(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 August 2015 (06.08.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/116753 A1

(51) International Patent Classification:
C07K 16/30 (2006.01) *A61K 39/395* (2006.01)

(21) International Application Number:
PCT/US2015/013410

(22) International Filing Date:
29 January 2015 (29.01.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/933,001 29 January 2014 (29.01.2014) US

(71) Applicants: **DANA-FARBER CANCER INSTITUTE, INC.** [US/US]; 450 Brookline Avenue, Boston, MA 02215-5450 (US). **GENUS ONCOLOGY, LLC.** [US/US]; 1550 N. Cleveland Ave., Chicago, IL 60610 (US).

(72) Inventors: **KUFE, Donald, W.**; 179 Grove Street, Wellesley, MA 02482 (US). **KHARBANDA, Surender**; 8 Jacqueline Circle, Natick, MA 01760 (US).

(74) Agent: **HIGHLANDER, Steven, L.**; Parker Highlander PLLC, 1120 S. Capital of Texas Highway, Building One, Suite 200, Austin, TX 78746 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2015/116753 A1

(54) Title: ANTIBODIES AGAINST THE MUC1-C/EXTRACELLULAR DOMAIN (MUC1-C/ECD)

(57) Abstract: The present invention is directed to antibodies binding to MUC1-C/extracellular domain (MUC1-C/ECD) and methods of using such antibodies to treat cancers that express the MUC1 antigen.

DESCRIPTION

ANTIBODIES AGAINST THE MUC1-C/EXTRACELLULAR DOMAIN (MUC1-C/ECD)

BACKGROUND OF THE INVENTION

The application claims benefit of priority to U.S. Provisional Application Serial No. 61/933,001, filed January 29, 2014, the entire contents of which are hereby incorporated by reference.

The sequence listing that is contained in the file named "GENUP0032WO_ST25.txt", which is 40 KB (as measured in Microsoft Windows®) and was created on January 29, 2015, is filed herewith by electronic submission and is incorporated by reference herein.

1. Field of the Invention

The present invention relates generally to the fields of medicine, oncology and immunotherapeutics. More particularly, it concerns the development of immunoreagents for use in detecting and treating MUC1-positive cancers.

2. Background of the Invention

Mucins are extensively *O*-glycosylated proteins that are predominantly expressed by epithelial cells. The secreted and membrane-bound mucins form a physical barrier that protects the apical borders of epithelial cells from damage induced by toxins, microorganisms and other forms of stress that occur at the interface with the external environment. The transmembrane mucin 1 (MUC1) can also signal to the interior of the cell through its cytoplasmic domain. MUC1 has no sequence similarity with other membrane-bound mucins, except for the presence of a sea urchin sperm protein-enterokinase-agrin (SEA) domain (Duraisamy *et al.*, 2006). In that regard, MUC1 is translated as a single polypeptide and then undergoes autocleavage at the SEA domain Macao, 2006).

MUC1 has been studied extensively by the inventors and others for its role in cancer. As discussed above, human MUC1 is heterodimeric glycoprotein, translated as a single polypeptide and cleaved into N- and C-terminal subunits (MUC1-N and MUC1-C) in the endoplasmic reticulum (Ligtenberg *et al.*, 1992; Macao *et al.*, 2006; Levitin *et al.*, 2005). Aberrant overexpression of MUC1, as found in most human carcinomas (Kufe *et al.*, 1984), confers anchorage-independent growth and tumorigenicity (Li *et al.*, 2003a; Huang *et al.*,

2003; Schroeder *et al.*, 2004; Huang *et al.*, 2005). Other studies have demonstrated that overexpression of MUC1 confers resistance to apoptosis induced by oxidative stress and genotoxic anti-cancer agents (Yin and Kufe, 2003; Ren *et al.*, 2004; Raina *et al.*, 2004; Yin *et al.*, 2004; Raina *et al.*, 2006; Yin *et al.*, 2007).

The family of tethered and secreted mucins functions in providing a protective barrier of the epithelial cell surface. With damage to the epithelial layer, the tight junctions between neighboring cells are disrupted, and polarity is lost as the cells initiate a heregulin-induced repair program (Vermeer *et al.*, 2003). MUC1-N is shed from the cell surface (Abe and Kufe, 1989), leaving MUC1-C to function as a transducer of environmental stress signals to the interior of the cell. In this regard, MUC1-C forms cell surface complexes with members of the ErbB receptor family, and MUC1-C is targeted to the nucleus in the response to heregulin stimulation (Li *et al.*, 2001; Li *et al.*, 2003c). MUC1-C also functions in integrating the ErbB receptor and Wnt signaling pathways through direct interactions between the MUC1 cytoplasmic domain (CD) and members of the catenin family (Huang *et al.*, 2005; Li *et al.*, 2003c; Yamamoto *et al.*, 1997; Li *et al.*, 1998; Li *et al.*, 2001; Li and Kufe, 2001). Other studies have demonstrated that MUC1-CD is phosphorylated by glycogen synthase kinase 3β, c-Src, protein kinase Cδ, and c-Abl (Raina *et al.*, 2006; Li *et al.*, 1998; Li *et al.*, 2001; Ren *et al.*, 2002). Inhibiting any of the foregoing interactions represents a potential point of therapeutic intervention for MUC1-related cancers.

SUMMARY OF THE INVENTION

Thus, in accordance with the present invention, there is provided an antibody that binds selectively to MUC1-C/extracellular domain (MUC1-C/ECD) defined by SEQ ID NO: 2, wherein said antibody:

(a) is an IgG antibody;

(b) inhibits cancer cell growth;

(c) induces cancer cell death;

(d) comprises a variable heavy chain comprising CDR1, CDR2 and CDR3 regions each having 90% or more homology to SEQ ID NOS: 3, 4, and 5, or 6, 7 and 8, or 27, 28 and 29, or 42, 43 and 44, or 45, 46 and 47, or 48, 49 and 50, and a variable light chain comprising CDR1, CDR2 and CDR3 regions each having 90% or more homology to SEQ ID NOS: 9, 10 and 11 or 12, 13 and 14, or 30, 31 and 32, or 51, 52, and 53, or 54, 55 and 56, or 57, 58 and 59, respectively;

(e) comprises a variable heavy chain having 80% or more homology to SEQ ID NO: 15, 19, 23, 60, 64 or 68, and a variable light chain having 80% or more homology to SEQ ID NO: 17, 21, 25, 62, 66, 70, respectively; and/or

(f) comprises a variable heavy chain encoded by a nucleic acid having 70% or more homology to SEQ ID NO: 16, 20, 24, 61, 65 or 69, and a variable light chain encoded by a nucleic acid having 70% or more homology to SEQ ID NO: 18, 22, 26, 63, 67 or 71, respectively.

The heavy and light chains may have 85%, 90%, 95% or 99% homology to SEQ ID NO:15, 19, 23, 60, 64 or 68, and 17, 21, 25, 62, 66 or 70, respectively. The heavy and light chains may be encoded by nucleic acids having 85%, 90%, 95% or 99% homology to SEQ ID NO: 16, 20, 24, 61, 65 or 69, and 18, 22, 26, 63, 67 or 71, respectively. In one embodiment, the antibody does not bind to SEQ ID NO: 33, 34 or 35.

The antibody may be a single chain antibody, a a single domain antibody, a chimeric antibody, or a Fab fragment. The antibody may be a recombinant antibody having specificity for the MUC1-C/ECD and a distinct cancer cell surface antigen. The antibody may be a murine antibody, an IgG, a humanized antibody or a humanized IgG. The antibody may further comprise a label, such as a peptide tag, an enzyme, a magnetic particle, a chromophore, a fluorescent molecule, a chemilluminescent molecule, or a dye. The antibody may further comprise an antitumor drug linked thereto, such as linked to said antibody through a photolabile linker or an enzymatically-cleaved linker. The antitumor drug may be

a toxin, a radioisotope, a cytokine or an enzyme. The antibody may be conjugated to a nanoparticle or a liposome. The induction of cell death may comprise antibody-dependent cell cytotoxicity or complement-mediated cytoxocity.

There is also provided a method of treating cancer comprising contacting a MUC1-positive cancer cell in a subject with an antibody as described above. The MUC1-positive cancer cell may be a solid tumor cell, such as a lung cancer cell, brain cancer cell, head & neck cancer cell, breast cancer cell, skin cancer cell, liver cancer cell, pancreatic cancer cell, stomach cancer cell, colon cancer cell, rectal cancer cell, uterine cancer cell, cervical cancer cell, ovarian cancer cell, testicular cancer cell, skin cancer cell, or esophageal cancer cell, or may be a leukemia or myeloma such as acute myeloid leukemia, chronic myelogenous leukemia or multiple myeloma.

The method may further comprise contacting said MUC1-positive cancer cell with a second anti-cancer agent or treatment, such as chemotherapy, radiotherapy, immunotherapy, hormonal therapy, or toxin therapy. The second anti-cancer agent or treatment may inhibit an intracellular MUC1 function. The second anti-cancer agent or treatment may be given at the same time as said first agent, or given before and/or after said first agent. The MUC1-positive cancer cell may be a metastatic cancer cell, a multiply drug resistant cancer cell or a recurrent cancer cell.

The antibody may be a single chain antibody, a a single domain antibody, a chimeric antibody, or a Fab fragment. The antibody may be a recombinant antibody having specificity for the MUC1-C/ECD and a distinct cancer cell surface antigen. The antibody may be a murine antibody, an IgG, a humanized antibody or a humanized IgG. The antibody may further comprise a label, such as a peptide tag, an enzyme, a magnetic particle, a chromophore, a fluorescent molecule, a chemilluminescent molecule, or a dye. The antibody may further comprise an antitumor drug linked thereto, such as linked to said antibody through a photolabile linker or an enzymatically-cleaved linker. The antitumor drug may be a toxin, a radioisotope, a cytokine or an enzyme. The antibody may be conjugated to a nanoparticle or a liposome.

Also provided is a fusion protein comprising:

(i) a first single chain antibody that binds selectively to MUC1-C/extracellular domain (MUC1-C/ECD) defined by SEQ ID NO: 2, wherein said antibody:

(a) is an IgG antibody;

(b) inhibits cancer cell growth;

(c) induces cancer cell death;

(d) comprises a variable heavy chain comprising CDR1, CDR2 and CDR3 regions each having 90% or more homology to SEQ ID NOS: 3, 4, and 5, or 6, 7 and 8, or 27, 28 and 29, or 42, 43 and 44, or 45, 46 and 47, or 48, 49 and 50, and a variable light chain comprising CDR1, CDR2 and CDR3 regions each having 90% or more homology to SEQ ID NOS: 9, 10 and 11 or 12, 13 and 14, or 30, 31 and 32, or 51, 52, and 53, or 54, 55 and 56, or 57, 58 and 59, respectively;

(e) comprises a variable heavy chain having 80% or more homology to SEQ ID NO: 15, 19, 23, 60, 64 or 68, and a variable light chain having 80% or more homology to SEQ ID NO: 17, 21, 25, 62, 66, 70, respectively; and/or

(f) comprises a variable heavy chain encoded by a nucleic acid having 70% or more homology to SEQ ID NO: 16, 20, 24, 61, 65 or 69, and a variable light chain encoded by a nucleic acid having 70% or more homology to SEQ ID NO: 18, 22, 26, 63, 67 or 71, respectively; and

(ii) a second single chain antibody that binds to a T or B cell.

The second single chain antibody may bind to to CD3, to a T cell or to a B cell. The fusion protein may further comprise a label or a therapeutic moiety. In one embodiment, the first single chain antibody does not bind to SEQ ID NO: 3, 4 or 5.

In another embodiment, there is provided a chimeric antigen receptor comprising:

(i) an ectodomain comprising single chain antibody variable region that binds selectively to MUC1-C/extracellular domain (MUC1-C/ECD) defined by SEQ ID NO: 2, wherein said antibody:

(a) is an IgG antibody;

(b) inhibits cancer cell growth;

(c) induces cancer cell death;

(d) comprises a variable heavy chain comprising CDR1, CDR2 and CDR3 regions each having 90% or more homology to SEQ ID NOS: 3, 4, and 5, or 6, 7 and 8, or 27, 28 and 29, or 42, 43 and 44, or 45, 46 and 47, or 48, 49 and 50, and a variable light chain comprising CDR1, CDR2 and CDR3 regions each having 90% or more homology to SEQ ID NOS: 9, 10 and 11 or 12, 13 and 14, or 30, 31 and 32, or 51, 52, and 53, or 54, 55 and 56, or 57, 58 and 59, respectively;

(e) comprises a variable heavy chain having 80% or more homology to SEQ ID NO: 15, 19, 23, 60, 64 or 68, and a variable light chain having 80% or more homology to SEQ ID NO: 17, 21, 25, 62, 66, 70, respectively; and/or

(f) comprises a variable heavy chain encoded by a nucleic acid having 70% or more homology to SEQ ID NO: 16, 20, 24, 61, 65 or 69, and a variable light chain encoded by a nucleic acid having 70% or more homology to SEQ ID NO: 18, 22, 26, 63, 67 or 71, respectively; and

with a flexible hinge attached at the C-terminus of said single chain antibody variable region;

(ii) a transmembrane domain; and

(iii) an endodomain,

wherein said endodomain comprises a signal transduction function when said single-chain antibody variable region is engaged with MUC1.

The transmembrane and endodomains may be derived from the same molecule. The endodomain comprises a CD3-zeta domain or a high affinity FcεRI. The flexible hinge may be from CD8α or Ig. Also provided is a cell expressing this chimeric antigen receptor. In one embodiment, the single chain variable region does not bind to SEQ ID NO: 3, 4 or 5.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one." The word "about" means plus or minus 5% of the stated number.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIGS. 1A-B. (FIG. 1A) Amino acid sequence of MUC1-C/ECD (58 aa: SEQ ID NO: 2). (FIG. 1B) The construct mFc-linker-MUC1-C/ECD-signal sequence was stably expressed in CHO-K1 cells. The protein is purified from the soup of CHO cells and used to immunize mice. Same protein was used to boost the mice. Serum titers were determined and following positive titer, spleen was fused to generate hybridomas.

FIG. 2. (RESERVED)

FIG. 3. Three overlapping peptides from the MUC1-C/ECD protein were synthesized to use in linear epitope mapping for positive Mab clones using ELISA assays. The sequences for the three peptides are: P1:SVVVQLTLAFREGTINVHDVET (SEQ ID NO: 33); P2:VETQFNQYKTEAASRYNLTISD (SEQ ID NO: 34); P3:TISDVSVSDVPFPFSAQSGAG (SEQ ID NO: 35).

FIG. 4. MUC1-C/ECD cDNAs with several point mutants (as described above) were generated (ECD-WT – SEQ ID NO: 2; ECD-L6A – SEQ ID NO: 36; ECD-L8A – SEQ ID NO: 37; ECD-L6,8A - SEQ ID NO: 38; ECD-Q23V – SEQ ID NO 39; ECD-Q26V – SEQ ID NO: 40; ECD-N36A – SEQ ID NO: 41). CHO-K1 cells were individually transfected with each of these cDNAs to express and secrete protein. Proteins were purified and will be used to define conformational epitope for positive Mab clones using ELISA assays. The positive control will be wt mFc-MUC1-C/ECD protein expressed and purified from CHO-K1 cells.

FIG. 5. (RESERVED)

FIGS. 6A-C. (FIG. 6A) Following immunizations, mice were bled and the immune sera was analyzed by immunoblotting with MUC1-C/ECD protein. MUC1-C/CD protein was used as negative control. (FIG. 6B) MAb clone 8E1, 8F1, 2A6 and 6A6 were analyzed by immunoblotting with mFc- and hFc-MUC1-C/ECD protein purified from CHO-K1 cells. Secondary Ab: anti-mouse-HRP (F(ab)2 specific) (1:5000) (FIG. 6C) MUC1-negative 293T cells, NSCLC H-1975 and MCF-7 & ZR-75-1 breast carcinoma cell lysates were analyzed by immunoblotting with clone 6A6 antibody.

FIG. 7. MAb clones 8E1, 6A6, 2G11 and 2H11 were analyzed by immunoblotting with mFc-MUC1-C/ECD (p58-mFc), MUC1-SEA domain (p62-mFc) and p62 only proteins produced and purified from bacteria.

FIG. 8. MAb clones 8E1, 6A6, 2G11 and 2H11 were analyzed by immunoblotting with mFc-MUC1-C/ECD (p58-mFc), MUC1-SEA domain (p62-mFc) and p62 only proteins purified from bacteria.

FIG. 9. MAb clones 8E1, 6A6, 2G11 and 2H11 were analyzed by immunoblotting with mFc-MUC1-C/ECD (p58-mFc), MUC1-SEA domain (p62-mFc) and p62 only proteins purified from bacteria.

FIG. 10. MAb clones 8E1, 6A6 and positive control DF3 were analyzed by Flow cytometry using MV4-11, MOLM-14 (AML); U266, RPMI8226 (Multiple Myeloma) and primary AML cells.

FIG. 11. MAb clones 8E1 and 6A6 were analyzed by Flow cytometry using K562/CsiRNA and K562/MUC1siRNA CML cells.

FIG. 12. Internalization of FITC-labeled anti-MUC1-C/ECD MAb 8E1 using H-1975 non-small cell lung carcinoma cells at 37°C for 3 hours. Capping and punctate staining in late endosoma/lysosomal vesicles (arrows).

FIG. 13. Internalization of FITC-labeled anti-MUC1-C/ECD MAb 6A6 using H-1975 non-small cell lung carcinoma cells at 37 °C for 3 hrs. Capping and punctate staining in late endosomal/lysosomal vesicles (arrows).

FIG. 14. Internalization of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 6A6 using ZR-75-1 breast carcinoma cells at 37 °C for 3 hours.

FIG. 15. Little, if any, staining of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 6A6 using MUC1-negative HEK-293T cells at 4 °C or 37 °C for 3 hours.

FIG. 16. Internalization of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 8E1 using MOLM-14 AML cells at 37 °C for 3 hours.

FIG. 17. Internalization of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 8E1 using K562/MUC1siRNA and K562/CsiRNA CML cells at 37 °C for 3 hrs.

FIG. 18. Plasma membrane staining of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 6A6 and its colocalization with RFP early endosomal marker in ZR-75-1 breast carcinoma cells at 4 °C.

FIG. 19. Internalization of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 6A6 and its colocalization with RFP-endocyte marker in ZR-75-1 breast carcinoma cells at 37 °C.

FIG. 20. Internalization of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 6A6 and its colocalization (arrows) with RFP-endocyte marker in H-1975 NSCLC cells at 37 °C.

FIG. 21. Internalization of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 8E1 and its colocalization (arrows) with RFP-endocyte marker in H-1975 NSCLC cells at 37 °C.

FIG. 22. Internalization of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 8E1 and its colocalization (arrows) with RFP-lysosomal marker in H-1975 NSCLC cells at 37 °C.

FIG. 23. Internalization of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 6A6 and its colocalization (arrows) with RFP-lysosomal marker in H-1975 NSCLC cells at 37 °C.

FIG. 24. Internalization of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 6A6 using mouse NSCLC (KW-814) cells at 37 °C for 3 hours.

FIG. 25. Internalization of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 8E1 and 6A6 using K562 CML cells at 37 °C for 3 hours. IgG labeling was used as negative control. RFP-Lysosomal IF was used as positive control.

FIG. 26. Immunofluorescence of RFP-endocyte marker transfected H-1975 NSCLC cells at 37 °C (right panel). Staining of RFP-endocyte marker transfectedH-1975 cells incubated with Alexa Fluor 488-labeled isotype control IgG antibody (middle panel).

FIG. 27. Immunofluorescence of RFP-lysosomal marker transfected H-1975 NSCLC cells at 37 °C (right panel). Staining of RFP-lysosomal marker transfected H-1975 cells incubated with Alexa Fluor 488-labeled isotype control IgG antibody (middle panel).

FIG. 28. Internalization of Alexa Fluor 488-labeled anti-MUC1-C/ECD antibody 8E1 and its co-localization with RFP-lysosomal marker in H-1975 NSCLC cells at 37 °C.

FIG. 29. Selection of antibody clones from mouse 384 immunization.

FIG. 30. Interaction of anti-MUC1-C/ECD antibodies with MUC1 proteins made in bacteria.

FIG. 31. HCT-116/vector and HCT-116/MUC1 cells were incubated with 7B8 or mouse IgG for 30 min, washed, incubated with goat anti-mouse immunoglobulin-flourescein-conjugated antibody (Santa Cruz Biotechnology), and fixed in 1% formaldehyde/PBS. Reactivity was detected by immunofluorescence FACScan. The results demonstrate that in contrast to HCT-116/vector cells (MUC1-negative), strong reactivity of 7B8 was seen in MUC1-positive HCT-116/MUC1 cells.

FIG. 32. ZR-75-1 breast carcinoma cells were incubated with 7B8 or mouse IgG for 30 min, washed, incubated with goat anti-mouse immunoglobulin-flourescein-conjugated antibody (Santa Cruz Biotechnology), and fixed in 1% formaldehyde/PBS. Reactivity was detected by immunofluorescence FACScan. The results demonstrate that in contrast to IgG control, 7B8 reacted strongly with ZR-75-1 cells.

FIG. 33. ZR-75-1 breast carcinoma cells, MCF-7 breast carcinoma cells and HCT-116/MUC1 colon carcinoma cells were incubated with 7B8 for 30 min, washed, incubated with goat anti-mouse immunoglobulin-flourescein-conjugated antibody (Santa Cruz Biotechnology), and fixed in 1% formaldehyde/PBS. Reactivity was detected by immunofluorescence FACScan. The results demonstrate that 7B8 reacted strongly with all these cell types.

FIG. 34. MDA-MB-468/CshRNA and MDA-MB-468/MUC1shRNA triple breast carcinoma cells MCF-7 breast carcinoma cells were incubated either with IgG or with 7B8 for 30 min, washed, incubated with goat anti-mouse immunoglobulin-flourescein-conjugated antibody (Santa Cruz Biotechnology), and fixed in 1% formaldehyde/PBS. Reactivity was detected by immunofluorescence FACScan. The results demonstrate that in contrast to MDA-MB-468/MUC1shRNA (MUC1-down regulated) cells, strong reactivity of 7B8 was seen in MUC1-positive MDA-MB-468/CshRNA cells.

FIG. 35. ZR-75-1 breast carcinoma cells, MCF-7 breast carcinoma cells and HCT-116/MUC1 colon carcinoma cells were incubated with 2B11 for 30 min, washed, incubated with goat anti-mouse immunoglobulin-flourescein-conjugated antibody (Santa Cruz Biotechnology), and fixed in 1% formaldehyde/PBS. Reactivity was detected by immunofluorescence FACScan. The results demonstrate that 2B11 reacted strongly with all three cell types.

FIG. 36. ZR-75-1 breast carcinoma cells, MCF-7 breast carcinoma cells and HCT-116/MUC1 colon carcinoma cells were incubated with 4G5 for 30 min, washed, incubated with goat anti-mouse immunoglobulin-flourescein-conjugated antibody (Santa Cruz Biotechnology), and fixed in 1% formaldehyde/PBS. Reactivity was detected by immunofluorescence FACScan. The results demonstrate that 4G5 reacted strongly with all three cell types.

FIG. 37. HCT116/MUC1-wild-type and HCT116/MUC1-CQC→AQA mutant cells 7B8 for 30 min, washed, incubated with goat anti-mouse immunoglobulin-flourescein-conjugated antibody (Santa Cruz Biotechnology), and fixed in 1% formaldehyde/PBS. Reactivity was detected by immunofluorescence FACScan. The results demonstrate

strong reactivity of 7B8 with both cell types indicating that MUC1-C/MUC1-C homodimerization is not required for binding of 7B8.

FIG. 38. ELISA assays were performed by coating plates with the antigen and reactivity with multiple different concentrations of purified antibodies 7B8, 4G5 and 2B11. The binding curves demonstrate similar reactivity with all the three antibodies.

FIG. 39. Summary of results from 7B8, 2B11 and 4G5 clones.

FIG. 40. ELISA assays were performed by coating plates with the wild type antigen and different mutant proteins (LTL→ATA; QFNQ→AFNQ and QFNA) reactivity with different purified antibodies 7B8, 4G5 and 2B11. The sensitivity of different antibody clones to mutant proteins is described in the figure.

FIG. 41. Sequence analysis of the CDR regions from heavy and light chains of 2B11 clone.

FIG. 42. Sequence analysis of the CDR regions from heavy and light chains of 4G5 clone.

FIG. 43. Sequence analysis of the CDR regions from heavy and light chains of 7B8 clone.

FIG. 44. IHC analysis using 7B8 antibody in FFPE section of colon carcinoma.

FIG. 45. The performance of 7B8 in Western blotting using whole cell lysates was analyzed and the results demonstrate the specific reactivity of the anti-MUC1-C/ECD antibodies with MUC1-C protein. 293T cells do not express MUC1 and hence negative in the western blot analysis.

FIG. 46. ELISA assays were performed using 2B11, 7B8 and 4G5 antibodies. The results demonstrate no inhibition of reactivity of three of these antibodies with any of the peptide indicating that the epitope is not linear.

FIG. 47. Linear epitope mapping of 7B8 and 3D1 clones: overlapping peptides. Three overlapping peptides spanning the entire MUC1-C/ECD region (58 amino acids) were synthesized. ELISA assays were performed coating the plates with the MUC1-C/ECD antigen and incubating with 7B8 or 3D1 purified antibodies in the presence or absence of P1, P2 or P3 peptides.

FIGS. 48A-B: Conformational epitope mapping of 7B8 and 3D1 using point mutants. Eight critical individual point mutants were generated in the MUC1-C/ECD region and respective proteins were purified. Separate 96-well plates were coated with these eight purified proteins. 7B8 and 3D1 purified antibodies were incubated with each of these plates and ELISA assays were performed.

FIG. 49. Conformational epitope mapping of 7B8 and 3D1. ZR-75-1 breast carcinoma cells were obtained from ATCC and maintained in DMEM with 10% heat-inactivated fetal bovine serum plus antibiotics. Cells were incubated either with purified MUC1-C/ECD protein or with corresponding volume of PBS. Following incubation, 7B8 or 3D1 antibodies were added and the cells prepared for flow cytometric analysis. DF3 antibody was used as a positive control and anti-MUC1-C/CD antibody (CD1) was used as a negative control. Following appropriate steps, cells were analyzed by FLOW.

FIG. 50. Monoclonal antibody sequencing of hybridoma 441.3.3D1.D6.D11.B1.F10. Total RNA was extracted from frozen hybridoma cells and cDNA was synthesized from the RNA. PCR was then performed to amplify the variable regions (heavy and light chains) of the antibody, which were then cloned into a standard cloning vector separately and sequenced. Five single colonies with correct VH and VL insert sizes were sequenced. Leader amino acid sequence (heavy chain) and leader amino acid sequence (light chain) are shown in the figure in normal text, CDR's are underlined, and framework regions are in bold. Heavy chain = SEQ ID NO: 82; Light chain = SEQ ID NO: 83.

FIGS. 51A-B. Antibody-Drug Conjugates (ADC) of 7B8 and 3D1 antibodies and biological activity of these antibody-drug-conjugates.

FIGS. 52A-B. Agarose gel electrophoresis of total RNA of the provided hybridoma 536064-1. (FIG. 52A) DNA Marker III. (FIG. 52B) Lane M, DNA Marker III; Lane R, total RNA of 536064-1.

FIG. 53. Agarose gel electrophoresis of PCR products of 536064-1. Lane M, DNA Marker III; Lane 1, V_H 536064-1; Lane 2, V_L 536064-1.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The inventors have raised antibodies against a 58 amino acid non-shed portion of the external domain of the MUC1-C protein. These antibodies have ben demonstrated to bind selectively to this portion of MUC1-C, and as such, present an opportunity to block the activity of MUC1 following cleavage of the N-terminal region. They also can be used to deliver therapeutic payloads to MUC1-expressing cancer cells even following the cleavage of the N-terminal MUC1 domain. These and other aspects of the invention are described in greater detail below.

I. MUC1

A. Structure

MUC1 is a mucin-type glycoprotein that is expressed on the apical borders of normal secretory epithelial cells (Kufe *et al.*, 1984). MUC1 forms a heterodimer following synthesis as a single polypeptide and cleavage of the precursor into two subunits in the endoplasmic reticulum (Ligtenberg *et al.*, 1992). The cleavage may be mediated by an autocatalytic process (Levitan *et al.*, 2005). The >250 kDa MUC1 N-terminal (MUC1 N-ter, MUC1-N) subunit contains variable numbers of 20 amino acid tandem repeats that are imperfect with highly conserved variations and are modified by O-linked glycans (Gendler *et al.*, 1988; Siddiqui *et al.*, 1988). MUC1-N is tethered to the cell surface by dimerization with the ~23 kDa C-terminal subunit (MUC1 C-ter, MUC1-C), which includes a 58 amino acid extracellular region, a 28 amino acid transmembrane domain and a 72-amino acid cytoplasmic domain (CD) (Merlo *et al.*, 1989). It is the 58 amino acid portion of the MUC1-C/ECD (italics) to which antibodies of the present invention bind. The human MUC1-C sequence is shown below:

*SVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG*VPG WGIALLVLVCVLVALAIVYLIALAVCQCRRKNYGQLDIFP**ARDTYHPMSEYPTYHT HGRYVPPSSTDRSPYEKVSAGNGGSSLSYTNPAVAATSANL** (SEQ ID NO: 1)

The bold sequence indicates the CD, and the underlined portion is an oligomer-inhibiting peptide. With transformation of normal epithelia to carcinomas, MUC1 is aberrantly overexpressed in the cytosol and over the entire cell membrane (Kufe *et al.*, 1984; Perey *et al.*, 1992). Cell membrane-associated MUC1 is targeted to endosomes by clathrin-mediated

endocytosis (Kinlough *et al.*, 2004). In addition, MUC1-C, but not MUC1-N, is targeted to the nucleus (Baldus *et al.*, 2004; Huang *et al.*, 2003; Li *et al.*, 2003a; Li *et al.*, 2003b; Li *et al.*, 2003c; Wei *et al.*, 2005; Wen *et al.*, 2003) and mitochondria (Ren *et al.*, 2004).

B. Function

MUC1-C interacts with members of the ErbB receptor family (Li *et al.*, 2001b; Li *et al.*, 2003c; Schroeder *et al.*, 2001) and with the Wnt effector, β-catenin (Yamamoto *et al.*, 1997). The epidermal growth factor receptor and c-Src phosphorylate the MUC1 cytoplasmic domain (MUC1-CD) on Y-46 and thereby increase binding of MUC1 and β-catenin (Li *et al.*, 2001a; Li *et al.*, 2001b). Binding of MUC1 and β-catenin is also regulated by glycogen synthase kinase 3β and protein kinase Cδ (Li *et al.*, 1998; Ren *et al.*, 2002). MUC1 colocalizes with β-catenin in the nucleus (Baldus *et al.*, 2004; Li *et al.*, 2003a; Li *et al.*, 2003c; Wen *et al.*, 2003) and coactivates transcription of Wnt target genes (Huang *et al.*, 2003). Other studies have shown that MUC1 also binds directly to p53 and regulates transcription of p53 target genes (Wei *et al.*, 2005). Notably, overexpression of MUC1-C is sufficient to induce anchorage-independent growth and tumorigenicity (Huang *et al.*, 2003; Li *et al.*, 2003b; Ren *et al.*, 2002; Schroeder *et al.*, 2004).

II. Producing Monoclonal Antibodies

A. General Methods

Antibodies to the MUC1-C/ECD may be produced by standard methods as are well known in the art (see, *e.g.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; U.S. Patent 4,196,265). The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. The first step for both these methods is immunization of an appropriate host or identification of subjects who are immune due to prior natural infection. As is well known in the art, a given composition for immunization may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine. As also is well known in the art, the immunogenicity of a particular immunogen

composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, also may be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate MAbs.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens or lymph nodes, or from circulating blood. The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized or human or human/mouse chimeric cells. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions. One particular murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line. More recently, additional fusion partner lines for use with human B cells have been described, including KR12 (ATCC CRL-8658; K6H6/B5 (ATCC CRL-1823 SHM-D33 (ATCC CRL-

1668) and HMMA2.5 (Posner *et al.*, 1987). The antibodies in this invention were generated using the SP2/0/mIL-6 cell line, an IL-6 secreting derivative of the SP2/0 line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.* (1977). The use of electrically induced fusion methods also is appropriate (Goding, pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, infused cells (particularly the infused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine. Ouabain is added if the B cell source is an Epstein Barr virus (EBV) transformed human B cell line, in order to eliminate EBV transformed lines that have not fused to the myeloma.

The preferred selection medium is HAT or HAT with ouabain. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells. When the source of B cells used for fusion is a line of EBV-transformed B cells, as here, ouabain is also used for drug selection of hybrids as EBV-transformed B cells are susceptible to drug killing, whereas the myeloma partner used is chosen to be ouabain resistant.

Culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays dot immunobinding assays, and the like.

The selected hybridomas are then serially diluted or single-cell sorted by flow cytometric sorting and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide mAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into an animal (*e.g.*, a mouse). Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection. When human hybridomas are used in this way, it is optimal to inject immunocompromised mice, such as SCID mice, to prevent tumor rejection. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. Alternatively, human hybridoma cells lines can be used *in vitro* to produce immunoglobulins in cell supernatant. The cell lines can be adapted for growth in serum-free medium to optimize the ability to recover human monoclonal immunoglobulins of high purity.

MAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as FPLC or affinity chromatography. Fragments of the monoclonal antibodies of the invention can be obtained from the purified monoclonal antibodies by methods which include digestion with enzymes, such as pepsin or papain, and/or by cleavage of disulfide bonds by chemical reduction. Alternatively, monoclonal antibody fragments encompassed by the present invention can be synthesized using an automated peptide synthesizer.

It also is contemplated that a molecular cloning approach may be used to generate monoclonals. For this, RNA can be isolated from the hybridoma line and the antibody genes obtained by RT-PCR and cloned into an immunoglobulin expression vector. Alternatively, combinatorial immunoglobulin phagemid libraries are prepared from RNA isolated from the cell lines and phagemids expressing appropriate antibodies are selected by panning using

viral antigens. The advantages of this approach over conventional hybridoma techniques are that approximately 10^4 times as many antibodies can be produced and screened in a single round, and that new specificities are generated by H and L chain combination which further increases the chance of finding appropriate antibodies.

Other U.S. patents, each incorporated herein by reference, that teach the production of antibodies useful in the present invention include U.S. Patent 5,565,332, which describes the production of chimeric antibodies using a combinatorial approach; U.S. Patent 4,816,567 which describes recombinant immunoglobulin preparations; and U.S. Patent 4,867,973 which describes antibody-therapeutic agent conjugates.

B. Antibodies of the Present Invention

Antibodies according to the present invention may be defined, in the first instance, by their binding specificity, which in this case is for MUC1-C/ECD, and in particular:

SVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG

(SEQ ID NO: 2). Those of skill in the art, by assessing the binding specificity/affinity of a given antibody using techniques well known to those of skill in the art, can determine whether such antibodies fall within the scope of the instant claims.

In on embodiment, the antibody is an Immunoglobulin G (IgG) antibody isotype. Representing approximately 75% of serum immunoglobulins in humans, IgG is the most abundant antibody isotype found in the circulation. IgG molecules are synthesized and secreted by plasma B cells. There are four IgG subclasses (IgG1, 2, 3, and 4) in humans, named in order of their abundance in serum (IgG1 being the most abundant). The range from having high to no affinity for the Fc receptor.

IgG is the main antibody isotype found in blood and extracellular fluid allowing it to control infection of body tissues. By binding many kinds of pathogens—representing viruses, bacteria, and fungi—IgG protects the body from infection. It does this via several immune mechanisms: IgG-mediated binding of pathogens causes their immobilization and binding together via agglutination; IgG coating of pathogen surfaces (known as opsonization) allows their recognition and ingestion by phagocytic immune cells; IgG activates the classical pathway of the complement system, a cascade of immune protein production that results in pathogen elimination; IgG also binds and neutralizes toxins. IgG also plays an important role in antibody-dependent cell-mediated cytotoxicity (ADCC) and intracellular antibody-

mediated proteolysis, in which it binds to TRIM21 (the receptor with greatest affinity to IgG in humans) in order to direct marked virions to the proteasome in the cytosol. IgG is also associated with Type II and Type III Hypersensitivity. IgG antibodies are generated following class switching and maturation of the antibody response and thus participate predominantly in the secondary immune response. IgG is secreted as a monomer that is small in size allowing it to easily perfuse tissues. It is the only isotype that has receptors to facilitate passage through the human placenta. Along with IgA secreted in the breast milk, residual IgG absorbed through the placenta provides the neonate with humoral immunity before its own immune system develops. Colostrum contains a high percentage of IgG, especially bovine colostrum. In individuals with prior immunity to a pathogen, IgG appears about 24–48 hours after antigenic stimulation.

In another aspect, the antibodies may be defined by their variable sequences that determine their binding specificity. Examples are provided below:

Table 1 – Antibody CDR Sequences

Antibody	Heavy Chain	Light Chain
6A6 CDR1	GFSLTTYG.... SEQ ID NO: 3	QSLVHNNGDTY. SEQ ID NO: 9
6A6 CDR2	IWSDGST... SEQ ID NO: 4	KVSNRFS.. SEQ ID NO: 10
6A6 CDR3	AKNYLGSLDY SEQ ID NO: 5	SQTTHVPLT SEQ ID NO: 11
8F1/8E1 CDR1	EYEFPSHD.... SEQ ID NO: 6	QSLVHSNGNTY. SEQ ID NO: 12
8F1/8E1 CDR2	INSDGGST.. SEQ ID NO: 7	KVSNRFS… SEQ ID NO: 13
8F1/8E1 CDR3	VRLYYGNVMDY SEQ ID NO: 8	SQSTHVPLT SEQ ID NO: 14
2H11 CDR1	GYTFTGYSMH SEQ ID NO: 27	RSSQSLVHSNGNTYLH SEQ ID NO: 30
2H11 CDR3	WINTETGEPTYDDFKG SEQ ID NO: 28	KVSNRFS SEQ ID NO: 31
2H11 CDR3	GTGGDD SEQ ID NO: 29	SQGTHVPPT SEQ ID NO: 32
7B8 CDR1	GHTFTSYWMH SEQ ID NO: 42	CRASESVQYSGTSLMH SEQ ID NO: 51
7B8 CDR3	EINPSNGRTYYNENFKT SEQ ID NO: 43	GASNVET SEQ ID NO: 52
7B8 CDR3	DGDYVSGFAY SEQ ID NO: 44	QQNWKVPWT SEQ ID NO: 53
4G5 CDR1	GFSLSTSGMGVS SEQ ID NO: 45	CKASQSVGNYVA SEQ ID NO: 54
4G5 CDR2	HIYWDDKRYNPSLKS SEQ ID NO: 46	FASNRYS SEQ ID NO: 55
4G5 CDR3	GVSSWFPY SEQ ID NO: 47	QQHYIFPYT SEQ ID NO: 56
2B11 CDR1	GFTFNYFWIE SEQ ID NO: 48	CKASENVGTYVS SEQ ID NO: 57
2B11 CDR2	EILPGTGSTNYNEKFKG SEQ ID NO: 49	GASNRYT SEQ ID NO: 58
2B11 CDR3	YDYTSSMDY SEQ ID NO: 50	GQSYSYPWT SEQ ID NO: 59
3D1 CDR1	NFWMN SEQ ID NO: 76	RASQSIGTSIH SEQ ID NO: 79
3D1 CDR2	QIYPGDGDTNYNGKFKG SEQ ID NO: 77	YASESIS SEQ ID NO: 80
3D1 CDR3	SYYRSAWFAY SEQ ID NO: 78	QQSNNWPLT SEQ ID NO: 81

Furthermore, the antibodies sequences may vary from the sequences provided above, optionally using methods discussed in greater detail below. For example, amino sequences

may vary from those set out above in that (a) the variable regions may be segregated away from the constant domains of the light chains, (b) the amino acids may vary from those set out above while not drastically affecting the chemical properties of the residues thereby (so-called conservative substitutions), (c) the amino acids may vary from those set out above by a given percentage, *e.g.*, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% homology. Alternatively, the nucleic acids encoding the antibodies may (a) be segregated away from the constant domains of the light chains, (b) vary from those set out above while not changing the residues coded thereby, (c) may vary from those set out above by a given percentage, *e.g.*, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% homology, or (d) vary from those set out above by virtue of the ability to hybridize under high stringency conditions, as exemplified by low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50°C to about 70°C.

In making conservative changes in amino acid sequence, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: basic amino acids: arginine (+3.0), lysine (+3.0), and histidine (-0.5); acidic amino acids: aspartate (+3.0 ± 1), glutamate (+3.0 ± 1), asparagine (+0.2), and glutamine (+0.2); hydrophilic, nonionic amino acids: serine (+0.3), asparagine (+0.2), glutamine (+0.2), and threonine (-0.4), sulfur containing amino acids: cysteine (-1.0) and methionine (-1.3); hydrophobic, nonaromatic amino acids: valine (-1.5), leucine (-1.8), isoleucine (-1.8), proline (-0.5 ± 1), alanine (-0.5), and glycine (0); hydrophobic, aromatic amino acids: tryptophan (-3.4), phenylalanine (-2.5), and tyrosine (-2.3).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity and produce a biologically or immunologically modified protein. In such

changes, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those that are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

As outlined above, amino acid substitutions generally are based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take into consideration the various foregoing characteristics are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

C. Engineering of Antibody Sequences

In various embodiments, one may choose to engineer sequences of the identified antibodies for a variety of reasons, such as improved expression, improved cross-reactivity, diminished off-target binding or abrogation of one or more natural effector functions, such as activation of complement or recruitment of immune cells (*e.g.*, T cells). In particular, IgM antibodies may be converted to IgG antibodies. The following is a general discussion of relevant techniques for antibody engineering.

Hybridomas may be cultured, then cells lysed, and total RNA extracted. Random hexamers may be used with RT to generate cDNA copies of RNA, and then PCR performed using a multiplex mixture of PCR primers expected to amplify all human variable gene sequences. PCR product can be cloned into pGEM-T Easy vector, then sequenced by automated DNA sequencing using standard vector primers. Assay of binding and neutralization may be performed using antibodies collected from hybridoma supernatants and purified by FPLC, using Protein G columns. Recombinant full length IgG antibodies can be generated by subcloning heavy and light chain Fv DNAs from the cloning vector into a Lonza pConIgG1 or pConK2 plasmid vector, transfected into 293 Freestyle cells or Lonza CHO cells, and collected and purified from the CHO cell supernatant.

