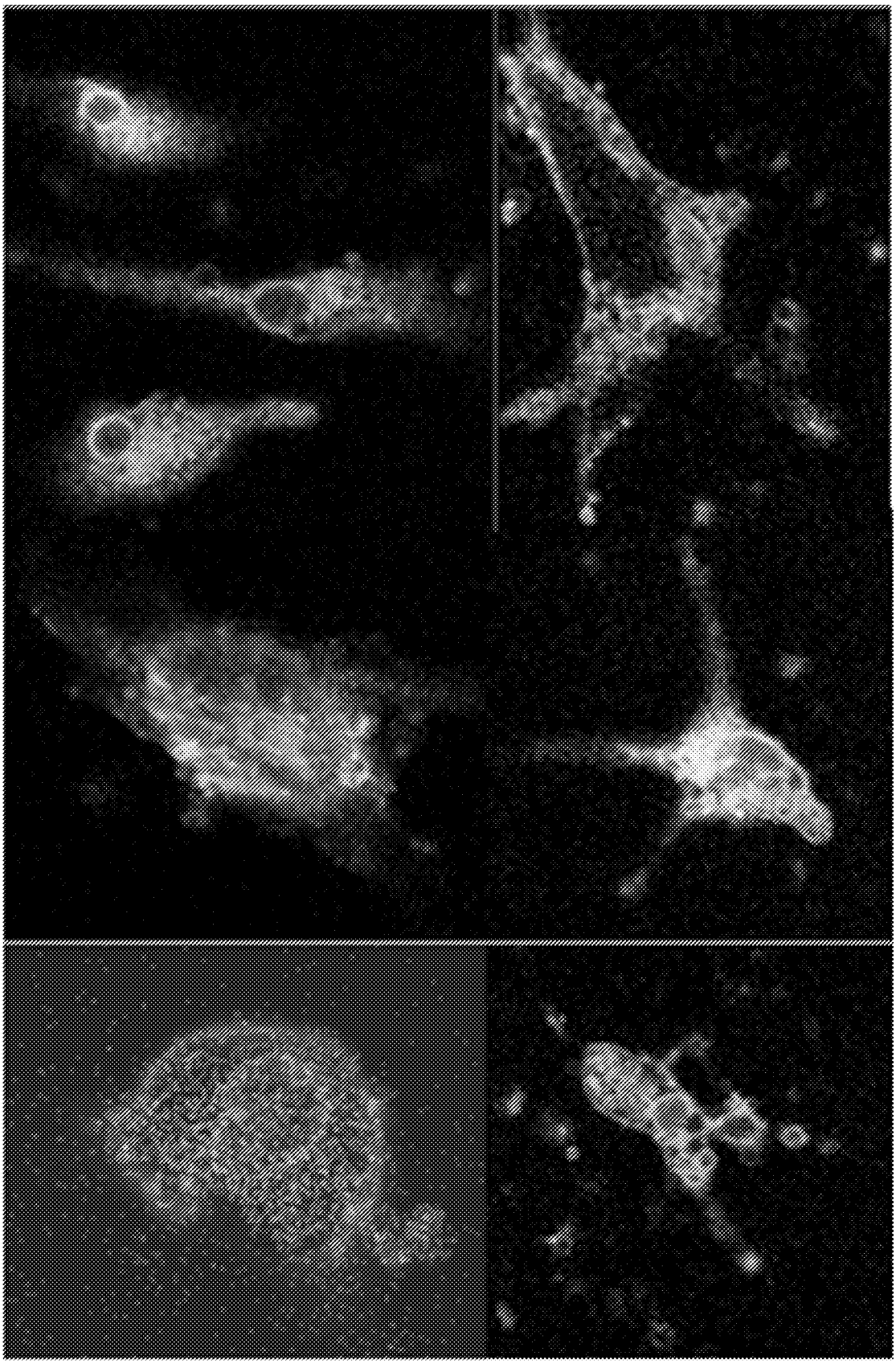


Figure 16

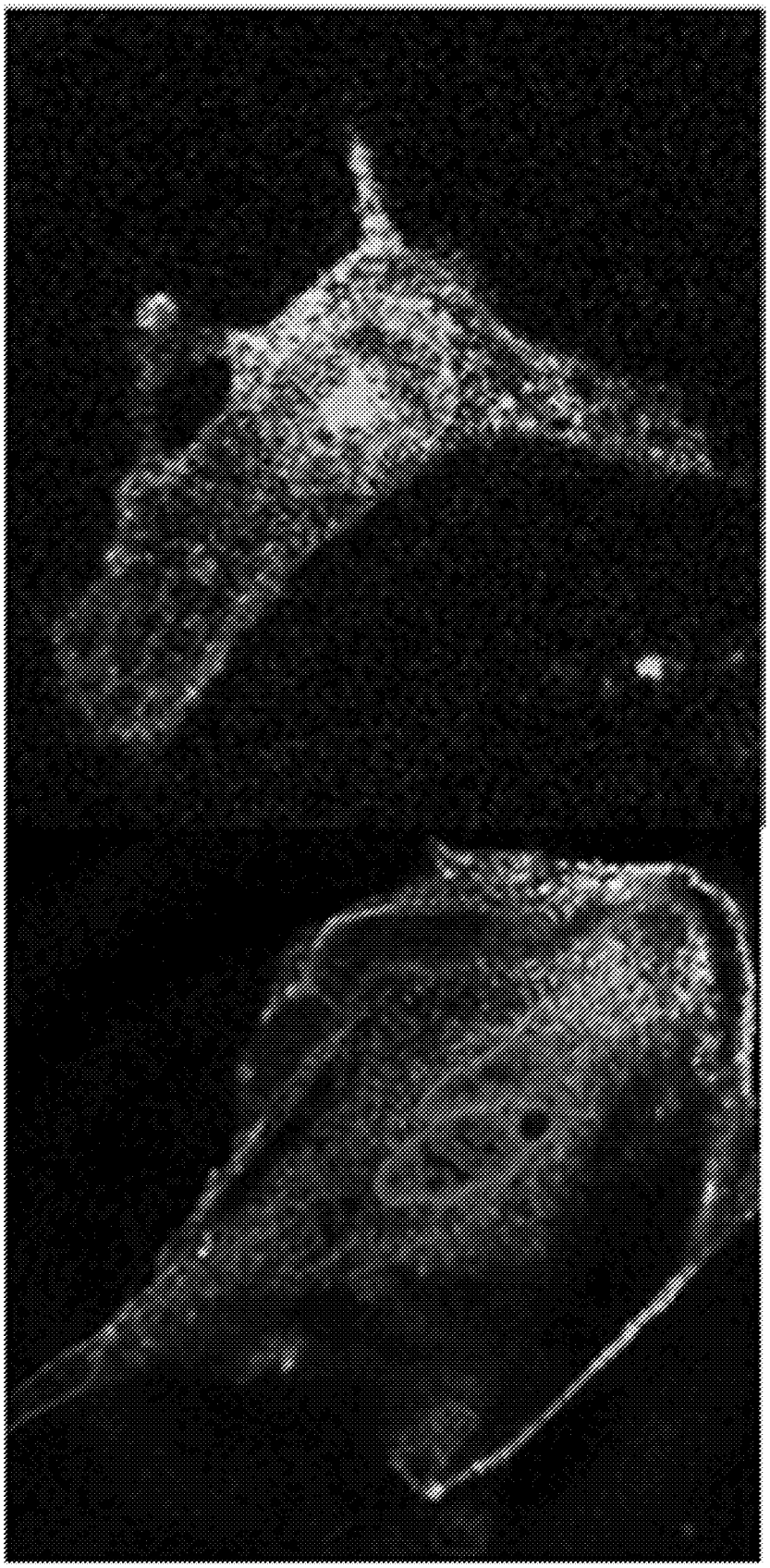


Figure 17

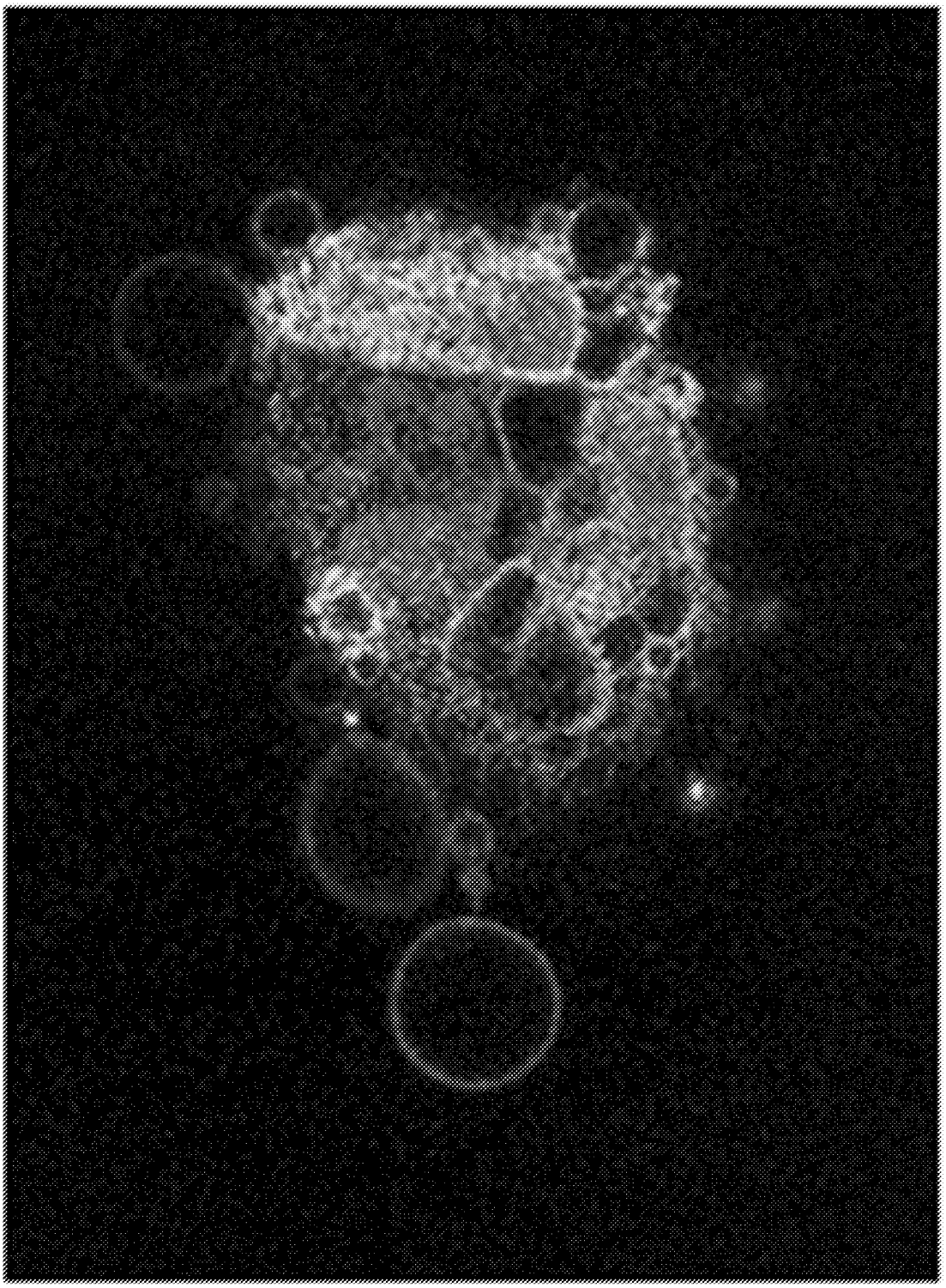


Figure 18

Figure 19

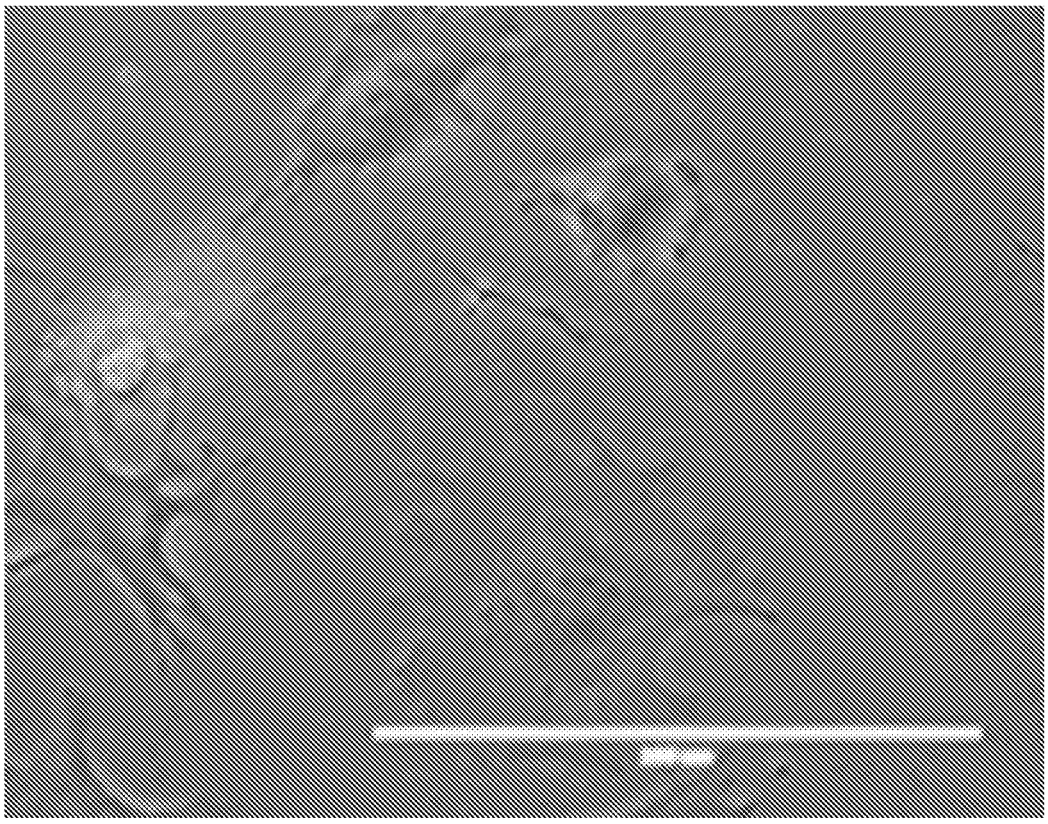
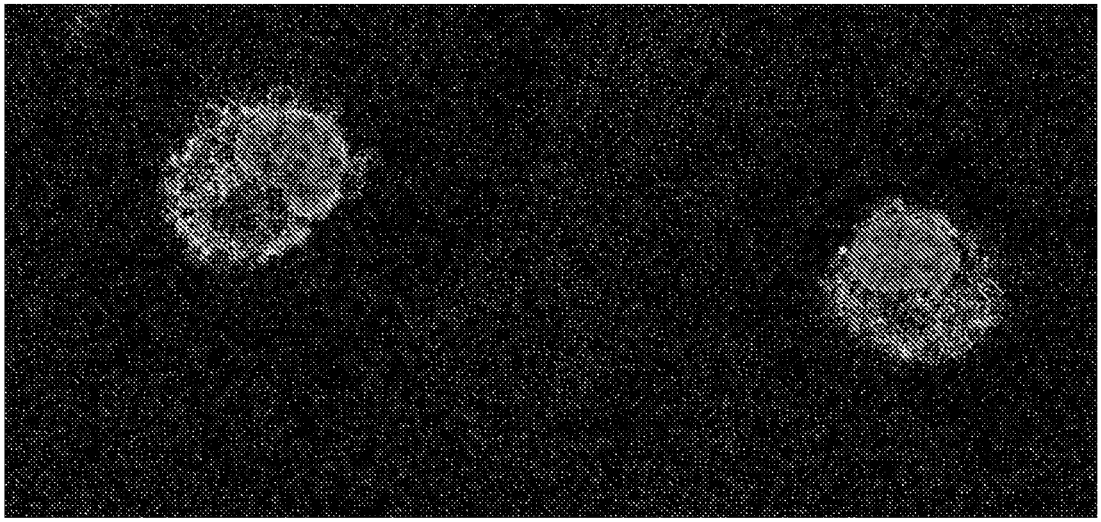


Figure 20



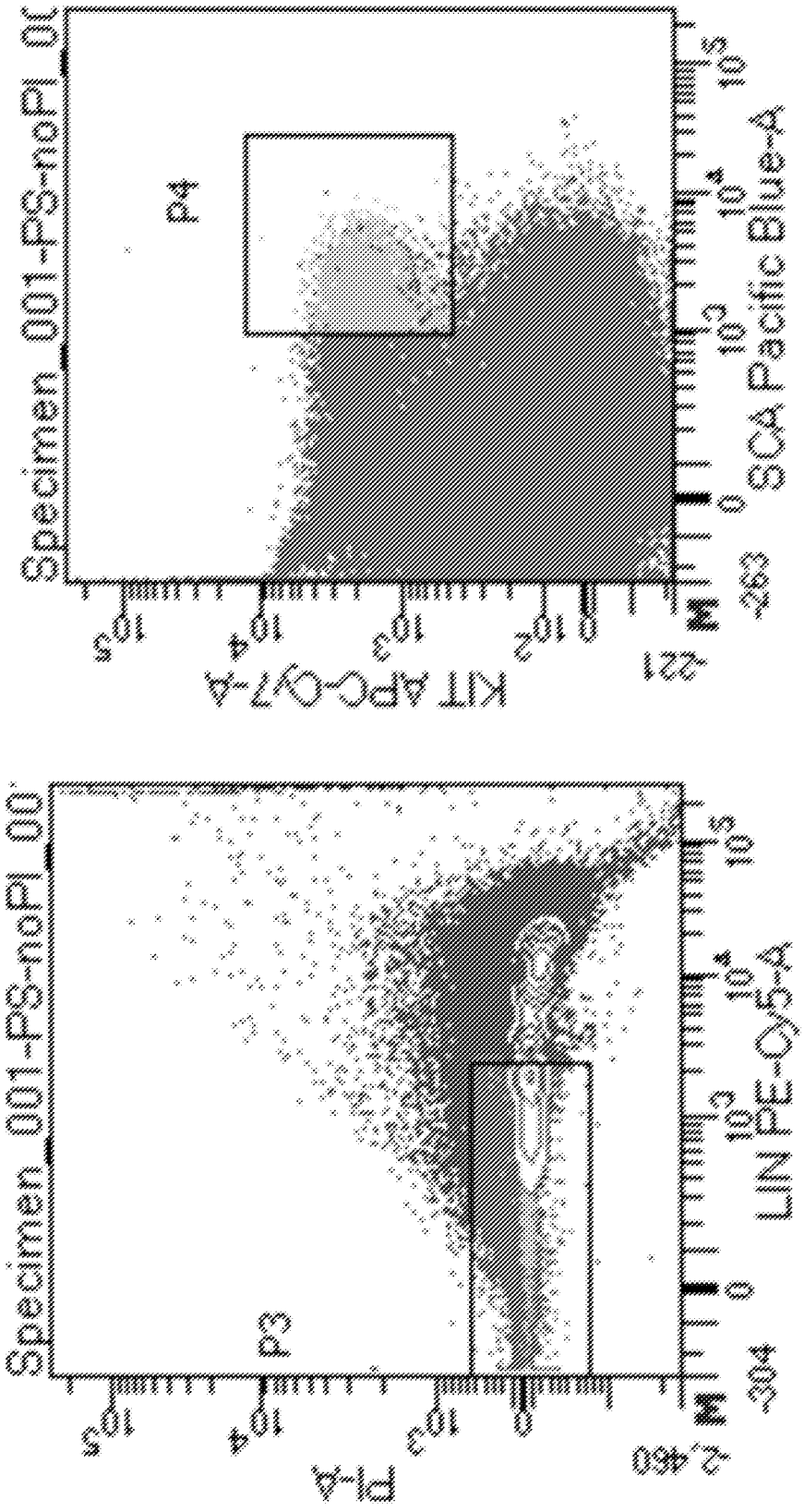

Specimen_001-PS-noPI_00
P4
SCA Pacific Blue-A
KIT APC-Cy7-A
-263
-221
Specimen_001-PS-noPI_00
P3
LIN PE-Cy5-A
PI-A
-304
-2,460

