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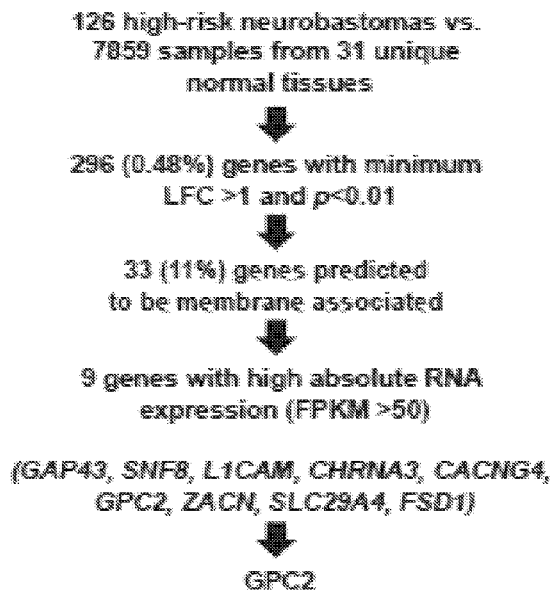


FIG. 1

(57) Abstract: The present disclosure is directed to antibodies binding to Glypican 2 and methods of using such antibodies to treat cancers that express or overexpress the Glypican 2 antigen.

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## **DESCRIPTION**

### **GLYPICAN 2 AS A CANCER MARKER AND THERAPEUTIC TARGET**

#### **STATEMENT OF FEDERAL FUNDING**

5           This invention was made with government support under Grant Numbers Genetics T32 T32GM008638 and ACC T32 32CA009615, awarded by the National Institutes of Health. The government has certain rights in the invention.

#### **PRIORITY CLAIM**

10           This application claims benefit of priority to U.S. Provisional Applications Serial Nos. 62/253,000, filed November 9, 2015, and 62/350,976, filed June 16, 2016, respectively, the entire contents of each application being incorporated by reference.

#### **BACKGROUND**

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##### **1. Field**

The present disclosure relates generally to the fields of medicine, oncology and immunotherapeutics. More particularly, it concerns the development of immunoreagents for use in detecting and treating glypican 2 (GPC2) -positive cancers.

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##### **2. Related Art**

Children with high-risk neuroblastoma have a poor prognosis despite intensive multimodal chemoradiotherapy. While monoclonal antibodies targeting the disialoganglioside GD2 improve outcomes in neuroblastoma, this therapy is associated with significant “on target-off tumor” toxicities. Thus, a major challenge remains in identifying novel cell surface molecules that meet the stringent criteria for modern immunotherapeutics, including unique tumor expression compared to normal childhood tissues, and preferably that these cell surface molecules be required for tumor sustenance.

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## SUMMARY

Thus, in accordance with the present disclosure, there is provided a method of treating cancer comprising contacting a Glypican 2-positive cancer cell in a subject with an antibody or antibody derivative that binds selectively to Glypican 2. The Glypican 2-positive cancer cell may be a solid tumor cancer cell. The solid tumor cancer cell may be 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, kidney cancer cell, rectal cancer cell, uterine cancer cell, cervical cancer cell, ovarian cancer cell, testicular cancer cell, skin cancer cell, or esophageal cancer cell. The Glypican 2-positive cancer cell may be an embryonal cancer cell. The solid tumor cancer cell may be a sarcoma cell, a neuroblastoma cell, a rhabdoid cancer cell, medulloblastoma cell or neuroblastoma cell. The cancer cell may be a pediatric cancer cell.

The method may further comprise contacting said Glypican 2-positive cancer cell with a second anti-cancer agent or treatment. The second anti-cancer agent or treatment may be selected from chemotherapy, radiotherapy, immunotherapy, hormonal therapy, or toxin therapy. The Glypican 2 antibody may be given before said second agent or treatment. 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 Glypican 2-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, the antibody may be a single domain antibody, the antibody may be a chimeric antibody, the antibody may be a Fab fragment. The antibody may be a recombinant antibody having specificity for the Glypican 2 and a distinct cancer cell surface antigen. The antibody may be a murine antibody, such as an IgG. The antibody may be a humanized or fully human antibody, such as an IgG.