The rapid availability of antibody produced in the same host cell and cell culture process as the final cGMP manufacturing process has the potential to reduce the duration of process development programs. Lonza has developed a generic method using pooled transfectants grown in CDACF medium, for the rapid production of small quantities (up to 50 g) of antibodies in CHO cells. Although slightly slower than a true transient system, the advantages include a higher product concentration and use of the same host and process as the production cell line. Example of growth and productivity of GS-CHO pools, expressing a

model antibody, in a disposable bioreactor: in a disposable bag bioreactor culture (5 L working volume) operated in fed-batch mode, a harvest antibody concentration of 2 g/L was achieved within 9 weeks of transfection.

pCon Vectors™ are an easy way to re-express whole antibodies. The constant region vectors are a set of vectors offering a range of immunoglobulin constant region vectors cloned into the pEE vectors. These vectors offer easy construction of full length antibodies with human constant regions and the convenience of the GS System™.

Antibody molecules will comprise fragments (such as F(ab'), $F(ab')_2$) that are produced, for example, by the proteolytic cleavage of the mAbs, or single-chain immunoglobulins producible, for example, via recombinant means. Such antibody derivatives are monovalent. In one embodiment, such fragments can be combined with one another, or with other antibody fragments or receptor ligands to form "chimeric" binding molecules. Significantly, such chimeric molecules may contain substituents capable of binding to different epitopes of the same molecule.

It may be desirable to "humanize" antibodies produced in non-human hosts in order to attenuate any immune reaction when used in human therapy. Such humanized antibodies may be studied in an *in vitro* or an *in vivo* context. Humanized antibodies may be produced, for example by replacing an immunogenic portion of an antibody with a corresponding, but non-immunogenic portion (*i.e.*, chimeric antibodies). PCT Application PCT/US86/02269; EP Application 184,187; EP Application 171,496; EP Application 173,494; PCT Application WO 86/01533; EP Application 125,023; Sun *et al.* (1987); Wood *et al.* (1985); and Shaw *et al.* (1988); all of which references are incorporated herein by reference. General reviews of "humanized" chimeric antibodies are provided by Morrison (1985); also incorporated herein by reference. "Humanized" antibodies can alternatively be produced by CDR or CEA substitution. Jones *et al.* (1986); Verhoeyen *et al.* (1988); Beidler *et al.* (1988); all of which are incorporated herein by reference.

In related embodiments, the antibody is a derivative of the disclosed antibodies, *e.g.*, an antibody comprising the CDR sequences identical to those in the disclosed antibodies (*e.g.*, a chimeric, humanized or CDR-grafted antibody). In yet a further embodiment, the antibody is a fully human recombinant antibody.

The present invention also contemplates isotype modification. By modifying the Fc region to have a different isotype, different functionalities can be achieved. For example, changing to IgG_4 can reduce immune effector functions associated with other isotypes.

Modified antibodies may be made by any technique known to those of skill in the art, including expression through standard molecular biological techniques, or the chemical synthesis of polypeptides. Methods for recombinant expression are addressed elsewhere in this document.

5

D. Expression

Nucleic acids according to the present invention will encode antibodies, optionally linked to other protein sequences. As used in this application, the term "a nucleic acid encoding a MUC1-C antibody" refers to a nucleic acid molecule that has been isolated free of
10 total cellular nucleic acid. In certain embodiments, the invention concerns antibodies that are encoded by any of the sequences set forth herein.

TABLE 2 - CODONS

Amino Acids			Codons
Alanine	Ala	A	GCA GCC GCG GCU
Cysteine	Cys	C	UGC UGU
Aspartic acid	Asp	D	GAC GAU
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	UUC UUU
Glycine	Gly	G	GGA GGC GGG GGU
Histidine	His	H	CAC CAU
Isoleucine	Ile	I	AUA AUC AUU
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC AAU
Proline	Pro	P	CCA CCC CCG CCU
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGU
Serine	Ser	S	AGC AGU UCA UCC UCG UCU
Threonine	Thr	T	ACA ACC ACG ACU
Valine	Val	V	GUA GUC GUG GUU
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAC UAU

15

The DNA segments of the present invention include those encoding biologically functional equivalent proteins and peptides of the sequences described above. Such sequences may arise as a consequence of codon redundancy and amino acid functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins

thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques or may be introduced randomly and screened later for the desired function, as described below.

Within certain embodiments, expression vectors are employed to express a MUC1-C ligand trap in order to produce and isolate the polypeptide expressed therefrom. In other embodiments, the expression vectors are used in gene therapy. Expression requires that appropriate signals be provided in the vectors, and which include various regulatory elements, such as enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in host cells. Elements designed to optimize messenger RNA stability and translatability in host cells also are defined. The conditions for the use of a number of dominant drug selection markers for establishing permanent, stable cell clones expressing the products are also provided, as is an element that links expression of the drug selection markers to expression of the polypeptide.

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding a gene of interest.

The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be "exogenous," which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (*e.g.,* YACs). One of skill in the art would be well equipped to construct a vector through standard recombinant techniques, which are described in Sambrook *et al.* (1989) and Ausubel *et al.* (1994), both incorporated herein by reference.

The term "expression vector" refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA

molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra.*

1. Regulatory Elements

A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence. A promoter may or may not be used in conjunction with an "enhancer," which refers to a *cis*-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally-associated with a gene or sequence, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment.

A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally-occurring," *i.e.,* containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in

connection with the compositions disclosed herein (see U.S. Patent 4,683,202, U.S. Patent 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

Table 3 lists several elements/promoters that may be employed, in the context of the present invention, to regulate the expression of a gene. This list is not intended to be exhaustive of all the possible elements involved in the promotion of expression but, merely, to be exemplary thereof. Table 4 provides examples of inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus.

TABLE 3

Promoter and/or Enhancer

Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl *et al.*, 1985; Atchinson *et al.*, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990
Immunoglobulin Light Chain	Queen *et al.*, 1983; Picard *et al.*, 1984
T-Cell Receptor	Luria *et al.*, 1987; Winoto *et al.*, 1989; Redondo *et al.*; 1990
HLA DQ a and/or DQ β	Sullivan *et al.*, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn *et al.*, 1988
Interleukin-2	Greene *et al.*, 1989

TABLE 3

Promoter and/or Enhancer

Promoter/Enhancer	References
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-DRa	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*; 1989
Muscle Creatine Kinase (MCK)	Jaynes *et al.*, 1988; Horlick *et al.*, 1989; Johnson *et al.*, 1989
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase I	Ornitz *et al.*, 1987
Metallothionein (MTII)	Karin *et al.*, 1987; Culotta *et al.*, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987
Albumin	Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere *et al.*, 1989
t-Globin	Bodine *et al.*, 1987; Perez-Stable *et al.*, 1990
β-Globin	Trudel *et al.*, 1987
c-fos	Cohen *et al.*, 1987
c-HA-*ras*	Triesman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh *et al.*, 1990
α_1-Antitrypain	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse and/or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor (PDGF)	Pech *et al.*, 1989

TABLE 3

Promoter and/or Enhancer

Promoter/Enhancer	References
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh *et al.*, 1985; Firak *et al.*, 1986; Herr *et al.*, 1986; Imbra *et al.*, 1986; Kadesch *et al.*, 1986; Wang *et al.*, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988
Polyoma	Swartzendruber *et al.*, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and/or Villarreal, 1988
Retroviruses	Kriegler *et al.*, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander *et al.*, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Choi *et al.*, 1988; Reisman *et al.*, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and/or Wilkie, 1983; Spalholz *et al.*, 1985; Lusky *et al.*, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens *et al.*, 1987; Glue *et al.*, 1988
Hepatitis B Virus	Bulla *et al.*, 1986; Jameel *et al.*, 1986; Shaul *et al.*, 1987; Spandau *et al.*, 1988; Vannice *et al.*, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber *et al.*, 1988; Jakobovits *et al.*, 1988; Feng *et al.*, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp *et al.*, 1989; Braddock *et al.*, 1989
Cytomegalovirus (CMV)	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking *et al.*, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

TABLE 4

Inducible Elements

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger *et al.*, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors *et al.*, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988
β-Interferon	poly(rI)x poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	ElA	Imperiale *et al.*, 1984
Collagenase	Phorbol Ester (TPA)	Angel *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angel *et al.*, 1987b
SV40	Phorbol Ester (TPA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	Hug *et al.*, 1988
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	ElA, SV40 Large T Antigen	Taylor *et al.*, 1989, 1990a, 1990b
Proliferin	Phorbol Ester-TPA	Mordacq *et al.*, 1989

TABLE 4

Inducible Elements

Element	Inducer	References
Tumor Necrosis Factor	PMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone α Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Examples of such regions include the human LIMK2 gene (Nomoto *et al.* 1999), the somatostatin receptor 2 gene (Kraus *et al.*, 1998), murine epididymal retinoic acid-binding gene (Lareyre *et al.*, 1999), human CD4 (Zhao-Emonet *et al.*, 1998), mouse alpha2 (XI) collagen (Tsumaki, *et al.*, 1998), D1A dopamine receptor gene (Lee, *et al.*, 1997), insulin-like growth factor II (Wu *et al.*, 1997), human platelet endothelial cell adhesion molecule-1 (Almendro *et al.*, 1996). Tumor specific promoters also will find use in the present invention. Some such promoters are set forth in Table 5.

TABLE 5 - CANDIDATE TISSUE-SPECIFIC PROMOTERS FOR CANCER GENE THERAPY

Tissue-specific promoter	Cancers in which promoter is active	Normal cells in which promoter is active
Carcinoembryonic antigen (CEA)*	Most colorectal carcinomas; 50% of lung carcinomas; 40-50% of gastric carcinomas; most pancreatic carcinomas; many breast carcinomas	Colonic mucosa; gastric mucosa; lung epithelia; eccrine sweat glands; cells in testes
Prostate-specific antigen (PSA)	Most prostate carcinomas	Prostate epithelium
Vasoactive intestinal peptide (VIP)	Majority of non-small cell lung cancers	Neurons; lymphocytes; mast cells; eosinophils
Surfactant protein A (SP-A)	Many lung adenocarcinomas cells	Type II pneumocytes; Clara
Human achaete-scute homolog (hASH)	Most small cell lung cancers	Neuroendocrine cells in lung
Mucin-1 (MUC1)**	Most adenocarcinomas (originating from any tissue)	Glandular epithelial cells in breast and in respiratory, gastrointestinal, and genitourinary tracts
Alpha-fetoprotein	Most hepatocellular carcinomas; possibly many testicular cancers	Hepatocytes (under certain conditions); testis
Albumin	Most hepatocellular carcinomas	Hepatocytes
Tyrosinase	Most melanomas	Melanocytes; astrocytes; Schwann cells; some neurons
Tyrosine-binding protein (TRP)	Most melanomas	Melanocytes; astrocytes, Schwann cells; some neurons

Keratin 14	Presumably many squamous cell carcinomas (*e.g.*, Head and neck cancers)	Keratinocytes
EBV LD-2	Many squamous cell carcinomas of head and neck	Keratinocytes of upper digestive Keratinocytes of upper digestive tract
Glial fibrillary acidic protein (GFAP)	Many astrocytomas	Astrocytes
Myelin basic protein (MBP)	Many gliomas	Oligodendrocytes
Testis-specific angiotensin-converting enzyme (Testis-specific ACE)	Possibly many testicular cancers	Spermatazoa
Osteocalcin	Possibly many osteosarcomas	Osteoblasts
E2F-regulated promoter	Almost all cancers	Proliferating cells
HLA-G	Many colorectal carcinomas; many melanomas; possibly many other cancers	Lymphocytes; monocytes; spermatocytes; trophoblast
FasL	Most melanomas; many pancreatic carcinomas; most astrocytomas possibly many other cancers	Activated leukocytes: neurons; endothelial cells; keratinocytes; cells in immunoprivileged tissues; some cells in lungs, ovaries, liver, and prostate
Myc-regulated promoter	Most lung carcinomas (both small cell and non-small cell); most colorectal carcinomas	Proliferating cells (only some cell-types): mammary epithelial cells (including non-proliferating)
MAGE-1	Many melanomas; some non-	Testis

	small cell lung carcinomas; some breast carcinomas	
VEGF	70% of all cancers (constitutive overexpression in many cancers)	Cells at sites of neovascularization (but unlike in tumors, expression is transient, less strong, and never constitutive)
bFGF	Presumably many different cancers, since bFGF expression is induced by ischemic conditions	Cells at sites of ischemia (but unlike tumors, expression is transient, less strong, and never constitutive)
COX-2	Most colorectal carcinomas; many lung carcinomas; possibly many other cancers	Cells at sites of inflammation
IL-10	Most colorectal carcinomas; many lung carcinomas; many squamous cell carcinomas of head and neck; possibly many other cancers	Leukocytes
GRP78/BiP	Presumably many different cancers, since GRP7S expression is induced by tumor-specific conditions	Cells at sites of ishemia
CarG elements from Egr-1	Induced by ionization radiation, so conceivably most tumors upon irradiation	Cells exposed to ionizing radiation; leukocytes

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the

necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

2. IRES

In certain embodiments of the invention, the use of internal ribosome entry sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5'-methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Patents 5,925,565 and 5,935,819, herein incorporated by reference).

3. Multi-Purpose Cloning Sites

Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector. See Carbonelli *et al.,* 1999, Levenson *et al.,* 1998, and Cocea, 1997, incorporated herein by reference. "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

4. Splicing Sites

Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression (see Chandler *et al.*, 1997, herein incorporated by reference).

5. Termination Signals

The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a stretch of about 200 A residues (polyA) to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that that terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The terminator and/or polyadenylation site elements can serve to enhance message levels and/or to minimize read through from the cassette into other sequences.

Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the termination sequences of genes, such as for example the bovine growth hormone terminator or viral termination sequences, such as for example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

6. Polyadenylation Signals

In expression, particularly eukaryotic expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention,

and/or any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal and/or the bovine growth hormone polyadenylation signal, convenient and/or known to function well in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

7. Origins of Replication

In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

8. Selectable and Screenable Markers

In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

9. Viral Vectors

The capacity of certain viral vectors to efficiently infect or enter cells, to integrate into a host cell genome and stably express viral genes, have led to the development and application of a number of different viral vector systems (Robbins *et al.*, 1998). Viral systems are currently being developed for use as vectors for *ex vivo* and *in vivo* gene transfer. For example, adenovirus, herpes-simplex virus, retrovirus and adeno-associated virus vectors are being evaluated currently for treatment of diseases such as cancer, cystic fibrosis, Gaucher disease, renal disease and arthritis (Robbins and Ghivizzani, 1998; Imai *et al.*, 1998; U.S. Patent 5,670,488). The various viral vectors described below, present specific advantages and disadvantages, depending on the particular gene-therapeutic application.

Adenoviral Vectors. In particular embodiments, an adenoviral expression vector is contemplated for the delivery of expression constructs. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a tissue or cell-specific construct that has been cloned therein.

Adenoviruses comprise linear, double-stranded DNA, with a genome ranging from 30 to 35 kb in size (Reddy *et al.*, 1998; Morrison *et al.*, 1997; Chillon *et al.*, 1999). An adenovirus expression vector according to the present invention comprises a genetically engineered form of the adenovirus. Advantages of adenoviral gene transfer include the ability to infect a wide variety of cell types, including non-dividing cells, a mid-sized genome, ease of manipulation, high infectivity and the ability to be grown to high titers (Wilson, 1996). Further, adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner, without potential genotoxicity associated with other viral vectors. Adenoviruses also are structurally stable (Marienfeld *et al.*, 1999) and no genome rearrangement has been detected after extensive amplification (Parks *et al.*, 1997; Bett *et al.*, 1993).

Salient features of the adenovirus genome are an early region (E1, E2, E3 and E4 genes), an intermediate region (pIX gene, Iva2 gene), a late region (L1, L2, L3, L4 and L5 genes), a major late promoter (MLP), inverted-terminal-repeats (ITRs) and a ψ sequence (Zheng, *et al.*, 1999; Robbins *et al.*, 1998; Graham and Prevec, 1995). The early genes E1, E2, E3 and E4 are expressed from the virus after infection and encode polypeptides that regulate viral gene expression, cellular gene expression, viral replication, and inhibition of cellular apoptosis. Further on during viral infection, the MLP is activated, resulting in the expression of the late (L) genes, encoding polypeptides required for adenovirus encapsidation.

The intermediate region encodes components of the adenoviral capsid. Adenoviral inverted terminal repeats (ITRs; 100-200 bp in length), are *cis* elements, and function as origins of replication and are necessary for viral DNA replication. The ψ sequence is required for the packaging of the adenoviral genome.

A common approach for generating adenoviruses for use as a gene transfer vectors is the deletion of the E1 gene ($E1^-$), which is involved in the induction of the E2, E3 and E4 promoters (Graham and Prevec, 1995). Subsequently, a therapeutic gene or genes can be inserted recombinantly in place of the E1 gene, wherein expression of the therapeutic gene(s) is driven by the E1 promoter or a heterologous promoter. The $E1^-$, replication-deficient virus is then proliferated in a "helper" cell line that provides the E1 polypeptides *in trans* (*e.g.*, the human embryonic kidney cell line 293). Thus, in the present invention it may be convenient to introduce the transforming construct at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. Alternatively, the E3 region, portions of the E4 region or both may be deleted, wherein a heterologous nucleic acid sequence under the control of a promoter operable in eukaryotic cells is inserted into the adenovirus genome for use in gene transfer (U.S. Patent 5,670,488; U.S. Patent 5,932,210, each specifically incorporated herein by reference).

Although adenovirus based vectors offer several unique advantages over other vector systems, they often are limited by vector immunogenicity, size constraints for insertion of recombinant genes and low levels of replication. The preparation of a recombinant adenovirus vector deleted of all open reading frames, comprising a full length dystrophin gene and the terminal repeats required for replication (Haecker *et al.*, 1996) offers some potentially promising advantages to the above mentioned adenoviral shortcomings. The vector was grown to high titer with a helper virus in 293 cells and was capable of efficiently transducing dystrophin in mdx mice, in myotubes *in vitro* and muscle fibers *in vivo*. Helper-dependent viral vectors are discussed below.

A major concern in using adenoviral vectors is the generation of a replication-competent virus during vector production in a packaging cell line or during gene therapy treatment of an individual. The generation of a replication-competent virus could pose serious threat of an unintended viral infection and pathological consequences for the patient. Armentano *et al.* (1990), describe the preparation of a replication-defective adenovirus vector, claimed to eliminate the potential for the inadvertent generation of a replication-competent adenovirus (U.S. Patent 5,824,544, specifically incorporated herein by reference). The

replication-defective adenovirus method comprises a deleted E1 region and a relocated protein IX gene, wherein the vector expresses a heterologous, mammalian gene.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes and/or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Adenovirus growth and manipulation is known to those of skill in the art, and exhibits broad host range *in vitro* and *in vivo* (U.S. Patent 5,670,488; U.S. Patent 5,932,210; U.S. Patent 5,824,544). This group of viruses can be obtained in high titers, *e.g.*, 10^9 to 10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. Many experiments, innovations, preclinical studies and clinical trials are currently under investigation for the use of adenoviruses as gene delivery vectors. For example, adenoviral gene delivery-based gene therapies are being developed for liver diseases (Han *et al.,* 1999), psychiatric diseases (Lesch, 1999), neurological diseases (Smith, 1998; Hermens and Verhaagen, 1998), coronary diseases (Feldman *et al.,* 1996), muscular diseases (Petrof, 1998), gastrointestinal diseases (Wu, 1998) and various cancers such as colorectal (Fujiwara and Tanaka, 1998; Dorai *et al.*, 1999), pancreatic, bladder (Irie *et al.*, 1999), head and neck (Blackwell *et al.*, 1999), breast (Stewart *et al.*, 1999), lung (Batra *et al.*, 1999) and ovarian (Vanderkwaak *et al.*, 1999).

Retroviral Vectors. In certain embodiments of the invention, the uses of retroviruses for gene delivery are contemplated. Retroviruses are RNA viruses comprising an RNA genome. When a host cell is infected by a retrovirus, the genomic RNA is reverse transcribed into a DNA intermediate which is integrated into the chromosomal DNA of infected cells. This integrated DNA intermediate is referred to as a provirus. A particular advantage of retroviruses is that they can stably infect dividing cells with a gene of interest (*e.g.,* a therapeutic gene) by integrating into the host DNA, without expressing immunogenic viral

proteins. Theoretically, the integrated retroviral vector will be maintained for the life of the infected host cell, expressing the gene of interest.

The retroviral genome and the proviral DNA have three genes: gag, pol, and env, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (matrix, capsid, and nucleocapsid) proteins; the pol gene encodes the RNA-directed DNA polymerase (reverse transcriptase) and the env gene encodes viral envelope glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of the virion RNAs. The LTR contains all other cis-acting sequences necessary for viral replication.

A recombinant retrovirus of the present invention may be genetically modified in such a way that some of the structural, infectious genes of the native virus have been removed and replaced instead with a nucleic acid sequence to be delivered to a target cell (U.S. Patent 5,858,744; U.S. Patent 5,739,018, each incorporated herein by reference). After infection of a cell by the virus, the virus injects its nucleic acid into the cell and the retrovirus genetic material can integrate into the host cell genome. The transferred retrovirus genetic material is then transcribed and translated into proteins within the host cell. As with other viral vector systems, the generation of a replication-competent retrovirus during vector production or during therapy is a major concern. Retroviral vectors suitable for use in the present invention are generally defective retroviral vectors that are capable of infecting the target cell, reverse transcribing their RNA genomes, and integrating the reverse transcribed DNA into the target cell genome, but are incapable of replicating within the target cell to produce infectious retroviral particles (*e.g.*, the retroviral genome transferred into the target cell is defective in gag, the gene encoding virion structural proteins, and/or in pol, the gene encoding reverse transcriptase). Thus, transcription of the provirus and assembly into infectious virus occurs in the presence of an appropriate helper virus or in a cell line containing appropriate sequences enabling encapsidation without coincident production of a contaminating helper virus.

The growth and maintenance of retroviruses is known in the art (U.S. Patent 5,955,331; U.S. Patent 5,888,502, each specifically incorporated herein by reference). Nolan *et al.* describe the production of stable high titre, helper-free retrovirus comprising a heterologous gene (U.S. Patent 5,830,725, specifically incorporated herein by reference). Methods for constructing packaging cell lines useful for the generation of helper-free recombinant retroviruses with amphoteric or ecotrophic host ranges, as well as methods of using the recombinant retroviruses to introduce a gene of interest into eukaryotic cells *in vivo* and *in vitro* are contemplated in the present invention (U.S. Patent 5,955,331).

Currently, the majority of all clinical trials for vector-mediated gene delivery use murine leukemia virus (MLV)-based retroviral vector gene delivery (Robbins *et al.*, 1998; Miller *et al.*, 1993). Disadvantages of retroviral gene delivery include a requirement for ongoing cell division for stable infection and a coding capacity that prevents the delivery of large genes. However, recent development of vectors such as lentivirus (*e.g.,* HIV), simian immunodeficiency virus (SIV) and equine infectious-anemia virus (EIAV), which can infect certain non-dividing cells, potentially allow the *in vivo* use of retroviral vectors for gene therapy applications (Amado and Chen, 1999; Klimatcheva *et al.*, 1999; White *et al.*, 1999; Case *et al.*, 1999). For example, HIV-based vectors have been used to infect non-dividing cells such as neurons (Miyatake *et al.,* 1999), islets (Leibowitz *et al.,* 1999) and muscle cells (Johnston *et al.*, 1999). The therapeutic delivery of genes *via* retroviruses are currently being assessed for the treatment of various disorders such as inflammatory disease (Moldawer *et al.*, 1999), AIDS (Amado and Chen, 1999; Engel and Kohn, 1999), cancer (Clay *et al.*, 1999), cerebrovascular disease (Weihl *et al.*, 1999) and hemophilia (Kay, 1998).

Herpesviral Vectors. Herpes simplex virus (HSV) type I and type II contain a double-stranded, linear DNA genome of approximately 150 kb, encoding 70-80 genes. Wild type HSV are able to infect cells lytically and to establish latency in certain cell types (*e.g.*, neurons). Similar to adenovirus, HSV also can infect a variety of cell types including muscle (Yeung *et al.*, 1999), ear (Derby *et al.*, 1999), eye (Kaufman *et al.*, 1999), tumors (Yoon *et al.*, 1999; Howard *et al.*, 1999), lung (Kohut *et al.*, 1998), neuronal (Garrido *et al.*, 1999; Lachmann and Efstathiou, 1999), liver (Miytake *et al.*, 1999; Kooby *et al.*, 1999) and pancreatic islets (Rabinovitch *et al.*, 1999).

HSV viral genes are transcribed by cellular RNA polymerase II and are temporally regulated, resulting in the transcription and subsequent synthesis of gene products in roughly three discernable phases or kinetic classes. These phases of genes are referred to as the Immediate Early (IE) or α genes, Early (E) or β genes and Late (L) or γ genes. Immediately following the arrival of the genome of a virus in the nucleus of a newly infected cell, the IE genes are transcribed. The efficient expression of these genes does not require prior viral protein synthesis. The products of IE genes are required to activate transcription and regulate the remainder of the viral genome.

For use in therapeutic gene delivery, HSV must be rendered replication-defective. Protocols for generating replication-defective HSV helper virus-free cell lines have been described (U.S. Patent 5,879,934; U.S. Patent 5,851,826, each specifically incorporated

herein by reference in its entirety). One IE protein, ICP4, also known as α4 or Vmw175, is absolutely required for both virus infectivity and the transition from IE to later transcription. Thus, due to its complex, multifunctional nature and central role in the regulation of HSV gene expression, ICP4 has typically been the target of HSV genetic studies.

Phenotypic studies of HSV viruses deleted of ICP4 indicate that such viruses will be potentially useful for gene transfer purposes (Krisky *et al.,* 1998a). One property of viruses deleted for ICP4 that makes them desirable for gene transfer is that they only express the five other IE genes: ICP0, ICP6, ICP27, ICP22 and ICP47 (DeLuca *et al.*, 1985), without the expression of viral genes encoding proteins that direct viral DNA synthesis, as well as the structural proteins of the virus. This property is desirable for minimizing possible deleterious effects on host cell metabolism or an immune response following gene transfer. Further deletion of IE genes ICP22 and ICP27, in addition to ICP4, substantially improve reduction of HSV cytotoxicity and prevented early and late viral gene expression (Krisky *et al.*, 1998b).

The therapeutic potential of HSV in gene transfer has been demonstrated in various *in vitro* model systems and *in vivo* for diseases such as Parkinson's (Yamada *et al.*, 1999), retinoblastoma (Hayashi *et al.,* 1999), intracerebral and intradermal tumors (Moriuchi *et al.*, 1998), B-cell malignancies (Suzuki *et al.*, 1998), ovarian cancer (Wang *et al.,* 1998) and Duchenne muscular dystrophy (Huard *et al.,* 1997).

Adeno-Associated Viral Vectors. Adeno-associated virus (AAV), a member of the parvovirus family, is a human virus that is increasingly being used for gene delivery therapeutics. AAV has several advantageous features not found in other viral systems. First, AAV can infect a wide range of host cells, including non-dividing cells. Second, AAV can infect cells from different species. Third, AAV has not been associated with any human or animal disease and does not appear to alter the biological properties of the host cell upon integration. For example, it is estimated that 80-85% of the human population has been exposed to AAV. Finally, AAV is stable at a wide range of physical and chemical conditions which lends itself to production, storage and transportation requirements.

The AAV genome is a linear, single-stranded DNA molecule containing 4681 nucleotides. The AAV genome generally comprises an internal non-repeating genome flanked on each end by inverted terminal repeats (ITRs) of approximately 145 bp in length. The ITRs have multiple functions, including origins of DNA replication, and as packaging signals for the viral genome. The internal non-repeated portion of the genome includes two large open reading frames, known as the AAV replication (rep) and capsid (cap) genes. The rep and cap genes code for viral proteins that allow the virus to replicate and package the

viral genome into a virion. A family of at least four viral proteins is expressed from the AAV rep region, Rep 78, Rep 68, Rep 52, and Rep 40, named according to their apparent molecular weight. The AAV cap region encodes at least three proteins, VP1, VP2, and VP3.

AAV is a helper-dependent virus requiring co-infection with a helper virus (*e.g.*, adenovirus, herpesvirus or vaccinia) in order to form AAV virions. In the absence of co-infection with a helper virus, AAV establishes a latent state in which the viral genome inserts into a host cell chromosome, but infectious virions are not produced. Subsequent infection by a helper virus "rescues" the integrated genome, allowing it to replicate and package its genome into infectious AAV virions. Although AAV can infect cells from different species, the helper virus must be of the same species as the host cell (*e.g.*, human AAV will replicate in canine cells co-infected with a canine adenovirus).

AAV has been engineered to deliver genes of interest by deleting the internal non-repeating portion of the AAV genome and inserting a heterologous gene between the ITRs. The heterologous gene may be functionally linked to a heterologous promoter (constitutive, cell-specific, or inducible) capable of driving gene expression in target cells. To produce infectious recombinant AAV (rAAV) containing a heterologous gene, a suitable producer cell line is transfected with a rAAV vector containing a heterologous gene. The producer cell is concurrently transfected with a second plasmid harboring the AAV rep and cap genes under the control of their respective endogenous promoters or heterologous promoters. Finally, the producer cell is infected with a helper virus.

Once these factors come together, the heterologous gene is replicated and packaged as though it were a wild-type AAV genome. When target cells are infected with the resulting rAAV virions, the heterologous gene enters and is expressed in the target cells. Because the target cells lack the rep and cap genes and the adenovirus helper genes, the rAAV cannot further replicate, package or form wild-type AAV.

The use of helper virus, however, presents a number of problems. First, the use of adenovirus in a rAAV production system causes the host cells to produce both rAAV and infectious adenovirus. The contaminating infectious adenovirus can be inactivated by heat treatment (56°C. for 1 hour). Heat treatment, however, results in approximately a 50% drop in the titer of functional rAAV virions. Second, varying amounts of adenovirus proteins are present in these preparations. For example, approximately 50% or greater of the total protein obtained in such rAAV virion preparations is free adenovirus fiber protein. If not completely removed, these adenovirus proteins have the potential of eliciting an immune response from the patient. Third, AAV vector production methods which employ a helper virus require the

use and manipulation of large amounts of high titer infectious helper virus, which presents a number of health and safety concerns, particularly in regard to the use of a herpesvirus. Fourth, concomitant production of helper virus particles in rAAV virion producing cells diverts large amounts of host cellular resources away from rAAV virion production, potentially resulting in lower rAAV virion yields.

Lentiviral Vectors. Lentiviruses are complex retroviruses, which, in addition to the common retroviral genes *gag, pol*, and *env*, contain other genes with regulatory or structural function. The higher complexity enables the virus to modulate its life cycle, as in the course of latent infection. Some examples of lentivirus include the Human Immunodeficiency Viruses: HIV-1, HIV-2 and the Simian Immunodeficiency Virus: SIV. Lentiviral vectors have been generated by multiply attenuating the HIV virulence genes, for example, the genes *env, vif, vpr, vpu* and *nef* are deleted making the vector biologically safe.

Recombinant lentiviral vectors are capable of infecting non-dividing cells and can be used for both *in vivo* and *ex vivo* gene transfer and expression of nucleic acid sequences. The lentiviral genome and the proviral DNA have the three genes found in retroviruses: *gag, pol* and *env,* which are flanked by two long terminal repeat (LTR) sequences. The *gag* gene encodes the internal structural (matrix, capsid and nucleocapsid) proteins; the *pol* gene encodes the RNA-directed DNA polymerase (reverse transcriptase), a protease and an integrase; and the *env* gene encodes viral envelope glycoproteins. The 5' and 3' LTR's serve to promote transcription and polyadenylation of the virion RNA's. The LTR contains all other *cis*-acting sequences necessary for viral replication. Lentiviruses have additional genes including *vif, vpr, tat, rev, vpu, nef* and *vpx.*

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the *Psi* site). If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the *cis* defect prevents encapsidation of genomic RNA. However, the resulting mutant remains capable of directing the synthesis of all virion proteins.

Lentiviral vectors are known in the art, see Naldini *et al.*, (1996); Zufferey *et al.*, (1997); U.S. Patents 6,013,516; and 5,994,136. In general, the vectors are plasmid-based or virus-based, and are configured to carry the essential sequences for incorporating foreign nucleic acid, for selection and for transfer of the nucleic acid into a host cell. The *gag, pol* and *env* genes of the vectors of interest also are known in the art. Thus, the relevant genes are cloned into the selected vector and then used to transform the target cell of interest.

Recombinant lentivirus capable of infecting a non-dividing cell wherein a suitable host cell is transfected with two or more vectors carrying the packaging functions, namely gag, pol and env, as well as rev and tat is described in U.S. Patent 5,994,136, incorporated herein by reference. This describes a first vector that can provide a nucleic acid encoding a viral *gag* and a *pol* gene and another vector that can provide a nucleic acid encoding a viral *env* to produce a packaging cell. Introducing a vector providing a heterologous gene, such as the STAT-1α gene in this invention, into that packaging cell yields a producer cell which releases infectious viral particles carrying the foreign gene of interest. The *env* preferably is an amphotropic envelope protein which allows transduction of cells of human and other species.

One may target the recombinant virus by linkage of the envelope protein with an antibody or a particular ligand for targeting to a receptor of a particular cell-type. By inserting a sequence (including a regulatory region) of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target-specific.

The vector providing the viral env nucleic acid sequence is associated operably with regulatory sequences, *e.g.*, a promoter or enhancer. The regulatory sequence can be any eukaryotic promoter or enhancer, including for example, the Moloney murine leukemia virus promoter-enhancer element, the human cytomegalovirus enhancer or the vaccinia P7.5 promoter. In some cases, such as the Moloney murine leukemia virus promoter-enhancer element, the promoter-enhancer elements are located within or adjacent to the LTR sequences.

The heterologous or foreign nucleic acid sequence, such as the STAT-1α encoding polynucleotide sequence herein, is linked operably to a regulatory nucleic acid sequence. Preferably, the heterologous sequence is linked to a promoter, resulting in a chimeric gene. The heterologous nucleic acid sequence may also be under control of either the viral LTR promoter-enhancer signals or of an internal promoter, and retained signals within the retroviral LTR can still bring about efficient expression of the transgene. Marker genes may be utilized to assay for the presence of the vector, and thus, to confirm infection and integration. The presence of a marker gene ensures the selection and growth of only those host cells which express the inserts. Typical selection genes encode proteins that confer resistance to antibiotics and other toxic substances, *e.g.*, histidinol, puromycin, hygromycin, neomycin, methotrexate, *etc.*, and cell surface markers.

The vectors are introduced via transfection or infection into the packaging cell line. The packaging cell line produces viral particles that contain the vector genome. Methods for transfection or infection are well known by those of skill in the art. After cotransfection of the packaging vectors and the transfer vector to the packaging cell line, the recombinant virus is recovered from the culture media and titered by standard methods used by those of skill in the art. Thus, the packaging constructs can be introduced into human cell lines by calcium phosphate transfection, lipofection or electroporation, generally together with a dominant selectable marker, such as neo, DHFR, Gln synthetase or ADA, followed by selection in the presence of the appropriate drug and isolation of clones. The selectable marker gene can be linked physically to the packaging genes in the construct.

Lentiviral transfer vectors Naldini *et al.* (1996), have been used to infect human cells growth-arrested *in vitro* and to transduce neurons after direct injection into the brain of adult rats. The vector was efficient at transferring marker genes in vivo into the neurons and long term expression in the absence of detectable pathology was achieved. Animals analyzed ten months after a single injection of the vector showed no decrease in the average level of transgene expression and no sign of tissue pathology or immune reaction (Blomer *et al.*, 1997). Thus, in the present invention, one may graft or transplant cells infected with the recombinant lentivirus *ex vivo*, or infect cells *in vivo*.

Other Viral Vectors. The development and utility of viral vectors for gene delivery is constantly improving and evolving. Other viral vectors such as poxvirus; *e.g.*, vaccinia virus (Gnant *et al.,* 1999; Gnant *et al.*, 1999), alpha virus; *e.g.*, sindbis virus, Semliki forest virus (Lundstrom, 1999), reovirus (Coffey *et al.*, 1998) and influenza A virus (Neumann *et al.*, 1999) are contemplated for use in the present invention and may be selected according to the requisite properties of the target system.

In certain embodiments, vaccinia viral vectors are contemplated for use in the present invention. Vaccinia virus is a particularly useful eukaryotic viral vector system for expressing heterologous genes. For example, when recombinant vaccinia virus is properly engineered, the proteins are synthesized, processed and transported to the plasma membrane. Vaccinia viruses as gene delivery vectors have recently been demonstrated to transfer genes to human tumor cells, *e.g.*, EMAP-II (Gnant *et al.*, 1999), inner ear (Derby *et al.,* 1999), glioma cells, *e.g.*, p53 (Timiryasova *et al.*, 1999) and various mammalian cells, *e.g.,* P_{450} (U.S. Patent 5,506,138). The preparation, growth and manipulation of vaccinia viruses are described in U.S. Patent 5,849,304 and U.S. Patent 5,506,138 (each specifically incorporated herein by reference).

In other embodiments, sindbis viral vectors are contemplated for use in gene delivery. Sindbis virus is a species of the alphavirus genus (Garoff and Li, 1998) which includes such important pathogens as Venezuelan, Western and Eastern equine encephalitis viruses (Sawai *et al.*, 1999; Mastrangelo *et al.*, 1999). *In vitro*, sindbis virus infects a variety of avian, mammalian, reptilian, and amphibian cells. The genome of sindbis virus consists of a single molecule of single-stranded RNA, 11,703 nucleotides in length. The genomic RNA is infectious, is capped at the 5' terminus and polyadenylated at the 3' terminus, and serves as mRNA. Translation of a vaccinia virus 26S mRNA produces a polyprotein that is cleaved co- and post-translationally by a combination of viral and presumably host-encoded proteases to give the three virus structural proteins, a capsid protein (C) and the two envelope glycoproteins (El and PE2, precursors of the virion E2).

Three features of sindbis virus suggest that it would be a useful vector for the expression of heterologous genes. First, its wide host range, both in nature and in the laboratory. Second, gene expression occurs in the cytoplasm of the host cell and is rapid and efficient. Third, temperature-sensitive mutations in RNA synthesis are available that may be used to modulate the expression of heterologous coding sequences by simply shifting cultures to the non-permissive temperature at various time after infection. The growth and maintenance of sindbis virus is known in the art (U.S. Patent 5,217,879, specifically incorporated herein by reference).

Chimeric Viral Vectors. Chimeric or hybrid viral vectors are being developed for use in therapeutic gene delivery and are contemplated for use in the present invention. Chimeric poxviral/retroviral vectors (Holzer *et al.*, 1999), adenoviral/retroviral vectors (Feng *et al.*, 1997; Bilbao *et al.*, 1997; Caplen *et al.*, 1999) and adenoviral/adeno-associated viral vectors (Fisher *et al.*, 1996; U.S. Patent 5,871,982) have been described.

These "chimeric" viral gene transfer systems can exploit the favorable features of two or more parent viral species. For example, Wilson *et al.*, provide a chimeric vector construct which comprises a portion of an adenovirus, AAV 5' and 3' ITR sequences and a selected transgene, described below (U.S. Patent 5,871,983, specifically incorporate herein by reference).

The adenovirus/AAV chimeric virus uses adenovirus nucleic acid sequences as a shuttle to deliver a recombinant AAV/transgene genome to a target cell. The adenovirus nucleic acid sequences employed in the hybrid vector can range from a minimum sequence amount, which requires the use of a helper virus to produce the hybrid virus particle, to only selected deletions of adenovirus genes, which deleted gene products can be supplied in the

hybrid viral production process by a selected packaging cell. At a minimum, the adenovirus nucleic acid sequences employed in the pAdA shuttle vector are adenovirus genomic sequences from which all viral genes are deleted and which contain only those adenovirus sequences required for packaging adenoviral genomic DNA into a preformed capsid head. More specifically, the adenovirus sequences employed are the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences of an adenovirus (which function as origins of replication) and the native 5' packaging/enhancer domain, that contains sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter. The adenovirus sequences may be modified to contain desired deletions, substitutions, or mutations, provided that the desired function is not eliminated.

The AAV sequences useful in the above chimeric vector are the viral sequences from which the rep and cap polypeptide encoding sequences are deleted. More specifically, the AAV sequences employed are the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences. These chimeras are characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host cell chromosome (U.S. Patent 5,871,983, specifically incorporate herein by reference). In the hybrid vector construct, the AAV sequences are flanked by the selected adenovirus sequences discussed above. The 5' and 3' AAV ITR sequences themselves flank a selected transgene sequence and associated regulatory elements, described below. Thus, the sequence formed by the transgene and flanking 5' and 3' AAV sequences may be inserted at any deletion site in the adenovirus sequences of the vector. For example, the AAV sequences are desirably inserted at the site of the deleted E1a/E1b genes of the adenovirus. Alternatively, the AAV sequences may be inserted at an E3 deletion, E2a deletion, and so on. If only the adenovirus 5' ITR/packaging sequences and 3' ITR sequences are used in the hybrid virus, the AAV sequences are inserted between them.

The transgene sequence of the vector and recombinant virus can be a gene, a nucleic acid sequence or reverse transcript thereof, heterologous to the adenovirus sequence, which encodes a protein, polypeptide or peptide fragment of interest. The transgene is operatively linked to regulatory components in a manner which permits transgene transcription. The composition of the transgene sequence will depend upon the use to which the resulting hybrid vector will be put. For example, one type of transgene sequence includes a therapeutic gene which expresses a desired gene product in a host cell. These therapeutic genes or nucleic acid sequences typically encode products for administration and expression in a patient *in*

vivo or *ex vivo* to replace or correct an inherited or non-inherited genetic defect or treat an epigenetic disorder or disease.

10. Non-Viral Transformation

Suitable methods for nucleic acid delivery for transformation of an organelle, a cell, a tissue or an organism for use with the current invention are believed to include virtually any method by which a nucleic acid (*e.g.*, DNA) can be introduced into an organelle, a cell, a tissue or an organism, as described herein or as would be known to one of ordinary skill in the art. Such methods include, but are not limited to, direct delivery of DNA such as by injection (U.S. Patents 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859, each incorporated herein by reference), including microinjection (Harland and Weintraub, 1985; U.S. Patent 5,789,215, incorporated herein by reference); by electroporation (U.S. Patent 5,384,253, incorporated herein by reference); by calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.,* 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer *et al.,* 1987); by liposome mediated transfection (Nicolau and Sene, 1982; Fraley *et al.,* 1979; Nicolau *et al.,* 1987; Wong *et al.,* 1980; Kaneda *et al.,* 1989; Kato *et al.,* 1991); by microprojectile bombardment (PCT Application Nos. WO 94/09699 and 95/06128; U.S. Patents 5,610,042; 5,322,783, 5,563,055, 5,550,318, 5,538,877 and 5,538,880, and each incorporated herein by reference); by agitation with silicon carbide fibers (Kaeppler *et al.,* 1990; U.S. Patents 5,302,523 and 5,464,765, each incorporated herein by reference); or by PEG-mediated transformation of protoplasts (Omirulleh *et al.,* 1993; U.S. Patents 4,684,611 and 4,952,500, each incorporated herein by reference); by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.,* 1985). Through the application of techniques such as these, organelle(s), cell(s), tissue(s) or organism(s) may be stably or transiently transformed.