Figure 21

Figure 22

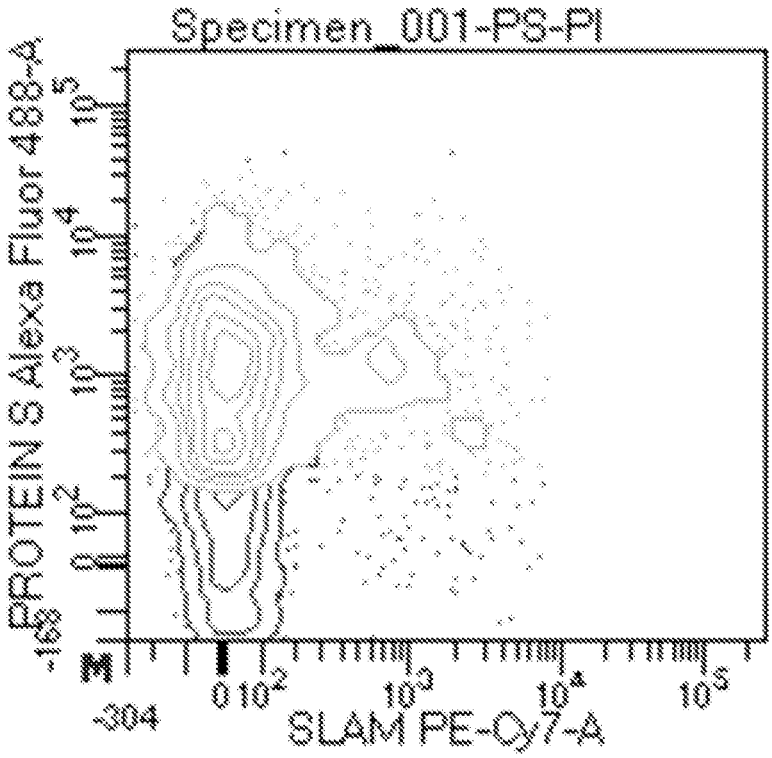


Figure 23

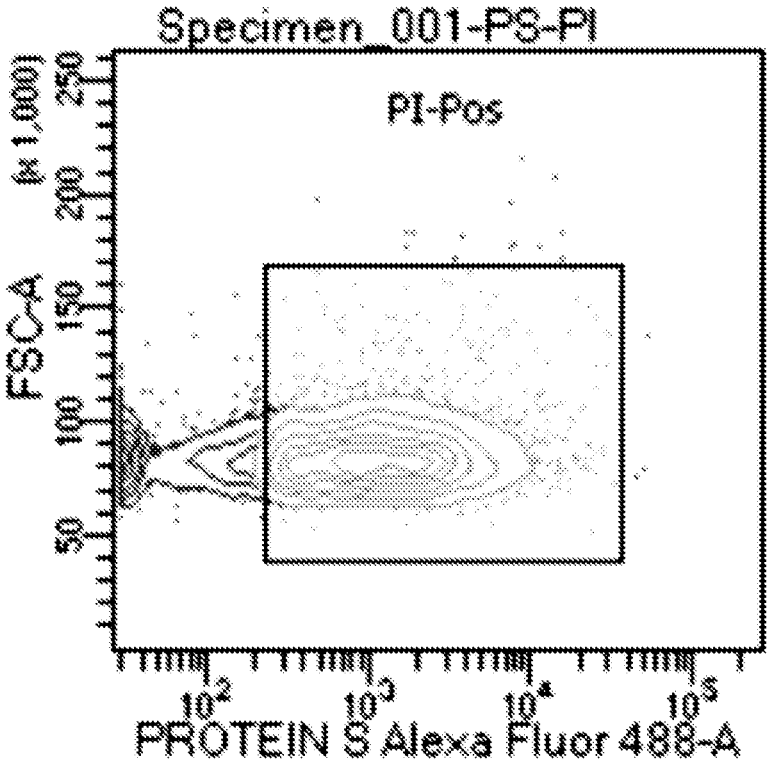


Figure 24

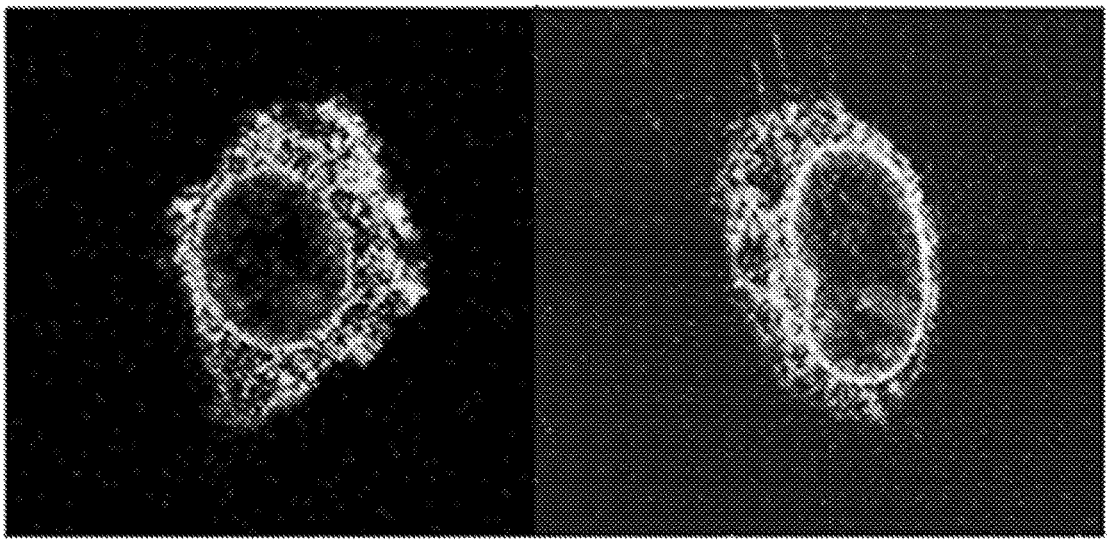
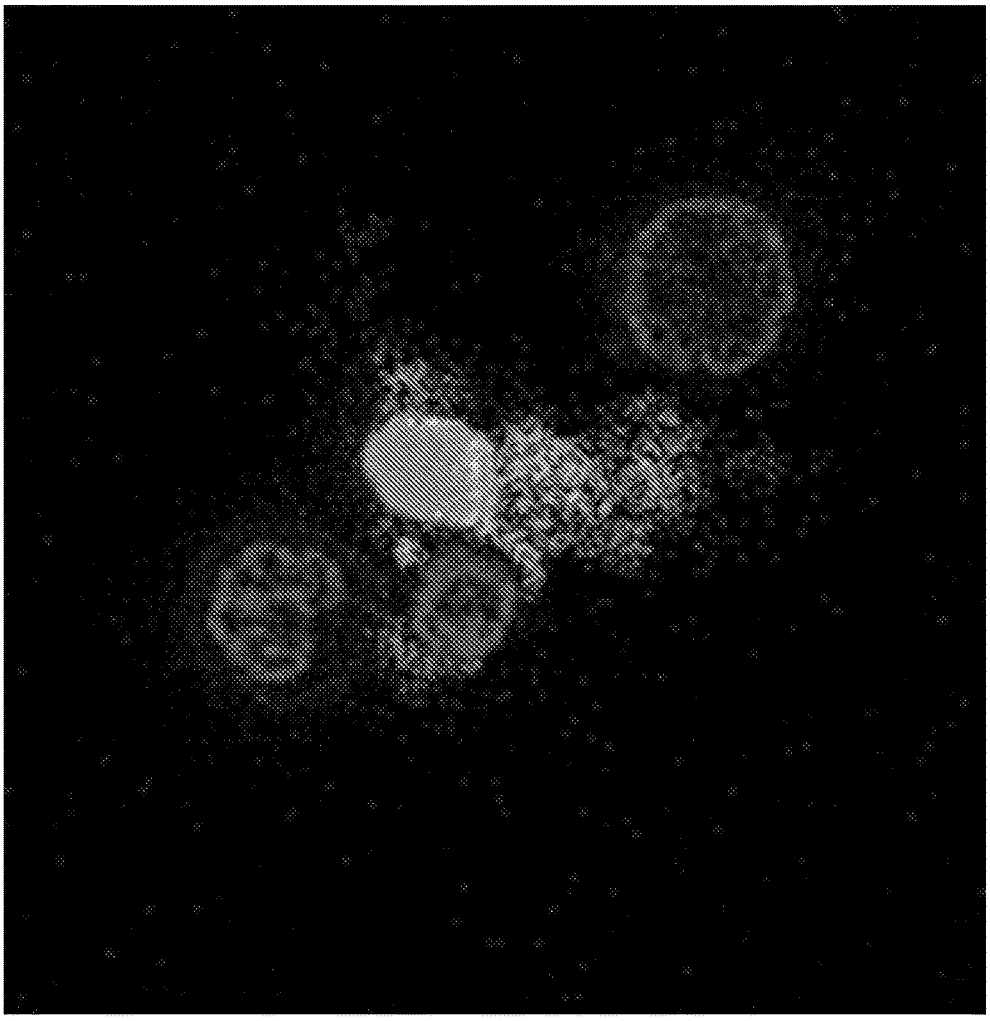


Figure 25



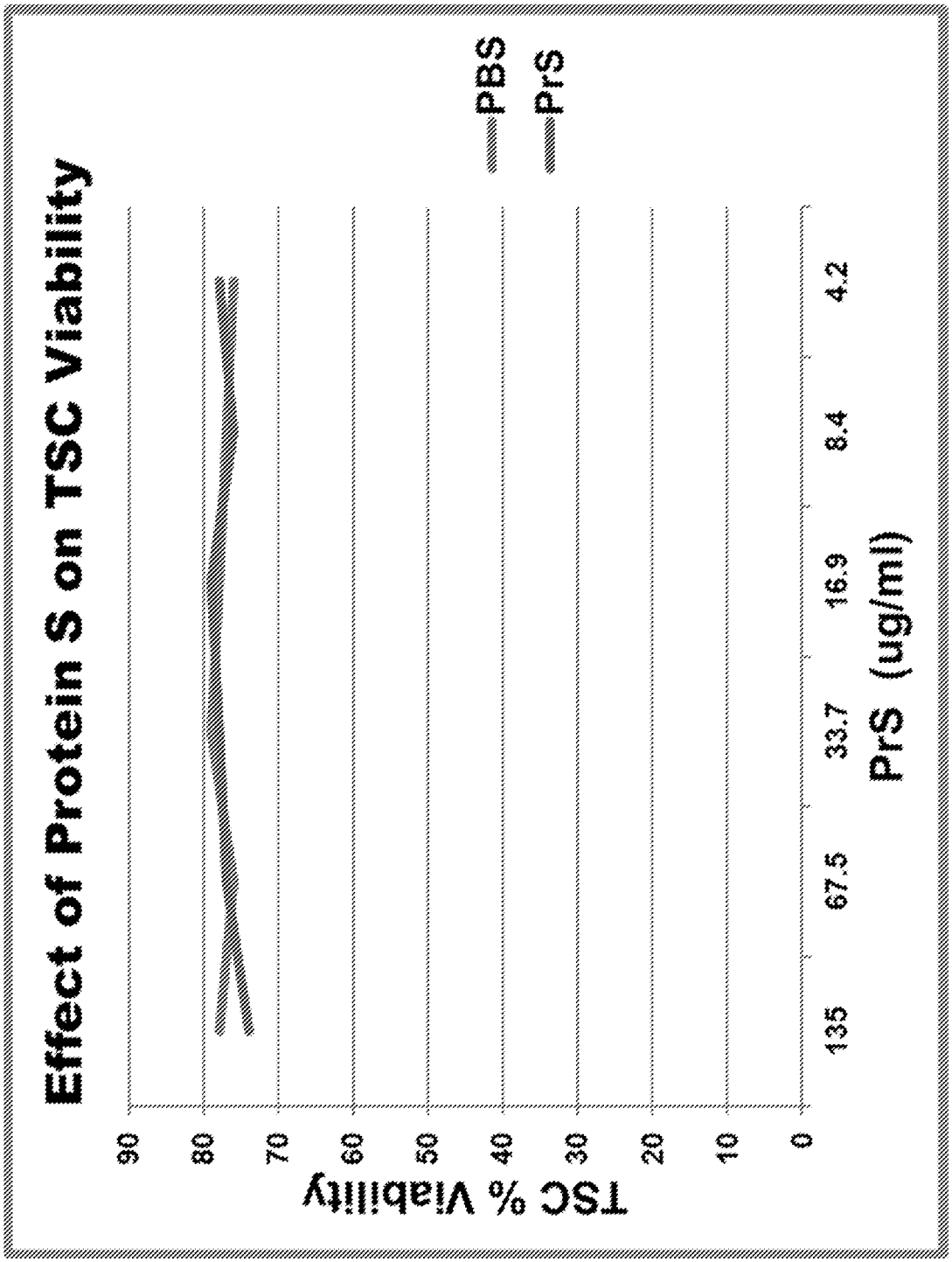

Effect of Protein S on TSC Viability
PBS
PrS
TSC % Viability
90
80
70
60
50
40
30
20
10
0
135
67.5
33.7
16.9
8.4
4.2
PrS (ug/ml)

Figure 26

DELIVERY OF PAYLOADS TO STEM CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/554,530 filed Sep. 5, 2017, U.S. Provisional Application No. 62/554,533 filed Sep. 5, 2017, U.S. Provisional Application No. 62/569,403 filed Oct. 6, 2017, U.S. Provisional Application No. 62/569,411 filed Oct. 6, 2017, U.S. Provisional Application No. 62/584,565 filed Nov. 10, 2017, and U.S. Provisional Application No. 62/593,014 filed Nov. 30, 2017, each of which applications is herein incorporated by reference in its entirety.

INCORPORATION OF SEQUENCE LISTING

[0002] This application contains a sequence listing submitted electronically via EFS-web, which serves as both the paper copy and the computer readable form (CRF) and consists of a file entitled "ST-CT1-PCT_sequence.txt", which was created on Sep. 5, 2018, which is 9,831 bytes in size, and which is herein incorporated by reference in its entirety.

[0003] The present disclosure relates to a method of targeting stems cells, in particular non-apoptotic stem cells, employing a GLA domain, for example to facilitate entry into the cell.

BACKGROUND

[0004] GLA domains are contained in a number of GLA proteins, such as Thrombin, Factor VII, Factor IX, Factor X, Protein C, Protein S (PrS), Protein Z, Osteocalcin, Matrix GLA protein, GAS6, Transthretin, Periostin, Proline rich GLA 1, Proline rich GLA 2, Proline rich GLA 3 and Proline rich GLA 4.

[0005] The GLA domains of so-called GLA proteins are able to bind phosphatidylserine (PtdS also referred to as PS) on the surface of apoptotic cells, such as cancer cells and pathogen infected cells. Molecules excluding the catalytic domain, which specifically bind phosphatidyl serine are disclosed in WO2014/151535 and WO2014/151683, incorporated herein by reference.

[0006] GLA domains (Vitamin K-dependent carboxylation/gamma-carboxyglutamic) are protein domains which have been modified by vitamin K dependent post-translational carboxylation of glutamate residues in the amino sequence to provide gamma-carboxyglutamate (Gla).

[0007] The GLA domain binds calcium ions by chelating them between two carboxylic acid residues. These residues are part of a region that starts at the N-terminal extremity of the mature form of GLA proteins, and that ends with a conserved aromatic residue. This results in a conserved Gla-x(3)-Gla-x-Cys motif that is found in the middle of the domain, which seems to be important for substrate recognition by the carboxylase.

[0008] Phosphatidyl serine was thought to be a conserved marker for apoptotic cells and part of the mechanism by which diseased cells reduce immune clearance or induced immune tolerance. Thus, it was hypothesized that the GLA domains only bound to apoptotic cells. However, surprisingly the present inventors have established that the GLA domains of the present disclosure can be employed to target stem cells, such as non-apoptotic stem cells and/or cancer stem cells. This is even more surprising because the inventors have evidence to suggest that normally healthy differentiated cells are not bound by the GLA domains employed in the present disclosure.

[0009] This has important implications, for example for stem therapy, which is used to treat conditions such as haematological cancers, such as leukemia. The stem cell therapy can only be given once the patient's own bone marrow/stems cells/immune system has been wiped clean.

[0010] This wiping clean process requires "obliteration therapies", for example high doses of chemo, radiation therapy, and/or B cell depletion therapy.

[0011] This "obliteration therapy" has many side effects, for example mouth and throat pain (which may make it difficult for the patient to eat), nausea and vomiting, susceptibility to infection, such as pneumonia and CMV infection, anemia, bleeding, infertility, cognitive dysfunction, etc. These side effects are very severe, and are difficult for patients, especially children to cope with. It would greatly improve patient quality of life if these side effects could be minimized or eliminated.

[0012] Wiping out the immune system and rebooting it has also been found to put aggressive forms of MS into the remission. However, the treatment is reserved for only the severest of cases because the risk associated with the treatment are significant. However, the present disclosure allows the chemotherapy to be specifically targette to the stems by employing the GLA-component.

[0013] The present invention provides a mechanism for "specifically" targeting stem cells, in particular non-apoptotic stem cells. Stem cells targeted by the method can, for example be isolated, treated (including genetic correction, augmentation, addition), labelled, transformed and/or eliminated. Thus, the method of the present disclosure can be employed to deliver therapeutics interventions to stem cells, for example genetic material and/or proteineous material and/or chemical therapies.

[0014] By linking the GLA domain of the present disclosure to a detectable label, such as fluorescent label, his-tag or a magnetic bead, then stems cells can isolated and sorted etc. This may be useful in a diagnostic or isolating stems cells for further manipulation to render them useful in therapeutic application.