Injection. In certain embodiments, a nucleic acid may be delivered to an organelle, a cell, a tissue or an organism via one or more injections (*i.e.*, a needle injection), such as, for example, either subcutaneously, intradermally, intramuscularly, intervenously or intraperitoneally. Methods of injection of vaccines are well known to those of ordinary skill in the art (*e.g.*, injection of a composition comprising a saline solution). Further embodiments of the present invention include the introduction of a nucleic acid by direct microinjection. Direct microinjection has been used to introduce nucleic acid constructs into Xenopus oocytes (Harland and Weintraub, 1985).

Electroporation. In certain embodiments of the present invention, a nucleic acid is introduced into an organelle, a cell, a tissue or an organism via electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge. In some variants of this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells (U.S. Patent 5,384,253, incorporated herein by reference). Alternatively, recipient cells can be made more susceptible to transformation by mechanical wounding.

Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human κ-immunoglobulin genes (Potter *et al.*, 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur-Kaspa *et al.*, 1986) in this manner.

To effect transformation by electroporation in cells such as, for example, plant cells, one may employ either friable tissues, such as a suspension culture of cells or embryogenic callus or alternatively one may transform immature embryos or other organized tissue directly. In this technique, one would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner. Examples of some species which have been transformed by electroporation of intact cells include maize (U.S. Patent 5,384,253; Rhodes *et al.*, 1995; D'Halluin *et al.*, 1992), wheat (Zhou *et al.*, 1993), tomato (Hou and Lin, 1996), soybean (Christou *et al.*, 1987) and tobacco (Lee *et al.*, 1989).

One also may employ protoplasts for electroporation transformation of plant cells (Bates, 1994; Lazzeri, 1995). For example, the generation of transgenic soybean plants by electroporation of cotyledon-derived protoplasts is described by Dhir and Widholm in International Patent Application No. WO 92/17598, incorporated herein by reference. Other examples of species for which protoplast transformation has been described include barley (Lazerri, 1995), sorghum (Battraw *et al.*, 1991), maize (Bhattacharjee *et al.*, 1997), wheat (He *et al.*, 1994) and tomato (Tsukada, 1989).

Calcium Phosphate. In other embodiments of the present invention, a nucleic acid is introduced to the cells using calcium phosphate precipitation. Human KB cells have been transfected with adenovirus 5 DNA (Graham and Van Der Eb, 1973) using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene (Chen and Okayama, 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe *et al.*, 1990).

DEAE-Dextran: In another embodiment, a nucleic acid is delivered into a cell using DEAE-dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells (Gopal, 1985).

Sonication Loading. Additional embodiments of the present invention include the introduction of a nucleic acid by direct sonic loading. LTK^- fibroblasts have been transfected with the thymidine kinase gene by sonication loading (Fechheimer *et al.*, 1987).

Liposome-Mediated Transfection. In a further embodiment of the invention, a nucleic acid may be entrapped in a lipid complex such as, for example, a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated is an nucleic acid complexed with Lipofectamine (Gibco BRL) or Superfect (Qiagen).

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987). The feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells has also been demonstrated (Wong *et al.*, 1980).

In certain embodiments of the invention, a liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, a liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, a liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In other embodiments, a delivery vehicle may comprise a ligand and a liposome.

Receptor-Mediated Transfection. Still further, a nucleic acid may be delivered to a target cell via receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis that will be occurring in a target cell. In view of the cell type-specific distribution of various receptors, this delivery method adds another degree of specificity to the present invention.

Certain receptor-mediated gene targeting vehicles comprise a cell receptor-specific ligand and a nucleic acid-binding agent. Others comprise a cell receptor-specific ligand to which the nucleic acid to be delivered has been operatively attached. Several ligands have

been used for receptor-mediated gene transfer (Wu and Wu, 1987; Wagner *et al.*, 1990; Perales *et al.*, 1994; Myers, EPO 0273085), which establishes the operability of the technique. Specific delivery in the context of another mammalian cell type has been described (Wu and Wu, 1993; incorporated herein by reference). In certain aspects of the present invention, a ligand will be chosen to correspond to a receptor specifically expressed on the target cell population.

In other embodiments, a nucleic acid delivery vehicle component of a cell-specific nucleic acid targeting vehicle may comprise a specific binding ligand in combination with a liposome. The nucleic acid(s) to be delivered are housed within the liposome and the specific binding ligand is functionally incorporated into the liposome membrane. The liposome will thus specifically bind to the receptor(s) of a target cell and deliver the contents to a cell. Such systems have been shown to be functional using systems in which, for example, epidermal growth factor (EGF) is used in the receptor-mediated delivery of a nucleic acid to cells that exhibit upregulation of the EGF receptor.

In still further embodiments, the nucleic acid delivery vehicle component of a targeted delivery vehicle may be a liposome itself, which will preferably comprise one or more lipids or glycoproteins that direct cell-specific binding. For example, lactosyl-ceramide, a galactose-terminal asialganglioside, have been incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes (Nicolau *et al.*, 1987). It is contemplated that the tissue-specific transforming constructs of the present invention can be specifically delivered into a target cell in a similar manner.

11. Expression Systems

Numerous expression systems exist that comprise at least a part or all of the compositions discussed above. Prokaryote- and/or eukaryote-based systems can be employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available.

The insect cell/baculovirus system can produce a high level of protein expression of a heterologous nucleic acid segment, such as described in U.S. Patents 5,871,986 and 4,879,236, both herein incorporated by reference, and which can be bought, for example, under the name MaxBac® 2.0 from Invitrogen® and BacPack™ Baculovirus Expression System From Clontech®.

Other examples of expression systems include Stratagene®'s Complete Control™ Inducible Mammalian Expression System, which involves a synthetic ecdysone-inducible receptor, or its pET Expression System, an *E. coli* expression system. Another example of an inducible expression system is available from Invitrogen®, which carries the T-Rex™ (tetracycline-regulated expression) System, an inducible mammalian expression system that uses the full-length CMV promoter. Invitrogen® also provides a yeast expression system called the *Pichia methanolica* Expression System, which is designed for high-level production of recombinant proteins in the methylotrophic yeast *Pichia methanolica*. One of skill in the art would know how to express a vector, such as an expression construct, to produce a nucleic acid sequence or its cognate polypeptide, protein, or peptide.

Primary mammalian cell cultures may be prepared in various ways. In order for the cells to be kept viable while *in vitro* and in contact with the expression construct, it is necessary to ensure that the cells maintain contact with the correct ratio of oxygen and carbon dioxide and nutrients but are protected from microbial contamination. Cell culture techniques are well documented.

One embodiment of the foregoing involves the use of gene transfer to immortalize cells for the production of proteins. The gene for the protein of interest may be transferred as described above into appropriate host cells followed by culture of cells under the appropriate conditions. The gene for virtually any polypeptide may be employed in this manner. The generation of recombinant expression vectors, and the elements included therein, are discussed above. Alternatively, the protein to be produced may be an endogenous protein normally synthesized by the cell in question.

Examples of useful mammalian host cell lines are Vero and HeLa cells and cell lines of Chinese hamster ovary, W138, BHK, COS-7, 293, HepG2, NIH3T3, RIN and MDCK cells. In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and process the gene product in the manner desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to insure the correct modification and processing of the foreign protein expressed.

A number of selection systems may be used including, but not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes, in *tk*-, *hgprt*- or *aprt*- cells, respectively. Also, anti-

metabolite resistance can be used as the basis of selection for *dhfr*, that confers resistance to; *gpt*, that confers resistance to mycophenolic acid; *neo*, that confers resistance to the aminoglycoside G418; and *hygro*, that confers resistance to hygromycin.

E. Purification

In certain embodiments, the antibodies of the present invention may be purified. The term "purified," as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein is purified to any degree relative to its naturally-obtainable state. A purified protein therefore also refers to a protein, free from the environment in which it may naturally occur. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. Other methods for protein purification include, precipitation with ammonium sulfate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; gel filtration, reverse phase, hydroxylapatite and affinity chromatography; and combinations of such and other techniques.

In purifying an antibody of the present invention, it may be desirable to express the polypeptide in a prokaryotic or eukaryotic expression system and extract the protein using denaturing conditions. The polypeptide may be purified from other cellular components using an affinity column, which binds to a tagged portion of the polypeptide. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

Commonly, complete antibodies are fractionated utilizing agents (*i.e.*, protein A) that bind the Fc portion of the antibody. Alternatively, antigens may be used to simultaneously purify and select appropriate antibodies. Such methods often utilize the selection agent bound

to a support, such as a column, filter or bead. The antibodies are bound to a support, contaminants removed (*e.g.*, washed away), and the antibodies released by applying conditions (salt, heat, *etc.*).

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. Another method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity. The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.,* 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

F. Single Chain/Single Domain Antibodies

A Single Chain Variable Fragment (scFv) is a fusion of the variable regions of the heavy and light chains of immunoglobulins, linked together with a short (usually serine, glycine) linker. This chimeric molecule, also known as a single domain antibody, retains the specificity of the original immunoglobulin, despite removal of the constant regions and the introduction of a linker peptide. This modification usually leaves the specificity unaltered. These molecules were created historically to facilitate phage display where it is highly convenient to express the antigen binding domain as a single peptide. Alternatively, scFv can be created directly from subcloned heavy and light chains derived from a hybridoma. Single domain or single chain variable fragments lack the constant Fc region found in complete antibody molecules, and thus, the common binding sites (*e.g.*, protein A/G) used to purify antibodies (single chain antibodies include the Fc region). These fragments can often be purified/immobilized using Protein L since Protein L interacts with the variable region of kappa light chains.

Flexible linkers generally are comprised of helix- and turn-promoting amino acid residues such as alaine, serine and glycine. However, other residues can function as well. Tang *et al.* (1996) used phage display as a means of rapidly selecting tailored linkers for

single-chain antibodies (scFvs) from protein linker libraries. A random linker library was constructed in which the genes for the heavy and light chain variable domains were linked by a segment encoding an 18-amino acid polypeptide of variable composition. The scFv repertoire (approx. 5×10^6 different members) was displayed on filamentous phage and subjected to affinity selection with hapten. The population of selected variants exhibited significant increases in binding activity but retained considerable sequence diversity. Screening 1054 individual variants subsequently yielded a catalytically active scFv that was produced efficiently in soluble form. Sequence analysis revealed a conserved proline in the linker two residues after the V_H C terminus and an abundance of arginines and prolines at other positions as the only common features of the selected tethers.

The recombinant antibodies of the present invention may also involve sequences or moieties that permit dimerization or multimerization of the receptors. Such sequences include those derived from IgA, which permit formation of multimers in conjunction with the J-chain. Another multimerization domain is the Gal4 dimerization domain. In other embodiments, the chains may be modified with agents such as biotin/avidin, which permit the combination of two antibodies.

In a separate embodiment, a single-chain antibody can be created by joining receptor light and heavy chains using a non-peptide linker or chemical unit. Generally, the light and heavy chains will be produced in distinct cells, purified, and subsequently linked together in an appropriate fashion (*i.e.*, the N-terminus of the heavy chain being attached to the C-terminus of the light chain via an appropriate chemical bridge).

Cross-linking reagents are used to form molecular bridges that tie functional groups of two different molecules, *e.g.*, a stablizing and coagulating agent. However, it is contemplated that dimers or multimers of the same analog or heteromeric complexes comprised of different analogs can be created. To link two different compounds in a step-wise manner, hetero-bifunctional cross-linkers can be used that eliminate unwanted homopolymer formation.

An exemplary hetero-bifunctional cross-linker contains two reactive groups: one reacting with primary amine group (*e.g.*, N-hydroxy succinimide) and the other reacting with a thiol group (*e.g.*, pyridyl disulfide, maleimides, halogens, *etc.*). Through the primary amine reactive group, the cross-linker may react with the lysine residue(s) of one protein (*e.g.*, the selected antibody or fragment) and through the thiol reactive group, the cross-linker, already tied up to the first protein, reacts with the cysteine residue (free sulfhydryl group) of the other protein (*e.g.*, the selective agent).

It is preferred that a cross-linker having reasonable stability in blood will be employed. Numerous types of disulfide-bond containing linkers are known that can be successfully employed to conjugate targeting and therapeutic/preventative agents. Linkers that contain a disulfide bond that is sterically hindered may prove to give greater stability *in vivo*, preventing release of the targeting peptide prior to reaching the site of action. These linkers are thus one group of linking agents.

Another cross-linking reagent is SMPT, which is a bifunctional cross-linker containing a disulfide bond that is "sterically hindered" by an adjacent benzene ring and methyl groups. It is believed that steric hindrance of the disulfide bond serves a function of protecting the bond from attack by thiolate anions such as glutathione which can be present in tissues and blood, and thereby help in preventing decoupling of the conjugate prior to the delivery of the attached agent to the target site.

The SMPT cross-linking reagent, as with many other known cross-linking reagents, lends the ability to cross-link functional groups such as the SH of cysteine or primary amines (*e.g.*, the epsilon amino group of lysine). Another possible type of cross-linker includes the hetero-bifunctional photoreactive phenylazides containing a cleavable disulfide bond such as sulfosuccinimidyl-2-(p-azido salicylamido) ethyl-1,3'-dithiopropionate. The N-hydroxy-succinimidyl group reacts with primary amino groups and the phenylazide (upon photolysis) reacts non-selectively with any amino acid residue.

In addition to hindered cross-linkers, non-hindered linkers also can be employed in accordance herewith. Other useful cross-linkers, not considered to contain or generate a protected disulfide, include SATA, SPDP and 2-iminothiolane (Wawrzynczak & Thorpe, 1987). The use of such cross-linkers is well understood in the art. Another embodiment involves the use of flexible linkers.

U.S. Patent 4,680,338, describes bifunctional linkers useful for producing conjugates of ligands with amine-containing polymers and/or proteins, especially for forming antibody conjugates with chelators, drugs, enzymes, detectable labels and the like. U.S. Patents 5,141,648 and 5,563,250 disclose cleavable conjugates containing a labile bond that is cleavable under a variety of mild conditions. This linker is particularly useful in that the agent of interest may be bonded directly to the linker, with cleavage resulting in release of the active agent. Particular uses include adding a free amino or free sulfhydryl group to a protein, such as an antibody, or a drug.

U.S. Patent 5,856,456 provides peptide linkers for use in connecting polypeptide constituents to make fusion proteins, *e.g.*, single chain antibodies. The linker is up to about 50

amino acids in length, contains at least one occurrence of a charged amino acid (preferably arginine or lysine) followed by a proline, and is characterized by greater stability and reduced aggregation. U.S. Patent 5,880,270 discloses aminooxy-containing linkers useful in a variety of immunodiagnostic and separative techniques.

G. Modified Antibodies

1. CARs

Artificial T cell receptors (also known as chimeric T cell receptors, chimeric immunoreceptors, chimeric antigen receptors (CARs)) are engineered receptors, which graft an arbitrary specificity onto an immune effector cell. Typically, these receptors are used to graft the specificity of a monoclonal antibody onto a T cell, with transfer of their coding sequence facilitated by retroviral vectors. In this way, a large number of cancer-specific T cells can be generated for adoptive cell transfer. Phase I clinical studies of this approach show efficacy.

The most common form of these molecules are fusions of single-chain variable fragments (scFv) derived from monoclonal antibodies, fused to CD3-zeta transmembrane and endodomain. Such molecules result in the transmission of a zeta signal in response to recognition by the scFv of its target. An example of such a construct is 14g2a-Zeta, which is a fusion of a scFv derived from hybridoma 14g2a (which recognizes disialoganglioside GD2). When T cells express this molecule (usually achieved by oncoretroviral vector transduction), they recognize and kill target cells that express GD2 (*e.g.*, neuroblastoma cells). To target malignant B cells, investigators have redirected the specificity of T cells using a chimeric immunoreceptor specific for the B-lineage molecule, CD19.

The variable portions of an immunoglobulin heavy and light chain are fused by a flexible linker to form a scFv. This scFv is preceded by a signal peptide to direct the nascent protein to the endoplasmic reticulum and subsequent surface expression (this is cleaved). A flexible spacer allows to the scFv to orient in different directions to enable antigen binding. The transmembrane domain is a typical hydrophobic alpha helix usually derived from the original molecule of the signalling endodomain which protrudes into the cell and transmits the desired signal.

Type I proteins are in fact two protein domains linked by a transmembrane alpha helix in between. The cell membrane lipid bilayer, through which the transmembrane domain passes, acts to isolate the inside portion (endodomain) from the external portion (ectodomain). It is not so surprising that attaching an ectodomain from one protein to an endodomain of

another protein results in a molecule that combines the recognition of the former to the signal of the latter.

Ectodomain. A signal peptide directs the nascent protein into the endoplasmic reticulum. This is essential if the receptor is to be glycosylated and anchored in the cell membrane. Any eukaryotic signal peptide sequence usually works fine. Generally, the signal peptide natively attached to the amino-terminal most component is used (*e.g.*, in a scFv with orientation light chain - linker - heavy chain, the native signal of the light-chain is used

The antigen recognition domain is usually an scFv. There are however many alternatives. An antigen recognition domain from native T-cell receptor (TCR) alpha and beta single chains have been described, as have simple ectodomains (*e.g.*, CD4 ectodomain to recognize HIV infected cells) and more exotic recognition components such as a linked cytokine (which leads to recognition of cells bearing the cytokine receptor). In fact almost anything that binds a given target with high affinity can be used as an antigen recognition region.

A spacer region links the antigen binding domain to the transmembrane domain. It should be flexible enough to allow the antigen binding domain to orient in different directions to facilitate antigen recognition. The simplest form is the hinge region from IgG1. Alternatives include the CH_2CH_3 region of immunoglobulin and portions of CD3. For most scFv based constructs, the IgG1 hinge suffices. However the best spacer often has to be determined empirically.

Transmembrane domain. The transmembrane domain is a hydrophobic alpha helix that spans the membrane. Generally, the transmembrane domain from the most membrane proximal component of the endodomain is used. Interestingly, using the CD3-zeta transmembrane domain may result in incorporation of the artificial TCR into the native TCR a factor that is dependent on the presence of the native CD3-zeta transmembrane charged aspartic acid residue. Different transmembrane domains result in different receptor stability. The CD28 transmembrane domain results in a brightly expressed, stable receptor.

Endodomain. This is the "business-end" of the receptor. After antigen recognition, receptors cluster and a signal is transmitted to the cell. The most commonly used endodomain component is CD3-zeta which contains 3 ITAMs. This transmits an activation signal to the T cell after antigen is bound. CD3-zeta may not provide a fully competent activation signal and additional co-stimulatory signaling is needed. For example, chimeric CD28 and OX40 can be used with CD3-Zeta to transmit a proliferative / survival signal, or all three can be used together.

"First-generation" CARs typically had the intracellular domain from the CD3 ξ- chain, which is the primary transmitter of signals from endogenous TCRs. "Second-generation" CARs add intracellular signaling domains from various costimulatory protein receptors (e.g., CD28, 41BB, ICOS) to the cytoplasmic tail of the CAR to provide additional signals to the T cell. Preclinical studies have indicated that the second generation of CAR designs improves the antitumor activity of T cells. More recent, "third-generation" CARs combine multiple signaling domains, such as CD3z-CD28-41BB or CD3z-CD28-OX40, to further augment potency.

Adoptive transfer of T cells expressing chimeric antigen receptors is a promising anti-cancer therapeutic as CAR-modified T cells can be engineered to target virtually any tumor associated antigen. There is great potential for this approach to improve patient-specific cancer therapy in a profound way. Following the collection of a patient's T cells, the cells are genetically engineered to express CARs specifically directed towards antigens on the patient's tumor cells, then infused back into the patient. Although adoptive transfer of CAR-modified T-cells is a unique and promising cancer therapeutic, there are significant safety concerns. Clinical trials of this therapy have revealed potential toxic effects of these CARs when healthy tissues express the same target antigens as the tumor cells, leading to outcomes similar to graft-versus-host disease (GVHD). A potential solution to this problem is engineering a suicide gene into the modified T cells. In this way, administration of a prodrug designed to activate the suicide gene during GVHD triggers apoptosis in the suicide gene-activated CAR T cells. This method has been used safely and effectively in hematopoietic stem cell transplantation (HSCT). Adoption of suicide gene therapy to the clinical application of CAR-modified T cell adoptive cell transfer has potential to alleviate GVHD while improving overall anti-tumor efficacy.

2. ADCs

Antibody Drug Conjugates or ADCs are a new class of highly potent biopharmaceutical drugs designed as a targeted therapy for the treatment of people with cancer. ADCs are complex molecules composed of an antibody (a whole mAb or an antibody fragment such as a single-chain variable fragment, or scFv) linked, via a stable chemical linker with labile bonds, to a biological active cytotoxic (anticancer) payload or drug. Antibody Drug Conjugates are examples of bioconjugates and immunoconjugates.

By combining the unique targeting capabilities of monoclonal antibodies with the cancer-killing ability of cytotoxic drugs, antibody-drug conjugates allow sensitive

discrimination between healthy and diseased tissue. This means that, in contrast to traditional chemotherapeutic agents, antibody-drug conjugates target and attack the cancer cell so that healthy cells are less severely affected.

In the development ADC-based anti-tumor therapies, an anticancer drug (*e.g.*, a cell toxin or cytotoxin) is coupled to an antibody that specifically targets a certain tumor marker (*e.g.*, a protein that, ideally, is only to be found in or on tumor cells; in this case MUC1). Antibodies track these proteins down in the body and attach themselves to the surface of cancer cells. The biochemical reaction between the antibody and the target protein (antigen) triggers a signal in the tumor cell, which then absorbs or internalizes the antibody together with the cytotoxin. After the ADC is internalized, the cytotoxic drug is released and kills the cancer. Due to this targeting, ideally the drug has lower side effects and gives a wider therapeutic window than other chemotherapeutic agents.

A stable link between the antibody and cytotoxic (anti-cancer) agent is a crucial aspect of an ADC. Linkers are based on chemical motifs including disulfides, hydrazones or peptides (cleavable), or thioethers (noncleavable) and control the distribution and delivery of the cytotoxic agent to the target cell. Cleavable and noncleavable types of linkers have been proven to be safe in preclinical and clinical trials. Brentuximab vedotin includes an enzyme-sensitive cleavable linker that delivers the potent and highly toxic antimicrotubule agent Monomethyl auristatin E or MMAE, a synthetic antineoplastic agent, to human specific CD30-positive malignant cells. Because of its high toxicity MMAE, which inhibits cell division by blocking the polymerization of tubulin, cannot be used as a single-agent chemotherapeutic drug. However, the combination of MMAE linked to an anti-CD30 monoclonal antibody (cAC10, a cell membrane protein of the tumor necrosis factor or TNF receptor) proved to be stable in extracellular fluid, cleavable by cathepsin and safe for therapy. Trastuzumab emtansine, the other approved ADC, is a combination of the microtubule-formation inhibitor mertansine (DM-1), a derivative of the Maytansine, and antibody trastuzumab (Herceptin®/Genentech/Roche) attached by a stable, non-cleavable linker.

The availability of better and more stable linkers has changed the function of the chemical bond. The type of linker, cleavable or noncleavable, lends specific properties to the cytotoxic (anti-cancer) drug. For example, a non-cleavable linker keeps the drug within the cell. As a result, the entire antibody, linker and cytotoxic (anti-cancer) agent enter the targeted cancer cell where the antibody is degraded to the level of an amino acid. The resulting complex – amino acid, linker and cytotoxic agent – now becomes the active drug. In

contrast, *cleavable linkers* are catalyzed by enzymes in the cancer cell where it releases the cytotoxic agent. The difference is that the cytotoxic payload delivered via a cleavable linker can escape from the targeted cell and, in a process called "bystander killing," attack neighboring cancer cells.

Another type of cleavable linker, currently in development, adds an extra molecule between the cytotoxic drug and the cleavage site. This linker technology allows researchers to create ADCs with more flexibility without worrying about changing cleavage kinetics. Researchers are also developing a new method of peptide cleavage based on Edman degradation, a method of sequencing amino acids in a peptide. Future direction in the development of ADCs also include the development of site-specific conjugation (TDCs) to further improve stability and therapeutic index and α emitting immunoconjugates and antibody-conjugated nanoparticles.

3. BitES

Bi-specific T-cell engagers (BiTEs) are a class of artificial bispecific monoclonal antibodies that are investigated for the use as anti-cancer drugs. They direct a host's immune system, more specifically the T cells' cytotoxic activity, against cancer cells. *BiTE* is a registered trademark of Micromet AG.

BiTEs are fusion proteins consisting of two single-chain variable fragments (scFvs) of different antibodies, or amino acid sequences from four different genes, on a single peptide chain of about 55 kilodaltons. One of the scFvs binds to T cells via the CD3 receptor, and the other to a tumor cell via a tumor specific molecule, in this case MUC1.

Like other bispecific antibodies, and unlike ordinary monoclonal antibodies, BiTEs form a link between T cells and tumor cells. This causes T cells to exert cytotoxic activity on tumor cells by producing proteins like perforin and granzymes, independently of the presence of MHC I or co-stimulatory molecules. These proteins enter tumor cells and initiate the cell's apoptosis. This action mimics physiological processes observed during T cell attacks against tumor cells.

BiTEs that were in clinical trials as of July 2010 include Blinatumomab (MT103) for the treatment of non-Hodgkin's lymphoma and acute lymphoblastic leukemia, directed towards CD19, a surface molecule expressed on B cells; and MT110 for the treatment of gastrointestinal and lung cancers, directed towards the EpCAM antigen.

Utilizing the same technology, melanoma (with MCSP specific BiTEs) and acute myeloid leukemia (with CD33 specific BiTEs) can be targeted. Research in this area is

currently ongoing. Another avenue for novel anti-cancer therapies is re-engineering some of the currently used conventional antibodies like trastuzumab (targeting HER2/neu), cetuximab and panitumumab (both targeting the EGF receptor), using the BiTE approach. BiTEs against CD66e and EphA2 are being developed as well.

III. Pharmaceutical Formulations and Treatment of Cancer

A. Cancers

Cancer results from the outgrowth of a clonal population of cells from tissue. The development of cancer, referred to as carcinogenesis, can be modeled and characterized in a number of ways. An association between the development of cancer and inflammation has long-been appreciated. The inflammatory response is involved in the host defense against microbial infection, and also drives tissue repair and regeneration. Considerable evidence points to a connection between inflammation and a risk of developing cancer, *i.e.*, chronic inflammation can lead to dysplasia.

Cancer cells to which the methods of the present invention can be applied include generally any cell that expresses MUC1, and more particularly, that overexpresses MUC1. An appropriate cancer cell can be a breast cancer, lung cancer, colon cancer, pancreatic cancer, renal cancer, stomach cancer, liver cancer, bone cancer, hematological cancer (*e.g.*, leukemia or lymphoma), neural tissue cancer, melanoma, ovarian cancer, testicular cancer, prostate cancer, cervical cancer, vaginal cancer, or bladder cancer cell. In addition, the methods of the invention can be applied to a wide range of species, *e.g.*, humans, non-human primates (*e.g.*, monkeys, baboons, or chimpanzees), horses, cattle, pigs, sheep, goats, dogs, cats, rabbits, guinea pigs, gerbils, hamsters, rats, and mice. Cancers may also be recurrent, metastatic and/or multi-drug resistant, and the methods of the present invention may be particularly applied to such cancers so as to render them resectable, to prolong or re-induce remission, to inhibit angiogenesis, to prevent or limit metastasis, and/or to treat multi-drug resistant cancers. At a cellular level, this may translate into killing cancer cells, inhibiting cancer cell growth, or otherwise reversing or reducing the malignant phenotype of tumor cells.

B. Formulation and Administration

The present invention provides pharmaceutical compositions comprising anti-MUC1-C antibodies. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more

particularly in humans. The term "carrier" refers to a diluent, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Other suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, saline, dextrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like.

The compositions can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, *etc.*, and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, *etc.*

The antibodies of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions, described *supra.* Of particular interest is direct intratumoral administration, perfusion of a tumor, or admininstration local or regional to a tumor, for example, in the local or regional vasculature or lymphatic system, or in a resected tumor bed.

The active compounds may also be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

C. Combination Therapies

In the context of the present invention, it also is contemplated that anti-MUC1-C antibodies described herein could be used similarly in conjunction with chemo- or radiotherapeutic intervention, or other treatments. It also may prove effective, in particular, to combine anti-MUC1-C/ECD antibodies with other therapies that target different aspects of

MUC1 function, such as peptides and small molecules that target the MUC1 cytoplasmic domain.

To kill cells, inhibit cell growth, inhibit metastasis, inhibit angiogenesis or otherwise reverse or reduce the malignant phenotype of tumor cells, using the methods and compositions of the present invention, one would generally contact a "target" cell with an anti-MUC1-C antibody according to the present invention and at least one other agent. These compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with the anti-MUC1-C antibody according to the present invention and the other agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the anti-MUC1-C antibody according to the present invention and the other includes the other agent.

Alternatively, the anti-MUC1-C antibody therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and the anti-MUC1 antibody are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both modalities within about 12-24 hours of each other and, more preferably, within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either anti-MUC1 antibody or the other agent will be desired. Various combinations may be employed, where an anti-MUC1-C antibody according to the present invention therapy is "A" and the other therapy is "B", as exemplified below:

A/B/A B/A/B B/B/A A/A/B B/A/A A/B/B B/B/B/A B/B/A/B
A/A/B/B A/B/A/B A/B/B/A B/B/A/A B/A/B/A B/A/A/B B/B/B/A
A/A/A/B B/A/A/A A/B/A/A A/A/B/A A/B/B/B B/A/B/B B/B/A/B

Other combinations are contemplated. Again, to achieve cell killing, both agents are delivered to a cell in a combined amount effective to kill the cell.

Agents or factors suitable for cancer therapy include any chemical compound or treatment method that induces DNA damage when applied to a cell. Such agents and factors include radiation and waves that induce DNA damage such as, irradiation, microwaves, electronic emissions, and the like. A variety of chemical compounds, also described as "chemotherapeutic" or "genotoxic agents," may be used. This may be achieved by irradiating the localized tumor site; alternatively, the tumor cells may be contacted with the agent by administering to the subject a therapeutically effective amount of a pharmaceutical composition.

Various classes of chemotherapeutic agents are comtemplated for use with the present invention. For example, selective estrogen receptor antagonists ("SERMs"), such as Tamoxifen, 4-hydroxy Tamoxifen (Afimoxfene), Falsodex, Raloxifene, Bazedoxifene, Clomifene, Femarelle, Lasofoxifene, Ormeloxifene, and Toremifene.

Chemotherapeutic agents contemplated to be of use, include, *e.g.*, camptothecin, actinomycin-D, mitomycin C,. The invention also encompasses the use of a combination of one or more DNA damaging agents, whether radiation-based or actual compounds, such as the use of X-rays with cisplatin or the use of cisplatin with etoposide. The agent may be prepared and used as a combined therapeutic composition, or kit, by combining it with a MUC1 peptide, as described above.

Heat shock protein 90 is a regulatory protein found in many eukaryotic cells. HSP90 inhibitors have been shown to be useful in the treatment of cancer. Such inhibitors include Geldanamycin, 17-(Allylamino)-17-demethoxygeldanamycin, PU-H71 and Rifabutin.

Agents that directly cross-link DNA or form adducts are also envisaged. Agents such as cisplatin, and other DNA alkylating agents may be used. Cisplatin has been widely used to treat cancer, with efficacious doses used in clinical applications of 20 mg/m^2 for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered via injection intravenously, subcutaneously, intratumorally or intraperitoneally.

Agents that damage DNA also include compounds that interfere with DNA replication, mitosis and chromosomal segregation. Such chemotherapeutic compounds include adriamycin, also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like. Widely used in a clinical setting for the treatment of neoplasms, these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m^2 at 21 day intervals for doxorubicin, to 35-50 mg/m^2 for etoposide intravenously or double the

intravenous dose orally. Microtubule inhibitors, such as taxanes, also are contemplated. These molecules are diterpenes produced by the plants of the genus *Taxus*, and include paclitaxel and docetaxel.

Epidermal growth factor receptor inhibitors, such as Iressa, mTOR, the mammalian target of rapamycin, also known as FK506-binding protein 12-rapamycin associated protein 1 (FRAP1) is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription. Rapamycin and analogs thereof ("rapalogs") are therefore contemplated for use in cancer therapy in accordance with the present invention.

Another possible therapy is TNF-α (tumor necrosis factor-alpha), a cytokine involved in systemic inflammation and a member of a group of cytokines that stimulate the acute phase reaction. The primary role of TNF is in the regulation of immune cells. TNF is also able to induce apoptotic cell death, to induce inflammation, and to inhibit tumorigenesis and viral replication.

Agents that disrupt the synthesis and fidelity of nucleic acid precursors and subunits also lead to DNA damage. As such a number of nucleic acid precursors have been developed. Particularly useful are agents that have undergone extensive testing and are readily available. As such, agents such as 5-fluorouracil (5-FU), are preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Although quite toxic, 5-FU, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to 15 mg/kg/day being commonly used.

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, x-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage DNA, on the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes. Dosage ranges for x-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

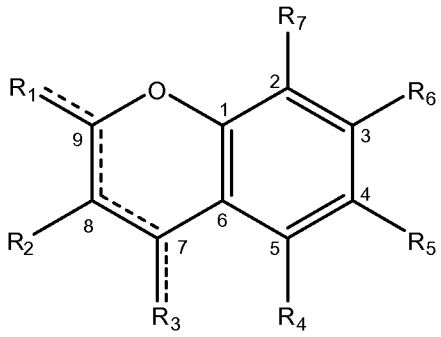
In addition, it also is contemplated that immunotherapy, hormone therapy, toxin therapy and surgery can be used. In particular, one may employ targeted therapies such as Avastin, Erbitux, Gleevec, Herceptin and Rituxan.

One particularly advantageous approach to combination therapy is to select a second agent that targets MUC1. In copending application filed by the present inventors, there are disclosed methods of inhibiting a MUC1-positive tumor cell in a subject comprising administering to said subject a MUC1 peptide of at least 4 consecutive MUC1 residues and no more than 20 consecutive MUC1 residues and comprising the sequence CQC, wherein the amino-terminal cysteine of CQC is covered on its NH_2-terminus by at least one amino acid residue that need not correspond to the native MUC-1 transmembrane sequence. The peptide may comprise at least 5 consecutive MUC1 residues, at least 6 consecutive MUC1 residues, at least 7 consecutive MUC1 residues, at least 8 consecutive MUC1 residues and the sequence may more specifically comprise CQCR (SEQ ID NO: **XX**), CQCRR (SEQ ID NO: **XX**), CQCRRR (SEQ ID NO: **XX**), CQCRRRR (SEQ ID NO: **XX**), CQCRRK (SEQ ID NO: **XX**), CQCRRKN (SEQ ID NO: **XX**), or RRRRRRRRRCQCRRKN (SEQ ID NO: **XX**). The peptide may contain no more than 10 consecutive residues, 11 consecutive residues, 12 consecutive residues, 13 consecutive residues, 14 consecutive residues, 15 consecutive residues, 16 consecutive residues, 17 consecutive residues, 18 consecutive residues or 19 consecutive residues of MUC1. The peptide may be fused to a cell delivery domain, such as poly-D-R, poly-D-P or poly-D-K. The peptide may comprise all L amino acids, all D amino acids, or a mix of L and D amino acids. See U.S. Patent No. 8,524,669.

A variation on this technology is described in U.S. Patent Application Serial No. 13/026,858. In that application, methods of inhibiting a MUC1-positive cancer cell are disclosed comprising contacting the cell with a MUC1 peptide of at least 4 consecutive MUC1 residues and no more than 20 consecutive MUC1 residues and comprising the sequence CQC, wherein (i) the amino-terminal cysteine of CQC is covered on its NH_2-terminus by at least one amino acid residue that need not correspond to the native MUC1 transmembrane sequence; and (ii) the peptide comprises 3-5 consecutive positively-charged amino acid residues in addition to those positively-charged amino acid residues corresponding to native MUC1 residues. The MUC1-positive cell may be a solid tumor cell, such as a lung cancer cell, a brain cancer cell, a head & neck cancer cell, a breast cancer cell, a skin cancer cell, a liver cancer cell, a pancreatic cancer cell, a stomach cancer cell, a colon cancer cell, a rectal cancer cell, a uterine cancer cell, a cervical cancer cell, an ovarian cancer cell, a testicular cancer cell, a skin cancer cell or a esophageal cancer cell. The MUC1-positive cell may be a leukemia or myeloma cell, such as acute myeloid leukemia, chronic myelogenous leukemia or multiple myeloma. The peptide may be a stapled peptide, a cyclized peptide, a peptidomimetic or peptoid. The method may further comprise contacting

the cell with a second anti-cancer agent, such as where the second anti-cancer agent is contacted prior to the peptide, after the peptide or at the same time as the peptide. Inhibiting may comprise inhibiting cancer cell growth, cancer cell proliferation or inducing cancer cell death, such as by apoptosis.

Another technology advanced by the inventors (see U.S. Patent Application Serial No. 13/045,033) involves methods of inhibiting inflammatory signaling in a cell comprising contacting said cell with a flavone having the structure of:



or a salt thereof, wherein:

R_1 is H, –OH, =O, substituted or unsubstituted alkyl(C_{1-8}), alkoxy(C_{1-8}), haloalkyl(C_{1-8}), substituted phenyl or unsubstituted phenyl, wherein if R_1 is =O, C_7–C_8 is a double bond;

R_2 is H,–OH, alkyl(C_{1-8}), substituted phenyl, unsubstituted phenyl, phenyl, phenyl thiazole, imidazole, pyrazole or furan;

R_3 is H, –OH, =O, halogen, haloalkyl(C_{1-8}), substituted or unsubstituted alkyl(C_{1-8}), substituted phenyl or unsubstituted phenyl, wherein if R_3 is =O, C_8–C_9 is a double bond;

R_4 is H or –OH;

R_5 is H,–OH, substituted or unsubstituted alkyl(C_{1-8}) or alkoxy(C_{1-8}), or OR_8, wherein R_8 is alkyl(C_{1-8}), an ester or an amide;

R_6 is H,–OH, substituted or unsubstituted alkyl(C_{1-8}) or alkoxy(C_{1-8}), or OR_8, wherein R_8 is alkyl(C_{1-8}), an ester or an amide; and

R_7 is H, –OH, or substituted or unsubstituted alkyl(C_{1-8}),

with the proviso that R_1 and R_3 cannot both be =O.

R_1 may be =O. R_3 may be =O. The flavone in Morin, Apigenin, Kaempferol, Fisetin, PD98059, 7-(benzyloxy)-4-(trifluoromethyl)-2H-chromen-2-one or 7-[(3-oxobutan-2-yl)oxy]-4-phenyl-2H-chromen-2-one, or a salt of any of the foregoing.

The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, Chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

IV. Antibody Conjugates

Antibodies may be linked to at least one agent to form an antibody conjugate. In order to increase the efficacy of antibody molecules as diagnostic or therapeutic agents, it is conventional to link or covalently bind or complex at least one desired molecule or moiety. Such a molecule or moiety may be, but is not limited to, at least one effector or reporter molecule. Effector molecules comprise molecules having a desired activity, *e.g.*, immunosuppression/anti-inflammation. Non-limiting examples of such molecules are set out above. Such molecules are optionally attached via cleavable linkers designed to allow the molecules to be released at or near the target site.

By contrast, a reporter molecule is defined as any moiety which may be detected using an assay. Non-limiting examples of reporter molecules which have been conjugated to antibodies include enzymes, radiolabels, haptens, fluorescent labels, phosphorescent molecules, chemiluminescent molecules, chromophores, photoaffinity molecules, colored particles or ligands, such as biotin.

Antibody conjugates are generally preferred for use as diagnostic agents. Antibody diagnostics generally fall within two classes, those for use in *in vitro* diagnostics, such as in a variety of immunoassays, and those for use *in vivo* diagnostic protocols, generally known as "antibody-directed imaging." Many appropriate imaging agents are known in the art, as are methods for their attachment to antibodies (see, for *e.g.*, U.S. Patents 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.

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).

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 often being preferred for use in certain embodiments, and technicium^{99m} and/or indium^{111} are also often preferred due to their low energy and suitability for long range detection. Radioactively labeled monoclonal antibodies may be produced according to well-known methods in the art. For instance, monoclonal antibodies 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. Monoclonal antibodies 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 antibody 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 antibody. Intermediary functional groups are often used to bind radioisotopes to antibody and exist as metallic ions are diethylenetriaminepentaacetic acid (DTPA) or ethylene diaminetetracetic acid (EDTA).

Among the fluorescent labels contemplated for use as conjugates 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, TAMRA, TET, Tetramethylrhodamine, and/or Texas Red.

Another type of antibody conjugates contemplated are those intended primarily for use *in vitro*, where the antibody 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 are described, for example, in U.S. Patents 3,817,837, 3,850,752, 3,939,350, 3,996,345, 4,277,437, 4,275,149 and 4,366,241.

Yet another known method of site-specific attachment of molecules to antibodies comprises the reaction of antibodies with hapten-based affinity labels. Essentially, hapten-based affinity labels react with amino acids in the antigen binding site, thereby destroying this site and blocking specific antigen reaction. However, this may not be advantageous since it results in loss of antigen binding by the antibody conjugate.

Molecules containing azido groups may also be used to form covalent bonds to proteins through reactive nitrene intermediates that are generated by low intensity ultraviolet light (Potter and Haley, 1983). In particular, 2- and 8-azido analogues of purine nucleotides have been used as site-directed photoprobes to identify nucleotide binding proteins in crude cell extracts (Owens & Haley, 1987; Atherton *et al.,* 1985). The 2- and 8-azido nucleotides have also been used to map nucleotide binding domains of purified proteins (Khatoon *et al.,* 1989; King *et al.,* 1989; Dholakia *et al.,* 1989) and may be used as antibody binding agents.

Several methods are known in the art for the attachment or conjugation of an antibody 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. Patents 4,472,509 and 4,938,948). Monoclonal antibodies 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. In U.S. Patent 4,938,948, imaging of breast tumors is achieved using monoclonal antibodies and the detectable imaging moieties are bound to the antibody using linkers such as methyl-p-hydroxybenzimidate or N-succinimidyl-3-(4-hydroxyphenyl)propionate.

In other embodiments, derivatization of immunoglobulins by selectively introducing sulfhydryl groups in the Fc region of an immunoglobulin, using reaction conditions that do not alter the antibody combining site are contemplated. Antibody conjugates produced according to this methodology are disclosed to exhibit improved longevity, specificity and sensitivity (U.S. Patent 5,196,066, incorporated herein by reference). Site-specific attachment of effector or reporter molecules, wherein the reporter or effector molecule is conjugated to a carbohydrate residue in the Fc region have also been disclosed in the literature (O'Shannessy *et al.,* 1987). This approach has been reported to produce diagnostically and therapeutically promising antibodies which are currently in clinical evaluation.

V. Immunodetection Methods

In still further embodiments, there are immunodetection methods for binding, purifying, removing, quantifying and otherwise generally detecting MUC1 and its associated antigens. Some immunodetection methods include enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunoradiometric assay, fluoroimmunoassay, chemiluminescent assay, bioluminescent assay, and Western blot to mention a few. In particular, a competitive assay for the detection and quantitation of MUC1-C antibodies also is provided. The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Doolittle and Ben-Zeev (1999), Gulbis and Galand (1993), De Jager *et al.* (1993), and Nakamura *et al.* (1987). In general, the immunobinding methods include obtaining a sample and contacting the sample with a first antibody in accordance with embodiments discussed herein, as the case may be, under conditions effective to allow the formation of immunocomplexes.

Contacting the chosen biological sample with the antibody under effective conditions and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the antibody composition to the sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with, *i.e.*, to bind to MUC1 present. After this time, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or Western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any of those radioactive, fluorescent, biological and enzymatic tags. Patents concerning the use of such labels include U.S. Patents 3,817,837, 3,850,752, 3,939,350, 3,996,345, 4,277,437, 4,275,149 and 4,366,241. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody and/or a biotin/avidin ligand binding arrangement, as is known in the art.

The antibody employed in the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined. Alternatively, the first antibody that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the antibody. In these cases, the second binding

ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under effective conditions and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody that has binding affinity for the antibody, is used to form secondary immune complexes, as described above. After washing, the secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again under effective conditions and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if this is desired.

One method of immunodetection uses two different antibodies. A first biotinylated antibody is used to detect the target antigen, and a second antibody is then used to detect the biotin attached to the complexed biotin. In that method, the sample to be tested is first incubated in a solution containing the first step antibody. If the target antigen is present, some of the antibody binds to the antigen to form a biotinylated antibody/antigen complex. The antibody/antigen complex is then amplified by incubation in successive solutions of streptavidin (or avidin), biotinylated DNA, and/or complementary biotinylated DNA, with each step adding additional biotin sites to the antibody/antigen complex. The amplification steps are repeated until a suitable level of amplification is achieved, at which point the sample is incubated in a solution containing the second step antibody against biotin. This second step antibody is labeled, as for example with an enzyme that can be used to detect the presence of the antibody/antigen complex by histoenzymology using a chromogen substrate. With suitable amplification, a conjugate can be produced which is macroscopically visible.

Another known method of immunodetection takes advantage of the immuno-PCR (Polymerase Chain Reaction) methodology. The PCR method is similar to the Cantor method up to the incubation with biotinylated DNA, however, instead of using multiple rounds of streptavidin and biotinylated DNA incubation, the DNA/biotin/streptavidin/antibody complex is washed out with a low pH or high salt buffer that releases the antibody. The resulting wash

solution is then used to carry out a PCR reaction with suitable primers with appropriate controls. At least in theory, the enormous amplification capability and specificity of PCR can be utilized to detect a single antigen molecule.

A. ELISAs

Immunoassays, in their most simple sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and western blotting, dot blotting, FACS analyses, and the like may also be used.

In one exemplary ELISA, the antibodies of the invention are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition suspected of containing the MUC1 is added to the wells. After binding and washing to remove non-specifically bound immune complexes, the bound antigen may be detected. Detection may be achieved by the addition of another anti-MUC1-C antibody that is linked to a detectable label. This type of ELISA is a simple "sandwich ELISA." Detection may also be achieved by the addition of a second anti-MUC1-C antibody, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

In another exemplary ELISA, the samples suspected of containing the MUC1 antigen are immobilized onto the well surface and then contacted with anti-MUC1-C antibody. After binding and washing to remove non-specifically bound immune complexes, the bound anti-MUC1-C antibodies are detected. Where the initial anti-MUC1-C antibodies are linked to a detectable label, the immune complexes may be detected directly. Again, the immune complexes may be detected using a second antibody that has binding affinity for the first anti-MUC1-C antibody, with the second antibody being linked to a detectable label.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating and binding, washing to remove non-specifically bound species, and detecting the bound immune complexes. These are described below.

In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific

protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein or solutions of milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

In ELISAs, it is probably more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of a protein or antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, and a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or a third binding ligand.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and/or antibodies with solutions such as BSA, bovine gamma globulin (BGG) or phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background.

The "suitable" conditions also mean that the incubation is at a temperature or for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours or so, at temperatures preferably on the order of 25 °C to 27 °C, or may be overnight at about 4 °C or so.

Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween, or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immune complexes may be determined.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact or incubate the first and second immune complex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions that favor the development of further immune complex formation (*e.g.*, incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea, or bromocresol purple, or 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid (ABTS), or H_2O_2, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generated, *e.g.*, using a visible spectra spectrophotometer.

B. Western Blot

The Western blot (alternatively, protein immunoblot) is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein.

Samples may be taken from whole tissue or from cell culture. In most cases, solid tissues are first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), or by sonication. Cells may also be broken open by one of the above mechanical methods. However, it should be noted that bacteria, virus or environmental samples can be the source of protein and thus Western blotting is not restricted to cellular studies only. Assorted detergents, salts, and buffers may be employed to encourage lysis of cells and to solubilize proteins. Protease and phosphatase inhibitors are often added to prevent the digestion of the sample by its own enzymes. Tissue preparation is often done at cold temperatures to avoid protein denaturing.

The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point (pI), molecular weight, electric charge, or a combination of these factors. The nature of the separation depends on the treatment of the sample and the nature of the gel. This is a very useful way to determine a protein. It is also possible to use a two-dimensional (2-D) gel which spreads the proteins from a single sample out in two dimensions. Proteins are separated according to isoelectric point (pH at which they have neutral net charge) in the first dimension, and according to their molecular weight in the second dimension.

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF). The membrane is placed on top of the gel, and a stack of filter papers placed on top of that.

The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. Another method for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this blotting process, the proteins are exposed on a thin surface layer for detection (see below). Both varieties of membrane are chosen for their non-specific protein binding properties (*i.e.*, binds all proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeated probings. The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with Coomassie Brilliant Blue or Ponceau S dyes. Once transferred, proteins are detected using labeled primary antibodies, or unlabeled primary antibodies followed by indirect detection using labeled protein A or secondary labeled antibodies binding to the Fc region of the primary antibodies.

C. Immunohistochemistry

The antibodies may also be used in conjunction with both fresh-frozen and/or formalin-fixed, paraffin-embedded tissue blocks prepared for study by immunohistochemistry (IHC). The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and is well known to those of skill in the art (Brown *et al.*, 1990; Abbondanzo *et al.*, 1990; Allred *et al.*, 1990).

Briefly, frozen-sections may be prepared by rehydrating 50 ng of frozen "pulverized" tissue at room temperature in phosphate buffered saline (PBS) in small plastic capsules; pelleting the particles by centrifugation; resuspending them in a viscous embedding medium (OCT); inverting the capsule and/or pelleting again by centrifugation; snap-freezing in -70°C isopentane; cutting the plastic capsule and/or removing the frozen cylinder of tissue; securing the tissue cylinder on a cryostat microtome chuck; and/or cutting 25-50 serial sections from the capsule. Alternatively, whole frozen tissue samples may be used for serial section cuttings.

Permanent-sections may be prepared by a similar method involving rehydration of the 50 mg sample in a plastic microfuge tube; pelleting; resuspending in 10% formalin for 4 hours fixation; washing/pelleting; resuspending in warm 2.5% agar; pelleting; cooling in ice water to harden the agar; removing the tissue/agar block from the tube; infiltrating and/or

embedding the block in paraffin; and/or cutting up to 50 serial permanent sections. Again, whole tissue samples may be substituted.

D. Immunodetection Kits

In still further embodiments, there are immunodetection kits for use with the immunodetection methods described above.. The immunodetection kits will thus comprise, in suitable container means, a first antibody that binds to MUC1 antigen, and optionally an immunodetection reagent.

In certain embodiments, the MUC1-C antibody may be pre-bound to a solid support, such as a column matrix and/or well of a microtitre plate. The immunodetection reagents of the kit may take any one of a variety of forms, including those detectable labels that are associated with or linked to the given antibody. Detectable labels that are associated with or attached to a secondary binding ligand are also contemplated. Exemplary secondary ligands are those secondary antibodies that have binding affinity for the first antibody.

Further suitable immunodetection reagents for use in the present kits include the two-component reagent that comprises a secondary antibody that has binding affinity for the first antibody, along with a third antibody that has binding affinity for the second antibody, the third antibody being linked to a detectable label. As noted above, a number of exemplary labels are known in the art and all such labels may be employed in connection with embodiments discussed herein.

The kits may further comprise a suitably aliquoted composition of the MUC1 antigen, whether labeled or unlabeled, as may be used to prepare a standard curve for a detection assay. The kits may contain antibody-label conjugates either in fully conjugated form, in the form of intermediates, or as separate moieties to be conjugated by the user of the kit. The components of the kits may be packaged either in aqueous media or in lyophilized form.

The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the antibody may be placed, or preferably, suitably aliquoted. The kits will also include a means for containing the antibody, antigen, and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

VI. Examples

The following examples are included to demonstrate preferred embodiments. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of embodiments, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1 – ANTIBODY PRODUCTION AND SCREENING METHODS

Immunization and Testing the Immune Sera. C57Bl/6 mice were immunized with the protein that contained the ECD of MUC1-C fused to Fc portion of mouse immunoglobulin (MUC1-C/ECD-mFc). Two mice were immunized with 100 µg of MUC1-C/ECD-mFc mixed with Freund's complete adjuvant (FCA) and after 3 days, with 100 µg of MUC1-C/ECD-mFc in PBS. The mice were repeatedly boosted 8 times every 3 days with 50 µg of MUC1-C/ECD-mFc in FCA alternating with 50 µg of MUC1-C/ECD-mFc in PBS as shown in Table 6. Final boosting was performed with 50 µg of antigen intravenously after checking the immune sera. Preimmune serum was collected to be used as negative control. Immune serum was collected after the 7^{th} injection as per the schedule and the serum was tested by both Western blotting and ELISA as per the methods described below.

ELISA. ELISA was performed by coating the plates with 100 µl of 1 µg/ml MUC1-C/ECD-hFc or MUC1-C/ECD-mFc or hFc (as negative control). Hybridoma supernatants or immune sera were screened against the coated proteins by incubating with the plate for 1 hr. The bound antibody was detected by incubating with specific secondary antibody (anti-mouse Ig, $F(ab)_2$ specific) conjugated to HRP (horse radish peroxidase). Further, the reaction was developed with HRP-specific substrate for 30 min and the plate was read at 405 nm.

Western blotting. The performance of antibodies in Western blotting using whole cell lysates was performed as follows. Total cell lysates from ZR-75-1 breast carcinoma, MCF-7 breast carcinoma, H441 non-small cell lung carcinoma and 293T cells were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Purified monoclonal antibody was incubated with the membrane and the antibody bound to the antigen was detected with HRP-conjugated goat anti-mouse Ig, F(ab)2 specific (1:5000 dilution; GE Healthcare) followed by enhanced chemiluminescence (GE Healthcare).

Table 6 - Immunization Schedule and Details

Mouse Strain: Swiss-webser, C57B1/6			**Age: 6 weeks Sex: Female N = 2**
Date	**Dose/animal**	**Adjuvant**	**Comments**
Day 0	100 µg	Complete	Pre-immunization bleed #1-N, #2-N (black) on 4/26/10
Day 3	100 µg	PBS	
Day 5	50 µg	Incomplete	
Day 7	50 µg	PBS	4th injection
Day 10	50 µg	Incomplete	5th injection
Day 12	50 µg	PBS	6th injection
Day 14	50 µg	Incomplete	7th injection
Day 17	50 µg	PBS	Bleeding
Day 17			8th injection
Day 31	100 µg	Incomplete	1st ext.injection i.p.

Fusion for Hybridoma and Primary Screening. Based on the results obtained from the ELISA, spleen from both mice was used for fusion with myeloma cells to generate hybridoma. Spleen-myeloma fusion was performed by mixing mouse myeloma cells sp2/0-Ag14 and splenocytes in 1:3 ratio in the presence of polyethylene glycol (PEG). Post fusion cell culture was carried out in selective HAT medium. Hybridomas selected by indirect ELISA or Western blot using recombinant MUC-C/ECD-mFc or MUC1-C/ECD-hFc as the antigen and were subjected to subcloning by limiting dilution protocol until the clones become stable. The fusion clones obtained from the spleen of mouse 1 is termed as 315.1 clones and similarly the one from mouse 2 is called 315.2 clones. The fusion clones obtained from the spleen of mouse 1 from other immunization batch is termed as 384 clones. From the 315.2 clones, 7 parental positive clones (3G1. 3H7, 4A11, 4H3, 5A4, 8E1 and 8F1) were identified. From mouse 315.1, 6 parental positive clones (1A4, 1G4, 2A6, 6D12, 6A6, 5G6) were selected. From mouse 384, two parental positive clones (2G11,2H11) were selected. These clones were reconfirmed by ELISA and while screening, we excluded any clones that

is reactive to hFc protein alone as the mice were immunized with MUC1-C/ECD-hFc as the antigen.

Secondary Screening. The selected parental clones were subjected to subcloning by limiting dilution in 96-well plates and the supernatants from these wells were subjected to further screening by ELISA. The subclones which had higher absorbance values were expanded in larger wells and the supernatants were selected for confirmation by ELISA. At this stage a maximum of 3 sub-clones from each parental clone were chosen. These selected sub-clones were confirmed to be reactive only to MUC1-C/ECD protein but not hFc protein. Selected sub-clones from 315.1 and 315.2 mouse:

315.1 clones	**315.2 clones**
315.1.2A6.E7	315.2.8E1.F7
315.1.2A6.H5	315.2.8F1.B5
315.1.2A6.H6	315.2.8F1.D2
315.1.6A6.A3	315.2.8F1.F12
315.1.6A6.C8	
315.1.6A6.C11	
315.1.6D12.H3	
315.1.6D12.G6	
315.1.6D12.H10	

Mouse 384 immunized with mammalian mFc-MUC1-C/ECD resulted in two additional clones, 2G11 and 2H11 (FIG. 29). These clones reacted positively with bacterially-produced MUC1-C/ECD protein in western blot analysis (FIG. 30). Hence, the characteristics of these clones are different than 6A6, 8E1, 2A6 and 8F1 (FIG. 30).

Selection of Final Clones. The sub-clones were chosen as per the above mentioned criteria and proceeded either for production and purification of mAb or further sub-cloning depending on the purity (single cell clone) and stability of the clones. Accordingly, the following sub-clones were finally selected for production and purification, after sub-cloning to stability:

315.1.6D12.G6

315.1.**2A6**.H6.F7

315.1.**6A6**.C11.G5

315.2.**8F1**.D2.D1

315.2.**8E1**.F7.C3.H7.H8.F5

384.1.2G11.H5.E4

384.1.2H11.F2.G6

Purification of anti-MUC1-C/ECD Monoclonal Antibodies. Hybridomas were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS containing low bovine IgG. Culture supernatants were passed through proteinA sepharose equilibrated with 50 mM sodium phosphate/300 mM NaCl using an Akta Xpress FPLC system (Amersham Pharmacia, Piscataway, NJ). After washing, antibodies were eluted using 0.1M citrate buffer (pH 3.0). Eluted fractions were neutralized, pooled, dialyzed against PBS, and concentrated using an Amicon Ultracel 10K filter (Millipore, Billerica, MA). All these purified antibodies were tested in ELISA at different dilutions for their reactivity against Muc1-ECD.

Nomenclature of the anti MUC1-C/ECD antibodies. The inventors have developed and purified 7 different monoclonal antibodies directed against the ECD of MUC1-C protein which are identified after the name of the subclone from which they are purified. However for simplicity reason, they have named them after their parental clones as indicate below.

Table 7 – Clone Nomenclature

Name of the clone	Simple name
315.1.6D12.G6 (IgM)	6D12 (IgM)
315.1.2A6.H6.F7	2A6 (IgG)
315.1.6A6.C11.G5	6A6 (IgG)
315.2.8F1.D2.D1	8F1 (IgG)
315.2.8E1.F7.C3.H7.H8.F5	8E1 (IgG)
384.2G11.H5.E4	2G11 (IgG)
384.2H11.F2.G6	2H11 (IgG)
400.6.**2B11**.D5.F6	2B11 (IgG1)
400.6.**4G5**.E11.G2	4G5 (IgG1)
400.6.**7B8**.G3.G1	7B8 (IgG2a)

Western Blot Analysis. The performance of the antibodies in Western blotting was analyzed. MUC1-ECD-mFc and/or MUC1-ECD-hFc (0.5 and 1 µg/lane) was subjected to SDSPAGE and transferred to nitrocellulose membrane at 20V for 60 min. The monoclonal

antibodies were incubated with the membrane and the antibody bound to the antigen was detected with HRP-conjugated goat anti-mouse Ig, F(ab)2 specific (1:5000 dilution; GE Healthcare) followed by enhanced chemiluminescence (GE Healthcare).

Flow Cytometric Analysis. The ability of the antibodies to bind the cell surface MUC1-C protein was analyzed by flow cytometry. Cells were incubated with anti-MUC1-C/ECD antibody or mouse IgG for 30 min, washed, incubated with goat anti-mouse immunoglobulinflourescein-conjugated antibody (Santa Cruz Biotechnology), and fixed in 1% formaldehyde/PBS. Reactivity was detected by immunofluorescence FACScan. Reactivity was detected by immunofluorescence FACScan (Table 8).

Table 8 – Flow Cytometry Data

Cell Line	-ve control	8E1	8F1	+ve (DF3)
KU812/Con siRNA *MUC1-Positive*	1.08	17.9	17.18	95.18
KU812/MUC1siRNA *MUC1-negative*	1.18	6.64	7.6	35.36
U266/Con siRNA *MUC1-positive*	0.94	24.62	16.86	94.68
U266/MUC1siRNA *MUC1-negative*	0.92	4.36	3.04	13.5
K562/Con siRNA *MUC1-positive*	0.86	22.82	34.44	40.61
K562/MUC1siRNA *MUC1-negative*	0.94	6.91	6.37	17.07

Cell Growth Inhibition Assay/Trypan Blue Exclusion. Trypan Blue Exclusion Assay. Estimated number of cells based on their growth rate was plated in a 24-well plate. Following overnight growing of the cells in the wells, 2-4 μg/ml of the antibody was added and mixed to the culture at various intervals (*e.g.*, once in three days) at 37 °C, 5% CO_2. After 6 days of incubation with the antibody, cells were harvested by trypsinization and the viable cells were counted by trypan blue exclusion method.

Cell Growth Inhibition Assay/Alamar Blue. Alternatively, cells were plated in 96-well plates. After overnight growth, the cells were treated with various concentration of the

antibody starting from 10 μg/ml with two fold serial dilution. After 4-6 days of treatment with the antibody(s), alamarBlue reagent was added to the wells and incubated for 3-5 hrs and the absorbance measured by reading the plate at 570 nm (excitation wavelength) and 600 nm (emission wave length) using Thermomax plate reader (Molecular Devices). The percentage reduction of alamarBlue is proportional to the percentage of the live cells in the assay and it was computed using SoftMax Pro software and plotted as a 4-parameter curve.

Cellular internalization of anti-MUC1-C/ECD MAbs 8E1 and 6A6 in ZR-75-1 breast cancer cells and H-1975 lung cancer cells. MAbs 6A6 and 8E1 were directly labeled with either FITC or Alexa Fluor 488 and following labeling, the antibodies were purified according to the manufacturer's instructions. Immunofluorescence was assessed on ZR-75-1 or H-1975 cells grown on glass bottom culture plates. FITC- or Alexa Fluor 488-conjugated MAbs 6A6 or 8E1 were used at concentrations of 4 and 2 μg/ml. MUC1-negative HEK-293T cells were used as a negative control. Initially, surface labeling was carried out at 4 °C for 60 min. Internalization of surface-bound antibodies was initiated by incubation at 37 °C in media for additional 3 hr. Live cells were subsequently analyzed with an epifluorescent microscope using appropriate wavelengths.

Co-localization of MAbs 6A6 and 8E1 with endosomal markers in ZR-75-1 breast carcinoma and H-1975 NSCLC cells. Human ZR-75-1 and H-1975 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, streptomycin (100 mg/ml), penicillin (100 U/ml), and 2 Mm L-glutamine. Cells were seeded at 15,000 cells per plate with RPMI-1640 into glass bottom culture plates 48 h prior to labeling. Alexa Fluor 488-conjugated MAbs 6A6 or 8E1 were used at concentrations of 4 and 2 μg/ml. Alexa Fluor 488-labeled non-specific IgG (isotype control antibody) was used as a negative control. MUC1-negative HEK-293T cells were used as an additional negative control. ZR-75-1 or H-1975 cells were transfected with RFP-endocyte marker (Invitrogen) for 16-18 h prior to incubation with the MAbs. At the appropriate time, cells were washed with PBS and blocked with 0.5% BSA in PBS. Alexa Fluor 488-labeled MAb 8E1 or 6A6 (2 μg/ml) were used to label the cells at 4 °C for 60 min. Following incubation, cells were subsequently washed thrice with PBS andallowed to incubate in culture media for an additional 3 h at either 4 °C to monitor membrane binding or 37 °C for internalization. At the end of treatment period cells were washed thrice with PBS and were examined using a Nikon deconvolution wide-field epiflourescence system using 60X oil

immersion objective and images were captured using NIS-element software (Nikon). All the Images were analyzed using Image J software.

EXAMPLE 2 – ANTIBODY PRODUCTION AND SCREENING RESULTS

The results of Western blotting demonstrated the specific reactivity of the anti-MUC1-C/ECD antibodies with MUC1-C/ECD protein and did not react with MUC1-CD protein (negative control). Cell viability assays obtained by trypan blue exclusion method have been presented in Tables 9 to 27. The results demonstrated clearly that the anti-MUC1-C/ECD antibodies have a distinct and selective effect on cell growth inhibition. The viability of the cells decreased with the increasing concentrations of the antibodies. However, when the antibody concentration is increased to more than 2 mg/ml, it did not affect the viability. This data corroborates with an assay performed simultaneously using trypan blue exclusion method. The selectivity of the antibody in inhibiting certain type cells was demonstrated using MCF-7 cells that did not show any major cell growth inhibition when treated with 6D12 antibody.

TABLE 9

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
Cell only (control)	90	76	84	90	82	84.4	2	1688000	0.09	151920	156240	100.0
	94	96	108	106	104	101.6	2	2032000	0.09	182880		
	98	58	74	77	65	74.4	2	1488000	0.09	133920		
Cells + 4 ug/ml anti-p59 2A6	30	17	30	41	23	28.2	2	564000	0.09	50760	100320	64.2
	69	66	68	67	69	67.8	2	1356000	0.09	122040		
	69	67	70	77	73	71.2	2	1424000	0.09	128160		
Cells + 4 ug/ml anti-p59 6A6	37	51	59	54	46	49.4	2	988000	0.2	197600	200266.7	128.2
	63	54	44	43	48	49.4	2	988000	0.2	197600		
	49	54	55	40	59	51.4	2	1028000	0.2	205600		

Cell Control	100
2A6 (4 ug/ml)	64.2
6A6 (4 ug/ml)	128.2

Treatment of H1975 cells with 4 µg/ml anti-p59 antibody 2A6 and 6A6. Anti-MUC1 CD (4 µg/ml) was used as control. 8000 cells/well/ml RPMI were plated in a 24 well plate. Treatment with the antibody was started next day. Cells were treated every day for 6 days.

TABLE 10

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
Cell only (control)	90	76	84	90	82	84.4	2	1688000	0.09	151920	156240	**100.0**
	94	96	108	106	104	101.6	2	2032000	0.09	182880		
	98	58	74	77	65	74.4	2	1488000	0.09	133920		
Cells + 4 ug/ml anti-p59-2A6	30	17	30	41	23	28.2	2	564000	0.09	50760	100320	**64.2**
	69	66	68	67	69	67.8	2	1356000	0.09	122040		
	69	67	70	77	73	71.2	2	1424000	0.09	128160		
Cells + 4 ug/ml anti-p59-6A6	37	51	59	54	46	49.4	2	988000	0.2	197600	200267	**128.2**
	63	54	44	43	43	49.4	2	988000	0.2	197600		
	49	54	55	40	59	51.4	2	1028000	0.2	205600		

Cell Control	100
2A6 (4 ug/ml)	64.2
6A6 (4 ug/ml)	128.2

Treatment of H-1975 cells with 4 µg/ml anti-p59 antibody 2A6 and 6A6. 8000 cells/well/ml RPMI were plated in a 24 well plate. Treatment with the antibody was started next day. Cells were treated every day for 6 days.

TABLE 11

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
Cell only (control)	67	68	66	59	63	64.6	2	1292000	0.7	904400	1029467	**100.0**
	74	76	76	77	81	76.8	2	1536000	0.7	1075200		
	64	75	97	81	79	79.2	2	1584000	0.7	1108800		
Cells + 4 ug/ml anti-p59-2A6	63	89	81	88	83	80.8	2	1616000	0.7	1131200	1134933	**110.2**
	87	84	90	84	72	83.4	2	1668000	0.7	1167600		
	74	85	89	68	79	79	2	1580000	0.7	1106000		
Cells + 4 ug/ml anti-p59-6A6	75	90	89	74	72	80	2	1600000	0.7	1120000	1249733	**121.4**
	82	73	72	102	79	81.6	2	1632000	0.7	1142400		
	99	110	115	95	112	106.2	2	2124000	0.7	1486800		

Cell Control	100
2A6 (4 ug/ml)	110.2
6A6 (4 ug/ml)	121.4

Treatment of 293T cells with 4 µg/ml anti-p59 antibodies 2A6 and 6A6. Anti-MUC1-CD (4 µg/ml) was used as control. 4500 cells/well/ml in DMEM were plated in a 24 well plate. Treatment with the antibody was started next day Cells were treated every day for 6 days.

TABLE 12

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
Cell only (control)	90	82	107	92	91	92.4	2	1848000	0.1	184800	208133	100.0
	94	135	135	93	109	113.2	2	2264000	0.1	226400		
	98	110	142	89	94	106.6	2	2132000	0.1	213200		
Cells + 4 ug/ml anti-p59-2A6	113	121	118	141	132	125	2	2500000	0.1	250000	268000	128.8
	98	126	141	151	128	128.8	2	2576000	0.1	257600		
	159	131	161	171	119	148.2	2	2964000	0.1	296400		
Cells + 4 ug/ml anti-p59-6A6	54	72	81	60	45	62.4	2	1248000	0.1	124800	141600	68.0
	83	64	67	64	56	66.8	2	1336000	0.1	133600		
	92	86	63	79	96	83.2	2	1664000	0.1	166400		

Cell Control	100
2A6 (4 ug/ml)	128.8
6A6 (4 ug/ml)	68

Treatment of ZR75-1 cells with 4 µg/ml anti-p59 antibody -2A6 and 6A6. Anti-MUC1-CD (4 µg/ml) was used as control. 10,000 cells/well/ml RPMI were plated in a 24 well plate. Treatment was started next day. Cells were treated every day for 6 days.

TABLE 13

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
Cell only (control)	150	169	19	147	158	128.6	2	2572000	0.2	514400	456267	100.0
	117	105	102	100	109	106.6	2	2132000	0.2	426400		
	110	71	107	104	143	107	2	2140000	0.2	428000		
Cells + 4 ug/ml anti-p59-8F1	108	126	113	91	104	108.4	2	2168000	0.2	433600	407733	89.4
	103	76	109	131	111	106	2	2120000	0.2	424000		
	80	66	86	112	113	91.4	2	1828000	0.2	365600		
Cells + 4 ug/ml anti-P59-2A6	103	80	103	101	95	96.4	2	1928000	0.2	385600	340800	74.7
	88	104	93	73	68	85.2	2	1704000	0.2	340800		
	87	87	59	68	69	74	2	1480000	0.2	296000		
Cells + 4 ug/ml anti-P59-6A6	75	67	131	109	91	94.6	2	1892000	0.3	567600	506000	110.9
	83	99	85	87	109	92.6	2	1852000	0.3	555600		
	68	61	62	59	79	65.8	2	1316000	0.3	394800		

Cell Control	100
8F1 (4 ug/ml)	89.4
2A6 (4 ug/ml)	74.7
6A6 (4 ug/ml)	110.9

Treatment of ZR75-1 cells with 4 µg/ml anti-p59 antibodies 8F1, 2A6 and 6A6. Anti-MUC1-CD 4 µg/ml was used as control. 10,000 cells/well/ml RPMI were plated in a 24 well plate. Treat ment of the antibody was done on the same day (the day of seeding cells). Cells harvested on the 7th day.

TABLE 14

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
Cell only (control)	76	86	89	54	63	73.6	2	1472000	0.2	294400	452800	**100.0**
	192	115	147	145	128	145.4	2	2908000	0.2	581600		
	120	131	159	87	106	120.6	2	2412000	0.2	482400		
Cells + 4 ug/ml anti-p59-8F1	35	31	58	68	41	46.6	2	932000	0.25	233000	299333.3	**66.1**
	81	68	56	63	54	64.4	2	1288000	0.25	322000		
	66	72	60	74	71	68.6	2	1372000	0.25	343000		
Cells + 4 ug/ml anti-p59-2A6	51	47	51	43	41	46.6	2	932000	0.25	233000	363000	**80.2**
	64	77	67	107	71	77.2	2	1544000	0.25	386000		
	102	107	93	78	90	94	2	1880000	0.25	470000		
Cells + 4 ug/ml anti-P59-6A6	59	96	106	65	59	77	2	1540000	0.25	385000	417333.3	**92.2**
	96	141	151	96	91	115	2	2300000	0.25	575000		
	66	56	56	51	63	58.4	2	1168000	0.25	292000		

Cell Control	100
8F1 (4 ug/ml)	66.1
2A6 (4 ug/ml)	80.2
6A6 (4 ug/ml)	92.2

Treatment of ZR75-1 cells with 4 µg/ml anti-p59 antibodies 8F1, 2A6 and 6A6. Anti-MUC1-CD (4 µg/ml) was used as control. 5000 cells/well/ml RPMI were plated in a 24 well plate. Treatment of the antibody was done (only on the day of seeding cells). Harvested on the 9th day. the same day.

TABLE 15

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
Cell only (control)	21	37	36	36	31	32.2	2	644000	0.3	193200	198160	100.0
	33	31	43	38	35	36	2	720000	0.24	172800		
	45	47	42	53	51	47.6	2	952000	0.24	228480		
Cells + 2 ug/ml anti-p59	37	25	50	38	34	36.8	2	736000	0.24	176640	179520	90.6
	44	34	43	39	36	39.2	2	784000	0.24	188160		
	32	28	34	44	43	36.2	2	724000	0.24	173760		
Cells + 4 ug/ml anti-p59	39	28	36	28	31	32.4	2	648000	0.24	155520	160000	80.7
	39	48	36	44	30	39.4	2	788000	0.24	189120		
	26	29	31	28	27	28.2	2	564000	0.24	135360		

Cell Control	100
8F1 (2 ug/ml)	80.7
8F1 (4 ug/ml)	90.6

Treatment of H1650 Cells with 2 and 4 µg/ml anti-p59 antibody (315.2.8F1.D2.D1). Anti-MUC1-CD antibody was used at 2 and 4 µg/ml as isotype matched control (IgG1kappa). 8,000 cells/well/800 µl RPMI were plated in a 24 well plate. Treatment was performed for 6 days.

TABLE 16

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
Cell only (control)	40	40	44	44	43	42.2	2	844000	0.3	253200	282000	**100.0**
	35	42	54	51	47	45.8	2	916000	0.3	274800		
	43	61	61	52	48	53	2	1060000	0.3	318000		
Cells + 4 ug/ml anti-p59	38	44	40	41	37	40	2	800000	0.3	240000	274400	**97.3**
	50	55	39	42	54	48	2	960000	0.3	288000		
	39	52	42	61	52	49.2	2	984000	0.3	295200		

Cell Control	100
8F1 (4 ug/ml)	97.3

Treatment of H1975 cells with 4 µg/ml anti-p59 antibody (315.2.8F1.D2.D1). Anti-MUC1-CD (4 µg/ml) was used as control. 8,000 cells/well/800 µl RPMI were plated in a 24 well plate. Treatment with the antibody was started next day. Cells were treated every day for 6 days.

TABLE 17

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
	40	40	44	44	43	42.2	2	844000	0.3	253200		
	35	42	54	51	47	45.8	2	916000	0.3	274800	282000	100.0
Cell only (control)	43	61	61	52	48	53	2	1060000	0.3	318000		
	38	44	40	41	37	40	2	800000	0.3	240000		
	50	55	39	42	54	48	2	960000	0.3	288000	274400	97.3
Cells + 4 ug/ml anti-p59	39	52	42	61	52	49.2	2	984000	0.3	295200		

Treatment of H1975 cells with 4 µg/ml anti-p59 antibody (315.2.8F1.D2.D1). Anti-MUC1-CD (4 µg/ml was used as control. 8,000 cells/well/800 µl RPMI were plated in a 24 well plate. Treatment with the antibody was started next day. Cells were treated every day for 6 days.

TABLE 18

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
	83	66	81	78	76	76.8	2	1536000	0.8	1228800		
	56	59	80	64	49	61.6	2	1232000	0.8	985600	1155200	100.0
Cell only (control)	79	76	85	82	69	78.2	2	1564000	0.8	1251200		
	45	58	55	65	72	59	2	1180000	0.8	944000		
	75	72	77	64	71	71.8	2	1436000	0.8	1148800	1091200	94.5
Cells + 4 ug/ml anti-p59	69	79	70	85	66	73.8	2	1476000	0.8	1180800		

Cell control	100
8F1 (4 ug/ml)	94.5

Treatment of HEK293T cells with 4 µg/ml anti-p59 antibody (315.2.8F1.D2.D1). Anti-MUC1- CD (4 µg/ml) was used as control. 4,500 cells/well/800 µl RPMI were plated in a 24 well plate. Treatment with the antibody was started next day. Cells were planned to be treated every day for 6 days. However, the cells were growing fast and therefore harvested after 5 days of treatment.

TABLE 19

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
	85	92	96	85	79	87.4	2	1748000	0.24	419520		
	82	80	70	65	86	76.6	2	1532000	0.24	367680	390080	100.0
Cell only (control)	70	82	86	80	81	79.8	2	1596000	0.24	383040		
	41	31	45	44	33	38.8	2	776000	0.24	186240		
	48	48	44	41	63	48.8	2	976000	0.24	234240	216000	55.4
Cells + 2 ug/ml anti-p59	40	64	39	54	40	47.4	2	948000	0.24	227520		
	37	32	49	41	27	37.2	2	744000	0.24	178560		
	55	48	43	53	61	52	2	1040000	0.24	249600	210240	53.9
Cells + 4 ug/ml anti-p59	29	50	40	43	49	42.2	2	844000	0.24	202560		

Cell Control	100
2 ug/ml 8F1	55.4
4 ug/ml 8F1	53.9

Treatment of ZR-75.1 Cells with 2 and 4 µg/ml anti-p59 antibody (315.2.8F1.D2.D1). 10,000 cells/well/800 µl RPMI were plated in a 24 well plate. Treatment was performed for 6 days.

TABLE 20

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
	110	164	113	129	108	124.8	2	2496000	0.1	249600		
	70	98	100	107	88	92.6	2	1852000	0.125	231500	251200	100.0
Cell only (control)	118	78	106	121	122	109	2	2180000	0.125	272500		
	85	81	79	90	99	86.8	2	1736000	0.125	217000		
	59	72	63	62	49	61	2	1220000	0.125	152500	159333	63.4
Cells + 4 ug/ml anti-p59	51	48	39	33	46	43.4	2	868000	0.125	108500		

Cell Control	100
8F1 (4 ug/m	63.4

Treatment of ZR 75-1 cells with 4 µg/ml anti-p59 antibody (315.2.8F1.D2.D1). Anti-MUC1-CD (4 µg/ml) was used as control. 10,000 cells/well/800 ul RPMI were plated in a 24 well plate. Treatment with the antibody was started next day. Cells were treated every day for 6 days.

TABLE 21 - Surface Staining: Antibody Testing (FLOW Cytometry) on MM, AML and CML Cell Lines Flow with Clone 6A6

	NC	PE	p58-6A6	DF-3
MOLM-14	1.09	5.24	71.49	99.55
MV4-11	1.01	2.6	96.93	74.19
ME559203	1.09	2.1	69.01	57.03
K562	1.13	1.63	11.56	78.75
KU812	1.09	1.57	11.39	98.06
RPMI8266	1.16	6.35	89.64	94.97
U266	1.11	4.17	98.55	99.19
KMS28PE	1.12	2.23	2.72	58.18

* 2 µg antibody/sample; PE-conjugated goat anti-mouse 2nd antibody

TABLE 22 - Surface Staining: Antibody Testing (FLOW Cytometry) on Breast Cancer Cell Lines and Primary Breast Cancer Patient Cells

	NC	PE	Clone 2A6	Clone 6A6	Clone 8E1	Clone 8F1	DF-3
ZR-75-1	0.98	0.82	0.98	0.92		0.94	98.52
ZR-75-1	1.06					1.34	6.84
ZR-75-1	1.13	1.43	1.78	2.16	1.61	1.57	89.48
JB564765	1.17	2.59	14.25	16.85		8.26	21.06
RG563999	1.19	15.42	42.14	68.24		42.16	60.54
RG563999	0.45	1.14			14.63		11.87

* 2 µg antibody/sample; PE-conjugated goat anti-mouse 2nd antibody

TABLE 23 – Surface Staining: Antibody Testing (FLOW Cytometry) on Chronic Myelogeneous Leukemia (CML) Cell Lines

	NC	PE	Clone 2A6	Clone 6A6	Clone 8E1	Clone 8F1	DF-3
K562	0.9	1.4	10.5	9.31	8.65	9.58	77.9
KU812	1.09	24.93	41.19	31.62	39.59	39.86	90.55

* 2 µg antibody/sample; PE-conjugated goat anti-mouse 2nd antibody.

TABLE 24 - Surface Staining: Antibodies Testing (FLOW Cytometry)

K562/siRNA

	NC	PE	p58-2A6	p58-6A6	P58-8E1	P58-8F1	DF-3
CTL-siRNA	1.13	14.01	29.74	22.65	22.36	34.44	40.61
MUC1-siRNA	0.94	5.81	6.98	6.26	6.91	6.37	17.07

* 2 µg antibody/sample; PE-conjugated goat anti-mouse 2nd antibody.
Fixed samples and kept at 4 °C for 4 days.

TABLE 25 - Surface Staining: Antibodies Testing (FLOW Cytometry)

	NC	PE	p58-2A6	p58-6A6	P58-8E1	P58-8F1	DF-3
KU812/siRNA							
CTL-siRNA	1.09	1.31	14.79	20.08			96.57
MUC1-siRNA	0.97	1.14	4.75	4.08			69.25
U266/siRNA							
CTL-siRNA	1.01	2.2	31.36	84.2			94.67
MUC1-siRNA	1.21	4.78	36.71	93.47			16.23
KU812/siRNA							
CTL-siRNA	1	0.68			17.9	15	95.18
MUC1-siRNA	1.18	1.54			6.64	7.6	85.36
U266/siRNA							
CTL-siRNA	1.18	0.8			24.62	16.86	96.5
MUC1-siRNA	0.92	5.74			4.36	3.04	13.5

* 2 µg antibody/sample; PE-conjugated goat anti-mouse 2nd antibody.

TABLE 26

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
Cell only (control)	90	76	84	90	82	84.4	2	1688000	0.09	151920	156240	**100.0**
	94	96	108	106	104	101.6	2	2032000	0.09	182880		
	98	58	74	77	65	74.4	2	1488000	0.09	133920		
Cells + 4 ug/ml anti-p59-2A6	30	17	30	41	23	28.2	2	564000	0.09	50760	100320	**64.2**
	69	66	68	67	69	67.8	2	1356000	0.09	122040		
	69	67	70	77	73	71.2	2	1424000	0.09	128160		
Cells + 4 ug/ml anti-p59-6A6	37	51	59	54	46	49.4	2	988000	0.2	197600	200267	**128.2**
	63	54	44	43	43	49.4	2	988000	0.2	197600		
	49	54	55	40	59	51.4	2	1028000	0.2	205600		
Cells + 4 ug/ml anti-MUC1-CD	1	0	0	0	0	0.2	2	4000	0.015	60	480	**0.3**
	3	3	3	1	2	2.4	2	48000	0.015	720		
	3	1	4	1	2	2.2	2	44000	0.015	660		

Treatment of H1975 cells with 4 µg/ml anti-p59 antibody 2A6 and 6A6. Anti-MUC1-CD (4 µg/ml) was used as control. 8000 cells/well/ml RPMI were plated in a24 well plate. Treatment with the antibody was started next day. Cells were treated every day for 6 days.

TABLE 27

Treatment	well 1	well 2	well 3	well 4	well 5	Mean	DF	cells/ml	Volume (ml)	Total cells	Mean Cell number	% Live cells
Cell only (control)	32	32	37	45	39	37	2	740000	0.05	37000	38666.67	**100.0**
	48	43	46	43	45	45	2	900000	0.05	45000		
	35	30	40	31	34	34	2	680000	0.05	34000		
Cells + 5 ug/ml IgM control	29	33	39	44	42	37.4	2	748000	0.05	37400	41066.67	**106.2**
	40	42	36	45	42	41	2	820000	0.05	41000		
	45	49	43	49	38	44.8	2	896000	0.05	44800		
Cells + 5 ug/ml anti-p59	26	32	39	39	34	34	2	680000	0.05	34000	37333.33	**96.6**
	38	46	32	41	43	40	2	800000	0.05	40000		
	40	38	36	34	42	38	2	760000	0.05	38000		

Treatment of SKOV3 Cells with 5 µg/ml anti-p59 antibody or IgM control antibody. 10,000 cells/800 µl RPMI was plated in 24 well plates. Treatment with the antibody was started next day. Cells were treated every day for 6 days.

The internalization of MAbs 6A6 and 8E1 conjugated either to FITC or Alexa Fluor 488 was assessed using ZR-75-1 breast carcinoma or H-1975 NSCLC cell lines. As a control, MUC1-negative HEK293T cells were used to define selectivity for binding and internalization. The results demonstrate significant internalization of FITC-labeled 6A6 and 8E1 antibodies when H-1975 NSCLC cells were incubated with the tagged antibodies at 37oC for 3 h (FIGS. 12 and 13). Similar results were obtained when Alexa Fluor 488-labeled 6A6 antibody was used in ZR-75-1 breast carcinoma cells at 37 °C for 3 h (FIG. 14). Neither antibody bound to the MUC1-negative HEK-293T cells when incubated either at 4 °C or 37 °C for 3 h (FIG. 15 and data not shown).