SUMMARY OF THE DISCLOSURE

[0015] The present disclosure will now be summarised in the "numbered" paragraphs below:

[0016] 1a. A method of targeting a stem cell said method comprising the step of contacting cells with a molecule comprising a payload linked to a gamma-carboxyglutamic acid component (GLA-component) wherein said GLA-component comprises a GLA domain or an active fragment thereof, and does not comprise an active catalytic domain from a GLA protein.

[0017] 1b. A molecule comprising a payload linked to a gamma-carboxyglutamic acid component (GLA-component), wherein said GLA-component comprises a GLA domain or an active fragment thereof, and does not comprise an active catalytic domain from a GLA protein for use in treatment or diagnosis of a stem cell

[0018] 1c. A molecule comprising a payload linked to a gamma-carboxyglutamic acid component (GLA-component), wherein said GLA-component comprises a GLA domain or an active fragment thereof, and does not

comprise an active catalytic domain from a GLA protein for use in the manufacture of a medicament for treatment or diagnosis of a stem cell.

[0019] 2. A method or molecule for use according to paragraph 1a, 1b or 1c, wherein GLA domain or active fragment thereof is independently selected from thrombin, factor VII, factor IX, factor X, protein C, protein S, protein Z, osteocalcin, matrix GLA protein (MGP), GAS6, transthyretin (TTR), inter-alpha-trypsin-inhibitor, periostin, proline rich gla 1 (PRRG1), proline rich gla 2 (PRRG2), proline rich gla 3(PRRG3), and proline rich gla 4 (PRRG4).

[0020] 3. A method or molecule for use according to paragraph 2, wherein the GLA domain or active fragment thereof is independently selected from thrombin, factor VII, factor IX, factor X, protein C, protein S, protein Z and GAS6, for example the GLA domain from protein S, in particular a sequence shown in SEQ ID NO: 1.

[0021] 4. A method or molecule for use according to paragraph 1a, 1b or 1c to 3, wherein the GLA-component further comprises an EGF domain, for example a calcium binding EGF domain.

[0022] 5. A method or molecule for use according to paragraph 1a, 1b or 1c to 4, wherein the construct comprises an EGF domain selected from thrombin, factor VII, factor IX, factor X, protein C, protein S, protein Z, Osteocalcin, Matrix GLA protein, GAS6, Transthretin, Periostin, Proline rich GLA 1, Proline rich GLA 2, Proline rich GLA 3 and Proline rich GLA 4.

[0023] 6. A method or molecule for use according to paragraph 5 wherein the EGF domain selected from thrombin, factor VII, factor IX, factor X, protein C, protein S, protein Z and GAS6, for example the EGF domain from protein S

[0024] 7. A method or molecule for use according to any one of paragraphs 1a, 1b or 1c to 6, wherein the GLA-component comprises a sequence shown in SEQ ID NO: 6 or a derivative thereof excluding the his-tag.

[0025] 8. A method or molecule for use according to paragraph 1a, 1b or 1c to 7, wherein the GLA-domain component further comprises a Kringle domain.

[0026] 9. A method or molecule for use according to paragraph 8, wherein the Kringle domain is from a protein selected from the group comprising Activating transcription factor 2 (ATF); Factor XII (F12); thrombin (F2); Hyaluronan-binding protein 2 (HABP2); Hepatocyte growth factor (HGF); Hepatocyte growth factor activator (HGFAC); Kremen protein 1 (KREMEN1); KREMEN2; Lipoprotein(a) (LPA); LPAL2; Macrophage-stimulating protein (MSP or MST1); Phosphoinositide-3-kinase-interacting protein 1 (PIK3IP1); Tissue plasminogen activator (PLAT); Urokinase (PLAU); Plasmin (PLG); PRSS12; Tyrosine-protein kinase transmembrane receptor ROR1 (ROR1); and Tyrosine-protein kinase transmembrane receptor ROR2 (ROR2).

[0027] 10. A method according to any one of paragraphs 1a, 1b, 1c to 9, wherein the method is performed in vitro.

[0028] 11. A method according to any one of paragraphs 1a, 1b, 1c to 10, wherein the delivery is to a cell in vivo, for example wherein the molecule comprising the GLA component and the payload are administered to a patient, for example a human patient.

[0029] 12. A method or molecule for use according to paragraph 1a, 1b or 1c to 11, wherein the molecule targets the exterior of a stem cell.

[0030] 13. A method or molecule for use according to paragraph 1a, 1b or 1c to 12, wherein the molecule is internalized in a stem cell.

[0031] 14. A method or molecule for use according to paragraph 1a, 1b or 1c to 13, wherein the cell is non-apoptotic (i.e. a healthy stem cell).

[0032] 15. A method or molecule for use according to paragraph 1a, 1b or 1c to 13, wherein the cell is apoptotic, for example a diseased stem cell.

[0033] 16. A method or molecule for use according to paragraph 1a, 1b or 1c to 15, wherein the stem cell is an adult stems cell, for example including progenitor cells, and haemotopoietic stem cells, myogenic stem cells, osteoprogenitor stem cells, neural stem cells, mesenchymal stem cell, such as satellite cells, radial glial cells, bone marrow stromal cells, periosteum, pancreatic progenitor cells, endothelial progenitor cells, blast cells and trophoblast stem cells.

[0034] 17. A method or molecule for use according to paragraph 1a, 1b or 1c to 16, wherein the stem cell expresses a surface marker CD34.

[0035] 18. A method or molecule for use according to paragraph 1a, 1b or 1c to 17, wherein the stem cell is negative for lineage positive surface markers (i.e. is Lin −ve).

[0036] 19. A method or molecule for use according to paragraph 1a, 1b or 1c to 18, wherein the stem cell is Lin −ve, CD34 +ve, CD38 −ve, CD45RA −ve, CD90 positive and CD49f +ve.

[0037] 20. A method or molecule for use according to paragraph 1a, 1b or 1c to 17, wherein the stem cell is a haemotopoietic stem cell.

[0038] 21. A method or molecule for use according to paragraph 20, wherein the stem cell expresses a surface marker from CD48, CD150, CD244, CD34, CD38, SCA-1, Thy1.1, C-kit, lin, CD135, slam1/CD150, Mac-1 (CD11b), CD4, stem cell factor (SCF) and combinations of two or more of the same.

[0039] 22. A method or molecule for use according to paragraph 1a, 1b or 1c to 19, wherein the stem cell is an osteoprogenitor cell.

[0040] 23. A method or molecule for use according to paragraph 22, wherein the stem cell expresses a surface marker selected from Gremlin-1, TGF-beta, bFGF, BMP-2, ALPP, MCAM, Collagen I, Collagen 1 alpha 1, Collagen II, RUNX2, Decorin, and combinations of two or more of the same (such as all said markers).

[0041] 24. A method or molecule for use according to paragraphs 22 or 23, wherein the stem cells are osteoblasts or a progenitor thereof.

[0042] 25. A method or molecule for use according to paragraph 24, wherein the stem cell expresses a surface marker selected from Runx2, alkaline phosphatase/ALPP/ALPI, osteocalcin, BAP1, OPN, BAP31, Collagen I, SCUBE3, Fibronectin, SPARC, IGFBP-3, and combinations of two or more of the same (such as all said markers).

[0043] 26. A method or molecule for use according to paragraph 1a, 1b or 1c to 16, wherein the stem cell is an osteocyte or progenitor thereof.

[0044] 27. A method or molecule for use according to paragraph 26, wherein the stem cell expresses a surface marker selected from TGF beta, RANKL, MCSF, Sclerostin, DKK, and combinations of two or more of the same (such as all said markers).

[0045] 28. A method or molecule for use according to paragraph 26, wherein the stem cell expresses a surface marker selected from Osterix +ve, CD90 +ve, osteocalcin +ve, collagen I +ve, bone sialoprotein +ve and combinations of two or more of the same (such as all said markers).

[0046] 29. A method or molecule for use according to paragraph 26, wherein the stem cell expresses a surface marker selected from alkaline phosphatase/ALPP(alkaline phosphatase placental)/ALPI +ve, collagen I +ve, collagen II +ve, decorin +ve, MCAM/CD146 +ve, MEPE/OF45 +ve, osterix +ve, CD90 +ve, osterix/Sp7 +ve, RUNX2/CBFA1 +ve, thrombopoietin/Tpo +ve, and combinations of two or more of the same (such as all said markers).

[0047] 30. A method or molecule for use according to any one of paragraphs 1a, 1b or 1c to 19, wherein the stem cell is a myogenic stem cell.

[0048] 31. A method or molecule for use according to paragraph 30, wherein the stem cell expresses a marker selected from CD56, CD146, VE-cadherin, alpha-smooth muscle actin, FABP3, integrin alpha 7, desmin, myosin heavy chain, UEA-1 receptor, and combinations of two or more of the same (such as all said markers).

[0049] 32. A method or molecule for use according to paragraph 1a, 1b or 1c to 19, wherein the stem cell is a neural stem cell.

[0050] 33. A method or molecule for use according to paragraph 32, wherein the stem cell expresses a marker selected from CD133, CD15, CD24 low or −ve, GCTM-2, CD45, CD34, Nestin, Sox-2, ABCG2, FGF R4, Frizzled-9, and combinations of two or more of the same (such as all said markers).

[0051] 34. A method or molecule for use according to paragraph 33, wherein the CD24 marker is low or −ve.

[0052] 35. A method or molecule for use according to paragraphs 33 or 34, wherein the wherein the stem cell expresses a marker combination of CD133 +ve, 5E12 +ve, CD34 −ve, CD45 −ve, and CD24 low or −ve.

[0053] 36. A method or molecule for use according to paragraph 1a, 1b or 1c to 19, wherein the stem cell is a mesenchymal stem cell.

[0054] 37. A method or molecule for use according to paragraph 36, wherein the stem cell expresses a surface marker, selected from CD10, CD13, CD73, CD105, CD271, CD140b, CD240, frizzled-9, CD29, CD90, CD146, oct4, SSEA4, STRO-1, stem cell factor (SCF) and combinations of two or more of the same.

[0055] 38. A method or molecule for use according to paragraph 1a, 1b or 1c to 19, wherein the stem cell is adipose-derived.

[0056] 39. A method or molecule for use according to paragraph 38, wherein the stem cell expresses a surface marker selected from K15, CD34, Nestlin, follistatin, p63, integrin alpha 6, teacin C, EGFR, IGFR, frizzled factors, and combinations of two or more of the same.

[0057] 40. A method or molecule for use according to paragraphs 38 or 39, wherein the stem cell expresses a surface marker selected from CD44, ICAM/CD54, CD34, integrin family members and combinations of two or more of the same.

[0058] 41. A method or molecule for use according to paragraph 1a, 1b or 1c to 19, wherein stem cell is an ovary and tubal epithelial stem cell.

[0059] 42. A method according to paragraph 41, wherein the stem cell expresses a surface marker selected from Gremlin 1, Lrig1, Lgr5, Bmi1, Tert, HopX and combinations of two or more of the same.

[0060] 43. A method or molecule for use according to paragraph 1a, 1b or 1c to 15, wherein the stem cell is an embryonic stem cell.

[0061] 44. A method or molecule for use according to paragraph 43, wherein the stem cell can be identified on the basis of one or more a surface marker selected from CD24, CD29, CD31, CD59, CD90, CD117, CD133, CD324, CD326, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, frizzled5, stem cell factor, crypto (TDGF-1)

[0062] 45. A method or molecule for use according to paragraph 1a, 1b or 1c to 44, wherein the stem cell is non-cancerous.

[0063] 46. A method or molecule for use according to paragraph 1a, 1b or 1c to 44, wherein the stem cell is a cancer stem cell.

[0064] 47. A method or molecule for use according to paragraph 46, wherein the cancer stem cell is of epithelial origin.

[0065] 48. A method or molecule for use according to paragraph 46 or 47, wherein cancer stem cell expresses a surface marker selected from CD44 (which is over expressed in at least breast, ovary, prostate, pancreatic, squamous, and bladder cancer), CD133 (which is over expressed in at least brain, colon, lung, prostate cancer and medulloblastoma), CD24, CD90, CD271, CD4f, CD13 and combinations of two or more of the same (other markers include $ABCB5^{+}$, CD44+/CD24, $CD34^{+}/CD38^{-}$, and $CD44^{+}/ESA^{+}$).

[0066] 49. A method or molecule for use according to paragraph 46, wherein the stem cell is of haemotopoietic origin.

[0067] 50. A method or molecule for use according to paragraph 49, wherein the cell has a marker selected from CD19, WT-1 and combinations thereof

[0068] 51. A method or molecule for use according to any one of paragraphs 1 to 50 wherein the GLA-component is conjugated to the payload.

[0069] 52. A method or molecule for use according to paragraph 1a, 1b or 1c to 51, wherein the payload comprises a therapeutic agent, a targeting agent and/or a label.

[0070] 53. A method or molecule for use according to paragraph 52, wherein the therapeutic agent is a chemical entity, or biological molecule (such as a protein), for example, an anti-cancer drug, an anti-cancer therapy, a chemotherapeutic agent, virus or viral vector, such as an oncolytic virus.

[0071] 54. A method or molecule for use according to paragraph 1a, 1b or 1c to 53, wherein the payload is selected from a toxin, a polymer(for example synthetic or naturally occurring polymers), biologically active proteins (for example enzymes, other antibody or antibody fragments), a drug (for example, small molecule (chemical entity) or chemotherapeutic agent), nucleic acids and fragments thereof (for example DNA, RNA such as

shRNA and siRNA and fragments thereof, including gRNA for CRISPRCas9 and CRISPRa/i, radionuclides (particularly radioiodide, radioisotopes) a metal chelating agent, nanoparticles and reporter groups (such as fluorescent or luminescent labels or compounds which may be detected by NMR or ESR spectroscopy).

[0072] 55. A method or molecule for use according to paragraph 54, wherein the toxin is selected from an auristatin (for example MMAE (monomethyl auristatin E), MMAF (monomethyl auristatin F)), pyrrolobenzodiazepine (PBD), doxorubicin, duocarmycin, a maytansinoid (for example N 2'-deacetyl-N 2'-(3-mercapto-1-oxopropyl)-maytansine (DM1), N 2'-deacetyl-N2'-(4-mercapto-1-oxopentyl)-maytansine (DM3) and N 2'-deacetyl-N 2'(4-methyl-4-mercapto-1-oxopentyl)-maytansine (DM4)), calocheamicin, dolastatin, maytansine, α-amanitin, *Pseudomonas* exotoxin (PE38), ricin A chain, diphtheria toxin, Pokeweed antiviral protein (PAP), saporin, gelonin and a tubulysin.

[0073] 56. A method or molecule for use according paragraph 53, wherein the chemotherapeutic is selected from temozolomide, epothilones, melphalan, carmustine, busulfan, lomustine, cyclophosphamide, dacarbazine, polifeprosan, ifosfamide, chlorambucil, mechlorethamine, busulfan, cyclophosphamide, carboplatin, cisplatin, thiotepa, capecitabine, streptozocin, bicalutamide, flutamide, nilutamide, leuprolide acetate, doxorubicin hydrochloride, bleomycin sulfate, daunorubicin hydrochloride, dactinomycin, liposomal daunorubicin citrate, liposomal doxorubicin hydrochloride, epirubicin hydrochloride, idarubicin hydrochloride, mitomycin, doxorubicin, valrubicin, anastrozole, toremifene citrate, cytarabine, fluorouracil, fludarabine, floxuridine, interferon α-2b, plicamycin, mercaptopurine, methotrexate, interferon α-2a, medroxyprogersterone acetate, estramustine phosphate sodium, estradiol, leuprolide acetate, megestrol acetate, octreotide acetate, deithylstilbestrol diphosphate, testolactone, goserelin acetate, etoposide phosphate, vincristine sulfate, etoposide, vinblastine, etoposide, vincristine sulfate, teniposide, trastuzumab, gemtuzumab ozogamicin, rituximab, exemestane, irinotecan hydrocholride, asparaginase, gemcitabine hydrochloride, altretamine, topotecan hydrochloride, hydroxyurea, cladribine, mitotane, procarbazine hydrochloride, vinorelbine tartrate, pentrostatin sodium, mitoxantrone, pegaspargase, denileukin diftitix, altretinoin, porfimer, bexarotene, paclitaxel, docetaxel, arsenic trioxide, tretinoin and combinations of two or more of the same.

[0074] 57. A method or molecule for use according to any one of paragraphs 52 to 54, wherein the chemotherapeutic is selected from an alkylating agent, an antimetabolite including thymidylate synthase inhibitors, a taxane, an anthracycline, an anti-microtubule agent including plant alkaloids, and combinations of two or more of the same.

[0075] 58. A method or molecule for use according to paragraph 57, wherein the chemotherapeutic is selected from paclitaxel, docetaxel, abraxane, carbazitaxel, derivatives of any one of the same, and combinations of two or more of any of the aforementioned.

[0076] 59. A method according to paragraph 57 or 58, wherein the alkylating agent is selected from a nitrogen mustard, a nitrosourea (such as carmustine), a tetrazine, a aziridine, a platin and derivatives thereof, a non-classical alkylating agent and a combination of two or more of the same.

[0077] 60. A method or molecule for use according to paragraph 59, where the platin is selected from cisplatin, carboplatin, oxaliplatin, satraplatin, picoplatin, nedaplatin, triplatin, lipoplatin and a combination of two or more of the same. 61. A method or molecule for use according to any one of paragraphs 57 or 58, wherein the alkylating agent is an antimetabolite selected from anti-folates (for example methotrexate and pemetrexed), purine analogues (for example thiopurines, such as azathiopurine, mercaptopurine, thiopurine, fludarabine (including the phosphate form), pentostatin and cladribine), pyrimidine analogues (for example fluoropyrimidines, such as 5-fluorouracil and prodrugs thereof such as capecitabine [Xeloda®]), floxuridine, gemcitabine, cytarabine, decitabine, raltitrexed(tomudex) hydrochloride, cladribine and 6-azauracil and combination of two or more thereof.

[0078] 62. A method or molecule for use according to any one of paragraph 56 to 60, wherein the anthracycline is selected from daunorubicin (Daunomycin), daunorubicin (liposomal), doxorubicin (Adriamycin), doxorubicin (liposomal), epirubicin, idarubicin, mitoxantrone and a combination of two or more thereof, in particular doxorubicin.

[0079] 63. A method or molecule for use according to any one of paragraphs 53 to 62, wherein the drug is an anti-cancer drug, for example selected from a topoisomerase inhibitor, a PARP inhibitor and a combination of or more of the same.

[0080] 64. A method or molecule for use according to any one of paragraph 53 to 64, wherein the anti-cancer therapy is a radionuclide, for example selected from Y-90, P-32, I-131, In-111, Sr-89, Re-186, Sm-153, Sn-117m and a combination of two or more of the same.