The inventors initially assessed binding of Alexa Fluor 488-6A6 MAb to ZR-75-1 breast carcinoma cells at 4 °C. Immunoflourescence analysis confirmed binding of MAb 6A6 antibody to ZR-75-1 at the plasma membrane at 4 °C (FIG. 18). Similar immunofluorescence surface staining was observed when ZR-75-1 cells were incubated with the RFP endosomal marker and analyzed at 4 °C. Moreover, Alexa Fluor 488-labeled 6A6 (green) was detectable at the cell membrane, where it colocalizes with early endosomes (areas of orange/yellow) at 4 °C. Longer incubations for 3 h at 37 °C resulted in movement of MAb 6A6 into the cytoplasm, where it colocalized with the RFP endosomal marker. This internalization and localization of Alexa Fluor 488-tagged 6A6 antibody within the late endosomes at 37 °C after 3 h was evident by the orange color in the overlay (white arrow, FIG. 19). These findings suggests that the majority of fluorescently labeled 6A6 antibody may have moved out of early endocytic compartments (at 4 °C) and colocalized with late endosomes/lysosomes at 37 °C.

Internalization and colocalization of anti-MUC1-C/ECD Mabs with an endosomal marker in H-1975 non-small cell carcinoma cells. The inventors next assessed internalization of Alexa Fluor 488-6A6 and 8E1 MAbs in H-1975 NSCLC cells. For colocalization studies, H-1975 cells were transfected with RFP-endocyte marker for 16-18 h prior to incubation with the anti-MUC1-C/ECD Mabs 6A6 or 8E1. Furthermore, as control, RFP-endocyte marker transfected H-1975 cells were also incubated separately with Alexa Fluor 488-labeled isotype control antibody (IgG). Immunoflourescence analysis confirmed staining of endosomes after transfection of cells with RFP-endocyte marker (FIG. 26). Importantly, the results demonstrate little, if any, staining of cells when incubated with an IgG isotype control antibody (FIG. 26). Furthermore, immunoflourescence analysis demonstrated internalization of Alexa Fluor 488-labeled MAb 6A6 in H-1975 cells when incubated at 37 °C for three hours (FIG. 20). Similar staining was observed when H-1975 cells were transfected with the RFP-endocyte marker and analyzed at 37 °C. More

importantly, the results demonstrate that Alexa Fluor 488-labeled 6A6 clearly co-localized with endosomes at 37 °C. This internalization and localization of Alexa Fluor 488-tagged 6A6 antibody within the late endosomes at 37 °C after 3 h was evident by the orange/yellow color in the overlay (FIG. 20). Similar results were obtained when RFP-endocyte marker transfected H-1975 cells were incubated with Alexa Fluor 488-labeled anti-MUC1-C/ECD 8E1 Mab (FIG. 21).

The specificity of the conjugates was further examined by evaluating the binding of fluorescently-labeled 6A6 in MUC1-negative HEK-293T cells. The results demonstrate little, if any, staining of Alexa Fluor 488-labeled 6A6 antibody when HEK-293T cells were incubated with the tagged antibody at 4 °C or at 37 °C for 3 hr (FIG. 26). Furthermore, no immunofluorescence was observed when Alexa Fluor 488-labeled isotype control (IgG) antibody was used in ZR-75-1 cells at 4 °C or at 37 °C for 3 hr (data not shown).

Internalization and colocalization of anti-MUC1-C/ECD Mabs with a lysosomal marker in H-1975 non-small cell carcinoma cells. The inventors next assessed internalization of Alexa Fluor 488-6A6 and 8E1 MAbs and their colocalization with lysosomes in H-1975 NSCLC cells. For colocalization studies, H-1975 cells were transfected with RFP-lysosomal marker for 16-18 h prior to incubation with the anti-MUC1-C/ECD Mabs 6A6 or 8E1. Furthermore, as control, RFP-lysosomal marker transfected H-1975 cells were also incubated separately with Alexa Fluor 488-labeled isotype control antibody (IgG).

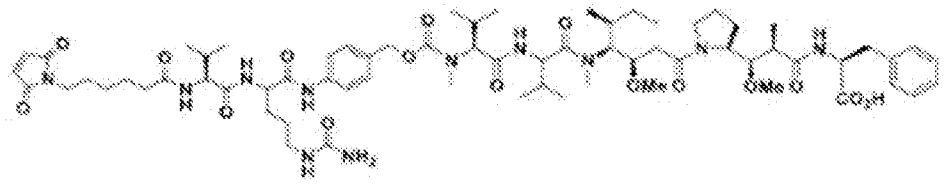
Immunoflourescence analysis confirmed staining of lysosomes after transfection of cells with RFP-lysosomal marker (FIG. 27). Interestingly, the results demonstrate little, if any, staining of cells when incubated with an IgG isotype control antibody (FIG. 27). Furthermore, immunoflourescence analysis demonstrated internalization of Alexa Fluor 488-labeled MAb 8E1 in H-1975 cells when incubated at 37 °C for three hours (FIG. 28). Similar staining was observed when H-1975 cells were transfected with the RFP-lysosomal marker and analyzed at 37 °C. More interestingly, the results demonstrate that Alexa Fluor 488-labeled 8E1 clearly co-localized with lysosomes at 37 °C. This internalization and localization of Alexa Fluor 488-tagged 8E1 antibody within lysosomes at 37 °C after 3 h was evident by the orange/yellow color in the overlay (FIG. 28). Similar results were obtained when RFP-lysosomal marker transfected H-1975 cells were incubated with Alexa Fluor 488-labeled anti-MUC1-C/ECD 6A6 MAb (FIG. 23).

FIG. 3 shows sequence of three overlapping peptides from the MUC1-C/ECD (58 aa). All of the described antibodies fail to react with any of these three peptides.

In conclusion, ELISA data on reactivity and isotype, flow data (specificity and selectivity), Western blot analysis (purified proteins and cell lysates), immunofluorescence (internalization; co-localization with endosomes & lysosomes), linear and conformational epitope mapping (using overlapping ECD peptides and multiple single point mutants) and biological activity studies (multiple cell lines *in vitro*) have been conducted. Multiple IgG clones were identified identified (6A6, 8E1/8F1, 2A6, 2G11, 2H11) and are described further below.

Characterized of 7B8 and 3D1 antibodies. Linear epitope mapping of 7B8 and 3D1 clones was performed using three overlapping peptides spanning the entire MUC1-C/ECD region (58 amino acids) were synthesized. ELISA assays were performed using 7B8 or 3D1 purified antibodies in the presence or absence of P1, P2 or P3 peptides. The results, shown in FIG. 47, demonstrate that the binding of 7B8 and 3D1 to the antigen was not inhibited by any of these three overlapping peptides. Conformational epitope mapping of 7B8 and 3D1 was performed using eight critical individual point mutants in the MUC1-C/ECD region by ELISA. The results demonstrate that antibody 3D1 is sensitive, at least in part, to the D19 amino acid present in the MUC1-C/ECD (FIGS. 48A-B). These findings indicate that D19 is a part of the conformational core/pocket for 3D1 binding. ZR-75-1 breast carcinoma cells were used determine 7B8 or 3D1 antibody binding in a competitive format with MUC1-C/ECD and analyzed by FLOW. The results demonstrate that in contrast to 7B8, incubation of cells with MUC1-C/ECD protein completely abrogates the binding of 3D1 (FIG. 49). These findings indicate that the conformational binding epitopes of 7B8 and 3D1 are different. The sequencing results for hybridoma 3D1 is shown in FIG. 50 and is discussed in greater detail below.

Antibody-Drug Conjugates (ADC) of 7B8 and 3D1 were generated by conjugating 2 mg each of the monoclonal antibodies to monomethyl auristatin F (MMAF) via a cleavable linker valine-citrulline–p-aminobenzyl (Val-Cit-PAB). MMAF is a new auristatin derivative with a charged C-terminal phenylalanine that attenuates its cytotoxic activity. The structure of the generated ADC is as shown below.



The inventors determined *in vitro* efficacy of 7B8-MMAF and 3D1-MMAF conjugates using cleavable linker. Anti-proliferative effects of 7B8 and 3D1 conjugates were evaluated on multiple cultured MUC1-positive cell lines. Importantly, 7B8 and 3D1 linked to MMAF through a cleavable linker displayed significant anti-proliferative activity compared with CD1 (an antibody that target the inside cytoplasmic domain of MUC1-C) linked to MMAF. 7B8 and 3D1 antibody conjugates induced a dose-dependent inhibition of proliferation in ZR-75-1 breast carcinoma cells with IC_{50} values of ~860 pM and 1.32 nM respectively (FIG. 51A). Similar results were obtained when MDA-MB-468 triple negative breast carcinoma cells were treated with 7B8 or 3D1 MMAF conjugates (FIG. 51B).

EXAMPLE 3 – ANTIBODY SEQUENCING METHODS AND RESULTS

8E1/8F1. Total RNA was extracted from hybridomas using Qiagen kit. QIAGEN® OneStep RT-PCR Kit (Cat No. 210210) was used. RT-PCR was performed with primer sets specific for the heavy and light chains. For each RNA sample, 12 individual heavy chain and 11 light chain RT-PCR reactions were set up using degenerate forward primer mixtures covering the leader sequences of variable regions. Reverse primers are located in the constant regions of heavy and light chains. No restriction sites were engineered into the primers.

Reaction setup was as follows:

5x QIAGEN® OneStep RT-PCR Buffer	5.0 µl
dNTP Mix (containing 10 mM of each dNTP)	0.8 µl
Primer set	0.5 µl
QIAGEN® OneStep RT-PCR Enzyme Mix	0.8 µl
Template RNA	2.0 µl
RNase-free water	to 20.0 µl
Total volume	20.0 µl

PCR conditions were as follows:

Reverse transcription:	50 °C, 30 min
Initial PCR activation:	95 °C, 15 min
Cycling: 20 cycles of	94 °C, 25 sec
	54 °C, 30 sec
	72 °C, 30 sec
Final extension:	72 °C, 10 min

The RT-PCR products from the first-round reactions were further amplified in the second-round PCR. 12 individual heavy chain and 11 light chain RT-PCR reactions were set up using semi-nested primer sets specific for antibody variable regions.

Reaction setup was as follows:

2x PCR mix	10 µl
Primer set	2 µl
First-round PCR product	8 µl
Total volume	20 µl

PCR conditions were as follows:

Initial denaturing of 5 min at 95 °C,

25 cycles of 95 °C for 25 sec,
57 °C for 30 sec,
68 °C for 30 sec,

Final extension is 10 min 68 °C.

After PCR is finished, PCR reaction samples were run onto agarose gel to visualize DNA fragments amplified. The correct antibody variable region DNA fragments should have a size between 400-500 base pair.

PCR positive bands are cloned by TOPO, then PCR-amplified, followed by gel electrophoresis and recovery from agarose gel. Approximately 24 clones were sequenced and CDR analysis was performed using sequencing data, and two heavy chains and one light chain were identified and are shown below:

The heavy chain amino acid and nucleotide sequences are show below:

EVQLVESGGGLVQPGESLKLSCESNEYEFPSHDMSWVRKTPEKRLELVAAINSDGGSTYY
PDTMERRFIISRDNTKKTLYLQMSSLRSEDTALYYCVRLYYGNVMDYWGQGTSVTVSS

(SEQ ID NO: 15)

GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGGAGAGTCCCTGAAACTC
TCCTGTGAATCCAATGAATACGAATTCCCTTCCCATGACATGTCTTGGGTCCGCAAGACT
CCGGAGAAGAGGCTGGAGTTGGTCGCAGCCATTAATAGTGATGGTGGTAGCACCTACTAT
CCAGACACCATGGAGAGACGATTCATCATCTCCAGAGACAATACCAAGAAGACCCTGTAC
CTGCAAATGAGCAGTCTGAGGTCTGAGGACACAGCCTTGTATTACTGTGTAAGACTCTAC
TATGGTAATGTTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA

(SEQ ID NO: 16)

The light chain amino acid and nucleotide sequences are shown below:

DVVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLYWYLQKPGQSPKLLIYKVSNRF
SGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPLTFGAGTKLELK
(SEQ ID NO: 19)

TCAGAGCCTTGTACACAGTAATGGAAACACCTATTTATATTGGTACCTACAGAAGCCAGG
CCAGTCTCCAAAGCTCCTGATCTACAAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAG
GTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGA
GGATCTGGGAGTTTATTTCTGCTCTCAAAGTACACATGTTCCTCTCACGTTCGGTGCTGG
GACCAAGCTGGAGCTGAAAC
(SEQ ID NO: 20)

6A6. Total RNA was extracted from hybridoma cells using QIAGEN® RNeasy Mini Kit (Cat No. 74104). First-round RT-PCR QIAGEN® OneStep RT-PCR Kit (Cat No. 210210) was used. RT-PCR was performed with primer sets specific for the heavy and light chains. For each RNA sample, 12 individual heavy chain and 11 light chain RT-PCR reactions were setup using degenerate forward primer mixtures covering the leader sequences of variable regions. Reverse primers are located in the constant regions of heavy and light chains. No restriction sites were engineered into the primers.

Reaction Setup was as follows:

5x QIAGEN® OneStep RT-PCR Buffer	5.0 µl
dNTP Mix (containing 10 mM of each dNTP)	0.8 µl
Primer set	0.5 µl
QIAGEN® OneStep RT-PCR Enzyme Mix	0.8 µl
Template RNA	2.0 µl
RNase-free water	10.9 µl
Total volume	20.0 µl

PCR conditions were as follows:

Reverse transcription:	50 °C, 30 min
Initial PCR activation:	95 °C, 15 min
20 cycles of	94 °C, 25 sec
	54 °C, 30 sec

	72 °C, 30 sec
Final extension:	72 °C, 10 min

Second-round semi-nested PCR The RT-PCR products from the first-round reactions were further amplified in the second-round PCR. 12 individual heavy chain and 11 light chain RT-PCR reactions were setup using semi-nested primer sets specific for antibody variable regions.

Reaction Setup was as follows:

2x PCR mix 10.0 µl

Primer set 2.0 µl

First-round PCR product 8.0 µl

Total volume 20.0 µl

PCR conditions were as follows:

Initial denaturing:	95°C, 5 min
25 cycles of	95°C, 25 sec
	57°C, 30 sec
	68°C, 30 sec
Final extension:	68°C, 10 min

After PCR was finished, PCR reaction samples were analyzed on an agarose gel to visualize the amplified DNA fragments. The correct antibody variable region DNA fragments should have a size between 400-500 base pairs. PCR positive bands were cloned by TOPO, and then PCR-amplified, followed by gel electrophoresis and recovery from agarose gel. Approximately 24 clones were sequenced and CDR analysis was performed using sequencing data (CDR regions were defined using VBASE2, world-wide-web at vbase2.org). One heavy chain and one light chain were identified:

The heavy chain amino acid and nucleotide sequences are show below:

QVQLKESGPGLVAPSQSLSMTCTVSGFSLTTYGVHWVRQPPGKGLEWLVVIWSDGSTT YNSPLKSRLSISRDNSKSQVFLKMNSLQADDTAIYYCAKNYLGSLDYWGQGTSVTVSS (SEQ ID NO: 17)

CAGGTGCAGCTGAAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCGCAGAGCCTGTCCA TGACATGCACCGTCTCAGGGTTTTCATTAACTACCTATGGTGTTCACTGGGTTCGCCA GCCTCCAGGAAAGGGTCTGGAGTGGCTGGTAGTGATATGGAGTGATGGAAGCACAACC TATAATTCACCTCTCAAGTCCAGACTGAGCATCAGCAGGGACAACTCCAAGAGCCAAG

TATTCTTAAAAATGAACAGTCTCCAAGCTGATGACACAGCCATCTACTACTGTGCCAA
AAATTACCTCGGTAGTCTGGACTACTGGGGTCAGGGAACCTCAGTCACCGTCTCCTCA
(SEQ ID NO: 18)

The light chain amino acid and nucleotide sequences are shown below:

DVVLTQTPLSLPVSLGDQASISCRSSQSLVHNNGDTYLHWYLQKPGQSPKLLIYKVSN
RFSGVPDRFSGSGSGTDFTFKISRVEAEDLGVYFCSQTTHVPLTFGAGTKLELK
(SEQ ID NO: 21)

GATGTTGTGTTGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCT
CCATCTCTTGCAGATCTAGTCAGAGCCTTGTACACAATAATGGAGACACCTATTTACA
TTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGATCTACAAAGTTTCCAAC
CGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACAT
TCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAAACTAC
ACATGTTCCGCTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAAC
(SEQ ID NO: 22)

2H11. The heavy chain amino acid and nucleotide sequences are show below:

QIQLVQSGPELKKPGETVKTSCKASGYTFTGYSMHWVKQAPGKGLKWMGWINTETGEPTY
ADDFKGRFALSLETSASTTYLQINNLKNEDTATYFCVRGTGGDDWGQGTTLTVSSAKTTP
(SEQ. ID NO: 23)

CAGATCCAGTTGGTGCAGTCTGGACCTGAGCTGAAGAAGCCTGGAGAGACAGTCAAGACC
TCCTGCAAGGCCTCTGGTTATACCTTCACAGGCTATTCAATGCACTGGGTGAAGCAGGCT
CCAGGAAAGGGTTTAAAGTGGATGGGCTGGATAAACACTGAGACTGGTGAGCCAACATAT
GCAGATGACTTCAAGGGACGGTTTGCCTTGTCTCTGGAAACCTCTGCCAGCACTACCTAT
TTGCAGATCAACAACCTCAAAAATGAGGACACGGCTACATATTTCTGTGTTAGGGGGACG
GGGGGTGACGACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGCCAAAACGACACCC
(SEQ. ID NO: 24)

The light chain amino acid and nucleotide sequences are shown below:

DVVMTQTPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYLQKPGQSPKLLIYKVSNRF
SGVPDRFSGSGSGTDFTLKINRVEAEDLGVYFCSQGTHVPPTFGGGTKLEIKRADAAPTV
(SEQ. ID No: 25)

GATGTTGTGATGACCCAAACTCCGCTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCC
ATCTCTTGCAGATCTAGTCAGAGCCTTGTACACAGTAATGGAAACACCTATTTACATTGG
TACCTTCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGATCTACAAAGTTTCCAACCGGTTT
TCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATC
AACAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAAGGTACACATGTTCCT
CCGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGGGCTGATGCTGCACCAACTGTA
(SEQ. ID No: 26)

2B11. The heavy chain amino acid and nucleotide sequences are shown below:

QVQLQQSGAELMKPGASVKISCKAIGFTFNYFWIEWVKQRPGHGLEWIGEILPGTGSTNY
NEKFKGKAIFTADTSSNTAYMQLRSLTSEDSAVYYCVRYDYTSSMDYWGQGTSVTVSS
(SEQ ID NO: 60)

CAGGTTCAGCTGCAGCAGTCTGGAGCTGAGCTGATGAAGCCTGGGGCCTCAGTGAAAATT
TCCTGCAAGGCTATTGGCTTCACATTCAATTACTTCTGGATAGAGTGGGTAAAACAGAGG
CCTGGGCATGGCCTTGAGTGGATTGGAGAGATTTTACCTGGAACTGGTAGTACTAACTAC
AATGAGAAGTTCAAGGGCAAGGCCATATTCACTGCAGATACATCCTCCAACACAGCCTAC
ATGCAACTCCGCAGCCTGACATCTGAGGACTCTGCCGTCTATTACTGTGTAAGATACGAC
TATACCTCTTCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAG
(SEQ ID NO: 61)

The light chain amino acid and nucleotide sequences are shown below:

NIVMTQSPKSMSMSVGERVTLTCKASENVGTYVSWYQQKPEQSPKLLIYGASNRYTGVPN
RFTGSGSATDFTLTISSVQAEDLADYYCGQSYSYPWTFGGGTKLEIK
(SEQ ID NO: 62)

AACATTGTAATGACCCAATCTCCCAAATCCATGTCCATGTCAGTAGGAGAGAGGGTCACC
TTGACCTGCAAGGCCAGTGAGAATGTGGGTACTTATGTATCCTGGTATCAACAGAAACCA
GAGCAGTCTCCTAAACTACTGATATACGGGGCATCCAACCGGTACACTGGGGTCCCCAAT
CGCTTCACGGGCAGTGGATCTGCAACAGATTTCACTCTGACCATCAGCAGTGTGCAGGCT

GAAGACCTTGCAGATTATTACTGTGGACAGAGTTACAGCTATCCGTGGACGTTCGGTGGA
GGCACCAAGCTGGAAATCAAAC

(SEQ ID NO: 63)

4G5. The heavy chain amino acid and nucleotide sequences are shown below:

QITLKESGPGILQPSQTLSLTCSFSGFSLSTSGMGVSWIRQPSGKGLEWLSHIYWDDDKR
YNPSLKSRLSISKDTSRNQVFLKITSVDTADTATYYCAPGVSSWFPYWGPGTLVTVSA

(SEQ ID NO: 64)

CAGATTACTCTGAAAGAGTCTGGCCCTGGGATATTGCAGCCCTCCCAGACCCTCAGTCTG
ACTTGTTCTTTCTCTGGGTTTTCACTGAGCACTTCTGGTATGGGTGTGAGCTGGATTCGT
CAGCCTTCAGGAAAGGGTCTGGAATGGCTGTCACACATTTACTGGGATGATGACAAGCGC
TATAACCCATCCCTGAAGAGCCGACTCTCAATCTCCAAGGATACCTCCAGAAACCAGGTA
TTCCTCAAGATCACCAGTGTGGACACTGCAGATACTGCCACATACTACTGTGCTCCCGGC
GTATCCTCATGGTTTCCTTACTGGGGCCCAGGGACTCTGGTCACTGTCTCTGCAG

(SEQ ID NO: 65)

The light chain amino acid and nucleotide sequences are shown below:

SIVMTQTPKFLPVSAGDRVTVTCKASQSVGNYVAWYQQKPGQSPKLLIYFASNRYSGVPD
RFTGSGSGTDFTFTISSVQVEDLAVYFCQQHYIFPYTFGSGTKLEIK

(SEQ ID NO: 66)

AGTATTGTGATGACCCAGACTCCCAAATTCCTGCCTGTATCAGCAGGAGACAGGGTTACC
GTGACCTGCAAGGCCAGTCAGAGTGTGGGTAATTATGTAGCCTGGTACCAACAGAAGCCA
GGACAGTCTCCTAAACTACTGATATACTTTGCATCCAATCGCTATAGTGGAGTCCCTGAT
CGCTTCACTGGCAGTGGATCTGGGACAGATTTCACTTTCACCATCAGCAGTGTGCAGGTT
GAAGACCTGGCAGTTTATTTCTGTCAGCAGCATTATATCTTTCCGTATACGTTCGGATCG
GGGACCAAGCTGGAAATAAAAC

(SEQ ID NO: 67)

7B8. The heavy chain amino acid and nucleotide sequences are shown below:

QVQLQQPGAELVKPGASEKLSCKASGHTFTSYWMHWVKQRPGQGLEWIGEINPSNGRTYY
NENFKTKATLTVDKYSSSASMQLRSLTSEDSAVYYCASDGDYVSGFAYWGQGTTLTVSS

(SEQ ID NO: 68)

CAGGTCCAACTGCAGCAGCCTGGGGCTGAACTGGTGAAGCCTGGGGCTTCAGAGAAGCTG
TCCTGCAAGGCTTCTGGGCACACCTTCACCAGCTACTGGATGCACTGGGTGAAGCAGAGG
CCTGGACAAGGCCTTGAGTGGATTGGAGAGATTAATCCTAGCAACGGTCGTACTTACTAC
AATGAGAACTTCAAGACCAAGGCCACACTGACTGTAGACAAATATTCCAGCTCAGCCTCC
ATGCAACTCCGCAGCCTGACATCTGAGGACTCTGCGGTCTATTACTGTGCAAGTGATGGT
GACTACGTCTCGGGCTTTGCCTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCAG

(SEQ ID NO: 69)

The light chain amino acid and nucleotide sequences are shown below:

DIVLTQSPGSLAVSLGQSVTISCRASESVQYSGTSLMHWYQQKPGQPPKLLIYGASNVET
GVPARFSGSGSGTDFSLNIHPVEEDDIAMYFCQQNWKVPWTFGGGTKLEIK

(SEQ ID NO: 70)

GACATTGTGCTCACCCAATCTCCAGGTTCTTTGGCTGTGTCTCTAGGGCAGAGTGTCACC
ATCTCCTGCAGAGCCAGTGAAAGTGTTCAATATTCTGGCACTAGTTTAATGCACTGGTAT
CAACAGAAACCAGGACAGCCACCCAAACTCCTCATCTATGGTGCATCCAACGTAGAGACT
GGGGTCCCTGCCAGGTTTAGTGGCAGTGGGTCTGGGACAGACTTCAGCCTCAACATCCAT
CCTGTGGAGGAGGATGATATTGCAATGTATTTCTGTCAGCAAAATTGGAAGGTTCCTTGG
ACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC

(SEQ ID NO: 71)

3D1. Total RNA was extracted from frozen hybridoma cells provided by the client following the technical manual of TRIzol® Reagent. The total RNA was analyzed by agarose gel electrophoresis. Total RNA was reverse transcribed into cDNA using isotype-specific anti-sense primers or universal primers following the technical manual of PrimeScript™ 1st Strand cDNA Synthesis Kit. The antibody fragments of V_H and V_L were amplified according to the standard operating procedure of RACE of GenScript.

Amplified antibody fragments were separately cloned into a standard cloning vector using standard molecular cloning procedures. Colony PCR screening was performed to identify clones with inserts of correct sizes. No less than five single colonies with inserts of correct sizes were sequenced for each antibody fragment.

The isolated RNA of the sample was run alongside a DNA Marker III (Tiangen Cat. No. MD103) on a 1.5% agarose/GelRed™ gel (FIG. 52). Four microliters of PCR products of

each sample were run alongside the DNA marker Marker III on a 1.5% agarose/GelRed™ gel (FIG. 53). The PCR products were purified and stored at -20 °C until further use.

Five single colonies with correct VH and VL insert sizes were sent for sequencing. The VH and VL genes of five different clones (see the attached file for sequence and sequence alignment for details) were found nearly identical. The consensus sequence, listed below, is believed to be the sequence of the antibody produced by the hybridoma 441.3.3D1.D6.D11.B1.F10 (Anti-Muc-1).

The heavy chain nucleotide and amino acid sequences are shown below:

CAGGTTCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAGGCCTGGGTCCTCAGTGAAGATT
TCCTGCAAGACTTCTGGCTATGCATTCAGTAACTTCTGGATGAACTGGGTGAAGCAGAGG
CCTGGACAGGGTCTAGAGTGGATTGGACAGATTTATCCTGGAGATGGTGACACTAACTAC
AATGGAAAGTTCAAGGGTAAAGCCACACTGACTGCAGACAAATCCTCCAGCACAGCCTAC
ATGCAGCTCAGCAGTCTAACATCTGAGGCCTCTGCGGTCTATTTCTGTGCAAGGTCCTAC
TATAGGTCGGCCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCTCTGTCTCTGCA

(SEQ ID NO: 72)

QVQLQQSGAELVRPGSSVKISCKTSGYAFSNFWMNWVKQRPGQGLEWIGQIYPGDGDTNY
NGKFKGKATLTADKSSSTAYMQLSSLTSEASAVYFCARSYYRSAWFAYWGQGTLVSVSA

(SEQ ID NO: 73)

The heavy chain nucleotide and amino acid sequences are shown below:

GACATCTTGCTAACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGT
TTCTCCTGCAGGGCCAGTCAGAGCATTGGCACAAGCATACACTGGTATCAGCAAAGAACA
AATGGTTCTCCAAGGCTTCTCATAAAGTATGCTTCTGAGTCTATCTCTGGGATCCCTTCC
AGGTTTAGTGGCAGTGGATCAGGGACAGATTTTACTCTTAGCATCAACAGTGTGGAGTCT
GAAGATATTGCAGATTATTACTGTCAACAAAGTAATAACTGGCCACTCACGTTCGGTGCT
GGGACCAAGCTGGAGCTGAAA

(SEQ ID NO: 74)

DILLTQSPAILSVSPGERVSFSCRASQSIGTSIHWYQQRTNGSPRLLIKYASESISGIPS
RFSGSGSGTDFTLSINSVESEDIADYYCQQSNNWPLTFGAGTKLELK

(SEQ ID NO: 75)

* * * * * * * * * * * * * * * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

VII. REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

U.S. Patent 3,817,837
U.S. Patent 3,850,752
U.S. Patent 3,939,350
U.S. Patent 3,996,345
U.S. Patent 4,196,265
U.S. Patent 4,275,149
U.S. Patent 4,277,437
U.S. Patent 4,366,241
U.S. Patent 4,472,509
U.S. Patent 4,554,101
U.S. Patent 4,680,338
U.S. Patent 4,683,202
U.S. Patent 4,684,611
U.S. Patent 4,816,567
U.S. Patent 4,867,973
U.S. Patent 4,879,236
U.S. Patent 4,938,948
U.S. Patent 4,952,500
U.S. Patent 5,021,236
U.S. Patent 5,141,648
U.S. Patent 5,196,066
U.S. Patent 5,217,879
U.S. Patent 5,302,523
U.S. Patent 5,322,783
U.S. Patent 5,384,253
U.S. Patent 5,464,765
U.S. Patent 5,506,138
U.S. Patent 5,538,877
U.S. Patent 5,538,880

U.S. Patent 5,550,318
U.S. Patent 5,563,055
U.S. Patent 5,563,250
U.S. Patent 5,565,332
U.S. Patent 5,580,859
U.S. Patent 5,589,466
U.S. Patent 5,610,042
U.S. Patent 5,656,610
U.S. Patent 5,670,488
U.S. Patent 5,702,932
U.S. Patent 5,736,524
U.S. Patent 5,739,018
U.S. Patent 5,780,448
U.S. Patent 5,789,215
U.S. Patent 5,824,544
U.S. Patent 5,830,725
U.S. Patent 5,849,304
U.S. Patent 5,851,826
U.S. Patent 5,856,456
U.S. Patent 5,858,744
U.S. Patent 5,871,982
U.S. Patent 5,871,983
U.S. Patent 5,871,986
U.S. Patent 5,879,934
U.S. Patent 5,880,270
U.S. Patent 5,888,502
U.S. Patent 5,925,565
U.S. Patent 5,928,906
U.S. Patent 5,932,210
U.S. Patent 5,935,819
U.S. Patent 5,945,100
U.S. Patent 5,955,331
U.S. Patent 5,981,274
U.S. Patent 5,994,136

U.S. Patent 5,994,624

U.S. Patent 6,013,516

U.S. Patent Serial No. 12/789,127

U.S. Patent Serial No. 13/026,858

U.S. Patent Serial No. 13/045,033

"Antibodies: A Laboratory Manual," Cold Spring Harbor Press, Cold Spring Harbor, NY, 1988.

Abbondanzo *et al.*, *Am. J. Pediatr. Hematol. Oncol.*, 12(4), 480-489, 1990.

Allred *et al.*, *Arch. Surg.*, 125(1), 107-113, 1990.

Almendro *et al.*, *J. Immunol.*, 157(12):5411-5421, 1996.

Amado and Chen, *Science*, 285(5428):674-676, 1999.

Angel *et al.*, *Cell,* 49:729, 1987a.

Angel *et al.*, *Cell*, 49:729, 1987b.

Armentano *et al., Proc. Natl. Acad. Sci. USA,* 87(16):6141-6145, 1990.

Atchison and Perry, *Cell*, 46:253, 1986.

Atchison and Perry, *Cell*, 48:121, 1987.

Atherton *et al., Biol. of Reproduction*, 32, 155-171, 1985.

Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, N.Y., 1994.

Baldus *et al., Clin. Cancer Res.*, 10(8):2790-2796, 2004.

Banerji *et al.*, *Cell*, 27:299, 1981.

Banerji *et al.*, *Cell*, 33(3):729-740, 1983.

Bates, *Mol. Biotechnol.*, 2(2):135-145, 1994.

Batra *et al.*, *Am. J. Respir. Cell Mol. Biol.*, 21(2):238-245, 1999.

Battraw and Hall, *Theor. App. Genet.*, 82(2):161-168, 1991.

Beidler *et al., J. Immunol.*, 141(11):4053-4060, 1988.

Berkhout *et al.*, *Cell*, 59:273-282, 1989.

Bett *et al.*, *J. Virololgy*, 67(10):5911-5921, 1993.

Bhattacharjee *et al.*, *J. Plant Bioch. Biotech.*, 6(2):69-73. 1997.

Bilbao *et al.*, *FASEB J.*, 11(8):624-634, 1997.

Blackwell *et al.*, *Arch. Otolaryngol Head Neck Surg.*, 125(8):856-863, 1999.

Blanar *et al.*, *EMBO J.,* 8:1139, 1989.

Blomer *et al.*, *J. Virol.*, 71(9):6641-6649, 1997.

Bodine and Ley, *EMBO J.*, 6:2997, 1987.

Boshart *et al.*, *Cell*, 41:521, 1985.

Bosze *et al.*, *EMBO J.*, 5(7):1615-1623, 1986.

Braddock *et al.*, *Cell*, 58:269, 1989.

Brown *et al.*, *J. Immunol. Meth.*, 12;130(1), :111-121, 1990.

Bulla and Siddiqui, *J. Virol.*, 62:1437, 1986.

Campbell and Villarreal, *Mol. Cell. Biol.,* 8:1993, 1988.

Campbell, *In*: *Monoclonal Antibody Technology, Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 13, Burden and Von Knippenberg, Eds. pp. 75-83, Amsterdam, Elsevier, 1984.

Campere and Tilghman, *Genes and Dev.*, 3:537, 1989.

Campo *et al.*, *Nature*, 303:77, 1983.

Capaldi *et al.*, *Biochem. Biophys. Res. Comm.*, 74(2):425-433, 1977.

Caplen *et al.*, *Gene Ther.*, 6(3):454-459, 1999.

Carbonelli *et al.*, *FEMS Microbiol. Lett.*, 177(1):75-82, 1999.

Case *et al.*, *Proc. Natl. Acad. Sci. USA*, 96(6):2988-2993, 1999.

Celander and Haseltine, *J. Virology*, 61:269, 1987.

Celander *et al.*, *J. Virology,* 62:1314, 1988.

Chandler *et al.*, *Proc. Natl. Acad. Sci. USA*, 94(8):3596-601, 1997.

Chang *et al.*, *Mol. Cell. Biol.*, 9:2153, 1989.

Chatterjee *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:9114, 1989.

Chen and Okayama, *Mol. Cell Biol.*, 7(8):2745-2752, 1987.

Chillon *et al., J. Virol.,* 73(3):2537-2540, 1999.

Choi *et al.*, *J. Mol. Biol.*, 262(2):151-167, 1996.

Christou *et al.*, *Proc. Natl. Acad. Sci. USA,* 84(12):3962-3966, 1987.

Clay *et al., J. Immunol.*, 162:1749, 1999.

Cocea, *Biotechniques*, 23(5):814-816, 1997.

Coffey *et al.*, *Science*, 282(5392):1332-1334, 1998.

Cohen *et al.*, *J. Cell. Physiol.*, 5:75, 1987.

Costa *et al.*, *Mol. Cell. Biol.*, 8:81-90, 1988.

Cripe *et al.*, *EMBO J.*, 6:3745, 1987.

Culotta and Hamer, *Mol. Cell. Biol.*, 9:1376-1380, 1989.

D'Halluin *et al.*, *Plant Cell*, 4(12):1495-1505, 1992.

Dandolo *et al.*, *J. Virology,* 47:55-64, 1983.

De Jager *et al.*, *Semin. Nucl. Med.* 23(2), 165-179, 1993.

DeLuca *et al.*, *J. Virol.*, 56(2):558-570, 1985.

Derby *et al.*, *Hear Res*, 134(1-2):1-8, 1999.

Deschamps *et al.*, *Science*, 230:1174-1177, 1985.

Dholakia *et al.*, *J. Biol. Chem.*, 264, 20638-20642, 1989.

Doolittle and Ben-Zeev, *Methods Mol. Biol.*, 109, :215-237, 1999.

Dorai *et al.*, *Int. J. Cancer*, 82(6):846-52, 1999.

Duraisamy *et al.*, *Gene*, 373:28-34, 2006.

Edbrooke *et al.*, *Mol. Cell. Biol.*, 9:1908-1916, 1989.

Edlund *et al.*, *Science*, 230:912-916, 1985.

Engel and Kohn, *Front Biosci*, 4:e26-33, 1999.

EP Application 125,023

EP Application 171,496

EP Application 173,494

EP Application 184,187

EPO 0273085

Fechheimer *et al.*, *Proc Natl. Acad. Sci. USA,* 84:8463-8467, 1987.

Feldman *et al.*, *Cardiovasc. Res.*, 32(2):194-207, 1996.

Feldman *et al.*, *Semin. Interv. Cardiol.*, 1(3):203-208,1996.

Feng and Holland, *Nature*, 334:6178, 1988.

Feng *et al.*, *Nat. Biotechnol.*, 15(9):866-870, 1997.

Firak and Subramanian, *Mol. Cell. Biol.*, 6:3667, 1986.

Fisher *et al.*, *Hum. Gene Ther.*, 7(17):2079-2087, 1996.

Foecking and Hofstetter, *Gene*, 45(1):101-105, 1986.

Fraley *et al.*, *Proc. Natl. Acad. Sci. USA,* 76:3348-3352, 1979.

Fujita *et al.*, *Cell,* 49:357, 1987.

Fujiwara and Tanaka, *Nippon Geka Gakkai Zasshi*, 99(7):463-468, 1998.

Garoff and Li, *Curr. Opin. Biotechnol.*, 9(5):464-469, 1998.

Garrido *et al.*, *J. Neurovirol.*, 5(3):280-288, 1999.

Gefter *et al.*, *Somatic Cell Genet.*, 3:231-236, 1977.

Gendler *et al.*, *J. Biol. Chem.*, 263:12820–12823, 1988.

Ghosh and Bachhawat, In: *Liver Diseases, Targeted Diagnosis and Therapy Using Specific Receptors and Ligands,* Wu *et al.* (Eds.), Marcel Dekker, NY, 87-104, 1991.

Gillies *et al.*, *Cell*, 33:717, 1983.

Gloss *et al.*, *EMBO J.*, 6:3735, 1987.

Gnant *et al.*, *Cancer Res.*, 59(14):3396-403, 1999.

Gnant *et al.*, *J. Natl. Cancer Inst.*, 91(20):1744-1750, 1999.

Godbout *et al.*, *Mol. Cell. Biol.*, 8:1169, 1988.

Goding, *In: Monoclonal Antibodies: Principles and Practice*, 2d ed., Orlando, Fla., Academic Press, 60-61, 65-66, 71-74, 1986.

Goodbourn and Maniatis, *Cell,* 41(2):509-520, 1985.

Goodbourn *et al.*, *Cell*, 45:601, 1986.

Gopal, *Mol. Cell Biol.*, 5:1188-1190, 1985.

Graham and Prevec, *Mol Biotechnol*, 3(3):207-220, 1995.

Graham and Van Der Eb, *Virology*, 52:456-467, 1973.

Greene *et al.*, *Immunology Today*, 10:272, 1989.

Grosschedl and Baltimore, *Cell*, 41:885, 1985.

Gulbis and Galand, *Hum. Pathol.* 24(12), 1271-1285, 1993.

Haecker *et al., Hum. Gene Ther.,* 7(15):1907-1914, 1996.

Han *et al., J. Infect. Dis.,* 179:230-233, 1999.

Harland and Weintraub, *J. Cell Biol.*, 101(3):1094-1099, 1985.

Haslinger and Karin, *Proc. Natl. Acad. Sci. USA*, 82:8572, 1985.

Hauber and Cullen, *J. Virology*, 62:673, 1988.

Hayashi *et al.*, *Neurosci. Lett.,* 267(1):37-40, 1999.

He *et al., Plant Cell Reports*, 14 (2-3):192-196, 1994.

Hen *et al.*, *Nature*, 321:249, 1986.

Hensel *et al.*, *Lymphokine Res.*, 8:347, 1989.

Hermens and Verhaagen, *Prog. Neurobiol.*, 55(4):399-432, 1998.

Herr and Clarke, *Cell*, 45:461, 1986.

Hirochika *et al.*, *J. Virol.*, 61:2599, 1987.

Hirsch *et al.*, *Mol. Cell. Biol.*, 10:1959, 1990.

Holbrook *et al.*, *Virology*, 157:211, 1987.

Holzer *et al. Virology*, 253(1):107-114, 1999.

Horlick and Benfield, *Mol. Cell. Biol.*, 9:2396, 1989.

Hou and Lin, *Plant Physiology*, 111:166, 1996.

Howard *et al.*, *Ann. NY Acad. Sci.*, 880:352-365, 1999.

Huang *et al.*, *Cell,* 27:245, 1981.

Huard *et al.*, *Neuromuscul Disord*, 7(5):299-313, 1997.

Hug *et al.*, *Mol. Cell. Biol.*, 8:3065-3079, 1988.

Hwang *et al.*, *Mol. Cell. Biol.*, 10:585, 1990.

Imagawa *et al.*, *Cell,* 51:251, 1987.

Imai *et al.*, *Nephrologie*, 19(7):397-402, 1998.

Imbra and Karin, *Nature*, 323:555, 1986.

Imler *et al.*, *Mol. Cell. Biol.*, 7:2558, 1987.

Imperiale and Nevins, *Mol. Cell. Biol.*, 4:875, 1984.

Irie *et al., Antisense Nucleic Acid Drug Dev.*, 9(4):341-349, 1999.

Jakobovits *et al.*, *Mol. Cell. Biol.,* 8:2555, 1988.

Jameel and Siddiqui, *Mol. Cell. Biol.*, 6:710, 1986.

Jaynes *et al.*, *Mol. Cell. Biol.*, 8:62, 1988.

Johnson *et al., Mol. Cell. Biol.*, 9(8):3393-3399, 1989.

Johnston *et al.*, *J. Virol.,* 73(6):4991-5000, 1999.

Jones *et al.*, *Nature,* 321:522-525, 1986.

Kadesch and Berg, *Mol. Cell. Biol.*, 6:2593, 1986.

Kaeppler *et al., Plant Cell Rep.*, 8:415–418, 1990.

Kaneda *et al.*, *Science*, 243:375-378, 1989.

Karin *et al.*, *Mol. Cell. Biol.*, 7:606, 1987.

Karin *et al.*, *Mol. Cell. Biol.*, 7:606, 1987.

Katinka *et al.*, *Cell*, 20:393, 1980.

Katinka *et al.*, *Nature*, 290:720, 1981.

Kato *et al, J. Biol. Chem.*, 266:3361-3364, 1991.

Kaufman *et al.*, *Arch. Ophthalmol.*, 117(7):925-928, 1999.

Kawamoto *et al.*, *Mol. Cell. Biol.*, 8:267, 1988.

Kay, *Haemophilia*, 4(4):389-392, 1998.

Khatoon *et al., Ann. of Neurology*, 26, 210-219, 1989.

Kiledjian *et al.*, *Mol. Cell. Biol.*, 8:145, 1988.

King *et al., J. Biol. Chem.*, 269, 10210-10218, 1989.

Kinlough *et al., J. Biol. Chem.,* 279(51):53071-53077, 2004.

Kinoshita *et al., Biochem. Biophys. Res. Commun.*, 394:205-210, 2010.

Klamut *et al.*, *Mol. Cell. Biol.*, 10:193, 1990.

Klimatcheva *et al.*, *Front Biosci,* 4:D481-496, 1999.

Koch *et al.*, *Mol. Cell. Biol.*, 9:303, 1989.

Kohler and Milstein, *Eur. J. Immunol.*, 6, 511-519, 1976.

Kohler and Milstein, *Nature*, 256, 495-497, 1975.

Kohut *et al.*, *Am. J. Physiol.*, 275(6Pt1):L1089-1094, 1998.

Kooby *et al.*, *FASEB J*, 13(11):1325-34, 1999.

Kraus *et al. FEBS Lett.*, 428(3):165-170, 1998.

Kriegler and Botchan, *Mol. Cell. Biol.*, 3:325, 1983.

Kriegler *et al.*, *Cell*, 38:483, 1984a.

Kriegler *et al.*, *In: Cancer Cells 2/Oncogenes and Viral Genes,* Van de Woude *et al.* eds, Cold Spring Harbor, Cold Spring Harbor Laboratory, 1984b.

Krisky *et al.*, *Gene Ther*, 5(11):1517-1530, 1998a.

Krisky *et al.*, *Gene Ther*, 5(12):1593-1603, 1998b.

Kuhl *et al.*, *Cell*, 50:1057, 1987.

Kunz *et al.*, *Nucl. Acids Res.*, 17:1121, 1989.

Kyte and Doolittle, *J. Mol. Biol.,* 157(1):105-132, 1982.

Lachmann and Efstathiou, *Curr. Opin. Mol. Ther.*, 1(5):622-632, 1999..

Lareyre *et al., J. Biol. Chem.*, 274(12):8282-8290, 1999.

Larsen *et al.*, *Proc. Natl. Acad. Sci. USA*, 83:8283, 1986.

Laspia *et al.*, *Cell*, 59:283, 1989.

Latimer *et al.*, *Mol. Cell. Biol.*, 10:760, 1990.

Lazzeri, *Methods Mol. Biol.*, 49:95-106, 1995.

Lee *et al.*, *Environ. Mol. Mutagen.*, 13(1):54-59, 1989.

Lee *et al.*, *Nature*, 294:228, 1981.

Lee *et al., Nucleic Acids Res.,* 12:4191-206, 1984.

Lee *et al.*, *DNA Cell Biol.*, 16(11):1267-1275, 1997.

Leibowitz *et al.*, *Diabetes*, 48(4):745-753, 1999.

Lesch, *Biol Psychiatry*, 45(3):247-253, 1999.

Levenson *et al.*, *Hum. Gene Ther.*, 9(8):1233-1236, 1998.

Li *et al., Cancer Biol. Ther.*, 2:187-193, 2003b.

Lin *et al.*, *Mol. Cell. Biol.*, 10:850, 1990.

Lundstrom, *J. Recept Signal Transduct. Res.*, 19(1-4):673-686, 1999.

Luria *et al.*, *EMBO J.*, 6:3307, 1987.

Lusky and Botchan, *Proc. Natl. Acad. Sci. USA*, 83:3609, 1986.

Lusky *et al.*, *Mol. Cell. Biol.*, 3:1108, 1983.

Macejak and Sarnow, *Nature*, 353:90-94, 1991.

Majors and Varmus, *Proc. Natl. Acad. Sci. USA*, 80:5866, 1983.

Marienfeld *et al., Gene Ther.*, 6(6):1101-1113, 1999.

Mastrangelo *et al.*, *Biotechnol. Bioeng.*, 65(3):298-305, 1999.

McNeall *et al.*, *Gene*, 76:81, 1989.

Miksicek *et al.*, *Cell*, 46:203, 1986.

Miller *et al.*, *J. Pharmacol. Exp. Ther.*, 264:11-16, 1993.

Miyatake *et al.*, *Gene Ther.*, 6:564-572, 1999.

Moldawer *et al.*, *Shock*, 12(2):83-101, 1999.

Mordacq and Linzer, *Genes and Dev.*, 3:760, 1989.

Moreau *et al.*, *Nucl. Acids Res.*, 9:6047, 1981.

Moriuchi *et al.*, *Cancer Res*, 58(24):5731-5737, 1998.

Morrison *et al., J. Gen. Virol.*, 78(Pt 4):873-878, 1997.

Morrison, *Science*, 229(4719):1202-1207, 1985.

Muesing *et al.*, *Cell*, 48:691, 1987.

Nakamura *et al.*, *In*: *Enzyme Immunoassays: Heterogeneous and Homogeneous Systems*, Chapter 27, 1987.

Naldini *et al.*, *Science*, 272(5259):263-267, 1996.

Neuberger *et al., Nucleic Acids Res.*, 16(14B):6713-6724, 1988.

Neumann *et al.*, *Proc. Natl. Acad. Sci. USA*, 96(16):9345-9350, 1999.

Ng *et al.*, *Nuc. Acids Res.*, 17:601, 1989.

Nicolau and Sene, *Biochim. Biophys. Acta,* 721:185-190, 1982.

Nicolau *et al.*, *Methods Enzymol.*, 149:157-176, 1987.

Nomoto *et al.*, *Gene*, 236(2):259-271, 1999.

Omirulleh *et al.*, *Plant Mol. Biol.,* 21(3):415-428, 1993.

Omitz *et al.*, *Mol. Cell. Biol.,* 7:3466, 1987.

Ondek *et al.*, *EMBO J.*, 6:1017, 1987.

O'Shannessy *et al., J. Immun. Meth.*, 99, 153-161, 1987.

Owens and Haley, *J. Biol. Chem.*, 259, 14843-14848, 1987.

Palmiter *et al.*, *Cell,* 29:701, 1982.

Parks *et al., J. Virol.*, 71(4):3293-8, 1997.

PCT Application PCT/US86/02269

PCT Application WO 86/01533

PCT Appln. WO 92/17598

PCT Appln. WO 94/09699

PCT Appln. WO 95/06128

Pech *et al.*, *Mol. Cell. Biol.*, 9:396, 1989.

Pelletier and Sonenberg, *Nature*, 334(6180):320-325, 1988.

Perales *et al., Proc. Natl. Acad. Sci. USA*, 91:4086-4090, 1994.

Perez-Stable and Constantini, *Mol. Cell. Biol.*, 10:1116, 1990.

Petrof, *Eur Respir J,* 11(2):492-497, 1998.

Picard and Schaffner, *Nature*, 307:83, 1984.

Pinkert *et al.*, *Genes and Dev.,* 1:268, 1987.

Ponta *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:1020, 1985.

Posner *et al.*, *Hybridoma* 6, 611-625, 1987.

Potrykus *et al.*, *Mol. Gen. Genet.,* 199(2):169-177, 1985.

Potter and Haley, *Meth. Enzymol.*, 91, 613-633, 1983.

Potter *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:7161-7165, 1984.

Queen and Baltimore, *Cell*, 35:741, 1983.

Quinn *et al.*, *Mol. Cell. Biol.*, 9:4713, 1989.

Rabinovitch*et al.*, *Diabetes*, 48(6):1223-1229, 1999.

Reddy *et al., Virology,* 251(2):414-26, 1998.

Redondo *et al.*, *Science*, 247:1225, 1990.

Reisman and Rotter, *Mol. Cell. Biol.*, 9:3571, 1989.

Remington's Pharmaceutical Sciences, 15th Ed., 33:624-652, 1990.

Resendez Jr. *et al.*, *Mol. Cell. Biol.*, 8:4579, 1988.

Rhodes *et al.*, *Methods Mol. Biol.,* 55:121-131, 1995.

Rippe *et al.*, *Mol. Cell. Biol.*, 9(5):2224-22277, 1989.

Rippe, *et al.*, *Mol. Cell Biol.*, 10:689-695, 1990.

Rittling *et al.*, *Nucl. Acids Res.*, 17:1619, 1989.

Robbins and Ghivizzani, *Pharmacol Ther*, 80(1):35-47, 1998.

Robbins *et al., Trends Biotechnol.,* 16(1):35-40, 1998.

Sambrook *et al.*, In: *Molecular cloning: a laboratory manual,* 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Sawai *et al. Mol. Genet. Metab.*, 67(1):36-42, 1999.

Schaffner *et al.*, *J. Mol. Biol.*, 201:81, 1988.

Searle *et al.*, *Mol. Cell. Biol.*, 5:1480, 1985.

Sharp and Marciniak, *Cell*, 59:229, 1989.

Shaul and Ben-Levy, *EMBO J.*, 6:1913, 1987.

Shaw *et al., J. Natl. Cancer Inst.*, 80(19):1553-1559, 1988.

Sherman *et al.*, *Mol. Cell. Biol.*, 9:50, 1989.

Sleigh and Lockett, *J. EMBO*, 4:3831, 1985.

Smith *et al.*, *Neuron.*, 20:1093-1102, 1998.

Spalholz *et al.*, *Cell*, 42:183, 1985.

Spandau and Lee, *J. Virology*, 62:427, 1988.

Spandidos and Wilkie, *EMBO J.*, 2:1193, 1983.

Stephens and Hentschel, *Biochem. J.*, 248:1, 1987.

Stewart *et al., Arch. Biochem. Biophys.* 365:71-74; 1999.

Stuart *et al.*, *Nature*, 317:828, 1985.

Sullivan and Peterlin, *Mol. Cell. Biol.*, 7:3315, 1987.

Sun *et al., J. Steroid Biochem.*, 26(1):83-92, 1987.

Suzuki *et al., Biochem Biophys Res Commun*, 252(3):686-90, 1998.

Swartzendruber and Lehman, *J. Cell. Physiology*, 85:179, 1975.

Takebe *et al.*, *Mol. Cell. Biol.*, 8:466, 1988.

Tavernier *et al.*, *Nature*, 301:634, 1983.

Taylor and Kingston, *Mol. Cell. Biol.*, 10:165, 1990a.

Taylor and Kingston, *Mol. Cell. Biol.*, 10:176, 1990b.

Taylor *et al.*, *J. Biol. Chem.*, 264:15160, 1989.

Thiesen *et al.*, *J. Virology*, 62:614, 1988.

Timiryasova *et al.*, *Int. J. Oncol.,* 14(5):845-854, 1999.

Treisman, *Cell*, 42:889, 1985.

Tronche *et al.*, *Mol. Biol. Med.*, 7:173, 1990.

Tronche *et al.*, *Mol. Cell. Biol.*, 9:4759, 1989.

Trudel and Constantini, *Genes and Dev.*, 6:954, 1987.

Tsukada *et al.*, *Plant Cell Physiol.*, 30(4)599-604, 1989.

Tsumaki *et al.*, *J. Biol. Chem.,* 273(36):22861-22864, 1998.

Tur-Kaspa *et al.*, *Mol. Cell Biol.*, 6:716-718, 1986.

Tyndall *et al.*, *Nuc. Acids. Res.*, 9:6231, 1981.

Vanderkwaak *et al.*, *Gynecol Oncol,* 74(2):227-234, 1999.

Vasseur *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:1068, 1980.

Verhoeyen *et al.*, *Science*, 239(4847):1534-1536, 1988.

Wagner *et al., Proc. Natl. Acad. Sci. USA* 87(9):3410-3414, 1990.

Wang and Calame, *Cell*, 47:241, 1986.

Wang *et al., Infect. Immun.*, 66:4193-202, 1998.

Wawrzynczak & Thorpe, *Cancer Treat Res.*, 37:239-51, 1988.

Weber *et al.*, *Cell*, 36:983, 1984.

Wei *et al., Cancer Cell*, 7:167-178, 2005.

Weihl *et al., Neurosurgery*, 44(2):239-252, 1999.

Wen *et al., J. Biol. Chem.*, 278:38029–38039, 2003.

White *et al. J. Virol.,* 73(4):2832-2840, 1999.

Wilson, *J. Clin. Invest.*, 98(11):2435, 1996.

Winoto and Baltimore, *Cell*, 59:649, 1989.

Wong *et al.*, *Gene*, 10:87-94, 1980.

Wood *et al., J. Clin. Lab. Immunol.*, 17(4):167-171, 1985.

Wu and Wu, *Adv. Drug Delivery Rev.*, 12:159-167, 1993.

Wu and Wu, *J. Biol. Chem.*, 262:4429-4432, 1987.

Wu *et al., Biochem. Biophys. Res. Commun.,* 233(1):221-226, 1997.

Wu *et al., Cancer Res.,* 58(8): 1605-8, 1998.

Yamada *et al., Brain Res.,* 833(2):302-307, 1999.

Yeung *et al., Gene Ther.,* 6(9):1536-1544, 1999.

Yoon *et al., J. Gastrointest. Surg.*, 3(1):34-48, 1999.

Yutzey *et al. Mol. Cell. Biol.*, 9:1397, 1989.

Zhao-Emonet *et al.*, *Biochim. Biophys. Acta*, 1442(2-3):109-119, 1998.

Zheng *et al., J. Gen. Virol.*, 80(Pt 7):1735-1742, 1999.

Zhou *et al., Nature*, 361(6412):543-547 , 1993.

Zufferey *et al.*, *Nat. Biotechnol.*, 15(9):871-875, 1997.

WHAT IS CLAIMED IS:

1. An antibody that binds selectively to MUC1-C extracellular domain (MUC1-C/ECD) defined by SEQ ID NO:1, wherein said antibody:

   (a) is an IgG antibody;

   (b) inhibits cancer cell growth;

   (c) induces cancer cell death;

   (d) comprises a variable heavy chain comprising CDR1, CDR2 and CDR3 regions each having 90% or more homology to SEQ ID NOS: 3, 4, and 5, or 6, 7 and 8, or 27, 28 and 29, or 42, 43 and 44, or 45, 46 and 47, or 48, 49 and 50, or 76, 77 and 78, and a variable light chain comprising CDR1, CDR2 and CDR3 regions each having 90% or more homology to SEQ ID NOS: 9, 10 and 11 or 12, 13 and 14, or 30, 31 and 32, or 51, 52, and 53, or 54, 55 and 56, or 57, 58 and 59,or 79, 80 and 81 respectively;

   (e) comprises a variable heavy chain having 80% or more homology to SEQ ID NO: 15, 19, 23, 60, 64, 68 or 73, and a variable light chain having 80% or more homology to SEQ ID NO: 17, 21, 25, 62, 66, 70 or 75, respectiely; and/or

   (f) comprises a variable heavy chain encoded by a nucleic acid having 70% or more homology to SEQ ID NO: 16, 20, 24, 61, 65, 69 or 72, and a variable light chain encoded by a nucleic acid having 70% or more homology to SEQ ID NO: 18, 22, 26, 63, 67, 71 or 74, respectively.

2. The antibody of claim 1, wherein said antibody is a single chain antibody.

3. The antibody of claim 1, wherein said antibody is a single domain antibody.

4. The antibody of claim 1, wherein said antibody is a chimeric antibody.

5. The antibody of claim 1, wherein said antibody a Fab fragment.

6. The antibody of claim 1, wherein said antibody is a recombinant antibody having specificity for the MUC1-C/ECD and a distinct cancer cell surface antigen.

7. The antibody of claim 1, wherein said antibody is a murine antibody.

8. The antibody of claim 7, wherein said murine antibody is an IgG.

9. The antibody of claim 1, wherein antibody is a humanized antibody.

10. The antibody of claim 9, wherein said humanized antibody is an IgG.

11. The antibody of claim 1, wherein said antibody further comprises a label.

12. The antibody of claim 11, wherein said label is a peptide tag, an enzyme, a magnetic particle, a chromophore, a fluorescent molecule, a chemilluminescent molecule, or a dye.

13. The antibody of claim 1, wherein said antibody further comprises an antitumor drug linked thereto.

14. The antibody of claim 13, wherein said antitumor drug is linked to said antibody through a photolabile linker.

15. The antibody of claim 13, wherein said antitumor drug is linked to said antibody through an enzymatically-cleaved linker.

16. The antibody of claim 13, wherein said antitumor drug is a toxin, a radioisotope, a cytokine or an enzyme.

17. The antibody of claim 1, wherein said heavy and light chains have 85%, 90%, 95% or 99% homology to SEQ ID NO: 15, 19, 23, 60, 64, 68 or 73 and 17, 21, 25, 62, 66, 70 or 75 respectively.

18. The antibody of claim 1, wherein said heavy and light chains are encoded by nucleic acids having 85%, 90%, 95% or 99% homology to SEQ ID NO: 14, 18, 24, 61, 65, 69 or 72, and 18, 22, 26, 63, 67, 71 or 74, respectively.

19. The antibody of claim 1, wherein said antibody is conjugated to a nanoparticle or a liposome.

20. The antibody of claim 1, wherein induction of cell death comprises antibody-dependent cell cytotoxicity or complement-mediated cytoxocity.

21. A method of treating cancer comprising contacting a MUC1-positive cancer cell in a subject with the antibody of claim 1.

22. The method of claim 21, wherein said MUC1-positive cancer cell is a solid tumor cell.

23. The method of claim 22, wherein said solid tumor cell is a lung cancer cell, brain cancer cell, head & neck cancer cell, breast cancer cell, skin cancer cell, liver cancer cell, pancreatic cancer cell, stomach cancer cell, colon cancer cell, rectal cancer cell, uterine cancer cell, cervical cancer cell, ovarian cancer cell, testicular cancer cell, skin cancer cell, or esophageal cancer cell.

24. The method of claim 21, wherein said MUC1-positive cancer cell is a leukemia or myeloma.

25. The method of claim 24, wherein said leukemia or myeloma is acute myeloid leukemia, chronic myelogenous leukemia or multiple myeloma.

26. The method of claim 21, further comprising contacting said MUC1-positive cancer cell with a second anti-cancer agent or treatment.

27. The method of claim 26, wherein said second anti-cancer agent or treatment is selected from chemotherapy, radiotherapy, immunotherapy, hormonal therapy, or toxin therapy.

28. The method of claim 26, wherein said second anti-cancer agent or treatment inhibits an intracellular MUC1 function.

29. The method of claim 26, wherein said second anti-cancer agent or treatment is given at the same time as said first agent.

30. The method of claim 26, wherein said second anti-cancer agent or treatment is given before and/or after said first agent.

31. The method of claim 21, wherein said MUC1-positive cancer cell is a metastatic cancer cell, a multiply drug resistant cancer cell or a recurrent cancer cell.

32. The method of claim 21, wherein said antibody is a single chain antibody.

33. The method of claim 21, wherein said antibody is a single domain antibody.

34. The method of claim 21, wherein said antibody is a chimeric antibody.

35. The method of claim 21, wherein said antibody a Fab fragment.

36. The method of claim 21, wherein said antibody is a recombinant antibody having specificity for the MUC1-C/ECD and a distinct cancer cell surface antigen.

37. The method of claim 21, wherein said antibody is a murine antibody.

38. The method of claim 37, wherein said murine antibody is an IgG.

39. The method of claim 21, wherein antibody is a humanized antibody.

40. The method of claim 39, wherein said humanized antibody is an IgG.

41. The method of claim 21, wherein said antibody further comprises an antitumor drug linked thereto.

42. The method of claim 41, wherein said antitumor drug is linked to said antibody through a photolabile linker.

43. The method of claim 41, wherein said antitumor drug is linked to said antibody through an enzymatically-cleaved linker.

44. The method of claim 41, wherein said antitumor drug is a toxin, a radioisotope, a cytokine, or an enzyme.

45. The method of claim 21, wherein said antibody further comprises a label.

46. The method of claim 45, wherein said label is a peptide tag, an enzyme, a magnetic particle, a chromophore, a fluorescent molecule, a chemilluminescent molecule, or a dye.

47. The method of claim 21, wherein said heavy and light chains have 85%, 90%, 95% or 99% homology to SEQ ID NO: 15, 19, 23, 60, 64, 68 or 73 and 17, 21, 25, 62, 66, 70 or 75, respectively.

48. The method of claim 21, wherein said heavy and light chains are encoded by nucleic acids having 85%, 90%, 95% or 99% homology to SEQ ID NO: 14, 18, 24, 61, 65, 69 or 72, and 18, 22, 26, 63, 67, 71 or 74, respectively.

49. The method of claim 21, wherein said antibody is conjugated to a liposome or nanoparticle.

50. The method of claim 21, wherein said antibody resuts in the induction of cell death, such as by antibody-dependent cell cytotoxicity or complement-mediated cytoxocity.

51. A fusion protein comprising:

(i) a first single chain antibody that binds selectively to MUC1-C/extracellular domain (ECD) defined by SEQ ID NO:1, wherein said antibody:

(a) is an IgG antibody;

(b) inhibits cancer cell growth;

(c) induces cancer cell death;

(d) comprises a variable heavy chain comprising CDR1, CDR2 and CDR3 regions each having 90% or more homology to SEQ ID NOS: 3, 4, and 5, or 6, 7 and 8, or 27, 28 and 29, or 42, 43 and 44, or 45, 46 and 47, or 48, 49 and 50, or 76, 77 and 78, and a variable light chain comprising CDR1, CDR2 and CDR3 regions each having 90% or more homology to SEQ ID NOS: 9, 10 and 11 or 12, 13 and 14, or 30, 31 and 32, or 51, 52, and 53, or 54, 55 and 56, or 57, 58 and 59, or 79, 80 and 81, respectively;

(e) comprises a variable heavy chain having 80% or more homology to SEQ ID NO: 15, 19, 23, 60, 64, 68 or 73, and a variable light chain having 80% or more homology to SEQ ID NO: 17, 21, 25, 62, 66, 70 or 75, respectively; and/or

(f) comprises a variable heavy chain encoded by a nucleic acid having 70% or more homology to SEQ ID NO: 16, 20, 24, 61, 65, 69 or 72, and a variable light chain encoded by a nucleic acid having 70% or

more homology to SEQ ID NO: 18, 22, 26, 63, 67, 71 or 74, respectively; and

(ii) a second single chain antibody that binds to a T or B cell.

52. The fusion protein of claim 51, wherein said second single chain antibody binds to to CD3.

53. The fusion protein of claim 51, wherein said second single chain antibody binds to a T cell.

54. The fusion protein of claim 51, wherein said second single chain antibody binds to a B cell.

55. The fusion protein of claim 51, wherein said fusion protein further comprises a label or a therapeutic moiety.

56. A chimeric antigen receptor comprising:

(i) an ectodomain comprising single chain antibody variable region that binds selectively to MUC1-C/extracellular domain (MUC1-C/ECD) defined by SEQ ID NO:1, wherein said antibody:

(a) is an IgG antibody;

(b) inhibits cancer cell growth;

(c) induces cancer cell death;

(d) comprises a variable heavy chain comprising CDR1, CDR2 and CDR3 regions each having 90% or more homology to SEQ ID NOS: 3, 4, and 5, or 6, 7 and 8, or 27, 28 and 29, or 42, 43 and 44, or 45, 46 and 47, or 48, 49 and 50, or 76, 77 and 78, and a variable light chain comprising CDR1, CDR2 and CDR3 regions each having 90% or more homology to SEQ ID NOS: 9, 10 and 11 or 12, 13 and 14, or 30, 31 and 32, or 51, 52, and 53, or 54, 55 and 56, or 57, 58 and 59, or 79, 80 and 81, respectively;

(e) comprises a variable heavy chain having 80% or more homology to SEQ ID NO: 15, 19, 23, 60, 64, 68 or 73, and a variable light chain having 80% or more homology to SEQ ID NO: 17, 21, 25, 62, 66, 70 or 75, respectiely; and/or

(f) comprises a variable heavy chain encoded by a nucleic acid having 70% or more homology to SEQ ID NO: 16, 20, 24, 61, 65, 69 or 72, and a variable light chain encoded by a nucleic acid having 70% or more homology to SEQ ID NO: 18, 22, 26, 63, 67, 71 or 74, respectively; and

with a flexible hinge attached at the C-terminus of said single chain antibody variable region;

(ii) a transmembrane domain; and

(iii) an endodomain,

wherein said endodomain comprises a signal transduction function when said single-chain antibody variable region is engaged with MUC1.

57. The receptor of claim 56, wherein said transmembrane and endodomains are derived from the same molecule.

58. The receptor of claim 56, where said endodomain comprises a CD3-zeta domain or a high affinity FcεRI.

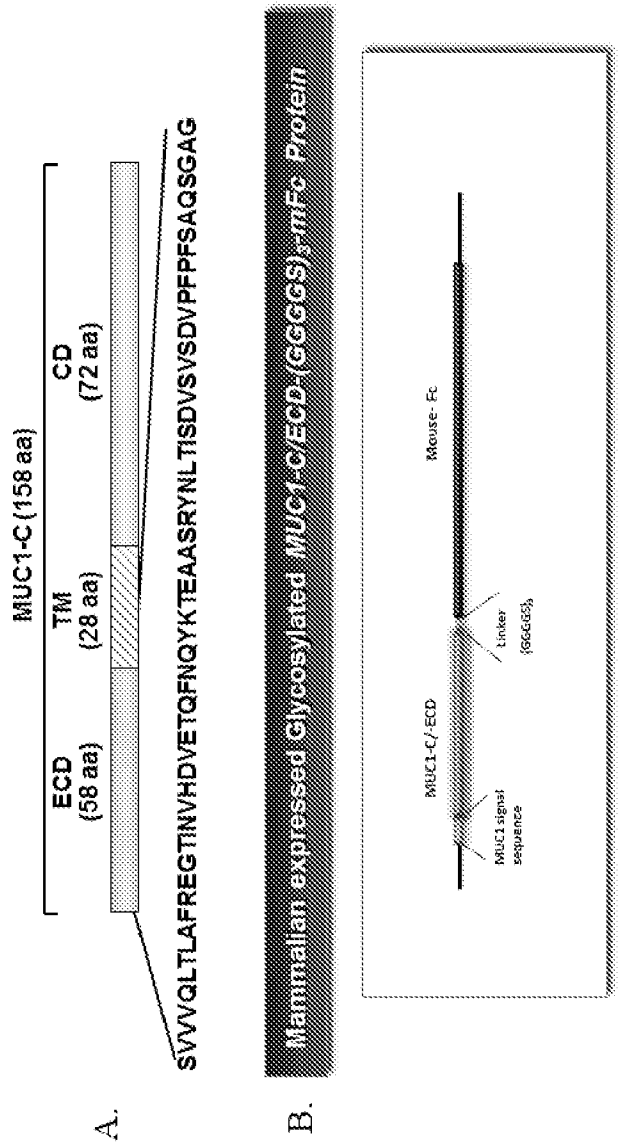
59. The receptor of claim 56, wherein the flexible hinge is from CD8α or Ig.

60. A cell expressing the chimeric antigen receptor of claim 56.

61. The cell of claim 60, wherein said transmembrane and endodomains are derived from the same molecule.

62. The cell of claim 60, where said endodomain comprises a CD3-zeta domain or a high affinity FcεRI.

63. The cell of claim 60, wherein the flexible hinge is from CD8α or Ig.


A.
MUC1-C (158 aa)
ECD (58 aa)
TM (28 aa)
CD (72 aa)
SVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG
B.
Mammalian expressed Glycosylated MUC1-C/ECD-(GGGGS)3-mFc Protein
Mouse- Fc
MUC1-C/-ECD
Linker (GGGGS)3
MUC1 signal sequence

FIGS. 1A-B

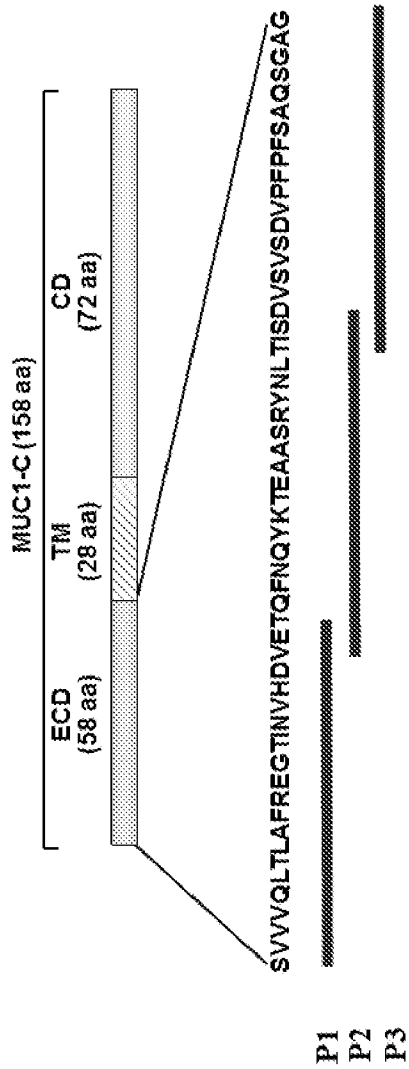

MUC1-C (158 aa)
ECD
(58 aa)
TM
(28 aa)
CD
(72 aa)
SVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG
P1
P2
P3

FIG. 3

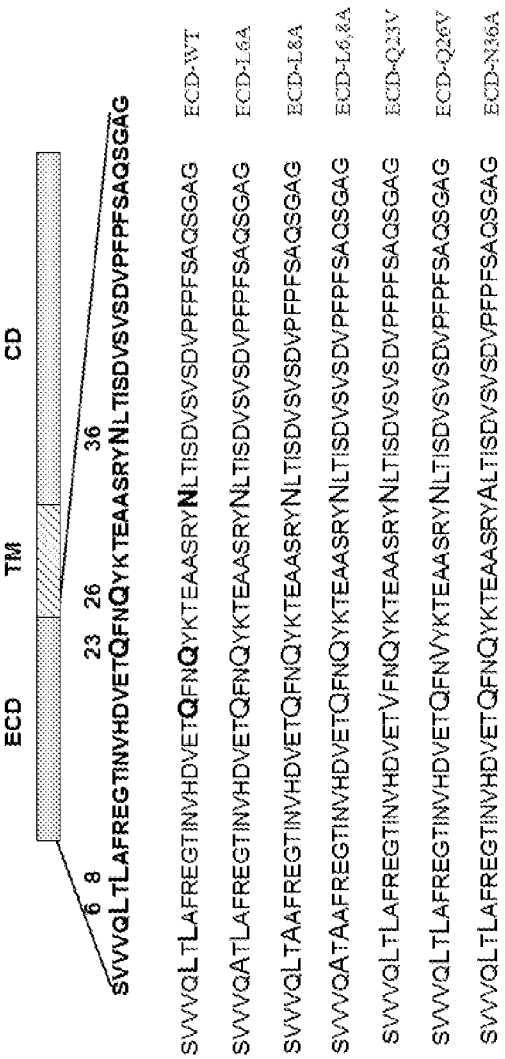
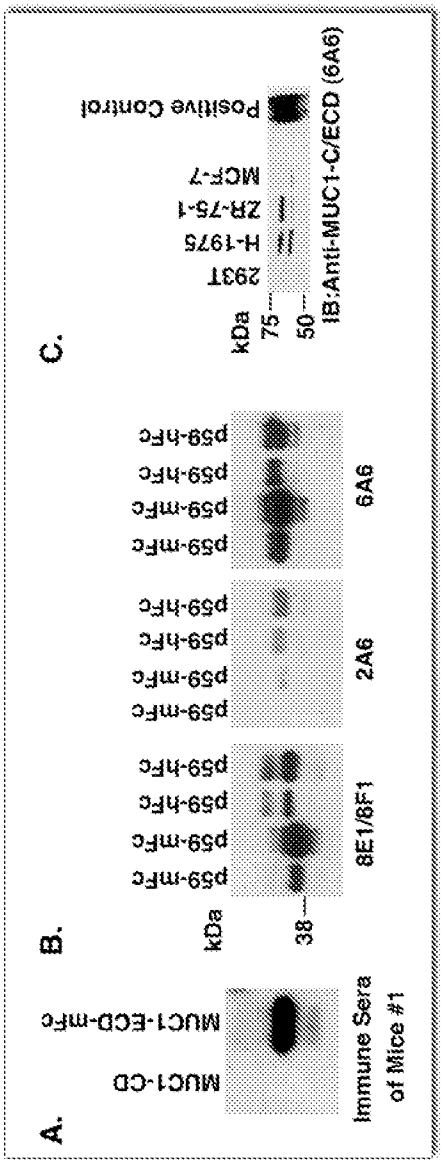

ECD
TM
CD
6 8
23 26
36
SVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG
SVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG
ECD-WT
SVVVQATLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG
ECD-L6A
SVVVQLTAAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG
ECD-L8A
SVVVQATAAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG
ECD-L6,8A
SVVVQLTLAFREGTINVHDVETVFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG
ECD-Q23V
SVVVQLTLAFREGTINVHDVETQFNVYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG
ECD-Q26V
SVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYALTISDVSVSDVPFPFSAQSGAG
ECD-N36A

FIG. 4


A.
MUC1-CD
MUC1-ECD-mFc
Immune Sera of Mice #1
B.
kDa
38
p59-mFc
p59-mFc
p59-hFc
p59-hFc
8E1/8F1
p59-mFc
p59-mFc
p59-hFc
p59-hFc
2A6
p59-mFc
p59-mFc
p59-hFc
p59-hFc
6A6
C.
kDa
75
50
IB:Anti-MUC1-C/ECD (6A6)
293T
H-1975
ZR-75-1
MCF-7
Positive Control

FIGS. 6A-C

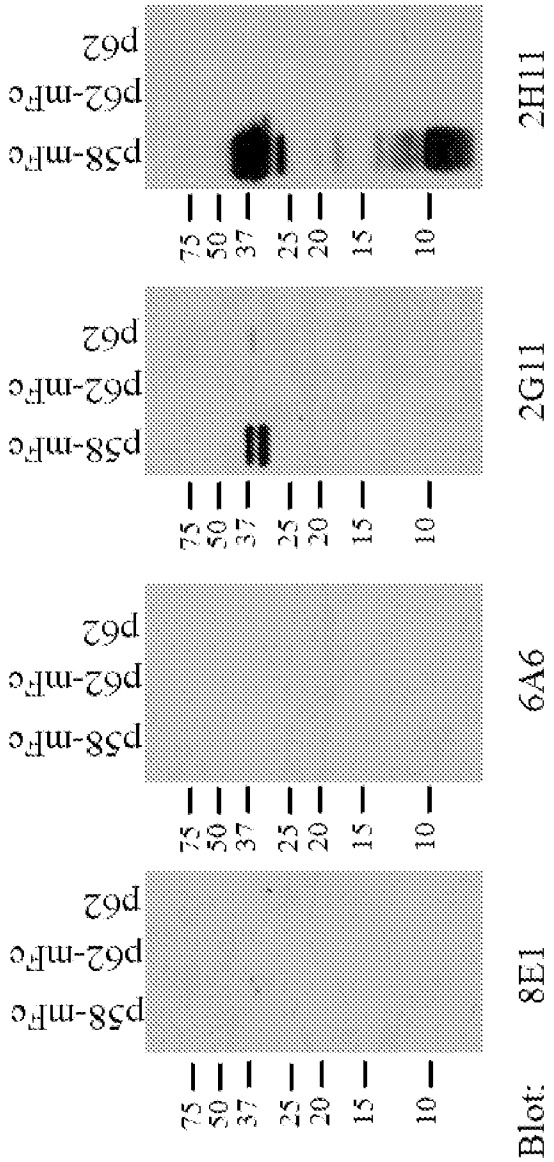


FIG. 7

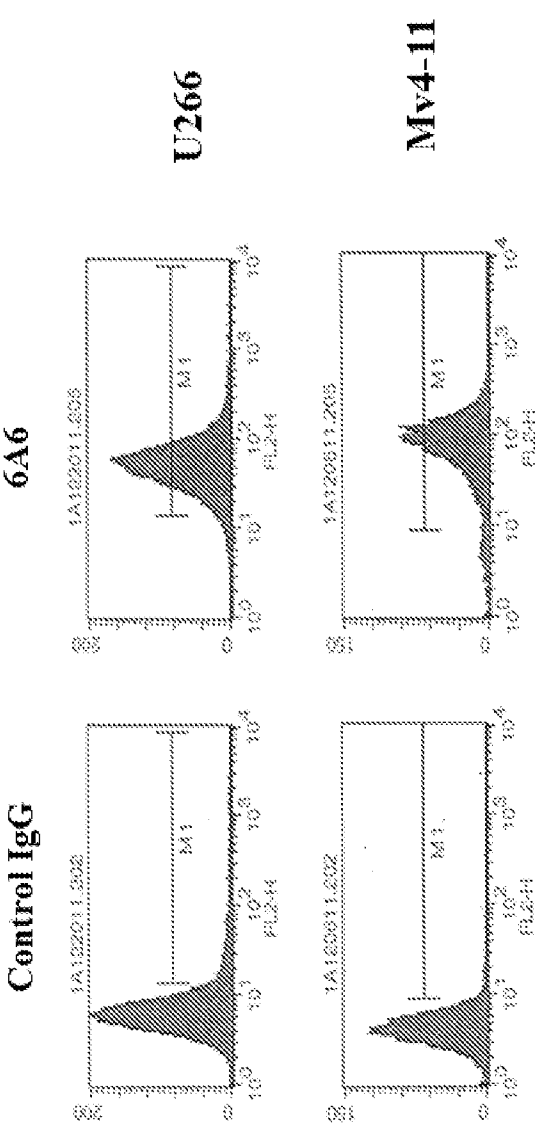

Control IgG
6A6
U266
Mv4-11
M1
FL2-H

FIG. 8

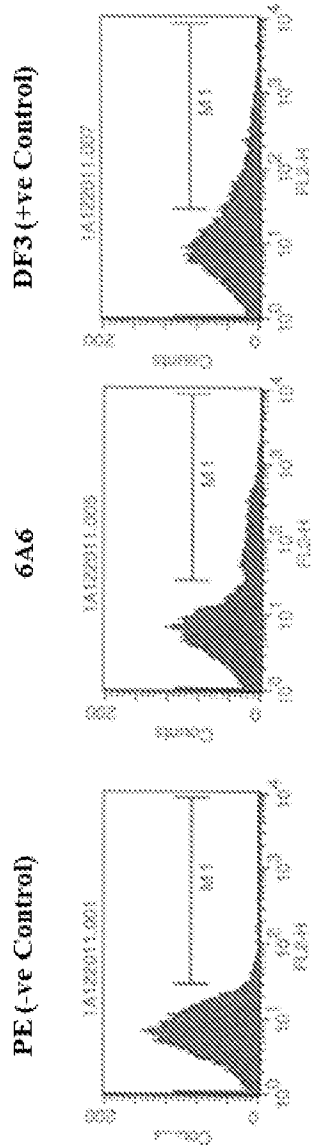

PE (-ve Control)
6A6
DF3 (+ve Control)
M1
FL2-H

FIG. 9

	Percent Positive Cells		
	8E1	6A6	DF3
U266	96.4	98.2	96.7
MOLM-14	71.4	72.5	99.5
Mv4-11	N/D	96.0	88.2
RPMI-8226	N/D	89.4	N/D
AML Primary Cells			
ME-559203		69.5	
RG-563999		68.3	