[0081] 65. A method or molecule for use according to any one of paragraphs 1a, **1***b* or 1c to 64 which comprises administering the molecule comprising the GLA component and payload to a cancer patient, for example where the cancer is refractory.

[0082] 66. A method or molecule for use according to paragraph 65, wherein the cancer is an epithelial cancer, for example colorectal cancer, testicular cancer, liver cancer, biliary tract cancer, glioblastomer, melanoma, prostate cancer, pancreatic cancer, breast cancer, ovarian cancer, cervical cancer, uterine cancer, gastric cancer, oesophageal cancer, thyroid cancer, renal cancer, bladder cancer, brain cancer, head and neck cancer or lung cancer or alternatively the cancer may be a haematological cancer, for example leukaemia, lymphoma, myeloma and chronic myeloproliferative diseases, such as AML, CML, ALL and CLL.

[0083] 67. A method or molecule for use according to any one of paragraphs 1 to 66, wherein the payload is converted to an active form inside the cell.

[0084] 68. A method or molecule for use according to paragraph 67, wherein conversion to the active form is performed by an enzyme.

[0085] 69. A method or molecule for use according to paragraph 68, wherein the enzyme is selected from a carboxylesterase, acetylecholinesterase, paraoxonase (such as paraoxonase 2), matrix metalloproteases, alkaline phosphatase, β-glucuronidase, purine-nucleoside

phosphorylase, beta-lactamase (for example produced by *Mycobacterium tuberculosis* and *kansasii*) and cytosine deminase.

[0086] 70. A method or molecule according to any one of claims **1** to **69**, wherein the GLA-component has the sequence shown in SEQ ID NO: 6 or an equivalent sequence excluding the His-tag.

[0087] In one embodiment the GLA-component binds surface exposed phosphatidylserine on the cells, before internalisation.

[0088] Whilst not wishing to be bound by theory the present inventors believe that not all phosphatidylserine is equivalent from a biological perspective. The inventors believe that the phosphatidylserine exposes by the enzyme TMEM16F is involved in immune suppression and is the one "seen" by the molecules of the present disclosure.

[0089] In one embodiment the stem cell is an adult stem cells or vesicle derived therefrom, for example a somatic stem cells, such as a hematopoetic stem cell, a mesenchymal stem cell, or a stromal stem cell.

[0090] In one embodiment the stem cell is an embryonic stem cell or a vesicle derived therefrom. In one embodiment the cell is not an embryonic stem cell.

[0091] In one embodiment the method relates to mammalian stem cells, for example human stem cells. The stem cell discussed herein are primarily human stem cells. However, the skilled person is able to identify the relevant or corresponding stem cell population for other mammals, as required. For example SSEA-1 is a marker for murine embryonic stem cells, human germline cells and embryonal carcinoma cells; SSEA-3 is a marker for primate embryonic stem cells, human embryonic germline cells, human embryonic stem cells and embryonal carcinoma cells; SSEA-4 is a marker for primate embryonic stem cells, human embryonic germ cells, human stem cells, embryonal carcinoma cells; CD324 is a marker for human & murine embryonic stem cells, embryonal cancer cells; CD90 is a marker for human & murine embryonic stems cells, hematopoietic stem cells, embryonal carcinoma cells; CD117 is a marker for human & murine embryonic stem cells, hematopoietic stem progenitor cells, neural crest-derived melanocytes, primordial germ cells, embryonal carcinoma cells; CD326 is a marker for human & murine embryonic stem cells, embryonal carcinoma cells; CD9 is a marker for human & murine embryonic stems; CD24 is a marker for human & murine embryonic stems; CD29 is a marker for human & murine embryonic stems; CD59 is a marker for human & murine embryonic stems; CD133 is a marker for human & murine embryonic stems, embryonal carcinoma cells, hematopoietic stem cells; CD31 is a marker for human & murine embryonic stems; TRA-1-60 is a marker for human embryonic stem cells, teracarcinoma, embryonic germ cells, embryonal carcinoma cells; TRA-1-81 is a marker for human embryonic stem cells, teracarcinoma, embryonic germ cells, embryonal carcinoma cells; Frizzled5 is a marker for human & murine embryonic stem cells; Stem cell factor (SCF) is a marker for human & embryonic stem cells, hematopoietic stem cells, mesenchymal stem cells, embryonal carcinoma cells; and Cripto is a marker for human & murine embryonic stem cells, cardiomyocytes and embryonal carcinoma cells.

[0092] In one embodiment the payload comprises a therapeutic agent.

[0093] In one embodiment the payload comprises a detectable label.

[0094] In one embodiment the payload comprises a DNA or RNA sequence, for example cDNA comprising a transgene or an RNAi sequence (such as miRNA, siRNA including shRNA). The DNA encoding a transgene may be delivered a transcriptionally active DNA or a plasmid for transient or stable expression.

[0095] In one embodiment the payload is suitable for inducing differentiation of the stem cell, for example to activate and/or mature the cell into a specific lineage.

[0096] In one embodiment the method of the present disclosure comprises a pre-treatment of a patient, for example to induce or augment expression of PS on stem cells, for example the pre-treatment step may be treatment with radiation therapy, in particular irradiation of bone marrow cells.

[0097] The disclosure also extends to pharmaceutical compositions comprising a molecule for use according to the present disclosure, in particular for use as described herein. Thus, in one embodiment the molecules according to the present disclosure are employed in the treatment of an intra-cellular target.

[0098] The present disclosure also extends to the use of a GLA-component comprises a GLA domain or an active fragment thereof, wherein said GLA-component does not comprise an active catalytic domain from a GLA protein, for intracellular targeting and delivery (including intracellular delivery of the payload).

[0099] The present disclosure also extends to the use of a GLA-component comprises a GLA domain or an active fragment thereof, wherein said GLA-component does not comprise an active catalytic domain from a GLA protein, for the manufacture of a medicament for intracellular targeting and delivery (including intracellular delivery of the payload, in particular where the payload comprises a therapeutic entity/molecule).

[0100] The present technology may be used to wipe out the immune cells of patient before stem cells transplantation, for example the payload will generally be a chemotherapy, for example comprising carmustine.

[0101] A current regime for immune cell ablation is (BCNU) 300 mg/m^2 on day −6, etoposide 200 mg/m^2 and cytarabine 200 mg/m^2 daily from day −5 to −2, and melphalan 140 mg/m^2 on day −1 (BEAM). Rabbit antithymocyte globulin (2.5 mg/kg/d) was administered on days −2 and −1. This regime can be adapted by conjugating each of the agents to a GLA molecule of the present disclosure.

[0102] In one embodiment the immune obliteration is for cancer, for a hematological cancer (for example is selected from myeloma, lymphoma, leukaemia, such as acute myeloid leukaemia (AML), chronic myeloproliferative disease, monoclonal gammopathy of uncertain significance, myelodysplastic syndrome and amyloidosis, such as AML, CML, CLL or ALL)

[0103] In one embodiment the myeloma is selected from multiple myeloma, amyloidosis and plasmacytoma.

[0104] In one embodiment the myeloma is selected from monoclonal gammopathy of undetermined significance, asymoptomatic myeloman, symptomatic myeloma and Kahler's disease.

[0105] In one embodiment the lymphoma is selected from anaplastic large cell lymphoma, Burkitt lymphoma, Burkitt-like lymphoma, cutaneous T-cell lymphoma, diffuse large B-cell lymphoma, diffuse large B-cell lymphoma, lymphoblastic lymphoma, MALT lymphoma, mantle cell lym-

phoma, mediastinal large B-cell lymphoma, nodal marginal zone B-cell lymphoma, small lymphocytic lymphoma, thyroid lymphoma, and Waldenstrom's macroglobulinaemia.

[0106] In one embodiment the chronic myeloproliferative disease is selected from essential thrombocythaemia, chronic idiopathic myelofibrosis, and polycythaemia *rubra* vera.

[0107] In one embodiment the leukaemia is selected from acute myeloid leukaemia (AML), hairy cell leukaemia, acute lymphoblastic leukaemia, and chronic lymphoblastic leukaemia, such as AML.

[0108] It has recently become apparent that for severe autoimmune diseases, such as severe multiple sclerosis and severe arthritis obliteration of immune cells followed by stem transplant can put the disease into remission. Thus, the ablation therapy of the present disclosure may be employed in an autoimmune disease, such as multiple sclerosis and arthritis.

[0109] In one embodiment the GLA molecule of the present disclosure is linked, for example conjugated, to a payload which comprises a detectable label. Examples of detectable labels are given below. The detectable label can be employed to sort or isolate the stem cells, for example employing FACs sorting, magnetic sorting or similar. Thus, in one embodiment there is provided a method isolating or enriching stem cells employing a GLA-molecule of the present disclosure. This is advantages because historically the isolation of the certain stem cell populations, such as cancer stem cells has been very difficult.

[0110] The labelled GLA molecule may also be employed in vivo as an imaging agent, in particular as a diagnostic tool, for example to identify cancer stem cells in primary tumors or metastasise. This may be important for monitoring patients after surgery and/or chemotherapy to ensure the cancer is in remission.

[0111] The DNA transgene payloads and/or RNA payloads linker to the GLA molecule can be employed as an alternative intracellular delivery to a viral vector delivery (transduction) or traditional transfection. This can be employed to in vitro to express exogenous or endogenous proteins in the cell (for example where the modified stem cells are for reinfusion into a patient) or can be effected in vivo. The genes can be expressed transiently or can be designed to be stably integrated into the stem cell.

[0112] Surprisingly the present inventors have shown that the molecules according to the present disclosure not only bind stem cells they are rapidly internalised therein along with the payload attached thereto.

DETAILED DISCLOSURE

[0113] Intra-cellular delivery as employed herein refers conveying, for example the payload to inside the cell.

[0114] In one embodiment the payload is not internalized.

[0115] In one embodiment the GLA-component and the payload is not internalised.

[0116] Payload as employed herein refers to a molecule which is linked to the GLA domain, in particular for the purpose of intracellular delivery. The link may be a link through chemical conjugation using, for example maleimide chemistry or click chemistry to anchor to moiety to a solvent exposed lysine. Alternatively, the link may be a fusion, for example a peptide bond where the linked entity is expressed as a fusion protein with the GLA component, for example this may be suitable for certain detectable labels, such as fluorescent proteins or antibodies. Linkers may be employed between the GLA-component and the payload. Payloads may comprising a drug, a toxin, a polymer, a biologically active protein, therapeutic virus, oncolytic virus, viral vector, radionuclides, a metal chelating agent and/or a reporter group (such as a label).

[0117] In one embodiment 1, 2, 3, 4 or 5 payloads are linked per GLA-component.

[0118] GLA-component (also referred to herein as a gamma-carboxyglutamic acid component) refers to a polypeptide comprising a GLA-domain in the absence of catalytic domain from a GLA protein, such as protein S. The polypeptide may further comprise an EGF domain and/kringle domain, for example from protein S. In one embodiment the GLA-component comprises 30 to 300 amino acid residues, for example 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290 or 300 residues. In one embodiment the GLA component is in the range of 4.5 to 30 kDa. In one embodiment the GLA-component comprises the sequence shown in SEQ ID NO: 1. In one embodiment the GLA-component comprises a sequence shown in SEQ ID NO: 6 or a derivative thereof excluding the his-tag.

[0119] GLA domains (Vitamin K-dependent carboxylation/gamma-carboxyglutamic) as employed herein are protein domains which have been modified by vitamin K dependent post-translational carboxylation of glutamate residues in the amino sequence to provide gamma-carboxyglutamate (Gla). In one embodiment the GLA domain employed in the molecules of the present disclosure comprises 30 to 45 consecutive residues from a native (wild-type) GLA domain. In one embodiment the GLA domain comprises 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 GLA residues.

[0120] In one embodiment 30% or less of the GLA-component is GLA residues.

[0121] In one embodiment the GLA-component comprises 1 to 5 disulfide bonds, for example 1, 2, 3, 4 or 5 disulfide bonds.

[0122] The GLA domain binds calcium ions by chelating them between two carboxylic acid residues. These residues are part of a region that starts at the N-terminal extremity of the mature form of Gla proteins, and that ends with a conserved aromatic residue. This results in a conserved Gla-x(3)-Gla-x-Cys motif that is found in the middle of the domain, and which seems to be important for substrate recognition by the carboxylase.

[0123] GLA domains are contained in a number of proteins, such as Thrombin, Factor VII, Factor IX, Factor X, Protein C, Protein S (PrS), Protein Z, Osteocalcin, Matrix GLA protein, GAS6, Transthretin, Periostin, Proline rich GLA 1, Proline rich GLA 2, Proline rich GLA 3, and Proline rich GLA 4.

[0124] GLA domain as employed herein also extends to proteins where 1 to 10 percent (such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10%) of the amino acids in the native GLA domain may be replaced and deleted, provided that modified domain retains at least 70% (such as 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%) of the native activity of the native (unmodified GLA domain) in a suitable in vitro assay.

[0125] EGF domain as employed herein refers is a conserved protein domain. It comprises about 30 to 40 amino-acid residues and has been found in a large number of mostly animal proteins. Most occurrences of the EGF-like domain

are found in the extracellular domain of membrane-bound proteins or in proteins known to be secreted. The EGF-like domain includes 6 cysteine residues. The main structure of EGF-like domains is a two-stranded β-sheet followed by a loop to a short C-terminal, two-stranded β-sheet. These two β-sheets are usually denoted as the major (N-terminal) and minor (C-terminal) sheets. EGF-like domains frequently occur in numerous tandem copies in proteins: these repeats typically fold together to form a single, linear solenoid domain block as a functional unit. In one embodiment the domain employed is the full-length native domain.

[0126] EGF domain as employed herein also extends to proteins where 1 to 10 percent (such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10%) of the amino acids in the native EGF domain may be replaced and deleted, provided that modified domain retains at least 70% (such as 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%) of the native activity of the native (unmodified EGF domain) in a suitable in vitro assay. In one embodiment the protein is the full-length native domain.

[0127] Kringle domain as employed herein refers to autonomous protein domains that fold into large loops stabilized by 3 disulfide bonds. They are characterized by a triple loop, 3-disulfide bridge structure, whose conformation is defined by a number of hydrogen bonds and small pieces of anti-parallel beta-sheet. They are found throughout the blood clotting and fibrinolytic proteins, in a varying number of copies, in some plasma proteins including prothrombin and urokinase-type plasminogen activator, which are serine proteases belonging to MEROPS peptidase family S1A.

[0128] Kringle domain as employed herein also extends to proteins where 1 to 10 percent (such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10%) of the amino acids in the native kringle domain may be replaced and deleted, provided that modified domain retains at least 70% (such as 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%) of the native activity of the native (unmodified Kringle domain) in a suitable in vitro assay. In one embodiment the domain employed is the full-length native domain.

[0129] An active fragment of a protein as employed herein is a less than the whole native protein (or relevant domain), which retains at least 50% (such as 60, 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%) of the active of the native full-length domain or protein in a relevant in vitro assay.

[0130] Catalytic domain as employed herein is a domain (or fragment) downstream of the EGF domain in the C-terminal direction, for example as illustrated in FIG. **1**A.

[0131] In vitro as employed herein refers to laboratory work not performed in a human or animal body.

[0132] In vivo as employed herein refer to work/testing/treatment in a living organism, in particular a human or animal.

[0133] Stem cell as employed herein refers to undifferentiated cells that are capable of differentiation and includes embryonic stem cells and adult stem cells, in particular adult stem cells.

[0134] Hematopoietic stem cells (HSCs) or hemocytoblasts are the stem cells that give rise to all the other blood cells through the process of haematopoiesis. They are derived from mesoderm and located in the red bone marrow, which is contained in the core of most bones.

[0135] Cancer stem cell as employed herein refers to tumorigenic cells (i.e. cancer cells found within tumors or hematological cancers) that possess characteristics associated with normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer sample. See, for example Identification and Targeting of Cancer Stem Cells, BioessayS 2009 October; 31 (10) 1038-1049. Cancer stem cells are defined by three distinct properties: i) a selective capacity to initiate tumour and drive neoplastic proliferation: ii) an ability to create endless copies of themselves through self-renewal, and iii) the potential to give rise to more mature non-stem cell cancer progeny though differentiation. Cancer stem cells are not necessarily derived from a healthy stem cell but may originate from a differentiated cell.

[0136] CD34 is also known as hematopoietic progenitor cells antigen CD34, has a function as cell-cell adhesion factor. It can be employed as a marker to enrich stem populations.

[0137] Molecule as employed herein is used in the broadest sense and includes a synthetic chemical molecule but also macromolecules such as proteins, polymers (natural or otherwise), ribonucleic acid molecules, labels etc.

[0138] Payloads may comprising a drug, a toxin, a polymer, a biologically active protein, radionuclides, a metal chelating agent and/or a reporter group.

[0139] A drug as employed herein, unless the context indicates otherwise, is intended to refer to a small chemical entity, for example which has been synthesised by organic chemistry methods, in particular a molecule approved or licensed or in the process of being licensed for therapeutic use, especially in humans. Drug as employed herein also includes an anti-viral compound, an antibiotic, and an anti-cancer therapy.

[0140] An antiviral compound (antiviral agent) as employed herein refers to the class of medicaments used specifically for treating viral infections, including broad spectrum anti-viral agents and also "narrow" spectrum specific to a particular virus or particular family of viruses.

[0141] Antibiotic as employed herein refers to medicine or agent that inhibits the growth of bacteria or destroys bacteria. Anti-bacterial and antibiotic are used interchangeable here unless the context indicates otherwise.

[0142] Anti-parasitic as employed herein in refers to a medicine or agent that inhibits the growth of parasite, destroys parasite or removes parasites from the host.

[0143] Anti-cancer therapy is a broad term which includes anti-cancer drugs, chemotherapy, radiotherapy, immune-oncology therapies, etc.

[0144] Anti-cancer drug as employed herein generally refers to a small molecule cancer therapy.

[0145] Chemotherapy as herein generally refers to a cytotoxic agent and includes antineoplastics.

[0146] A biological therapeutic (also referred to as a biopharmaceutical, biological or biologic) is a therapeutic product "derived" from a biological source, for example a recombinant proteins and fragments, including antibodies molecules, including antibodies, antibody binding fragments and multispecific antibody molecules and complex combinations of such materials. A biologically active protein is a subgroup of a biological therapeutics and includes recombinant proteins and active fragments thereof (including antibody molecules).

[0147] Antibody molecules as employed herein include a complete antibody having full length heavy and light chains or a fragment thereof and a molecule comprising any one of the same for example a Fab, modified Fab, Fab', modified

Fab', F(ab')2, Fv, Fab-Fv, Fab-dsFv, single domain antibodies (e.g. VH or VL or VHH), scFv, bi, tri or tetra-valent antibodies, Bis-scFv, diabodies, triabodies, tetrabodies and epitope-binding fragments of any of the above (see for example Holliger and Hudson, 2005, Nature Biotech. 23(9): 1126-1136; Adair and Lawson, 2005, Drug Design Reviews—Online 2(3), 209-217). The methods for creating and manufacturing these antibody fragments are well known in the art (see for example Verma et al., 1998, Journal of Immunological Methods, 216, 165-181). Other antibody fragments for use in the present invention include the Fab and Fab' fragments described in International patent applications WO2005/003169, WO2005/003170 and WO2005/003171. Multi-valent antibodies may comprise multiple specificities e.g bispecific or may be monospecific (see for example WO 92/22853 and WO05/113605). Bispecific and multispecific antibody variants are especially considered in this example since the aim is to neutralise two independent target proteins. Variable regions from antibodies disclosed herein may be configured in such a way as to produce a single antibody variant which is capable of binding to and neutralising two target antigens.

[0148] Antibody and binding fragments thereof, in particular small antibody fragments such as domain antibodies, VHHs, single chain Fvs (scFvs), ds-scFvs and dsFv, may be delivered intracellularly using the present technology.

[0149] In one embodiment the antibody or binding fragment thereof is a checkpoint inhibitor, for example an anti-PD-1 or anti-PD-L1 inhibitor.

[0150] In one embodiment the antibody molecule is human or humanised.

[0151] A toxin is a poisonous substance, especially derived from a natural source, in particular a protein. Many toxins, such as calicheamicin are used in cancer therapy. In addition chemotherapeutic agents can be considered toxic (or toxins). Thus the definition of toxin overlaps with other definitions herein. However, neurotoxins like snake venom are toxin but not a chemotherapeutic. However, those skilled in the art are familiar with these technical definitions and are capable of understanding the meaning the context of the present disclosure.

[0152] Diagnostic as employed herein is agent used in analysis or imaging to diagnose, label or monitor or understand a disease status. A diagnostic will generally comprise a reporter molecule, such as a label or similar that can visualised, measured or monitored in some way.

[0153] Radionuclides suitable for use the present disclosure include thallium-201, technetium-99m, Iodine-123, Iodine 131, Iodine-125, Fluorine-18 and Oxygen-15.

[0154] Also, of particular interest, is using GLA-component to deliver intrabodies, for example via GLA-fusions, for example where the intrabody is fused to the N or C terminus of the GLA-component. Intrabodies are able to target intracellular antigens.

[0155] In one embodiment antibodies that interact and inhibit RAS or proteins in the RAS signaling pathway are employed in the payload. RAS genes constitute a multigene family that includes HRAS, NRAS, and KRAS. RAS proteins are small guanosine nucleotide-bound GTPases that function as a critical signaling hub within the cell. The RAS/MAPK pathway has been studied extensively in the context of oncogenesis because its somatic dysregulation is one of the primary causes of cancer. RAS is somatically mutated in approximately 20% of malignancies (Bos J L, Cancer Res. 49: 4682-4689, 1989). In this particular case, it is envisioned that, for example the GLA-component is fuses to a RAS intrabody (described in Cetin M et al., J Mol Biol. 429:562-573, 2017).

[0156] Apoptosis as employed herein is cell death pathway which occurs as normal and controlled part an organism growth. Cell death by apoptosis is less damaging to surrounding tissue than cell death mechanisms, such as necrosis.

[0157] Necrosis as employed herein is cell death from disease or injury. It releases cytokines and factors into the surrounding tissue that may damage surrounding cells. Gangrene is an example of necrotic cell death.

Chemotherapeutic Agents

[0158] Chemotherapeutic agent and chemotherapy or cytotoxic agent are employed interchangeably herein unless the context indicates otherwise.

[0159] Chemotherapy as employed herein is intended to refer to specific antineoplastic chemical agents or drugs that are "selectively" destructive to malignant cells and tissues, for example alkylating agents, antimetabolites including thymidylate synthase inhibitors, anthracyclines, anti-microtubule agents including plant alkaloids, topoisomerase inhibitors, parp inhibitors and other antitumour agents. Selectively in this context is used loosely because of course many of these agents have serious side effects.

[0160] The preferred dose may be chosen by the practitioner, based on the nature of the cancer being treated.

[0161] Examples of alkylating agents, which may be employed in the method of the present disclosure include an alkylating agent nitrogen mustards, nitrosoureas, tetrazines, aziridines, platins and derivatives, and non-classical alkylating agents.

[0162] Example a platinum containing chemotherapeutic agent (also referred to as platins), such as cisplatin, carboplatin, oxaliplatin, satraplatin, picoplatin, nedaplatin, triplatin and lipoplatin (a liposomal version of cisplatin), in particular cisplatin, carboplatin and oxaliplatin.

[0163] The dose for cisplatin ranges from about 20 to about 270 mg/m^2 depending on the exact cancer. Often the dose is in the range about 70 to about 100 mg/m^2.

[0164] Nitrogen mustards include mechlorethamine, cyclophosphamide, melphalan, chlorambucil, ifosfamide and busulfan.

[0165] Nitrosoureas include N-Nitroso-N-methylurea (MNU), carmustine (BCNU), lomustine (CCNU) and semustine (MeCCNU), fotemustine and streptozotocin. Tetrazines include dacarbazine, mitozolomide and temozolomide.

[0166] Aziridines include thiotepa, mytomycin and diaziquone (AZQ). Examples of antimetabolites, which may be employed in the method of the present disclosure, include anti-folates (for example methotrexate and pemetrexed), purine analogues (for example thiopurines, such as azathiopurine, mercaptopurine, thiopurine, fludarabine (including the phosphate form), pentostatin and cladribine), pyrimidine analogues (for example fluoropyrimidines, such as 5-fluorouracil and prodrugs thereof such as capecitabine [Xeloda®]), floxuridine, gemcitabine, cytarabine, decitabine, raltitrexed(tomudex) hydrochloride, cladribine and 6-azauracil.

[0167] Examples of anthracyclines, which may be employed in the method of the present disclosure, include

daunorubicin (Daunomycin), daunorubicin (liposomal), doxorubicin (Adriamycin), doxorubicin (liposomal), epirubicin, idarubicin, valrubicin currenity used only to treat bladder cancer and mitoxantrone an anthracycline analog, in particular doxorubicin.

[0168] Examples of anti-microtubule agents, which may be employed in the method of the present disclosure, include include vinca alkaloids and taxanes.

[0169] Vinca alkaloids include completely natural chemicals for example vincristine and vinblastine and also semi-synthetic vinca alkaloids, for example vinorelbine, vindesine, and vinflunine

[0170] Taxanes include paclitaxel, docetaxel, abraxane, carbazitaxel and derivatives of thereof. Derivatives of taxanes as employed herein includes reformulations of taxanes like taxol, for example in a micelluar formulations, derivatives also include chemical derivatives wherein synthetic chemistry is employed to modify a starting material which is a taxane.

[0171] Topoisomerase inhibitors, which may be employed in a method of the present disclosure include type I topoisomerase inhibitors, type II topoisomerase inhibitors and type II topoisomerase poisons. Type I inhibitors include topotecan, irinotecan, indotecan and indimitecan. Type II inhibitors include genistein and ICRF 193 which has the following structure:

[0172] Type II poisons include amsacrine, etoposide, etoposide phosphate, teniposide and doxorubicin and fluoroquinolones.

[0173] In one embodiment the chemotherapeutic is a PARP inhibitor.

Labels

[0174] In one embodiment the payload comprises a fluorescent label, a chemi-lluminescent label, a radio label, an enzyme, a dye or a ligand.

[0175] A label in accordance with the present disclosure is defined as any moiety which may be detected using an assay. Non-limiting examples of reporter molecules include enzymes, radiolabels, haptens, fluorescent labels, phosphorescent molecules, chemiluminescent molecules, chromophores, photoaffinity molecules, colored particles or ligands, such as biotin.

[0176] Label conjugates are generally preferred for use as diagnostic agents. Diagnostic agents generally fall within two classes, those for use in in vitro diagnostics, and those for use in vivo diagnostic protocols, generally known as "directed imaging." Many appropriate imaging agents are known in the art, as are methods for their attachment to peptides and polypeptides (see, for e.g., U.S. Pat. Nos. 5,021,236, 4,938,948, and 4,472,509). The imaging moieties used can be paramagnetic ions, radioactive isotopes, fluorochromes, NMR-detectable substances, and X-ray imaging agents.

[0177] In the case of paramagnetic ions, one might mention by way of example ions such as chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and/or erbium (III), with gadolinium being particularly preferred. Ions useful in other contexts, such as X-ray imaging, include but are not limited to lanthanum (III), gold (III), lead (II), and especially bismuth (III).

[0178] In the case of radioactive isotopes for therapeutic and/or diagnostic application, one might mention astatine^{211}, ^{14}carbon, ^{51}chromium, ^{36}chlorine, ^{57}cobalt, ^{58}cobalt, copper^{67}, ^{152}Eu, gallium^{67}, ^{3}hydrogen, iodine^{123}, iodine^{125}, iodine^{131}, indium^{111}, ^{59}iron, ^{32}phosphorus, rhenium^{186}, rhenium^{188}, ^{75}selenium, ^{35}sulphur, technicium^{99m} and/or yttrium^{90}. ^{125}I is suitable for use in certain embodiments, and technicium^{99m} and/or indium^{111} are particularly suitable due to their low energy and suitability for long range detection. Radioactively labeled peptides and polypeptides may be produced according to well-known methods in the art. For instance, peptides and polypeptides can be iodinated by contact with sodium and/or potassium iodide and a chemical oxidizing agent such as sodium hypochlorite, or an enzymatic oxidizing agent, such as lactoperoxidase. Peptides may be labeled with technetium^{99m} by ligand exchange process, for example, by reducing pertechnate with stannous solution, chelating the reduced technetium onto a Sephadex column and applying the peptide to this column. Alternatively, direct labeling techniques may be used, e.g., by incubating pertechnate, a reducing agent such as $SNCl_2$, a buffer solution such as sodium-potassium phthalate solution, and the peptide. Intermediary functional groups which are often used to bind radioisotopes which exist as metallic ions to peptide are diethylenetriaminepentaacetic acid (DTPA) or ethylene diaminetetracetic acid (EDTA).

[0179] Fluorescent labels suitable for use as payloads include Alexa 350, Alexa 430, AMCA, BODIPY 630/650, BODIPY 650/665, BODIPY-FL, BODIPY-R6G, BODIPY-TMR, BODIPY-TRX, Cascade Blue, Cy3, Cy5,6-FAM, Fluorescein Isothiocyanate, HEX, 6-JOE, Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, REG, Rhodamine Green, Rhodamine Red, Renographin, ROX, TAM RA, TET, Tetramethylrhodamine, and/or Texas Red.

[0180] Another type of payload is that suitable for use in vitro, is where a peptide is linked to a secondary binding ligand and/or to an enzyme (an enzyme tag) that will generate a colored product upon contact with a chromogenic substrate. Examples of suitable enzymes include urease, alkaline phosphatase, (horseradish) hydrogen peroxidase or glucose oxidase. Preferred secondary binding ligands are biotin and avidin and streptavidin compounds. The use of such labels is well known to those of skill in the art and is described, for example, in U.S. Pat. Nos. 3,817,837, 3,850,752, 3,939,350, 3,996,345, 4,277,437, 4,275,149 and 4,366,241.

[0181] Other methods are known in the art for the attachment for linking a peptide to its conjugate moiety. Some attachment methods involve the use of a metal chelate complex employing, for example, an organic chelating agent such a diethylenetriaminepentaacetic acid anhydride (DTPA); ethylenetriaminetetraacetic acid; N-chloro-p-toluenesulfonamide; and/or tetrachloro-3α-6α-diphenylglycouril-3 attached to the antibody (U.S. Pat. Nos. 4,472,509 and 4,938,948). Peptides or polypeptides may also be reacted with an enzyme in the presence of a coupling agent such as glutaraldehyde or periodate. Conjugates with fluorescein markers are prepared in the presence of these coupling agents or by reaction with an isothiocyanate.

[0182] In one embodiment the label is able to stain or label the nucleus of a stem cell.

Viruses Suitable for Use as Payloads in the Present Disclosure

[0183] In one embodiment the virus employed in the present disclosure is an envelope virus, for example selected from a herpesvirus (such as Herpes simplex 1), a poxvirus (such as vaccina virus), a hepadnavirus, a flavivirus, a togavirus, a coronavirus, hepatitis D, orthomyxovirus, paramyxovirus (such as measles or Newcastle disease virus), rhabdovirus, bunyavirus, filovirus, and Rhabdoviridae (such as vesicular stomatitis Indiana virus (VSV).

[0184] In one embodiment the virus employed in the present disclosure is a non-envelope virus, for example selected from adenoviridae (such as an adenovirus), papilomaviridae, picornaviridae (such as coxsackie virus or Seneca Valley virus (eg Senecavirus)), reovirus.

[0185] In one embodiment the virus is an adenovirus, for example a human adenovirus, such as selected from a group B virus (in particular Ad3, Ad7, Ad11, Ad14, Ad16, Ad21, Ad34, Ad35, Ad51 or a chimeria thereof, such as Enadenotucirev), a group C virus (in particular Ad1, 2, 5, 6 or a chimeria thereof), a group D virus (in particular Ad8, Ad10, Ad13, Ad15, Ad17, Ad19, Ad20, Ad22, Ad30, Ad32, Ad33, Ad36, Ad37, Ad38, Ad39, Ad42, Ad43, Ad44, Ad45, A46, Ad47, Ad48, Ad49, Ad50 or a chimeria thereof), a group E virus (in particular Ad4), a group F virus (in particular Ad40, Ad41 or a chimeria thereof) and a chimeria of two or more of group B, C, D, E or F viruses.

[0186] The vast majority of viruses have well described proteins associated with target cell recognition and uptake. Modification of their tropism to re-direct or enable more selective tumor targeting into oncolytic viruses may be introduced using methods described in rev. in Verheije and Rottier, Adv. Virology 2012: 798526, 2012.

[0187] Additional viral cell surface proteins not involved in native viral targeting can have targeting motifs engineered onto them (e.g. Ad virion minor coat protein IX Salisch et al., PLoS One 12: e0174728, 2017).

[0188] Envelope viruses have an outer membrane (envelope) covering the virus capsid. The envelope is typically derived from the portions of the host cell membranes (phospholipids and proteins) but also include some viral proteins. Glycoproteins on the surface of the envelope serve to identify and bind to receptor sites on the host's membrane. The viral envelope then fuses with the host's membrane, allowing the capsid and viral genome to enter and infect the host.

[0189] Various oncolytic viruses are disclosed in WO2014/13834, incorporated herein by reference.

[0190] Herpes simplex virus (HSV) enters cells by means of four essential glycoproteins—gD, gH/gL, gB, activated in a cascade fashion by gD binding to one of its receptors, nectin1 and HVEM. Retargeting of HSV has been achieved by the insertion of ligands and scFvs into the gC and/or gD protein or gH (Campadelli-Fiume, G et al., Rev in Med Virol 21: 213-226, 2011, Gatta, V PLoS Pathog 11: e1004907, 2015). Oncolytic herpes simplex virus type 1 vectors have been developed for clinical use. These viruses are replication competent and have mutations in the genes that affect viral replication, neuropathogenicity, and immune evasiveness, and for example include first generation viruses such as NV1020 (R7020), dlsptk, d18.36tk, hrR3, R3616, 1716, second generation viruses such as G207 (MGH-1), 3616UB, SUP, NV1023, third generation viruses such as G47Δ, transcriptional expressing vectors such as G92A, d12.CALP, Myb34.5, transgene expressing vectors such as rRP450, and other viruses such as Talimogene laherparepvec (T-Vec). The HSV-1 vectors are the thought to be useful in the treatment of a wide of solid tumors, for example including glioma, melanoma, breast, prostate, colon, ovarian, and pancreatic cancers. The HSV-1 virus infects a broad range of cells types and species, it is cytolytic by nature, the replicative life cycle of the virus results in host cell destruction, it has a well characterised and large genome (152K) but contains many non-essential genes providing up to 30K of space for the insertion of therapeutic genes. Generally, HSV viruses are not mutated in the thymidine kinase gene for safety reasons. Talimogene laherparepvec is an oncolytic herpes virus, which is approved for use in the treatment of melanoma. Other herpes bases viruses include G207, SEPREHVIR (HSV-1716), by Virttu Biologics, HSV-1 R3616 mutant, HSV-1 1716 mutant, NV1020 (R7020), R3616 mutant (deleted RL1), KM100 mutant has insertions in UL48 (encodes the transactivator tegument protein pUL48 [VP16]) and RL2 genes, G92A, mutants, Myb34.5 and rQNestin34.5.

[0191] Poxvirus—Vaccina virus, such as Modified Vaccinia Ankara (MVA) may be employed (Galmiche M C et al., J Gen Virol 78: 3019-3027, 1997), MVA may be replaced with a p14 fusion molecule carrying an inserted scFv directed against the tumor associate antigen MUC-1 (Paul, S et al., Viral Immunol 20: 664-671, 2007) See also rev. in Liang L et al., Viruses 6: 3787-3808, 2014, Hsiao J C et al., J Virol 73: 8750-8761, 1999, rev. in Chen T L and Roffler S, Med Res. Rev. 28: 885-928, 2008 and Kinoshita T et al., J Biochem 144: 287-294, 2008. JX-594, by Jennerex, is a thymidine kinase-deleted Vaccinia virus plus GM-CSF. GL-ONC1 is an attenuated vaccinia virus (Lister strain) that causes regression and elimination of a wide range of solid tumors in preclincal mouse models

[0192] Paramyxovirus (such as measles or Newcastle disease virus),

[0193] Measles virus (MeV) is a single-stranded, negative-sense, enveloped (non-segmented) RNA virus of the genus Morbillivirus within the family Paramyxoviridae. Measles virus has two envelope glycoproteins: the hemagglutinin (H) attachment protein and the fusion (F) protein. Attachment, entry and subsequent cell-cell fusion is mediated via 2 measles receptors, CD46 and the signaling lymphocyte activation molecule (SLAM). See for example rev. in Msaouel P et al., Methods Mol Biol 797: 141-162, 2012, Robinson S. and Galanis, E. Expert Opin Biol Ther. 17: 353-363, 2017, Aref S et al., Viruses 8. Pii:E294, 2016); (rev. in Chen T L and Roffler S, Med Res. Rev. 28: 885-928, 2008 and Kinoshita T et al., J Biochem 144: 287-294, 2008), and (Russell S J and Peng K W, Curr Topic Microbiol. Immunol 330: 213-241, 2009, Robinson S and Galanis, E Expert Opin Biol. Ther 17: 353-363, 2017, Aref S et al., Viruses 8. Pii: E294, 2016). Measles virus encoding the human thyroidal sodium iodide symporter or MV-NIS is an attenuated oncolytic Edmonston (Ed) strain of measles virus. Radioactive Iodine imaging provides a novel technique for NIS gene expression monitoring.

[0194] Newcastle disease virus may also be employed.