FIG. 10

	Percent Positive Cells	
	8E1	6A6
K562/C-siRNA	34.4	40.6
K562/MUC1-siRNA	6.3	17.0

FIG. 11

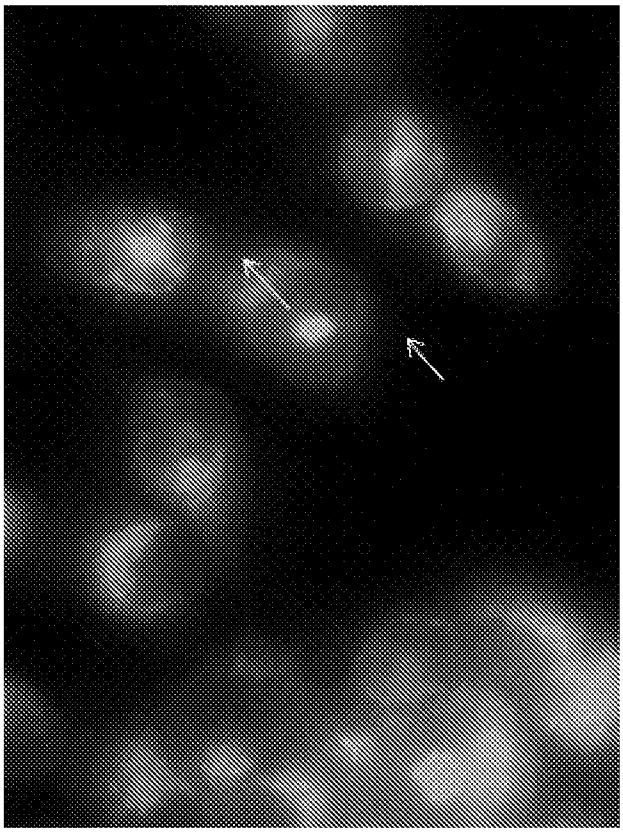


FIG. 12

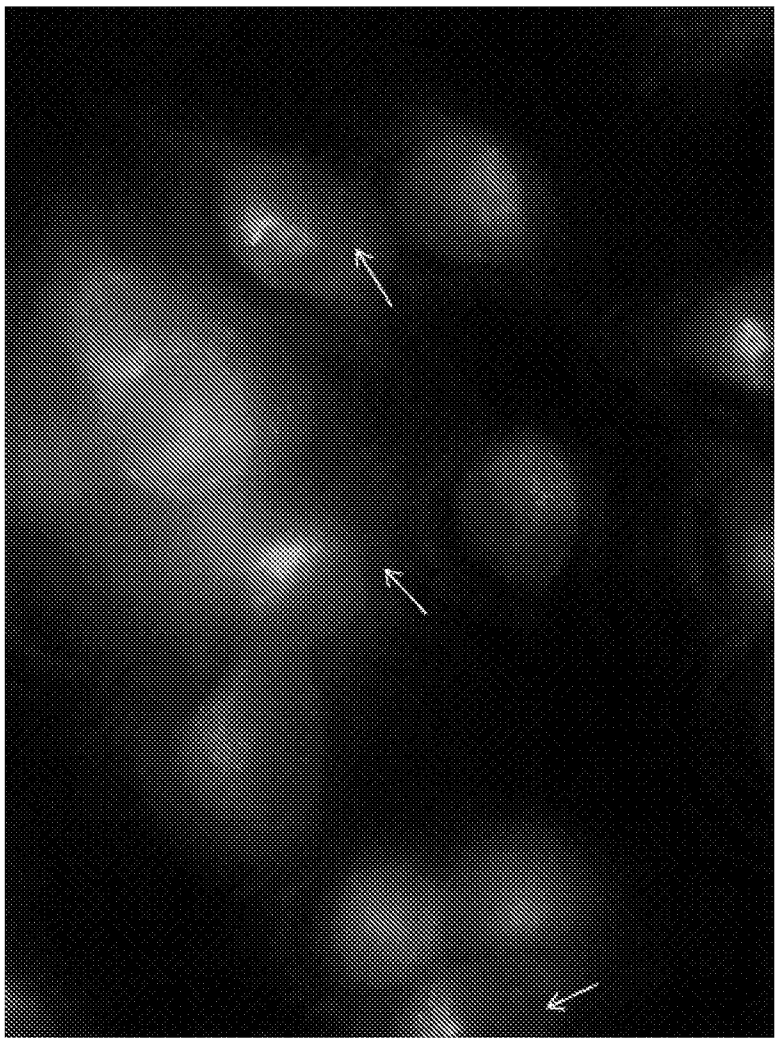


FIG. 13

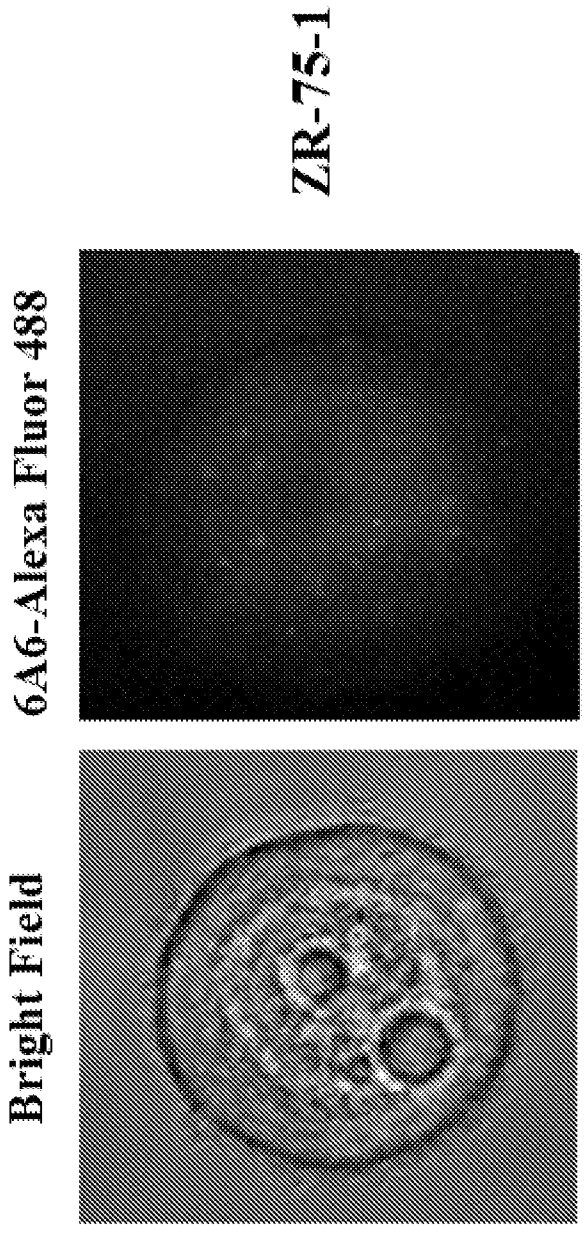


FIG. 14

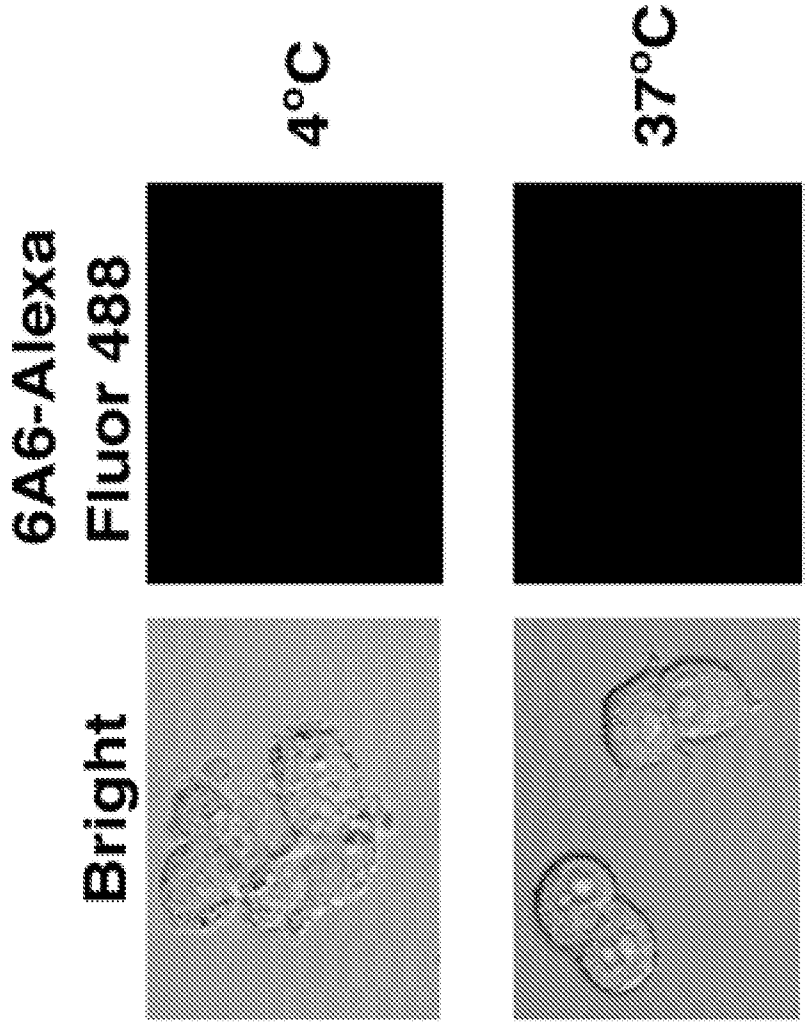


FIG. 15

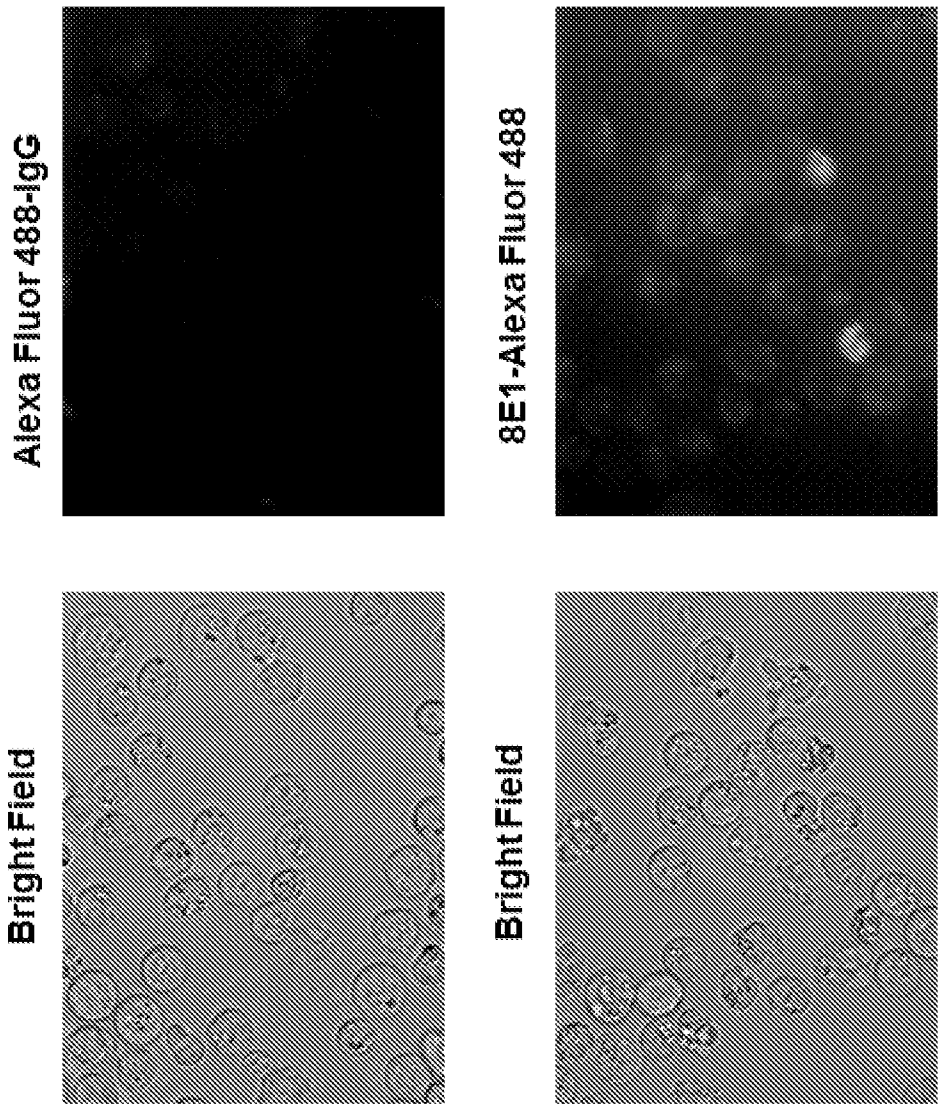

Alexa Fluor 488-IgG
8E1-Alexa Fluor 488
Bright Field
Bright Field

FIG. 16

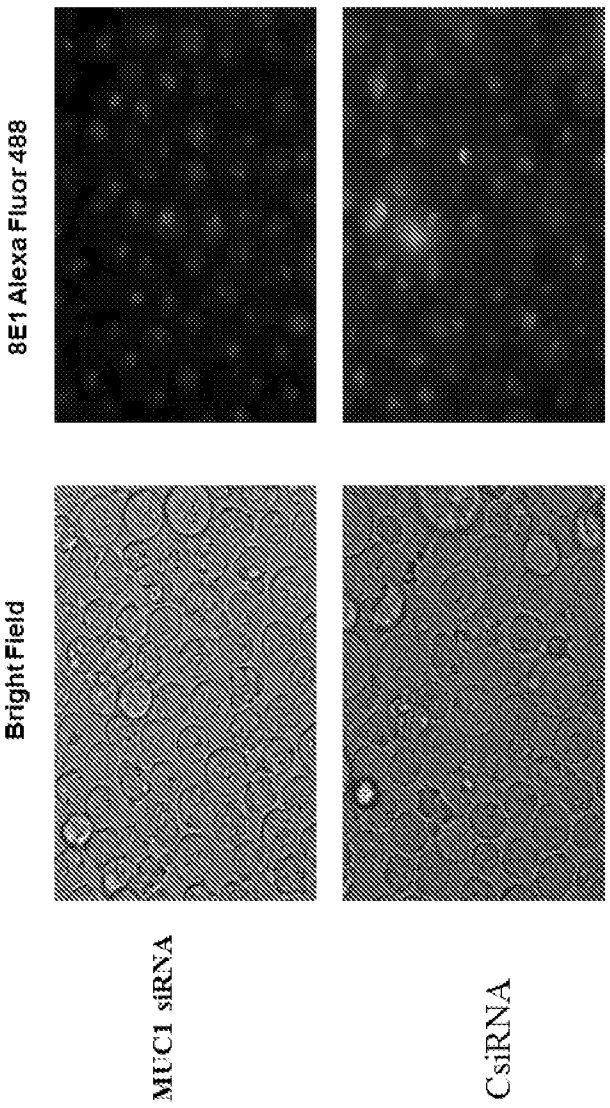


FIG. 17

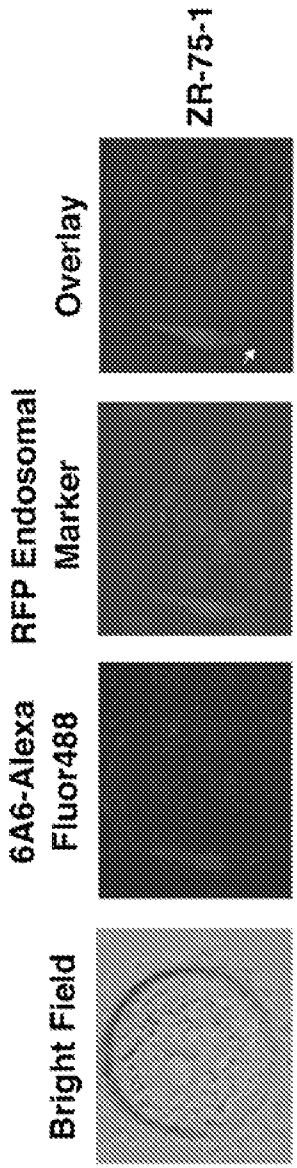

Bright Field
6A6-Alexa Fluor488
RFP Endosomal Marker
Overlay
ZR-75-1

FIG. 18

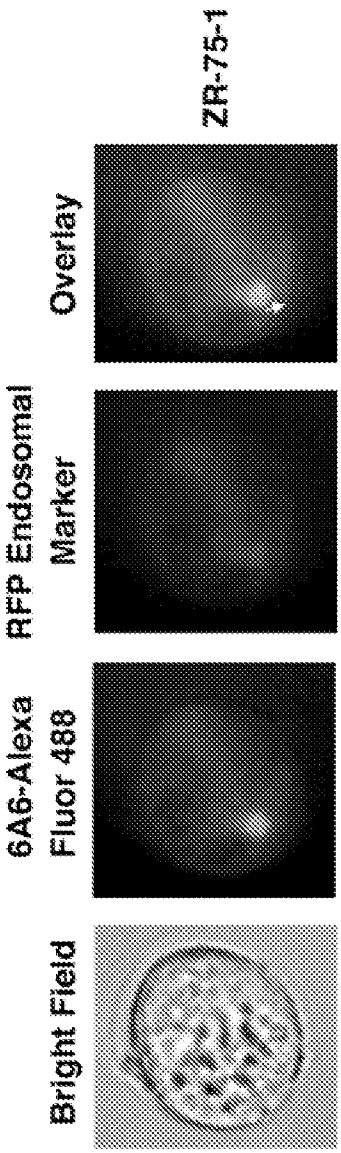

Bright Field
6A6-Alexa Fluor 488
RFP Endosomal Marker
Overlay
ZR-75-1

FIG. 19

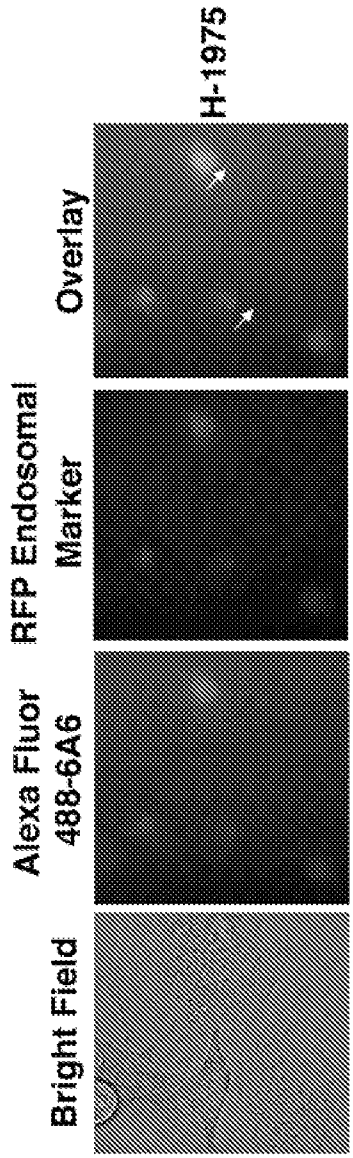

H-1975
Bright Field
Alexa Fluor 488-6A6
RFP Endosomal Marker
Overlay

FIG. 20

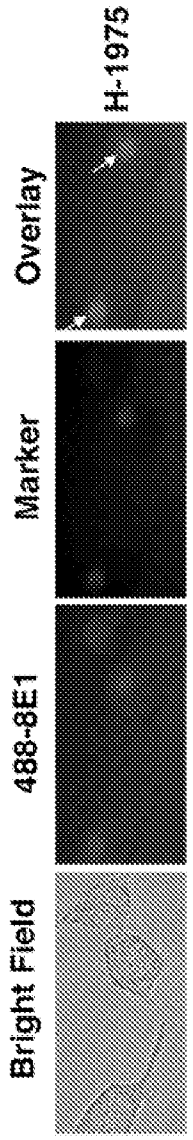

H-1975
Bright Field
Alexa Fluor 488-8E1
RFP Endosomal Marker
Overlay

FIG. 21

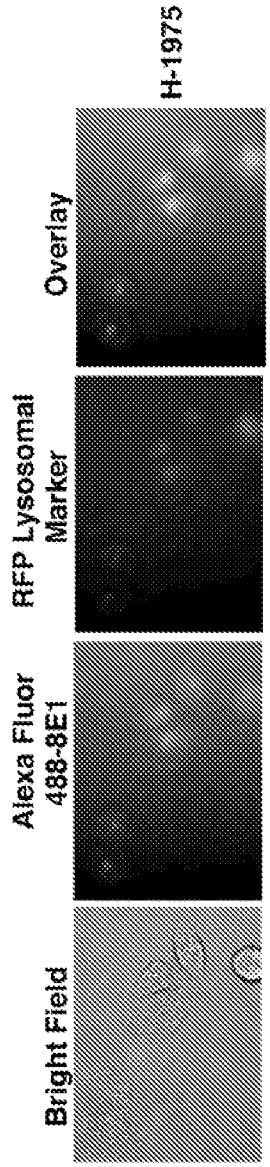

H-1975
Bright Field
Alexa Fluor 488-8E1
RFP Lysosomal Marker
Overlay

FIG. 22

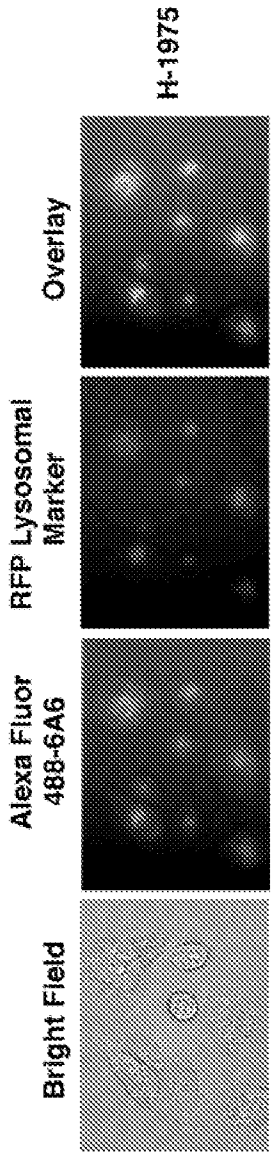

H-1975
Bright Field
Alexa Fluor 488-6A6
RFP Lysosomal Marker
Overlay

FIG. 23

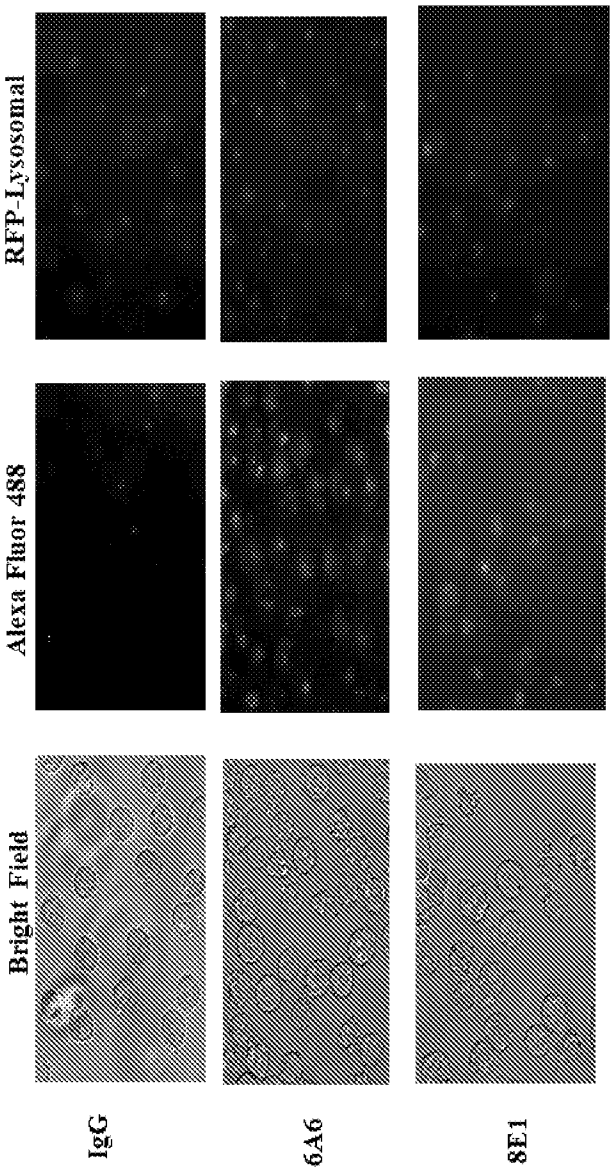


FIG. 25

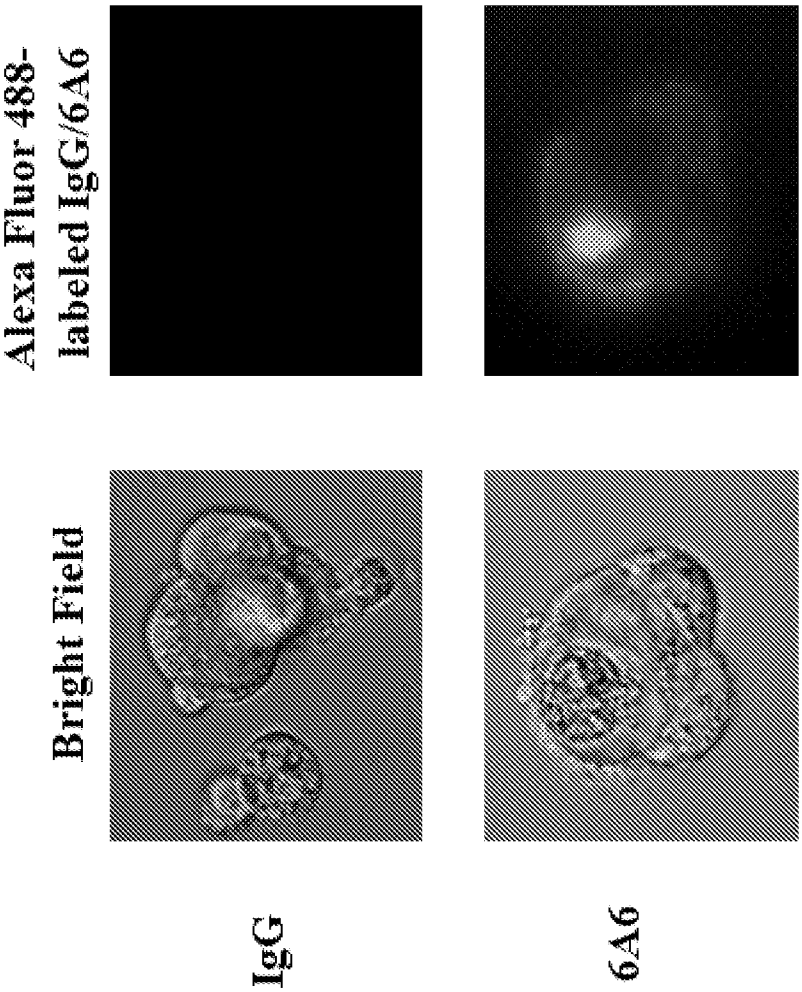


FIG. 24

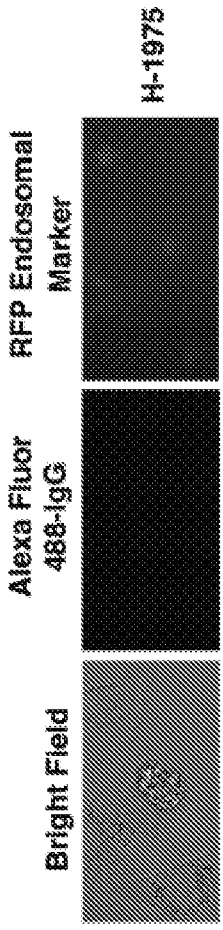

H-1975
RFP Endosomal Marker
Alexa Fluor 488-IgG
Bright Field

FIG. 26

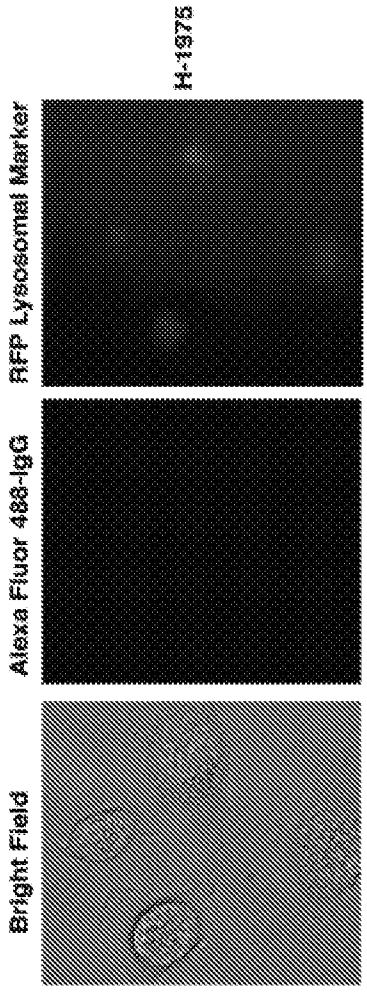

H-1975
RFP Lysosomal Marker
Alexa Fluor 488-IgG
Bright Field

FIG. 27

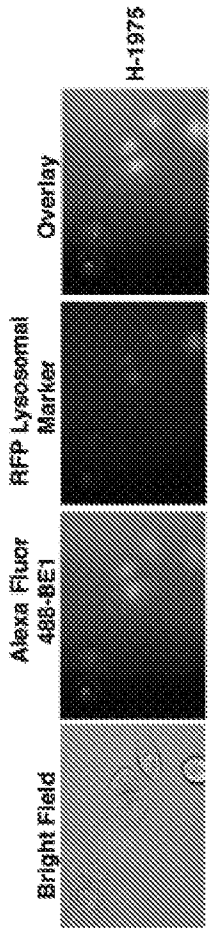

H-1975
Overlay
RFP Lysosomal Marker
Alexa Fluor 488-8E1
Bright Field

FIG. 28

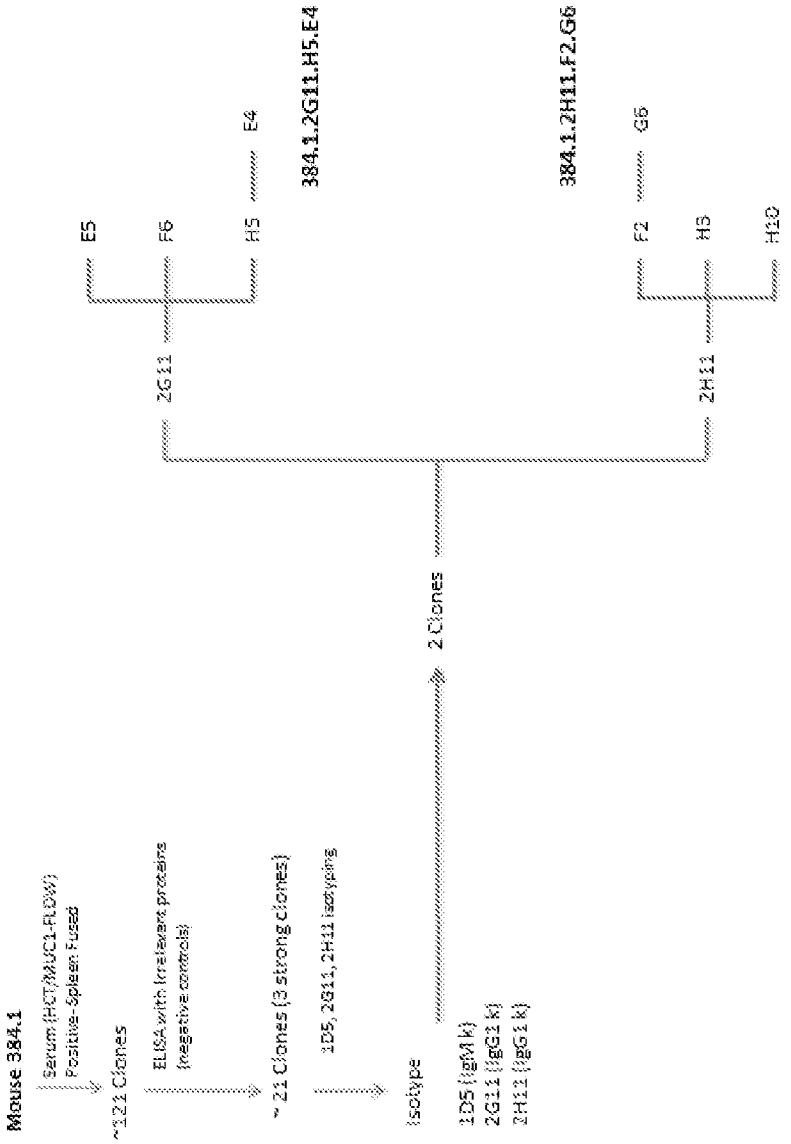

Mouse 384.1
Serum (HCT/MUC1-PDW)
Positive-Spleen Fused
~321 Clones
ELISA with breast cancer proteins
(negative controls)
~ 21 Clones (3 strong clones)
1D5, 2G11, 2H11 isotyping
Isotype
2 Clones
1D5 (IgM k)
2G11 (IgG1 k)
2H12 (IgG1 k)
384.1.2G11.H5.E4
384.1.2H11.F2.G6
E5
F6
H5
E4
2G11
F2
G6
H5
H10
2H11

FIG. 29

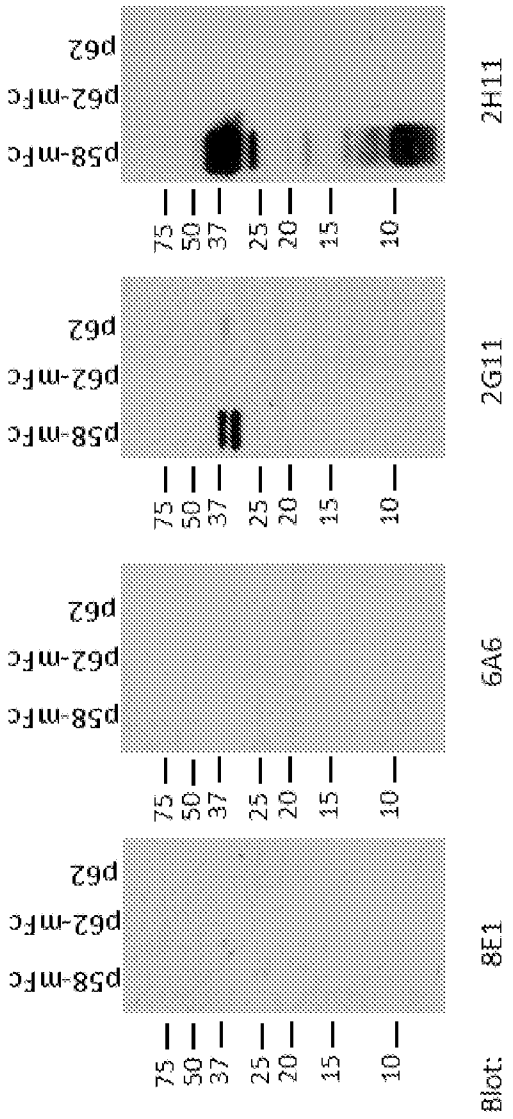


FIG. 30

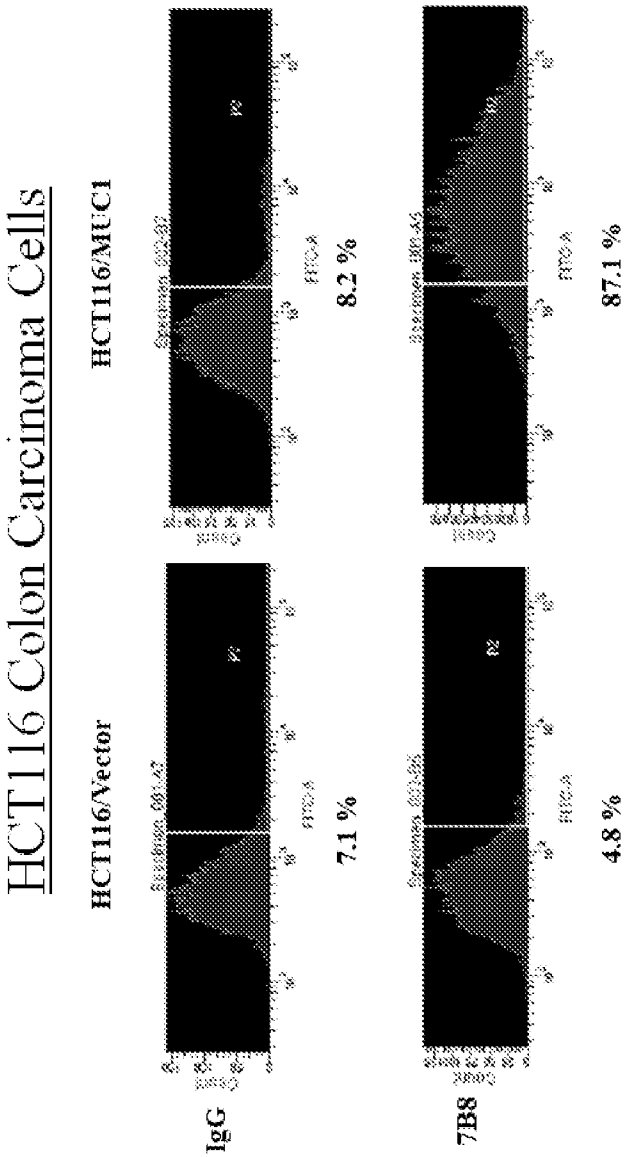

HCT116 Colon Carcinoma Cells
HCT116/Vector
HCT116/MUC1
IgG
7B8
7.1 %
8.2 %
4.8 %
87.1 %

FIG. 31

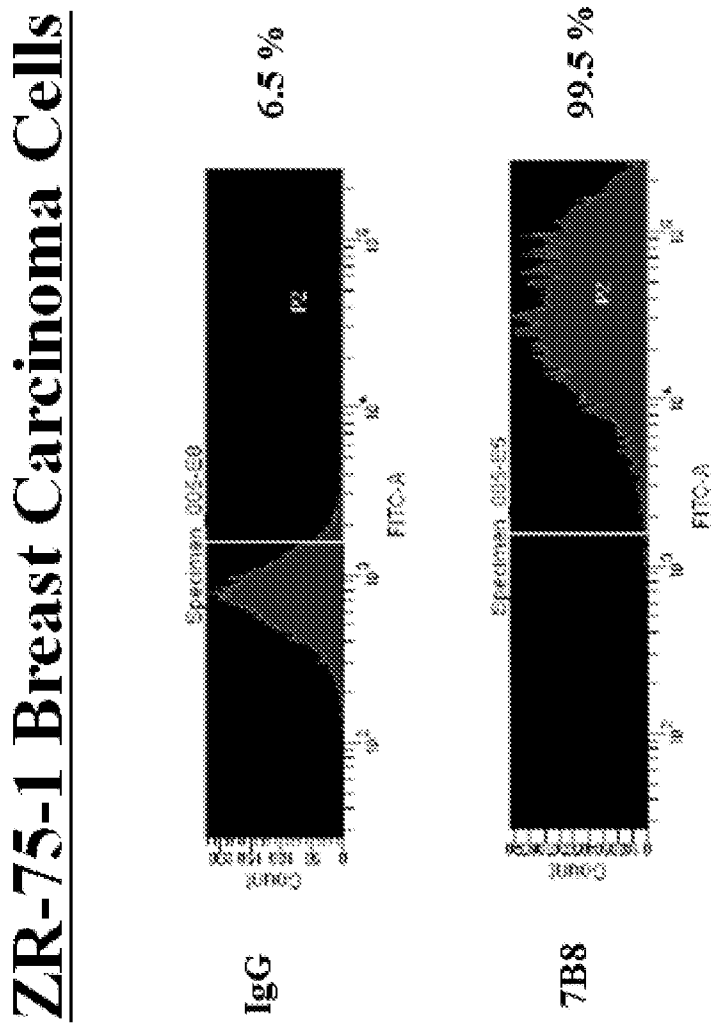


FIG. 32

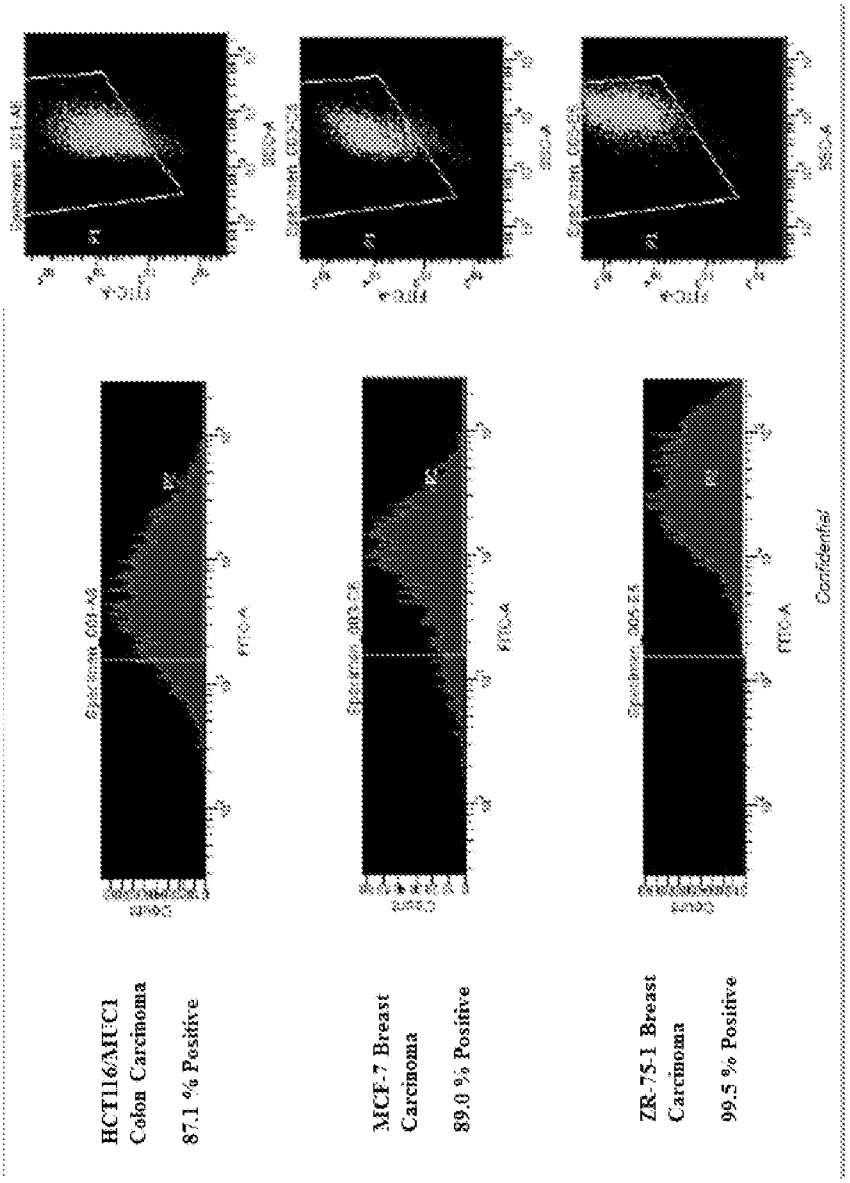

HCT116/MUC1
Colon Carcinoma
87.1 % Positive
MCF-7 Breast
Carcinoma
89.0 % Positive
ZR-75-1 Breast
Carcinoma
99.5 % Positive
FITC-A
SSC-A
Confidential

FIG. 33

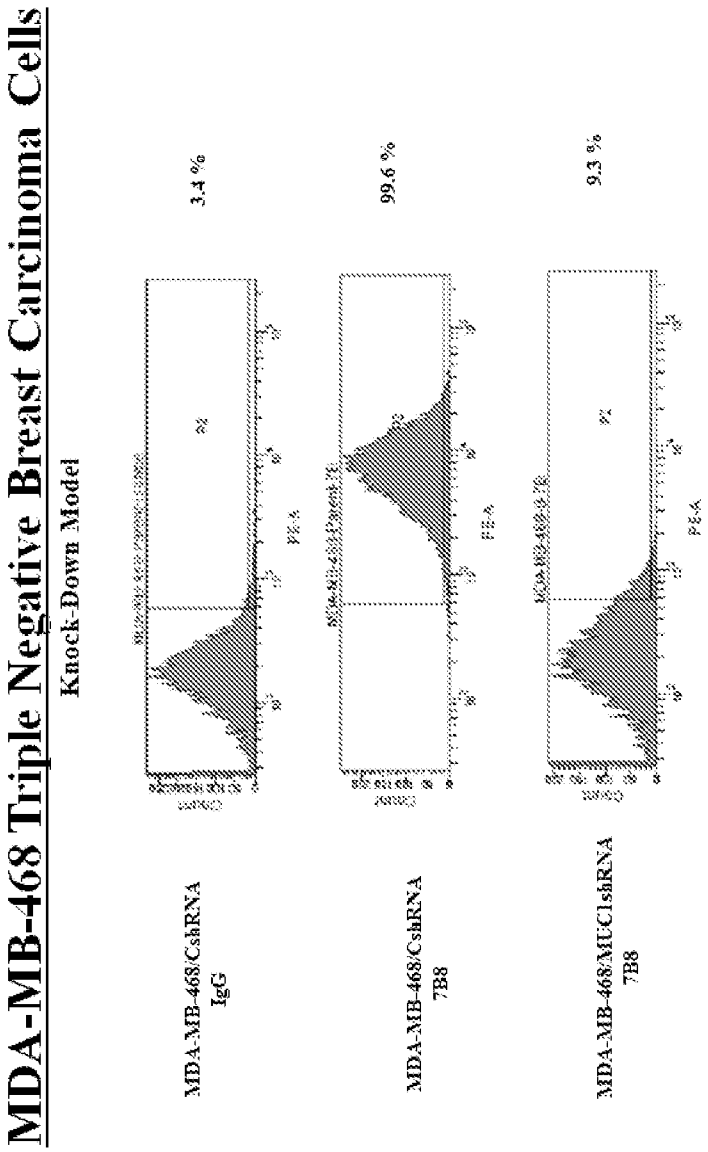

MDA-MB-468 Triple Negative Breast Carcinoma Cells
Knock-Down Model
MDA-MB-468/CshRNA IgG
3.4 %
MDA-MB-468/CshRNA 7B8
99.6 %
MDA-MB-468/MUC1shRNA 7B8
9.3 %