[0195] Adenoviridae Adenoviruses are among the most extensively studied viruses being used as oncolytic agents. An array of peptides and proteins have been engineered into virion associated viral proteins to alter the native tropism of the virus (rev. in Verheije M H and Rottier P J M Adv Virol

2012: 798526, 2012). However, all of these are dependent upon viral assembly in the nucleus which presents significant challenges.

[0196] Other non-enveloped viruses include Coxsackievirus, Poliovirus and Reovirus. See for example rev. in Altan-Bonnet, N, Curr Opin Microbiol 32: 77-81, 2016 and Chen Y H et al., Cell 160: 619-630, 2015, rev. in Chen T L and Roffler S, Med Res. Rev. 28: 885-928, 2008 and Kinoshita T et al., J Biochem 144: 287-294, 2008 and rev. in Verheije M H and Rottier P J M Adv Virol 2012: 798526, 2012).

[0197] There are a numerous adenoviruses for example Ad5-yCD/mutTKSR39rep-hIL12, such as for the treatment of prostate cancer was initiated, CGTG-102 (Ad5/3-D24-GMCSF), by Oncos Therapeutics, for example for the treatment soft tissue sarcomas, Oncorine (H101), CG0070, Enadenotucirev (EnAd) WO2005/118825, OvAd1 and OvAd2 disclosed in WO2008/080003, ONCOS-102, for example for Unresectable Malignant Pleural Mesothelioma, and DNX-2401 for example for glioma.

[0198] Cavatak is the trade name for a preparation of wild-type Coxsackievirus A21, useful in the treatment of malignant melanoma. Seneca Valley virus (NTX-010) and (SVV-001), for example for small cell lung cancer and neuroblastoma

[0199] Reovirus-Reolysin® (pelareorep; Wild-Type Reovirus; Serotype 3 Dearing; Oncolytics Biotech), for example for the treatment of various cancers and cell proliferative disorders.

[0200] Vesicular Stomatitis Virus (VSV) VSV is another enveloped virus being explored as on oncolytic agent. See for example Betancourt D et al., J Virol 89: 11786-11800, 2015) and rev. in Hastie E and Grdzelishvili V Z J Gen Virol 93: 2529-2545, 2012).

Proteins Encoded by a Virus

[0201] In one embodiment a virus or vector employed in the method of the present disclosure comprises a transgene, for example where the transgene is to replace defective genetic material in the cell, to provide a new or augmented function in the cell, to sensitize the cell to treatment, to block a function in the cell, or to express a therapeutic protein or peptide.

[0202] In one embodiment the virus employed as the payload according to the present disclosures, comprises a transgene or transgenes, for example encoding an agent independently selected from an RNAi sequence, a protein, polypeptide or peptide (for example an antibody molecule or binding fragment thereof, a chemokine, a cytokine, an immunomodulator, a fluorescent tag or an enzyme).

[0203] This includes but is not limited to unique formats that have shown preclinical promise but have lacked an effective and economical means for delivery e.g. peptides, intrabodies and alternative scaffolds (rev. in Boldicke T, Protein Sci 26: 925-945, 2017, Marschall and Dubel, Comput Struct Biotechnol J 14: 304-308, 2016, Miersch and Sidhu F1000Res 5.pii.F1000 Faculty Rev. 1947, 2016, Peptides, Tsomaia Eur J Med Chem 94:459-470, 2015, Marschall A L J et al, Mabs 7: 1010-1035, 2015, AlDeghaither D et al., J Clin Pharmacol. 55: S4-S20, 2015))) and includes agents with therapeutic effects on the tumor cells tumor stem cells, tumor associated endothelium and tumor associated stroma. Of special interest are molecules that could serve multiple functions, for example as therapeutics, biomarkers and/or diagnostics. The herpes simplex virus thymidine kinase (HSV-TK) gene is a well-established pro-drug converting enzyme with a clinically approved pro-drug (ganciclovir—GCV) see for example Holder et al., Cancer Res. 53: 3475-3485, 1993, Touraine R L et al., Gene Therapy 5: 1705-1711, 1998),

[0204] In addition, the thymidine kinase protein expression can also be exploited to image and track the activity of the virotherapy during the course of treatment. Positron emission tomography and single photon emission computed tomography are both methods that are routinely used for the detection and monitoring of cancer and cancer therapies and are both viable means to detect the expression of the thymidine kinase protein when an appropriate thymidine kinase substrate is administered (Wang J Q et al., Bioorg Med Chem 13: 549-556, 2005, Tjuvajev J G et al, J Nucl Med 43: 1072-1083, 2002). Alternatively, the NIS gene may be used and has been explored as an agent for diagnostic and therapeutic purposes in oncolytic viruses, much like TK (Miller A and Russell S Expert Opin Biol Ther 16: 15-32, 2016, Ravera S et al., Annu Rev Physiol 79: 261-289, 2017, Portulano et al., Endocr Rev. 35: 106-149, 2014).

[0205] In one embodiment antibodies that interact and inhibit RAS or proteins in the RAS signaling pathway are encoded in the virus of the present disclosure, for example as fusion protein with the GLA-component. RAS genes constitute a multigene family that includes HRAS, NRAS, and KRAS. See for example Bos J L, Cancer Res. 49: 4682-4689, 1989; and Cetin M et al., J Mol Biol. 429:562-573, 2017.

Combination Therapy

[0206] In one embodiment the GLA-component is employed in combination with a second therapy, for example an anti-cancer therapy. This is therapy that is administered separately to the GLA-component (i.e. is not linked to the GLA-component).

[0207] In one embodiment a combination of chemotherapeutic agents employed is a chemotherapeutic described herein, for example a platin and 5-FU or a prodrug thereof, for example cisplatin or oxaplatin and capecitabine or gemcitabine, such as FOLFOX.

[0208] In one embodiment the chemotherapy comprises a combination of chemotherapy agents, in particular cytotoxic chemotherapeutic agents.

[0209] In one embodiment the chemotherapy combination comprises a platin, such as cisplatin and fluorouracil or capecitabine.

[0210] In one embodiment the chemotherapy combination in capecitabine and oxaliplatin (Xelox).

[0211] In one embodiment the chemotherapy is a combination of folinic acid and 5-FU, optionally in combination with oxaliplatin.

[0212] In one embodiment the chemotherapy is a combination of folinic acid, 5-FU and irinotecan (FOLFIRI), optionally in combination with oxaliplatin (FOLFIRINOX). The regimen consists of: irinotecan (180 mg/m^2 IV over 90 minutes) concurrently with folinic acid (400 mg/m^2 [or 2×250 mg/m^2] IV over 120 minutes); followed by fluorouracil (400-500 mg/m^2 IV bolus) then fluorouracil (2400-3000 mg/m^2 intravenous infusion over 46 hours). This cycle is typically repeated every two weeks. The dosages shown above may vary from cycle to cycle.

[0213] In one embodiment the chemotherapy combination employs a microtubule inhibitor, for example vincristine

sulphate, epothilone A, N-[2-[(4-Hydroxyphenyl)amino]-3-pyridinyl]-4-methoxybenzenesulfonamide (ABT-751), a taxol derived chemotherapeutic agent, for example paclitaxel, abraxane, or docetaxel or a combination thereof.

[0214] In one embodiment the combination therapy employs an mTor inhibitor. Examples of mTor inhibitors include: everolimus (RAD001), WYE-354, KU-0063794, papamycin (Sirolimus), Temsirolimus, Deforolimus(MK-8669), AZD8055 and BEZ235(NVP-BEZ235).

[0215] In one embodiment the combination therapy employs a MEK inhibitor. Examples of MEK inhibitors include: AS703026, CI-1040 (PD184352), AZD6244 (Selumetinib), PD318088, PD0325901, AZD8330, PD98059, U0126-EtOH, BIX 02189 or BIX 02188.

[0216] In one embodiment the combination therapy employs an AKT inhibitor. Examples of AKT inhibitors include: MK-2206 and AT7867.

In one embodiment the combination employs an aurora kinase inhibitor. Examples of aurora kinase inhibitors include: Aurora A Inhibitor I, VX-680, AZD1152-HQPA (Barasertib), SNS-314 Mesylate, PHA-680632, ZM-447439, CCT129202 and Hesperadin.

[0217] In one embodiment the combination therapy employs a p38 inhibitor, for example as disclosed in WO2010/038086, such as N-[4-({4-[3-(3-tert-Butyl-1-p-tolyl-1H-pyrazol-5-yl) ureido]naphthalen-1-yloxy}methyl) pyridin-2-yl]-2-methoxyacetamide.

[0218] In one embodiment the combination employs a Bcl-2 inhibitor. Examples of Bcl-2 inhibitors include: obatoclax mesylate, ABT-737, ABT-263(navitoclax) and TW-37.

[0219] In one embodiment the combination therapy comprises a checkpoint inhibitor, for an anti-PD-1 inhibitor or an anti-PD-L1 inhibitor.

[0220] In one embodiment the chemotherapy combination comprises an antimetabolite such as capecitabine (xeloda), fludarabine phosphate, fludarabine (fludara), decitabine, raltitrexed (tomudex), gemcitabine hydrochloride and cladribine.

[0221] In one embodiment the chemotherapy combination comprises ganciclovir, which may assist in controlling immune responses and/or tumour vasculation.

[0222] In one embodiment the chemotherapy includes a PARP inhibitor.

[0223] In one embodiment the combination therapy includes an inhibitor of cancer metabolism with specific inhibition of the activity of the DHODH enzyme.

[0224] In one embodiment one or more therapies employed in the method herein are metronomic, that is a continuous or frequent treatment with low doses of anticancer drugs, often given concomitant with other methods of therapy.

[0225] In one embodiment, there is provided the use of multiple cycles of treatment (such as chemotherapy) for example 2, 3, 4, 5, 6, 7, 8.

[0226] In one embodiment the chemotherapy is employed in a 28 day cycle.

[0227] In one embodiment the molecules of the present disclosure are provided in a pharmaceutical composition comprising a excipient, diluent and/or carrier. In one embodiment the composition is as a parenteral formulation.

[0228] Parenteral formulation means a formulation designed not to be delivered through the GI tract. Typical parenteral delivery routes include injection, implantation or infusion.

[0229] In one embodiment the parenteral formulation is in the form of an injection. Injection includes intravenous, subcutaneous, intra-cranial, intrathecal, intra-tumoural or intramuscular injection. Injection as employed herein means the insertion of liquid into the body via a syringe.

[0230] In one embodiment the parenteral formulation is in the form of an infusion.

[0231] Infusion as employed herein means the administration of fluids at a slower rate by drip, infusion pump, syringe driver or equivalent device. In one embodiment, the infusion is administered over a period in the range of 1.5 minutes to 120 minutes, such as about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 15, 16, 17, 18, 19 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 65, 80, 85, 90, 95, 100, 105, 110 or 115 minutes.

[0232] In one embodiment, the formulation is for intravenous (i.v.) administration. This route is particularly effective because it allows rapid access to the majority of the organs and tissue and is particular useful for the treatment of metastases, for example established metastases especially those located in highly vascularised regions such as the liver and lungs.

[0233] Therapeutic formulations typically will be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other parenteral formulation suitable for administration to a human and may be formulated as a pre-filled device such as a syringe or vial, particular as a single dose.

[0234] As discussed above the formulation will generally comprise a pharmaceutically acceptable diluent or carrier, for example a non-toxic, isotonic carrier that is compatible with the virus, and in which the virus is stable for the requisite period of time.

[0235] The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a dispersant or surfactant such as lecithin or a non-ionic surfactant such as polysorbate 80 or 40. In dispersions the maintenance of the required particle size may be assisted by the presence of a surfactant. Examples of isotonic agents include sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition.

[0236] In one embodiment there is provided a kit of parts comprising a GLA-component according to the present disclosure and a payload, wherein the payload is linked or unlinked to said GLA-component.

[0237] "Comprising" in the context of the present specification is intended to mean "including".

[0238] Where technically appropriate, embodiments of the invention may be combined.

[0239] Embodiments are described herein as comprising certain features/elements. The disclosure also extends to separate embodiments consisting or consisting essentially of said features/elements.

[0240] Technical references such as patents and applications are incorporated herein by reference.

[0241] The technical backgrounds is part of the technical disclosure of the present specification and may be used as basis for amendments because the discussion therein is not limited to discussing the prior art as it also includes a discussion of the technical problems encountered in the field and the application of the present technology.

[0242] Any embodiments specifically and explicitly recited herein may form the basis of a disclaimer either alone or in combination with one or more further embodiments.

[0243] The present application claims priority from U.S. Ser. Nos. 62/554,530, 62/569,403, 62/554,533, 62/569,411, 62/584,565 and 62/593,014. Each of these applications are incorporated by reference. These applications may be employed as the basis for a correction to the present specification.

[0244] The invention will now be described with reference to the following examples, which are merely illustrative and should not in any way be construed as limiting the scope of the present invention.

EXAMPLES

[0245] FIG. **1**A-D Shows various representations of GLA protein structures.

[0246] FIG. **1**E Shows an embodiment of a GLA-component according to the present disclosure.

[0247] FIG. **2** Shows Protein S (PrS) and annexin staining of breast cancer cell lines treated with peroxide to induce apoptosis. A, human MDA-231 cells treated with peroxide and stained with FITC-PrS. B, untreated MDA-231 cells stained as in A. C, treated MDA-231 cells stained with annexin. D, human MCF-7 cells treated with peroxide and stained with PrS. E, murine MET-1 cells, as in D. F, murine 4T1 cells, as in D.

[0248] FIG. **3** Shows overlapping, yet distinct, cellular localization of PrS and annexin. A, murine 4T1 cells treated with peroxide and stained with Cy5 PrS (RED) and FITC annexin (GREEN). Light arrow, co-localized signals; red arrows, cells staining with PrS and not annexin; green arrow, cell staining relatively brighter with annexin but less bright with PrS, indicating distinct binding patterns (insets show PrS and annexin staining separately). B, treated 4T1 cells stained with FITC PrS and Cy5 annexin. Green arrows, cells staining with PrS and not annexin. C, Cy5 annexin staining of treated 4T1 cells pre-incubated with 1,000-fold excess of cold annexin.

[0249] FIG. **4** Shows staining of apoptotic COS-1 cells with PrS and annexin. Cells were treated with t-BHP as described and stained with FITC annexin (left) and Cy5 PrS (right). Arrows indicate subcellular structures presumed to be apoptotic bodies.

[0250] FIG. **5** Shows differential staining of extracellular vesicles with PrS and annexin. Extracellular vesicles were prepared from 4T1 cells and stained with FITC PrS (GREED) and Cy5 annexin (RED). Arrows indicate vesicles staining with annexin only (RED arrow), PrS only (GREEN arrow) and both proteins (light arrow).

[0251] FIG. **6** Shows subcellular localization of PrS and annexin. A, B, apoptotic 4T1 cells were stained with FITC PrS (GREEN arrows) and Cy5 annexin (RED arrows); light arrows, co-localization. C, Possible apoptotic bodies.

[0252] FIG. **7** Shows internalization of PrS within 5 minutes. Apoptotic 4T1 cells were stained with FITC PrS (green) and Cy5 annexin (red) and imaged within 10 min of the addition of the proteins. A, Merged image. B, Hoescht nuclear stain alone.

[0253] FIG. **8** Shows BLI images of 4T1 tumors in mice.

[0254] FIG. **9** SPECT imaging of effect of doxorubicin on 4T1 tumors, using radiolabeled PrS and annexin. Mice with 4T1 breast cancer tumors were imaged with 99mTc PrS (A and B), or annexin (C and D), before (A and C) and 24 h after doxorubicin (B and D).

[0255] FIG. **10** Shows SPECT imaging of cyclohexamide-treated mice. Five mice per panel are shown before (A and C) and 24 h after (B and D) treatment. The mice were imaged with either ^{99m}Tc PrS (A and B), or annexin (C and D), Arrows indicate increased liver signal.

[0256] FIG. **11** Shows localization of Cy5 PrS to infected spleen. CD1 mice were infected with bioluminescent *Listeria* and imaged on day 2 post infection. The mice were injected with Cy5 PrS 30 min before sacrifice, and the spleens removed and frozen. Modestly infected (A) and control uninfected (C) mice are shown. Sections of the infected (B) and uninfected (D) spleens of each mouse in the Cy5 channel are shown, merged with phase contrast images.

[0257] FIG. **12** Shows localization of Cy5 PrS to tumors treated with doxorubicin. Mice implanted with 4T1 breast cancer tumors were treated with doxorubicin (right panels) or left untreated (left panels). 24 hours later the mice were injected intravenously with Cy5 PrS and sacrificed 30 min later. The tumors were removed, frozen, and sectioned for fluorescence microscopy. Merged Cy5/phase contrast images from four different mice are shown.

[0258] FIG. **13** Shows differentiation of TSCs. TSCs were cultured in the presence (left) or absence (right) of growth factors. Arrows in the right panel indicate giant cells characteristic of differentiation.

[0259] FIG. **14** Shows PrS staining of trophoblast stem cells and differentiated trophoblasts. Trophoblast stem cells (left) were differentiated into trophoblast giant cells (right) by withdrawal of growth factors. The cells were stained with Cy5 PrS and imaged.

[0260] FIG. **15** Shows MSC differentiation. MSC were treated as described in the text, for differentiation into adipocytes (upper panels) or osteoblasts (lower panels). Differentiated cells exhibited the expected morphology in each case.

[0261] FIG. **16** Shows MSCs stained with PrS (green), annexin (red), and Hoechst (blue). Cells were imaged within 10 min of addition of the stain mixture.

[0262] FIG. **17** Shows TSCs stained with PrS (green, lightest area), annexin (red, light around the cell membrane), and Hoechst (blue). Cells were imaged within 5 min of addition of the stain mixture.

[0263] FIG. **18** Shows differential staining of TSC vesicles. TSCs were stained as in FIG. **17**. The group of cells are secreting large vesicles that stain with annexin (red) and not PrS (green).

[0264] FIG. **19** Shows PrS staining of C17.2 neural progenitor cells. The cells were stained with PrS-FITC and imaged with standard (non-confocal) microscopy.

[0265] FIG. **20** Shows internalization of PrS into TSC at 4C. FITC PrS (green) and Cy5 annexin (red) were added to TSC at 4C and imaged with confocal microscopy.

[0266] FIG. **21** Shows lineage-negative, SCA-1/c-kit staining cells from mouse bone marrow. The cells were not stained with either PI (propidium iodide; to detect dead

cells) or PrS at this point in the analysis. Absence of staining for hematopoietic lineages (left panel) and staining of c-kit and SCA1 (right panel) defines the population of HSC, shown in green (lightest areas).

[0267] FIG. **22** PrS staining of long-term HSC. HSC were isolated as in FIG. **1**, and stained with FITC PrS. SLAM pattern was determined with Cy7 (x-axis).

[0268] FIG. **23** PrS staining of short-term HSC. HSC were isolated as in FIG. **1**, and stained with FITC PrS. SLAM pattern was determined with Cy7 (x-axis).

[0269] FIG. **24** Shows internalization of PrS in long-term HSC. HSC were prepared as described, stained for PrS, and examined with confocal microscopy. Green (lightest areas), FITC PrS; blue, Hoescht nuclear stain; red, PI. Note that PI stain is excluded from the nucleus, indicating the cells are alive.

[0270] FIG. **25** Shows an example of dead HSC exhibiting nuclear PI.

[0271] FIG. **26** GLA-mediated delivery is non-toxic to cells

[0272] This specification also includes sequences 1 to 6, in the associated sequence listing.

[0273] This project initiated the testing of labeled recombinant PrS as an in vivo imaging agent for SPECT (Single Photon Computed Tomography). Surprisingly it was found that the molecule rapidly internalized into apoptotic cells. This unexpected finding led us to explore the phenomenon further, whereupon we found that PrS was also internalized into a subset of non-apoptotic stem cells of several types.

[0274] PrS is protein S GLA domain and protein S EGF domain as shown in SEQ ID NO: 6.

Methods

[0275] For fluorescence, conjugation of Cy5 and FITC was achieved using Amersham (GE Heathcare) and Molecular Probes (Invitrogen) labeling kits, respectively, according to the instructions of the manufacturers. Both kits provide columns for the removal of unconjugated fluorophore. Initially, 0.77 mg of PrS (Fraction 2) in 1 ml and 0.77 mg of annexin in 1 ml were labeled with FITC to test for specificity of binding to apoptotic cells. For co-localization and competition studies 0.68 mg of PrS (Fraction 3) in 1 ml and 0.68 mg of annexin were labeled with Cy5. For confocal microscopy, 0.76 mg of PrS from the second shipment was labeled with FITC and the previously labeled Cy5-conjugated annexin was used. It should be noted that the precise efficiency of labeling was not determined and the recovery from the columns was assumed to be 85%, according to the instructions of the manufacturers of the labeling kits. Thus, the relative staining intensity of the two proteins in any case may reflect these contingencies. The cells were stained for 30 min initially, but it was subsequently determined that less than 5 min was sufficient. To test PrS for apoptotic cell-specificity, four breast cancer cell lines were initially employed; human MDA-231 and MCF7 and murine 4T1 and MET-1. Subsequently, COS-1 monkey kidney cells were also used. Apoptosis was induced with hydrogen peroxide or tertiary-Butyl hydroperoxide (t-BHP). The cells were plated in 24-well plates at 6×10^4 cells per well or Eppendorf chamber slides at 1×10^4 cells per well, and apoptosis was induced the next day, using 2 mM H_2O_2, or t-BHP for time points from 30 min to 2 hrs. After induction, the wells were washed with Annexin Binding Buffer (AB; Santa Cruz Biotech), and stained with labeled protein. From past experience and the literature, 5.5 μg/ml of annexin protein was used for staining. This amount was adjusted for equimolar addition of PrS by assuming the molecular weights of annexin to be 36 kD and the recombinant PrS to be 30 kD, based on the gel images provided. The cells were stained for 15 min. Hoechst 33342 dye was used for visualizing nucleic acid. The wells were then washed with AB and observed using the EVOS fluorescence microscope while still viable. For confocal microscopy, the Leica SP8 microscope in the Stanford Cell Sciences Imaging Facility was employed. The wells were then washed with AB and observed using the Leica sp8 microscope. Hoechst 33342 dye was used for visualizing nuclei. For toxicity studies, PrS was added to trophoblast stem cells (TSCs) and the viability tested with trypan blue using a Nexcelom Cellometer.

[0276] To test the labeled proteins for the ability to detect tumors, 5×10^4 4 T1-luc cells were implanted into groups of 5 male BALB/c mice, in the left axillary fat pad. The mice were imaged with in vivo bioluminescence imaging (BLI) each day to monitor tumor growth, starting at 1 week post implantation. The mice were then treated on day 11 post implantation with 13 mg/kg body weight of intraperitoneal (IP) doxorubicin, and BLI was performed the next day. Control mice bearing tumors were left untreated with doxorubicin. 48 hrs post treatment the mice were imaged 1 hr after intravenous tracer injection (anesthesia 1.3 g/kg of urethane IP), with single head A-SPECT gamma camera (Gamma Medica); 1 mm pin hole collimator, 128 steps into a 128×128 imaging matrix, 15 seconds per step, 2.7 cm ROR; FOV=upper chest/neck. The injected dose of each protiein was 160 μl (800 μCO. The animals were then sacrificed and biodistribution was performed. For the cyclohexamide treatment experiment, groups of 5 young (7 week old) male Swiss Webster mice were anesthetized (1.3 g/kg of urethane IP) and injected intravenously with 50 mg/kg cycloheximide. 1 hr 45 min after cycloheximide injection, tracer was injected (PrS=180 ul/1.2 mCi per dose; annexin V =170 μl/1.05 mCi per dose). 45 min after tracer injection, the mice were imaged with 10 min static whole body images using a single head parallel hole collimator (128×128 matrix) on the A-SPECT gamma camera.

[0277] To test for the specific localization of fluorescent PrS to apoptotic sites due to infection in live animals, CD1 mice were injected intravenously with bioluminescent *Listeria monocytogenes*. This bacterial pathogen infects many organs including the spleen, in which extensive apoptosis of monocytes and granulocytes occurs. At certain times post infection, spleen is the primary site of bacterial replication and so splenic BLI signals from the bacteria can be correlated with the localization of probes for apoptosis. Mice were infected and imaged each day. When splenic signals were evident (day 2 post infection for 2×10^5 colony forming units of bacteria in 8 week old CD1 female mice), 300 mg/kg body mass of Cy5 PrS was injected into mice, the animals were sacrificed 30 min later, and the spleens removed, frozen in OCT, and sectioned for fluorescence microscopy. Uninfected control mice were employed.

[0278] Flow cytometry was performed. Freshly labeled FITC PrS, prepared as described above, was employed. Murine hematopoietic stem cells (HSCs) are routinely purified in this laboratory. The cells were isolated from normal mouse bone marrow by staining for c-Kit+, lineage-negative cells. To further characterize the cells, SLAM marker staining was also performed. These markers stain cells that

self-renew and differentiate, whereas non-staining HSCs can only differentiate. Subsequent staining with FITC PrS revealed the percent positive in SLAM-staining cells, as shown in the Results. The cells were then sorted for FITC and examined with confocal microscopy, using Hoechst 33342 for nuclear visualization.

Results

[0279] To assess PrS binding specificity in the context of apoptosis in cell culture, we employed several human and murine breast cancer cell lines. Apoptosis was induced with peroxide as described above, and FITC PrS binding was assessed. Examples of these experiments are shown in FIG. **2**. Untreated cells exhibited minimal binding, such as shown in panel B of FIG. **2**. Concentrations of peroxide and incubation times were chosen such that only a minority of cells would be affected, because at higher concentrations and/or longer incubation times the cells detached and staining and microscopy was not possible. In addition, the presence of many unaffected cells served as an internal negative control within each field. FITC-annexin showed specificity for apoptosis similar to PrS, serving as an internal positive control. We then tested the two proteins for co-localization and competitive binding. For co-localization, both FITC and Cy5 labeled PrS and annexin were prepared. 4T1 cells were treated with peroxide and stained with Cy5 and FITC labeled PrS and annexin, using both combinations of fluorophores. The cells were then visualized in the EVOS fluorescence microscope. The results are shown in FIG. **3**. Under the conditions tested, all the brightly staining cells exhibited staining with both proteins. However, whether using Cy5 or FITC, PrS appeared to stain some cells that annexin did not, albeit weakly (FIG. **3**). The relative staining intensity of different cells by each protein sometimes differed between the two probes, i.e., sometimes annexin stained two cells with equal intensity and PrS did not, and vice versa (FIG. **3**A, green arrow and insert). Thus, while both probes generally stained the same cells, they appeared to exhibit subtle differences. In the competition assay, increasing excess amounts of unlabeled annexin were pre-incubated with apoptotic 4T1 cells for 15 min and the cells were then stained with Cy5 PrS. Surprisingly, the staining of PrS was not blocked by even 1,000 fold excess of annexin, the highest excess amount tested (FIG. **3**C), although these proteins are thought to bind to the same target molecule, exposed PS. Co-staining of annexin and PrS was observed with many cell types. While the two proteins generally stained the same cells in each cell type, other differences became apparent. In particular, some objects smaller than cells were differentially stained (FIG. **4**). These objects, which were present in increased numbers after peroxide treatment, were interpreted as apoptotic bodies; membrane-bound cell fragments produced during the fragmentation of apoptotic cells. As shown in FIG. **4**, PrS stained these entities, whereas annexin did not, although some of these objects did stain with both proteins. This observation was unexpected. To further explore the differential staining of sub cellular entities, extracellular vesicles (EVs) were prepared from 4T1 murine tumor cells using a standard centrifugation protocol. The two proteins also differentially stained these vesicles (FIG. **5**), a result that may have biological and therapeutic implications.

[0280] EVs, specifically exosomes, microvesicles (MVs) and apoptotic bodies (ABs), are presumed to play key roles in cell-cell communication via transfer of biomolecules between cells. The biogenesis of these types of EVs differs, and they originate from either the endosomal (exosomes) or plasma membranes (MV) or are products of programmed cell death (ABs). All mammalian cells are thought to secrete EVs. Each type of EV can transfer molecular cargo to both neighboring and distant cells, affecting cellular behaviors such as those involved in tumor development and progression. In fact, EVs may play a role in nearly all the hallmarks of cancer, including sustaining proliferative signaling, evading growth suppression, resisting cell death, reprogramming energy metabolism, acquiring genomic instability, and developing the tumor microenvironment. They have also been implicated in the induction of angiogenesis, control of invasion, initiation of premetastatic niches, sustaining inflammation, and evading immune surveillance. Immune cells appear to also communicate through EVs and my recognize EVs as signals from tumor cells, infected tissues and wounds. A deeper understanding of the biology of EVs and their contribution to the hallmarks of cancer is leading to new possibilities for diagnosis and treatment of cancer. Development of additional EV surface markers is essential to advancing this field and PrS may be such a determinant.

[0281] Following these studies with fluorescence microscopy, the subcellular localization of the staining by PrS and annexin was then evaluated via confocal microscopy. Murine 4T1 cells (lacking the Luc-GFP reporters) were plated on 8-part chamber slides at 1×10^4 cells per chamber and apoptosis was induced with 2 mM H_2O_2 or t-BHP (2 hr exposure) the next day. The cells were then washed and stained for 15 min with PrS and annexin. Hoechst 33342 dye was used to stain nucleic acid. In all cases, the most brightly staining cells were stained with both probes. However, in many cells labeled PrS was observed in the cytoplasm, whereas the labeled annexin was not (FIG. **6**). Although annexin was internalized and appears in vesicles of a few cells, internalized annexin together with surface localized PrS in the same cell was not observed. These results were unexpected, because the two proteins are both presumed to bind PS. To further study the internalization of PrS, a time course experiment was performed. Apoptotic 4T1 cells were stained for 5 min with Cy5 annexin and FITC PrS, and observed within 5 min of the addition of the probes. PrS was observed in the cytoplasm of these cells immediately, indicating internalization within 5 min (FIG. **7**). The time course images also showed that PrS and annexin did not always stain the same cells equally at early time points. The cells in FIG. **7** appear to be in different stages of apoptosis, as the cell on the left shows an uncondensed nucleus surrounded by an apparently intact nuclear membrane, whereas the right cell exhibits the strong staining often characteristic of chromatin condensation that occurs later in the apoptotic process. Staining patterns such as these may indicate that PrS binds earlier in apoptosis than annexin. Although purely conjecture at this point, such a preference would explain many of the differences between these proteins that have been observed so far. For example, the staining of some cells by PrS and not annexin, such as in FIGS. **3**A and B may be due to PrS binding earlier in the process of apoptosis. To examine PrS localization in live animals, several experiments were performed. These studies employed chemical and infectious induction of apoptosis in vivo, as well as the localization of PrS to tumors treated with doxorubicin, which is known to induce apoptosis. SPECT imaging using

HYNIC-labeled PrS and annexin was performed in animals given 4T1luc breast tumors and treated with doxorubicin. Because the 4T1 tumors have been labeled with luciferase, they can be imaged in mice using in vivo bioluminescence imaging (BLI). One of the images from this experiment is shown in FIG. **8**. This method can be used to evaluate tumor implantation and to follow progression in individual animals over time. ^{99}mTc labeled PrS and annexin were then employed for SPECT imaging of animals treated with doxorubicin and controls. An example of the results is shown in FIG. **9**. The images of the head and thorax of the two animals show non-specific accumulation of the PrS probe in the salivary gland, and a low signal to noise ratio using this probe. Therefore, the threshold of the display in the PrS images shown was lowered to reveal more background, resulting in the brighter false-color of the images. The low signal-to-noise ratio is likely due to HYNIC labeling of only 1 mg of protein, which is sub-optimal, and also due to the inability to perform controlled studies of HYNIC:protein labeling ratio.

[0282] SPECT imaging of mice treated with cyclohexamide, which induces apoptosis in the liver, was also performed (FIG. **10**). In FIG. **10**, the whole-body images of 5 mice are shown in each panel. As with many radiolabeled probes, background is seen in the kidneys. Treatment of the mice with cyclohexamide increased the annexin SPECT signal in the liver. Again, the PrS showed low signal compared to annexin. Annexin was able to detect the apoptotic livers of cyclohexamide treated mice, whereas PrS showed only slight increase of signal in the liver due to treatment. To test the localization of PrS to apoptotic tissues and treated tumors independently of SPECT imaging and the concomitant complications of HYNIC labeling, mice infected with bacteria that induce apoptotic responses and tumor bearing mice were injected with Cy5 PrS. For infection, we employed *Listeria monocytogenes*, a bacterial pathogen labeled with luciferase and well characterized for BLI. Characteristic BLI signals from the spleen provide for excellent co-localization studies. CD1 mice were infected as described above and were imaged with BLI on day 2 post infection. The mice were then injected with Cy5 PrS and 30 min later sacrificed, and the spleens removed for sectioning and fluorescence microscopy (FIG. **11**). In all cases, splenic sections from infected mice showed much greater Cy5 fluorescence signals than controls. In FIG. **11**, the infected mouse shown displayed low photon counts, indicating the infection had not yet progressed very far in this animal. Many mice exhibit 10 times this signal intensity from the spleen on this day. However, the Cy5 channel fluorescence was still very strong relative to the uninfected control shown. This result may reflect the ongoing innate immune response to infection, as granulocytes and macrophages have been shown to be the main source of annexin signal in such animals (these cells are programmed for apoptosis to limit tissue destruction).

[0283] The localization of fluorescent PrS to 4T1 tumors treated with doxorubicin was then tested. Mice implanted with tumors were treated with doxorubicin as described above and Cy5 PrS was injected intravenously 30 min prior to sacrifice and removal of the tumors for sectioning and fluorescence microscopy. The results are shown in FIG. **12**. Areas of intense staining were observed in the treated animals, whereas more modest signal was observed from the untreated tumor sections. Although some untreated tumors did exhibit small areas of higher signal than background, no signals of similar intensity to the treated tumors were observed in any of the untreated sections.

[0284] Stem cells are distinct in phenotype from differentiated cells and may express PS non-apoptotically to avoid the induction of immune responses. Trophoblast stem cells (TSCs) differentiate into several types of trophoblasts in culture. TSCs are prepared from mouse uterine scrapings grown in the presence of fibroblast growth factor, activin, and heparin. TSCs spontaneously differentiate into giant cells when these factors are removed from the medium (FIG. **13**). TSCs stained with PrS, whereas differentiated trophoblasts derived from these cells in culture did not stain (FIG. **14**). We have also determined that PrS is internalized into stem cells without apoptotic induction. This result confirms observations made in tumor cell lines, in which apoptosis was induced. Without induction of apoptosis, minimal staining was observed in tumor cells. To test for internalization in stem cells, we employed mesenchymal stem cells (MSCs) and TSCs. MSCs were prepared from mouse bone marrow. The bone marrow was flushed from mice and cultured for 6 days in the absence of growth factors. During this incubation, MSCs and hematopoietic stem cells (HSCs) replicate, whereas fibroblasts adhere but do not multiply beyond a few generations. After 6 days, a monolayer is visible. Upon passage by trypsinization, the adherent MSCs are retained, whereas the HSCs, which grow in suspension, are lost. The fibroblasts do not persist due to absence of growth factors and are also not retained. Thus, this simple procedure results in a nearly homogeneous population of MSCs. To confirm the identity of these cells, we treated the cultures separately with dexamethasone and glycerol phosphate (to induce differentiation into osteoblasts) or dexamethasone and indomethacin (to induce differentiation into adipocytes). The results are shown in FIG. **15**. In response to the above treatments, differentiated cells showed the appearance of the respective cells. Adipocytes contained large fat vesicles and osteoblasts were dark with distinctive intracellular collagen and mineralization.

[0285] To assess subcellular staining pattern, undifferentiated MSC were stained with PrS and annexin, as well as Hoechst nuclear staining reagent, and observed with confocal microscopy. Results of the observations are shown in FIG. **16**. PrS was rapidly internalized. In the case of MSC, about 1 in 20 cells stained with PrS, consistent with previous data, however the precise percentage that stained was not determined. The morphology of MSCs is heterogeneous, and the cells secrete abundant material into the medium, some of which adheres to the surface of the chamber slide, making resulting in background in some of the images. Nonetheless, the data clearly show internalized PrS, within 5 minutes of addition and annexin on the surface. TSCs were also stained and imaged as was done with the MSCs. The observations confirm internalization into these cells as well, which also occurs within 5 minutes of addition of the protein. The results are shown in FIG. **17**. TSCs are morphologically quite variable, and can be multinucleate in the absence of differentiation, as can be seen in the figure. As with the MSCs, these primary cells shed abundant material into the medium, some of which we have established as extracellular vesicles (previous data). This material again makes the imaging difficult. Some EVs stain with annexin and not PrS, and this phenomenon can be seen in TSCs, in FIG. **18**. In this image of a cluster of TSCs, vesicles being

released by the cells stain with annexin and not PrS, which is internalized. These patterns raise interesting questions regarding the specificity and binding targets of PrS and annexin. The two proteins are both reputed to bind PS. However, the differential binding to EVs as well as distinct subcellular localization patterns suggest that they are not binding in exactly the same manner. Further studies will be required to establish the basis of this distinction, which may prove to be significant. We have also observed PrS staining of the neural progenitor cell line C17.2 (FIG. **19**), which is a transformed cell line capable of differentiation in vitro into astrocytes and other neuronal cells. Approximately 5% of these transformed cells stained, although this percentage is an estimate. Remarkably, entry into TSCs occurred even when the cells were chilled to 4° C. (FIG. **20**). However, it must be noted that the chamber could not be continually chilled once placed on the microscope. Nevertheless, the temperature could not have risen much within the 5 min time frame of the imaging procedure. This result, while provocative, must clearly be repeated under more controlled conditions. Should the finding be substantiated, the mechanism would have to be very interesting indeed.