FIG. 34

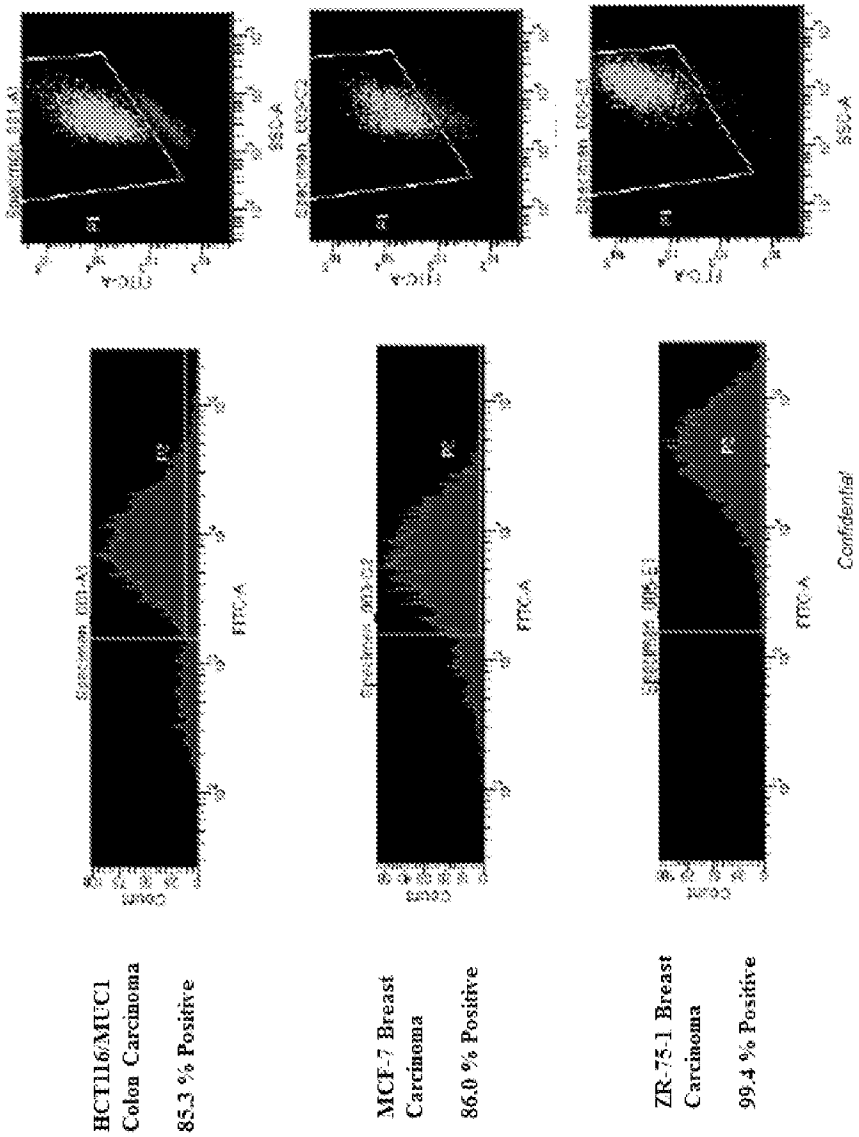

HCT116/MUC1
Colon Carcinoma
85.3 % Positive
MCF-7 Breast
Carcinoma
86.0 % Positive
ZR-75-1 Breast
Carcinoma
99.4 % Positive
FITC-A
SSC-A
Confidential

FIG. 35

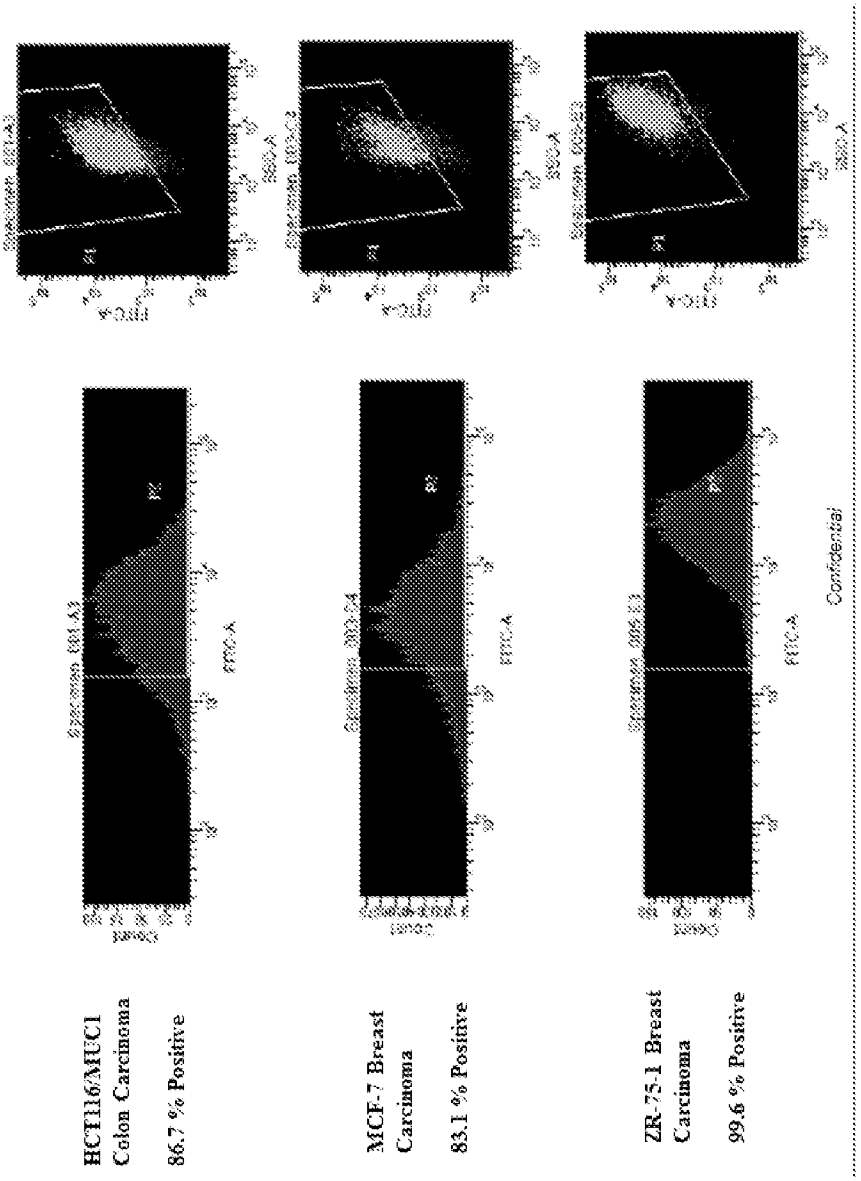

HCT116/MUC1
Colon Carcinoma
86.7 % Positive
MCF-7 Breast
Carcinoma
83.1 % Positive
ZR-75-1 Breast
Carcinoma
99.6 % Positive
Confidential

FIG. 36

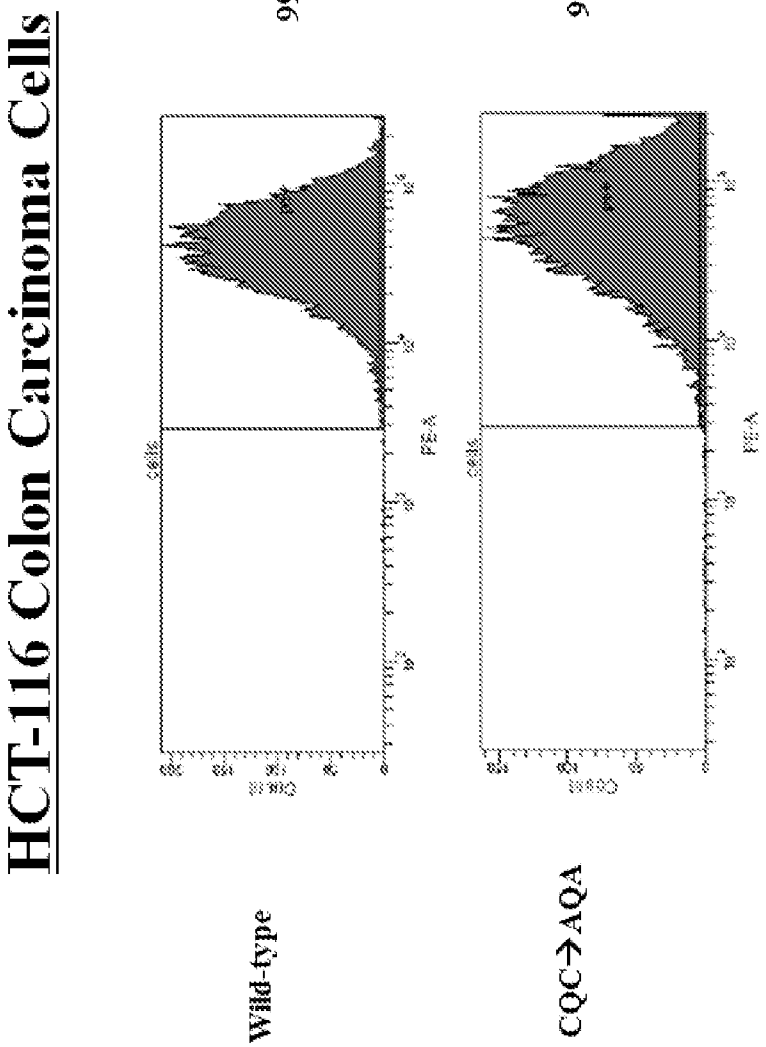


FIG. 37

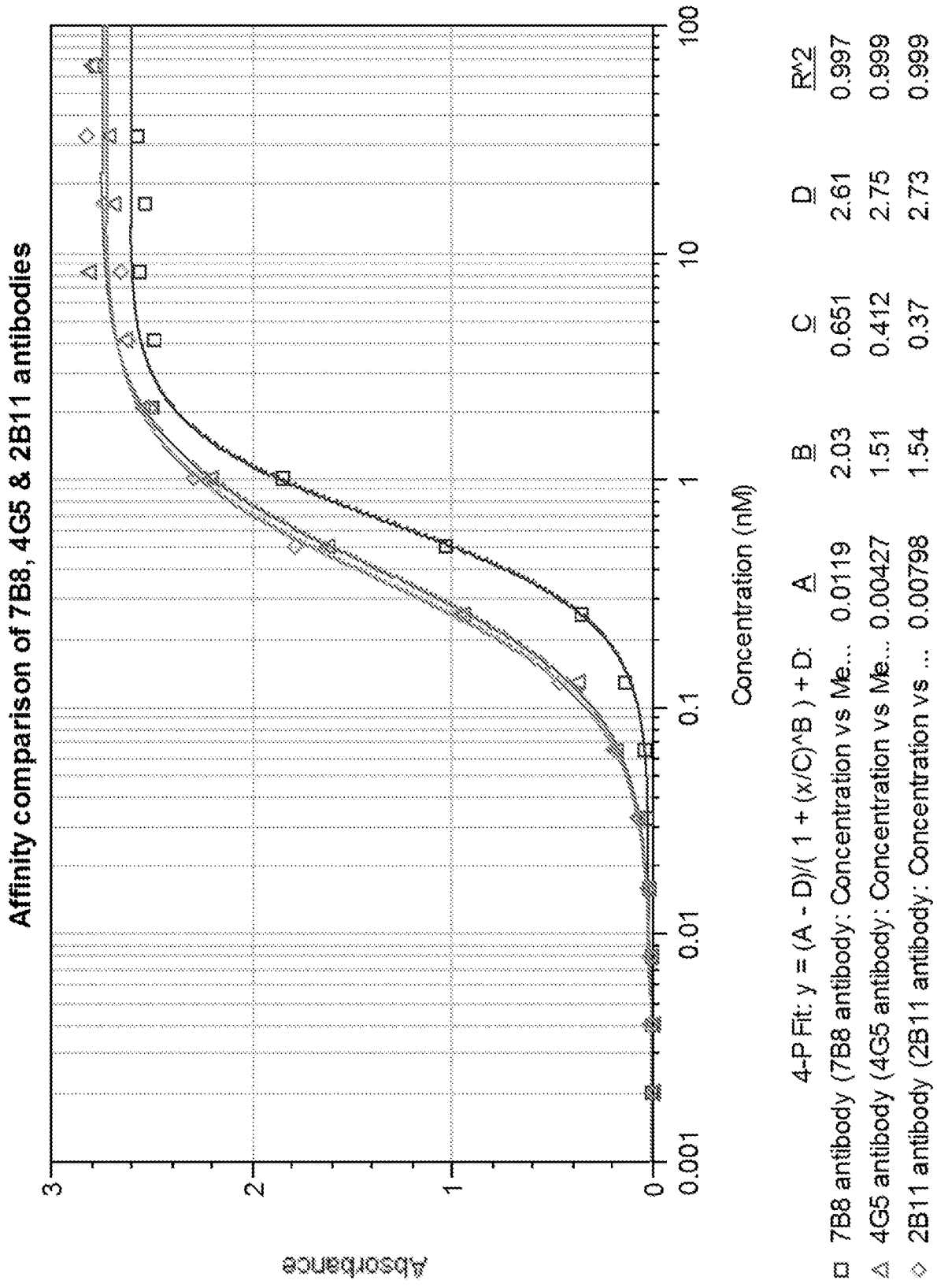

Affinity comparison of 7B8, 4G5 & 2B11 antibodies
Absorbance
Concentration (nM)
0
1
2
3
0.001
0.01
0.1
1
10
100
4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:
A
B
C
D
R^2
7B8 antibody (7B8 antibody: Concentration vs Me... 0.0119 2.03 0.651 2.61 0.997
4G5 antibody (4G5 antibody: Concentration vs Me... 0.00427 1.51 0.412 2.75 0.999
2B11 antibody (2B11 antibody: Concentration vs ... 0.00798 1.54 0.37 2.73 0.999

FIG. 38

Anti-MUC1-C/ECD MAbs

Clone	ELISA	Western	Flow	IHC	Internalization
7B8	+	+	+	+	ND
4G5	+	+	+	ND	ND
2B11	+	+	+	ND	ND

ND: Not Determined

FIG. 39

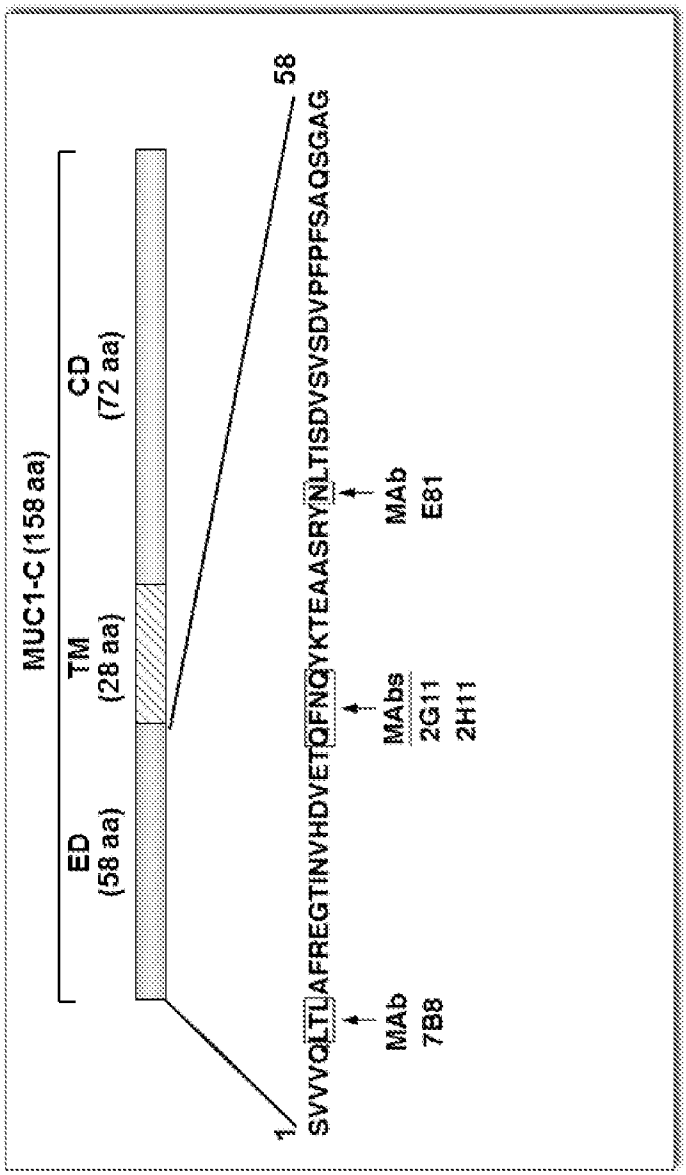

MUC1-C (158 aa)
ED
(58 aa)
TM
(28 aa)
CD
(72 aa)
1
58
SVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG
MAb
7B8
MAbs
2G11
2H11
MAb
E81

FIG. 40

2B11

CDR – H1	G F T F N Y F W I E
CDR – H2	E I L P G T G S T N Y N E K F K G
CDR – H3	Y D Y T S S M D Y
CDR – L1	C K A S E N V G T Y V S
CDR – L2	G A S N R Y T
CDR – L3	G Q S Y S Y P W T

FIG. 41

4G5

CDR – H1	G F S L S T S G M G V S
CDR – H2	H I Y W D D D K R Y N P S L K S
CDR – H3	G V S S W F P Y
CDR – L1	C K A S Q S V G N Y V A
CDR – L2	F A S N R Y S
CDR – L3	Q Q H Y I F P Y T

FIG. 42

7B8

CDR – H1	G H T F T S Y W M H
CDR – H2	E I N P S N G R T Y Y N E N F K T
CDR – H3	D G D Y V S G F A Y
CDR – L1	C R A S E S V Q Y S G T S L M H
CDR – L2	G A S N V E T
CDR – L3	Q Q N W K V P W T

FIG. 43

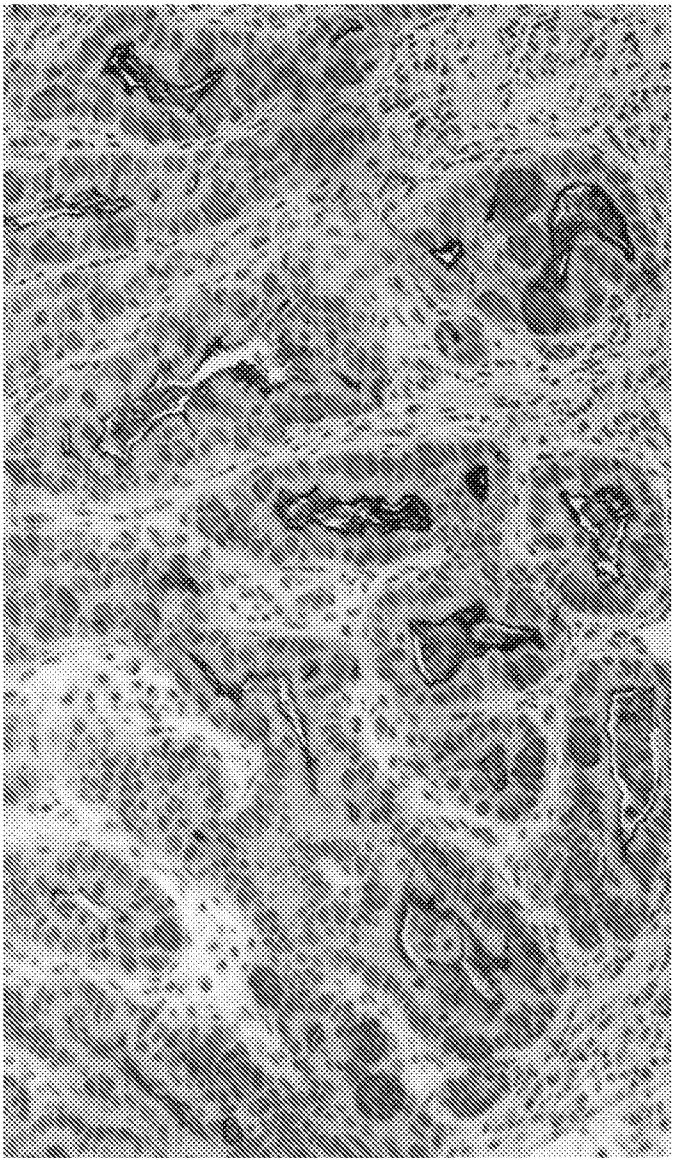


FIG. 44

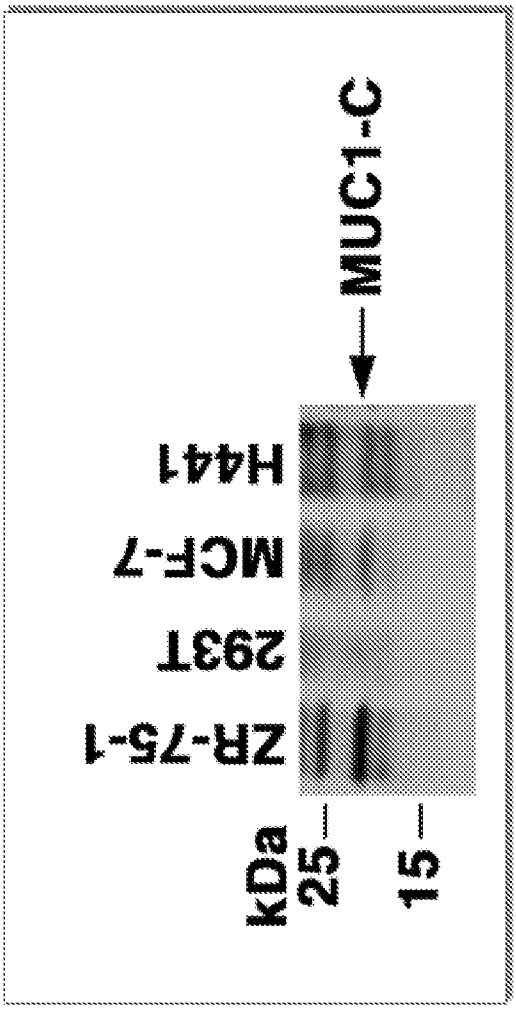
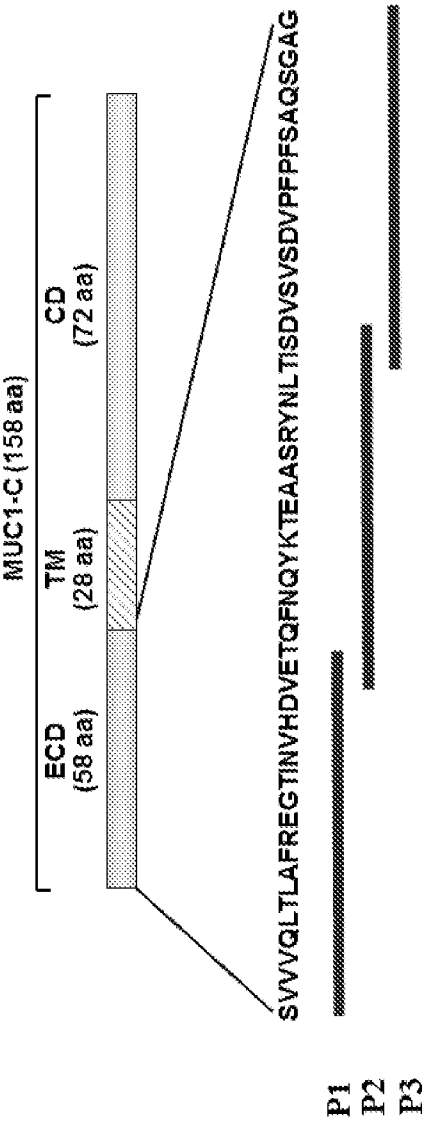


FIG. 45

* Linear Epitope Mapping
SVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG
P1
P2
P3
* No Peptide Reactivity
MAbs: 2B11, 4G5 and 7B8

FIG. 46



None of the three peptides blocks the reactivity of 7B8 group and 3D1 MAbs with MUC1-C/ECD protein in ELISA assays

FIG. 47

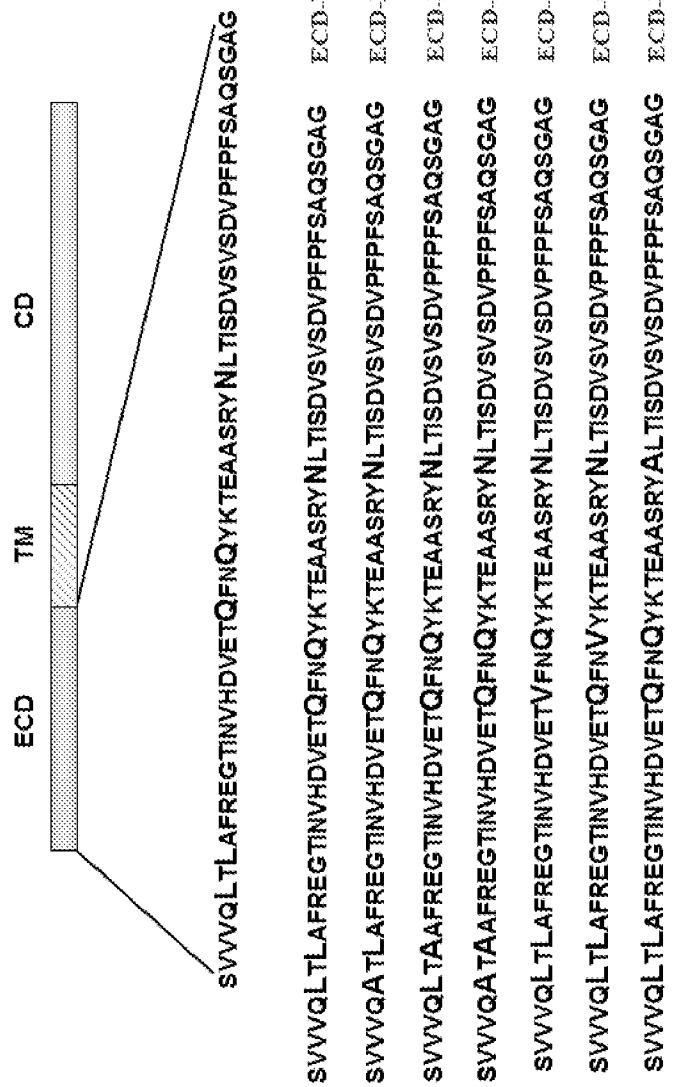
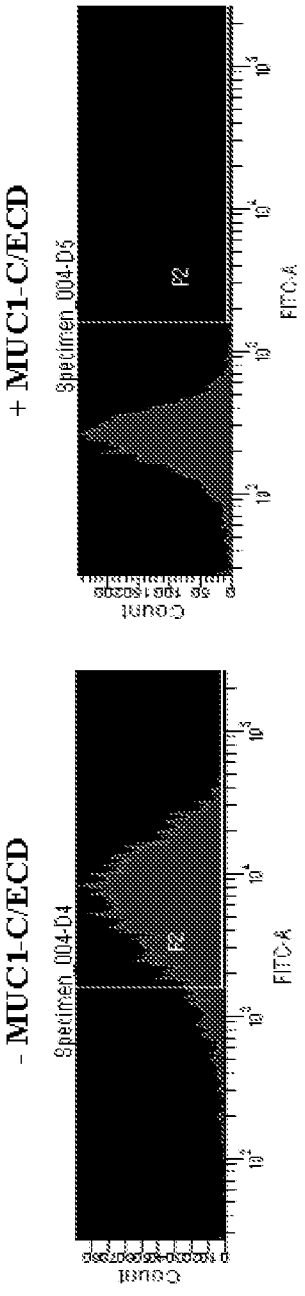

ECD
TM
CD
SVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG
SVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG ECD-WT
SVVVQATLAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG ECD-L6A
SVVVQLTAAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG ECD-L8A
SVVVQATAAFREGTINVHDVETQFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG ECD-L6,8A
SVVVQLTLAFREGTINVHDVETVFNQYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG ECD-Q23V
SVVVQLTLAFREGTINVHDVETQFNVYKTEAASRYNLTISDVSVSDVPFPFSAQSGAG ECD-Q26V
SVVVQLTLAFREGTINVHDVETQFNQYKTEAASRYALTISDVSVSDVPFPFSAQSGAG ECD-N36A

FIG. 48A

Mab/Coated protein		MUC1-C/ECD		MUC1-C/ECD (D19E)	
		1	2	11	12
7B8	5 ug/ml	3.064	3.054	3.195	3.296
3D1	5 ug/ml	3.087	3.075	1.556	1.694
CD1(-ve Control)	5 ug/ml	0.056	0.065	0.064	0.066

FIG. 48B



		ZR75-1	
Protein	Antibody	P1%Parent	MFI
-	CD1	1.2	5082
-	DF3 (+ve)	88.1	16317
-	7B8	95.1	13290
-	3D1	89.8	7087
MUC1-C/ECD Protein	3D1	0.1	2430

FIG. 49

Heavy chain: Amino acids sequence (138 AA)

Leader sequence-**FR1**-CDR1-**FR2**-CDR2-**FR3**-CDR3-**FR4**

MEWPCIFLFLLSVTEGVHS**QVQLQQSGAELVRPGSSVKISCKTSGYAFS**NFWMN**WVKQRPGQGLEWIG**QIYP
GDGDTNYNGKFKG**KATLTADKSSSTAYMQLSSLTSEASAVYFCAR**SYYRSAWFAY**WGQGTLVSVSA**

Light chain: Amino acids sequence (127 AA)

Leader sequence-**FR1**-CDR1-**FR2**-CDR2-**FR3**-CDR3-**FR4**

MVSTPQFLVFLLFWIPASRG**DILLTQSPAILSVSPGERVSFSC**RASQSIGTSIH**WYQQRTNGSPRLLIK**YASESIS**GI
PSRFSGSGSGTDFTLSINSVESEDIADYYC**QQSNNWPLT**FGAGTKLELK**

FIG. 50

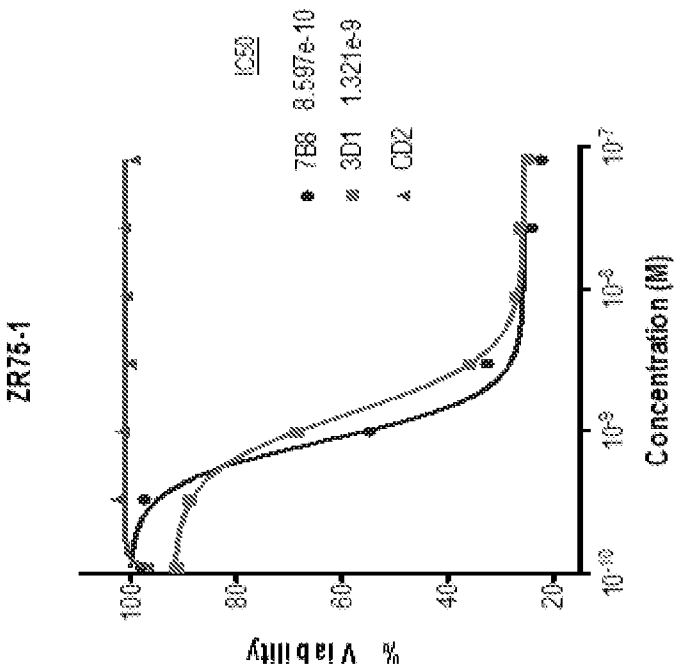

ZR75-1
IC50
7B8 8.597e-10
3D1 1.321e-9
CD2
% Viability
100
80
60
40
20
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
Concentration (M)

FIG. 51A

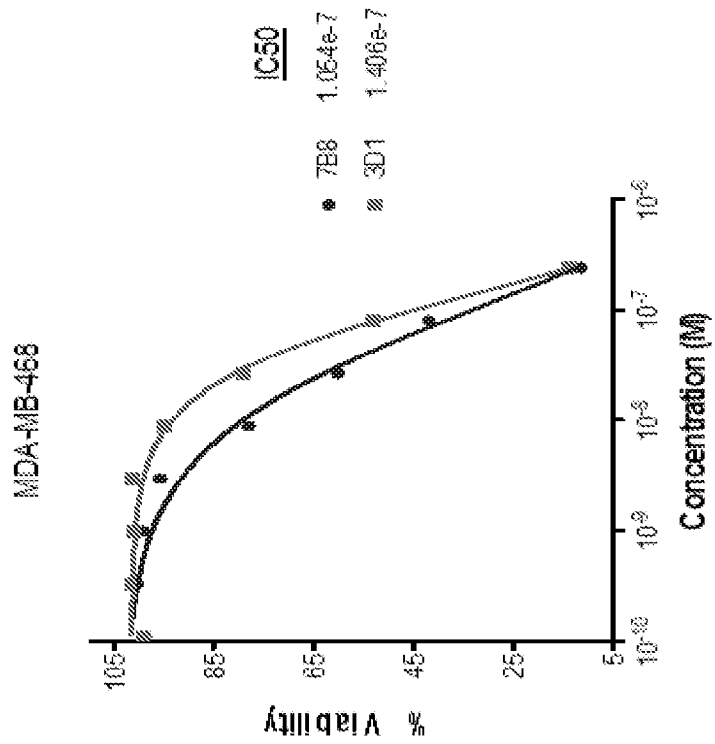
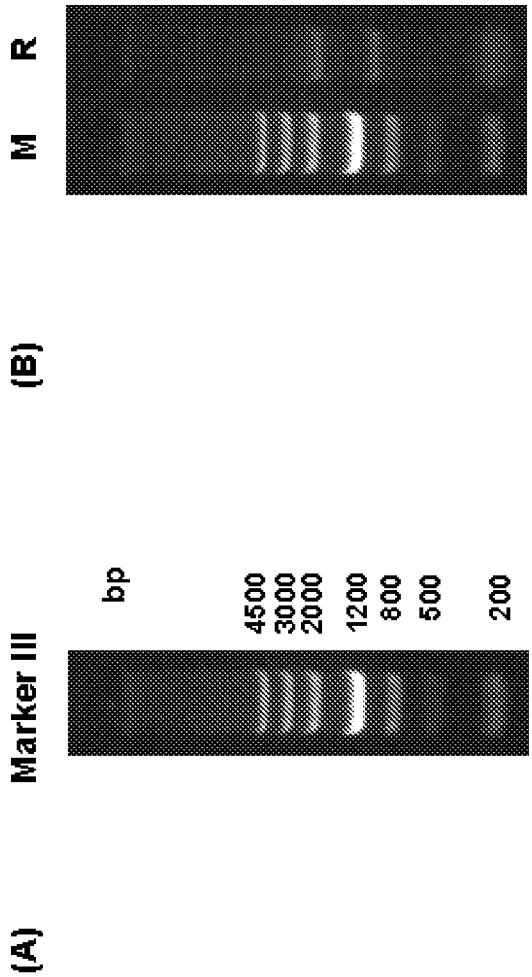

MDA-MB-468
IC50
7B8 1.05e-7
3D1 1.406e-7
% Viability
105
85
65
45
25
5
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
Concentration (M)

FIG. 51B



FIGS. 52A-B

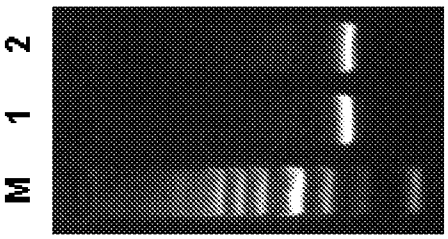


FIG. 53

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/013410

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/30 A61K39/395
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, MEDLINE, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/005470 A2 (IMMUNOGEN INC [US]) 15 January 2004 (2004-01-15) page 29; example 1 table 2 page 42; example 9 page 46; example 12 page 50 - page 51; example 16 page 36; example 4 -----	1-63
A	WO 91/16353 A1 (CORVAS INTERNATIONAL N V [BE]) 31 October 1991 (1991-10-31) -----	1
A	PICHINUK EDWARD ET AL: "Antibody targeting of cell-bound MUC1 SEA domain kills tumor cells.", CANCER RESEARCH 1 JUL 2012, vol. 72, no. 13, 1 July 2012 (2012-07-01), pages 3324-3336, XP002738243, ISSN: 1538-7445 -----	1

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 June 2015	03/07/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Sitch, David

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/013410

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-63(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-63(partially)

An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined at least as having the V H CDRs defined by sequences having at least 90% homology to SEQ ID NOS:3, 4 and 5 , together, in principle, with any of the VL chain CDRs having at least 90% homology to SEQ ID NOS: 9, 10, 11, 12, 13, 14, 30, 31, 32, 51, 52, 53, 54, 55, 56, 57, 58, 59, 79, 80 and 81;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain having at least 80% homology to SEQ ID NO:17 *, together, in principe, with any of the VL chains defined by SEQ ID NOS: 21, 19, 25, 70, 66, 62 and 75;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain encoded by a nucleic acid having at least 70% homology to SEQ ID NO:18 , together, in principe, with any of the VL chains encoded by a nucleic acid having least 70% homology to SEQ ID NOS: 22, 20, 26, 71, 67, 63 and 74; related products and uses;

---

2. claims: 1-63(partially)

An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined at least as having the V H CDRs defined by sequences having at least 90% homology to SEQ ID NOS:6, 7 and 8 , together, in principle, with any of the VL chain CDRs having at least 90% homology to SEQ ID NOS: 9, 10, 11, 12, 13, 14, 30, 31, 32, 51, 52, 53, 54, 55, 56, 57, 58, 59, 79, 80 and 81;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain having at least 80% homology to SEQ ID NO:15 , together, in principe, with any of the VL chains defined by SEQ ID NOS: 21, 19, 25, 70, 66, 62 and 75;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain encoded by a nucleic acid having at least 70% homology to SEQ ID NO:16 , together, in principe, with any of the VL chains encoded by a nucleic acid having least 70% homology to SEQ ID NOS: 22, 20, 26, 71, 67, 63 and 74;
related products and uses;

---

3. claims: 1-63(partially)

An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined at least as having the V H CDRs defined by sequences having at least 90% homology to SEQ ID NOS:27, 28 and 29 , together, in principle, with any of the VL chain CDRs having at least 90% homology to SEQ ID NOS: 9, 10, 11, 12, 13, 14, 30, 31, 32, 51, 52, 53, 54, 55, 56, 57, 58, 59, 79, 80 and 81;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain having at least 80% homology to SEQ ID NO:23 , together, in principe, with any of the VL chains defined by SEQ ID NOS: 21, 19, 25, 70, 66, 62 and 75;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain encoded by a nucleic acid having at least 70% homology to SEQ ID NO:24 , together, in principe, with any of the VL chains encoded by a nucleic acid having least 70% homology to SEQ ID NOS: 22, 20, 26, 71, 67, 63 and 74;
related products and uses;

---

4. claims: 1-63(partially)

An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined at least as having the V H CDRs defined by sequences having at least 90% homology to SEQ ID NOS:42, 43 and 44 , together, in principle, with any of the VL chain CDRs having at least 90% homology to SEQ ID NOS: 9, 10, 11, 12, 13, 14, 30, 31, 32, 51, 52, 53, 54, 55, 56, 57, 58, 59, 79, 80 and 81;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain having at least 80% homology to SEQ ID NO:68 , together, in principe, with any of the VL chains defined by SEQ ID NOS: 21, 19, 25, 70, 66, 62 and 75;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain encoded by a nucleic acid having at least 70% homology to SEQ ID NO:69 , together, in principe, with any of the VL chains encoded by a nucleic acid having least 70% homology to SEQ ID NOS: 22, 20, 26, 71, 67, 63 and 74;
related products and uses;

---

5. claims: 1-63(partially)

An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said

antibody is defined at least as having the V H CDRs defined by sequences having at least 90% homology to SEQ ID NOS:45, 46 and 47 , together, in principle, with any of the VL chain CDRs having at least 90% homology to SEQ ID NOS: 9, 10, 11, 12, 13, 14, 30, 31, 32, 51, 52, 53, 54, 55, 56, 57, 58, 59, 79, 80 and 81;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain having at least 80% homology to SEQ ID NO:64 , together, in principe, with any of the VL chains defined by SEQ ID NOS: 21, 19, 25, 70, 66, 62 and 75;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain encoded by a nucleic acid having at least 70% homology to SEQ ID NO:65 , together, in principe, with any of the VL chains encoded by a nucleic acid having least 70% homology to SEQ ID NOS: 22, 20, 26, 71, 67, 63 and 74;
related products and uses;

---

6. claims: 1-63(partially)

An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined at least as having the V H CDRs defined by sequences having at least 90% homology to SEQ ID NOS:48, 49 and 50 , together, in principle, with any of the VL chain CDRs having at least 90% homology to SEQ ID NOS: 9, 10, 11, 12, 13, 14, 30, 31, 32, 51, 52, 53, 54, 55, 56, 57, 58, 59, 79, 80 and 81;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain having at least 80% homology to SEQ ID NO:60 , together, in principe, with any of the VL chains defined by SEQ ID NOS: 21, 19, 25, 70, 66, 62 and 75;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain encoded by a nucleic acid having at least 70% homology to SEQ ID NO:61 , together, in principe, with any of the VL chains encoded by a nucleic acid having least 70% homology to SEQ ID NOS: 22, 20, 26, 71, 67, 63 and 74;
related products and uses;

---

7. claims: 1-63(partially)

An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined at least as having the V H CDRs defined by sequences having at least 90% homology to SEQ ID NOS:76, 77 and 78 , together, in principle, with any of the VL chain

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

CDRs having at least 90% homology to SEQ ID NOS: 9, 10, 11, 12, 13, 14, 30, 31, 32, 51, 52, 53, 54, 55, 56, 57, 58, 59, 79, 80 and 81;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain having at least 80% homology to SEQ ID NO:73 , together, in principe, with any of the VL chains defined by SEQ ID NOS: 21, 19, 25, 70, 66, 62 and 75;
An antibody able to selectively bind the extracellular domain of MUC1-C as defined by SEQ ID NO:1, and wherein said antibody is defined as having a V H chain encoded by a nucleic acid having at least 70% homology to SEQ ID NO:72 , together, in principe, with any of the VL chains encoded by a nucleic acid having least 70% homology to SEQ ID NOS: 22, 20, 26, 71, 67, 63 and 74;
related products and uses.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/013410

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004005470 A2	15-01-2004	AU 2003247762 A1	23-01-2004
		CA 2491017 A1	15-01-2004
		EP 1536814 A2	08-06-2005
		JP 2006502110 A	19-01-2006
		US 2004057952 A1	25-03-2004
		WO 2004005470 A2	15-01-2004
WO 9116353 A1	31-10-1991	AU 7766391 A	11-11-1991
		WO 9116353 A1	31-10-1991