[0286] We have succeeded in staining hematopoietic stem cells (HSC) with PrS. Using flow cytometry we determined that HSC stain with PrS, and have observed internalization of PrS in these cells with confocal microscopy. HSC were identified and isolated using fluorescence activated cell sorting (FACS). The cells were identified in bone marrow as lineage-negative, SCA/c-kit positive cells (FIG. **21**). These were then stained with FITC-PrS. Two populations of HSC, short-term and long-term, can be identified with the pattern of SLAM marker staining. The SLAM (Signaling Lymphocyte Activation Molecule) markers CD48, CD150, CD229 and CD244 differentially stain HSC with distinct patterns such that SLAM pattern-positive staining is indicative of the ability to both self-renew and differentiate, whereas SLAM pattern-negative HSC can only differentiate. PrS stained a subset of long-term HSC (FIG. **22**), and also short-term HSC (FIG. **23**). The cells shown are propidium iodide (PO-negative, meaning that they are all live cells. This result confirms previous experiments demonstrating that a subset of stem cells stains with PrS without the induction of apoptosis.

[0287] We then proceeded to test for internalization of PrS into HSC. This experiment was complicated by many factors. Perhaps the most difficult was the survival in culture of HSC, which die in large numbers in medium overnight. We therefore had to time the experiment such that flow cytometry analysis and confocal microscopy occurred on the same day. Furthermore, the cells are not adherent, making microscopy less than optimal. To make microscopy more efficient, the cells were resuspended in a small drop of medium. Finally, we needed to make sure that the PrS-stained cells analyzed by microscopy were still alive. Many HSC died during the processes of analysis and isolation. Therefore, PI was added and scanned in addition to the Hoescht nuclear stain, and another channel was employed. The presence of PI-bright nuclei indicated dead cells. Despite these difficulties and the complexities of timing, we were able to perform the experiment, and confirmed internalization of PrS into live HSC (FIG. **24**). The cells were confirmed as alive by lack of nuclear PI staining. However, some cells were dead or dying as shown in FIG. **25**. Despite the complexity and length of the experiment shown, the results show internalization.

[0288] Finally, in FIG. **26**, we have performed preliminary toxicity studies on TSC, and determined that at a concentration of 135 μg/ml, viability was reduced only by a very minimal extent after 30 min, from 78% to 74%, relative to PBS. Considering that, at this level, 10% of the culture volume was PrS-containing solution, this result confirmed our qualitative observations that PrS is basically non-toxic to stem cells, and the minor toxicity observed could well be due to contaminating contents of the preparation itself. Lower concentrations of PrS showed no effect on viability. The highest level of protein tested was more than 1000 times the concentration used for staining. While full toxicity studies, which were not formally part of this project, will require much more extensive tests, in our hands PrS exhibits very little toxicity.

Summary

[0289] The above results have shown that PrS is rapidly internalized into an array of cells expressing PrS, including stem cells of many types, which suggests that PrS possesses unique characteristics amenable to manipulation toward the goal of developing a therapeutic agent. In addition, the difference in specificity between PrS and annexin such as seen in FIGS. **3** and **7** suggests that binding itself is different between these two proteins. The mere fact that annexin is a tetramer and PrS is a monomer cannot explain these differences and these data suggest that some other component on the cell surface may be involved in PrS binding. The mechanism of binding, specificity, and internalization of PrS, as well as the capability of modular manipulation provide a host of possibilities.

Example 2

[0290] Stem cells are distinct in phenotype from differentiated cells and may express PS non-apoptotically to avoid the induction of immune responses. Stem cells were stained with a GLA domain molecule of the present disclosure comprising a payload of a fluorescent label, without the induction of apoptosis.

[0291] Trophoblast stem cells, (FIG. **14**) which differentiate into several types of trophoblasts in the placenta, stained with Protein S, whereas differentiated trophoblasts derived from these cells in culture did not stain. The stain was able to distinguish between in vivo differentiated stems cells and cells differentiated in vitro.

[0292] This data the molecules of the present disclosure may be employed to target cells in vivo or in ex vivo samples.

REFERENCES

[0293] 1. Thompson, W. W., et al., *Estimates of US influenza-associated deaths made using four different methods*. Influenza Other Respir Viruses, 2009.3(1): p. 37-49.

[0294] 2. Osterholm, M. T., et al., *Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis*. Lancet Infect Dis, 2012. 12(1): p. 36-44.

[0295] 3. Dixit, R., et al., *Emergence of oseltamivir resistance: control and management of influenza before, during and after the pandemic*. Infect Disord Drug Targets, 2013. 13(1): p. 34-45.

[0296] 4. Jefferson, T., et al., *Oseltamivir for influenza in adults and children: systematic review of clinical study reports and summary of regulatory comments*. BMJ, 2014. 348: p. g2545.

[0297] 5. Godfrey, C., et al., *Delivery is key: lessons learnt from developing splice-switching antisense therapies*. EMBO Mol Med, 2017. 9(5): p. 545-557.

[0298] 6. Kaczmarek, J. C., P. S. Kowalski, and D. G. Anderson, *Advances in the delivery of RNA therapeutics: from concept to clinical reality*. Genome Med, 2017.9(1): p. 60.

[0299] 7. Ling, H., *Non-coding RNAs: Therapeutic Strategies and Delivery Systems. Adv Exp Med Biol,* 2016. 937: p. 229-37.

[0300] 8. Poon, I. K., et al., *Apoptotic cell clearance: basic biology and therapeutic potential*. Nat Rev Immunol, 2014. 14(3): p. 166-80.

[0301] 9. Birge, R. B., et al., *Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer*. Cell Death Differ, 2016. 23(6): p. 962-78.

[0302] 10. Amara, A. and J. Mercer, *Viral apoptotic mimicry*. Nat Rev Microbiol, 2015. 13(8): p. 461-9.

[0303] 11. Moller-Tank, S. and W. Maury, *Phosphatidylserine receptors: enhancers of enveloped virus entry and infection*. Virology, 2014. 468-470: p. 565-80.

[0304] 12. Ludwig, S., et al., *MEK inhibition impairs influenza B virus propagation without emergence of resistant variants*. FEBS Lett, 2004. 561(1-3): p. 37-43.

[0305] 13. Sharma, R., et al., *Detection of phosphatidylserine-positive exosomes for the diagnosis of early-stage malignancies*. Br J Cancer, 2017. 117(4): p. 545-552.

[0306] 14. Azuma, K., et al., *Liver-specific gamma-glutamyl carboxylase-deficient mice display bleeding diathesis and short life span*. PLoS One, 2014.9(2): p. e88643.

[0307] 15. Hjortoe, G., et al., *Factor VIIa binding and internalization in hepatocytes*. J Thromb Haemost, 2005. 3(10): p. 2264-73.

[0308] 16. Meliopoulos, V. A., et al., *Host gene targets for novel influenza therapies elucidated by high-throughput RNA interference screens*. FASEB J, 2012. 26(4): p. 1372-86.

[0309] 17. Pleschka, S., et al., *Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade*. Nat Cell Biol, 2001. 3(3): p. 301-5.

[0310] 18. Makkoch, J., et al., *Human microRNAs profiling in response to influenza A viruses (subtypes pH1N1, H3N2, and H5N1)*. Exp Biol Med (Maywood), 2016. 241(4): p. 409-20.

[0311] 19. Wolf, S., et al., *MicroRNA Regulation of Human Genes Essential for Influenza A (H7N9) Replication*. PLoS One, 2016. 11(5): p. e0155104.

[0312] 20. Skalickova, S., et al., *Perspective of Use of Antiviral Peptides against Influenza Virus*. Viruses, 2015. 7(10): p. 5428-42.

[0313] 21. Matsubara, T., et al., *Sialic acid-mimic peptides as hemagglutinin inhibitors for anti-influenza therapy*. J Med Chem, 2010. 53(11): p. 4441-9.

[0314] 22. Wunderlich, K., et al., *Identification of a PA-binding peptide with inhibitory activity against influenza A and B virus replication*. PLoS One, 2009. 4(10): p. e7517.

[0315] 23. Ozcan, G., et al., *Preclinical and clinical development of siRNA-based therapeutics*. Adv Drug Deliv Rev, 2015. 87: p. 108-19.

[0316] 24. Mack, S., et al., *Pseudo-Ligandless Click Chemistry for Oligonucleotide Conjugation*. Curr Protoc Chem Biol, 2016. 8(2): p. 83-95.

[0317] 25. Paredes, E. and S. R. Das, *Click chemistry for rapid labeling and ligation of RNA*. Chembiochem, 2011. 12(1): p. 125-31.

[0318] 26. Zheng, Y. and P. A. Beal, *Synthesis and evaluation of an alkyne-modified ATP analog for enzymatic incorporation into RNA*. Bioorg Med Chem Lett, 2016. 26(7): p. 1799-802.

[0319] 27. Jain, N., et al., *Current ADC Linker Chemistry*. Pharm Res, 2015. 32(11): p. 3526-40.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 6

<210> SEQ ID NO 1
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (16)..(16)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(20)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature

-continued

<222> LOCATION: (25)..(26)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (29)..(29)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (32)..(32)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (36)..(36)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue

<400> SEQUENCE: 1

Ala Asn Ser Leu Leu Xaa Xaa Thr Lys Gln Gly Asn Leu Xaa Arg Xaa
1 5 10 15

Cys Ile Xaa Xaa Leu Cys Asn Lys Xaa Xaa Ala Arg Xaa Val Phe Xaa
20 25 30

Asn Asp Pro Xaa Thr Asp Tyr Phe Tyr Pro Lys Tyr Leu
35 40 45

<210> SEQ ID NO 2
<211> LENGTH: 46
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (7)..(8)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (11)..(11)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (20)..(21)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (26)..(27)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (30)..(30)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (33)..(33)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(35)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (40)..(40)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue

<400> SEQUENCE: 2

-continued

Ala Gly Ser Tyr Leu Leu Xaa Xaa Leu Phe Xaa Gly Asn Leu Xaa Lys
1 5 10 15

Xaa Cys Tyr Xaa Xaa Ile Cys Val Tyr Xaa Xaa Ala Arg Xaa Val Phe
20 25 30

Xaa Asn Xaa Val Val Thr Asp Xaa Phe Trp Arg Arg Tyr Lys
35 40 45

<210> SEQ ID NO 3
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (16)..(16)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(20)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (25)..(26)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (29)..(29)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (32)..(32)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue

<400> SEQUENCE: 3

Ala Asn Thr Phe Leu Xaa Xaa Val Arg Lys Gly Asn Leu Xaa Arg Xaa
1 5 10 15

Cys Val Xaa Xaa Thr Cys Ser Tyr Xaa Xaa Ala Phe Xaa Ala Leu Xaa
20 25 30

Ser Ser Thr Ala Thr Asp Val Phe Trp Ala Lys Tyr Thr
35 40 45

<210> SEQ ID NO 4
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (16)..(16)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:

-continued

<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(20)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (25)..(26)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (29)..(29)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(35)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue

<400> SEQUENCE: 4

Ala Asn Ala Phe Leu Xaa Xaa Leu Arg Pro Gly Ser Leu Xaa Arg Xaa
1 5 10 15

Cys Lys Xaa Xaa Gln Cys Ser Phe Xaa Xaa Ala Arg Xaa Ile Phe Lys
20 25 30

Asp Ala Xaa Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser
35 40 45

<210> SEQ ID NO 5
<211> LENGTH: 45
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (16)..(16)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(20)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (25)..(26)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (29)..(29)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (32)..(32)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (35)..(35)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue

<400> SEQUENCE: 5

Ala Asn Ala Phe Leu Xaa Xaa Leu Arg Gln Gly Ser Leu Xaa Arg Xaa
1 5 10 15

Cys Lys Xaa Xaa Gln Cys Ser Phe Xaa Xaa Ala Arg Xaa Ile Phe Xaa
20 25 30

Asp Ala Xaa Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser

-continued

```
        35                  40                  45

<210> SEQ ID NO 6
<211> LENGTH: 250
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (6)..(7)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (14)..(14)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (16)..(16)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(20)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (25)..(26)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (29)..(29)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (32)..(32)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (36)..(36)
<223> OTHER INFORMATION: X is gamma-carboxyglutamic acid residue

<400> SEQUENCE: 6

Ala Asn Ser Leu Leu Xaa Xaa Thr Lys Gln Gly Asn Leu Xaa Arg Xaa
1               5                   10                  15

Cys Ile Xaa Xaa Leu Cys Asn Lys Xaa Xaa Ala Arg Xaa Val Phe Xaa
            20                  25                  30

Asn Asp Pro Xaa Thr Asp Tyr Phe Tyr Pro Lys Tyr Leu Val Cys Leu
        35                  40                  45

Arg Ser Phe Gln Thr Gly Leu Phe Thr Ala Ala Arg Gln Ser Thr Asn
    50                  55                  60

Ala Tyr Pro Asp Leu Arg Ser Cys Val Asn Ala Ile Pro Asp Gln Cys
65                  70                  75                  80

Ser Pro Leu Pro Cys Asn Glu Asp Gly Tyr Met Ser Cys Lys Asp Gly
                85                  90                  95

Lys Ala Ser Phe Thr Cys Thr Cys Lys Pro Gly Trp Gln Gly Glu Lys
            100                 105                 110

Cys Glu Phe Asp Ile Asn Glu Cys Lys Asp Pro Ser Asn Ile Asn Gly
        115                 120                 125

Gly Cys Ser Gln Ile Cys Asp Asn Thr Pro Gly Ser Tyr His Cys Ser
    130                 135                 140

Cys Lys Asn Gly Phe Val Met Leu Ser Asn Lys Lys Asp Cys Lys Asp
145                 150                 155                 160

Val Asp Glu Cys Ser Leu Lys Pro Ser Ile Cys Gly Thr Ala Val Cys
                165                 170                 175
```

-continued

```
Lys Asn Ile Pro Gly Asp Phe Glu Cys Glu Cys Pro Glu Gly Tyr Arg
            180                 185                 190

Tyr Asn Leu Lys Ser Lys Ser Cys Glu Asp Ile Asp Glu Cys Ser Glu
        195                 200                 205

Asn Met Cys Ala Gln Leu Cys Val Asn Tyr Pro Gly Gly Tyr Thr Cys
    210                 215                 220

Tyr Cys Asp Gly Lys Lys Gly Phe Lys Leu Ala Gln Asp Gln Lys Ser
225                 230                 235                 240

Cys Glu Ser Arg His His His His His His
                245                 250
```

1. A method of targeting a stem cell said method comprising the step of contacting cells with a molecule comprising a payload linked to a gamma-carboxyglutamic acid component (GLA-component)

wherein said GLA-component comprises a GLA domain or an active fragment thereof, and said molecule does not comprise an active catalytic domain from a GLA protein.

2. A method according to claim 1, wherein GLA domain or active fragment thereof is independently selected from thrombin, factor VII, factor IX, factor X, protein C, protein S, protein Z, osteocalcin, matrix GLA protein (MGP), GAS6, transthyretin (TTR), inter-alpha-trypsin-inhibitor, periostin, proline rich gla 1 (PRRG1), proline rich gla 2 (PRRG2), proline rich gla 3(PRRG3), and proline rich gla 4 (PRRG4).

3. A method according to claim 2, wherein the GLA domain or active fragment thereof is from protein S.

4. A method according to claim 1, wherein the GLA-component further comprises an EGF domain, for example a calcium binding EGF domain.

5. A method according to claim 1, wherein the construct comprises an EGF domain selected from thrombin, factor VII, factor IX, factor X, protein C, protein S, protein Z, Osteocalcin, Matrix GLA protein, GAS6, Transthretin, Periostin, Proline rich GLA 1, Proline rich GLA 2, Proline rich GLA 3 and Proline rich GLA 4.

6. A method according to claim 5, wherein the EGF domain is from protein S.

7. A method according to claim 1, wherein the GLA-domain component further comprises a Kringle domain.

8. A method according to claim 7, wherein the Kringle domain is from a protein selected from the group comprising Activating transcription factor 2 (ATF); Factor XII (F12); thrombin (F2); Hyaluronan-binding protein 2 (HABP2); Hepatocyte growth factor (HGF); Hepatocyte growth factor activator (HGFAC); Kremen protein 1 (KREMEN1); KREMEN2; Lipoprotein(a) (LPA); LPAL2; Macrophage-stimulating protein (MSP or MST1); Phosphoinositide-3-kinase-interacting protein 1 (PIK3IP1); Tissue plasminogen activator (PLAT); Urokinase (PLAU); Plasmin (PLG); PRSS12; Tyrosine-protein kinase transmembrane receptor ROR1 (ROR1); and Tyrosine-protein kinase transmembrane receptor ROR2 (ROR2).

9. A method according to claim 1, wherein the GLA-component comprises a sequence shown in SEQ ID NO: 6.

10. A method according to claim 1, wherein the method is performed in vitro.

11. A method according to claim 1, wherein the delivery is to a cell in vivo, for example wherein the molecule comprising the GLA component and the payload are administered to a patient.

12. A method according to claim 1, wherein the molecule targets the exterior of the stem cell.

13. A method according to claim 1, wherein the molecule is internalised in the stem cell.

14. A method according to claim 1, wherein the cell is non-apoptotic.

15. A method according to claim 1, wherein the cell is apoptotic, for example a diseased stem cell.

16. A method according to claim 1, wherein the stem cell is an embryonic stem cell or an adult stems cells including progenitor cells, and haemotopoietic stem cells, myogenic stem cells, osteoprogenitor stem cells, neural stem cells, mesenchymal stem cell, such as satellite cells, radial glial cells, bone marrow stromal cells, periosteum, pancreatic progenitor cells, endothelial progenitor cells, blast cells and trophoblast stem cells.

17. A method according to claim 1, wherein the stem cell expresses a surface marker CD34.

18. A method according to claim 1, wherein the stem cell is negative for lineage positive surface markers (i.e. is Lin −ve).

19. A method according to claim 1, wherein the stem cell is CD90+ve, CD133+ve, CD105+ve, CD45+, Lin−ve, CD48−ve, and CD244−ve.

20. A method according to claim 1, wherein the stem cell is Lin−ve, CD34+ve, CD38−ve, CD45RA−ve, CD90+ve and CD49f_+ve.

21. A method according to claim 1, wherein the stem cell is non-cancerous.

22. A method according to claim 1, wherein the stem cell is a cancer stem cell.

23. A method according to claim 22, wherein the cancer stem cell is of epithelial origin.

24. A method according to claim 22, wherein cancer stem cell expresses a surface marker selected from CD44 (which is over expressed in at least breast, ovary, prostate, pancreatic, squamous, and bladder cancer), CD133 (which is over expressed in at least brain, colon, lung, prostate cancer and

medulloblastoma), CD24, CD90, CD271, CD4f, CD13 and combinations of two or more of the same.

25. A method according to claim **1**, wherein the stem cell is of haemotopoietic origin.

* * * * *