The antibody may further comprise an antitumor drug linked thereto. The antitumor drug may be linked to said antibody through a photolabile linker. The antitumor drug may be linked to said antibody through an enzymatically-cleaved linker. The antitumor drug may be a toxin, a radioisotope, a cytokine, or an enzyme. The antibody may further comprise a label, such as a peptide tag, an enzyme, a magnetic particle, a chromophore, a fluorescent molecule, a chemiluminescent molecule, or a dye. The antibody may be conjugated to a liposome or nanoparticle. The antibody or antibody derivative may result in the induction of cell death, such as by antibody-dependent cell cytotoxicity or complement-mediated cytotoxicity. The antibody derivative may be a chimeric antigen receptor. The antibody may be a bispecific antibody.

Also provided is a fusion protein comprising (i) a first single chain antibody that binds selectively to Glypican 2 (a) is an IgG antibody; (b) inhibits cancer cell growth; (c) induces cancer cell death; 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. The first single chain antibody may be characterized by by CDR sequences SEQ ID NOS: 5-10, 15-20 or 25-30. Still another embodiment comprises a cell expressing the fusion protein as defined above.

Another embodiment includes a chimeric antigen receptor comprising (i) an ectodomain comprising single chain antibody variable region that binds selectively to Glypican 2, wherein said antibody: (a) is an IgG antibody; (b) inhibits cancer cell growth; (c) induces cancer cell death, and has 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 Glypican 2. The transmembrane and endodomains may be derived from the same molecule. The endodomain may comprise a CD3-zeta domain or a high affinity Fc $\epsilon$ RI. The flexible hinge may be from CD8 $\alpha$  or Ig. The single chain GPC2 antibody may be characterized by CDR sequences SEQ ID NOS: 5-10, 15-20 or 25-30. Still another embodiment comprises a cell expressing the chimeric antigen receptor as defined above.

In still another embodiment, there is provided a monoclonal antibody, wherein the antibody or antibody fragment is characterized by CDR sequences SEQ ID NOS: 5-10, 15-20 or 25-30. The antibody or antibody fragment may be encoded by heavy and light chain variable sequences of SEQ ID NOS. 1 and 3, 11 and 13, and 21 and 23, respectively, may be encoded by heavy and light chain variable sequences having at least 70%, 80%, or 90% identity to SEQ ID NOS. 1 and 3, 11 and 13, and 21 and 23, respectively, or may be encoded by heavy and light chain variable sequences having at least 95% identity to SEQ ID NOS. 1 and 3, 11 and 13, and 21 and 23, respectively. The antibody or antibody fragment may comprise heavy and light chain variable sequences comprising SEQ ID NOS. 2 and 4, 12 and 14, and 22 and 24, respectively, or may comprise light and heavy chain variable sequences having 95% identity to comprising SEQ ID NOS. 2 and 4, 12 and 14, and 22 and 24, respectively. The antibody fragment may be a recombinant ScFv (single chain fragment variable) antibody, Fab fragment, F(ab')<sub>2</sub> fragment, or Fv fragment. The antibody may be a chimeric antibody, or is bispecific antibody. The monoclonal antibody may be an IgG. The antibody or antibody fragment may further comprises a label. Also provided are pharmaceutical compositions comprising the

foregoing antibodies, dispersed in pharmaceutically acceptable buffer, medium or diluent, or lyophilized.

In still yet another embodiment, there is provided a hybridoma or engineered cell encoding an antibody or antibody fragment, wherein the antibody or antibody fragment is characterized by CDR sequences SEQ ID NOS: 5-10, 15-20 or 25-30. The antibody or antibody fragment may be encoded by heavy and light chain variable sequences of SEQ ID NOS. 1 and 3, 11 and 13, and 21 and 23, respectively, may be encoded by heavy and light chain variable sequences having at least 70%, 80%, or 90% identity to SEQ ID NOS. 1 and 3, 11 and 13, and 21 and 23, respectively, or may be encoded by heavy and light chain variable sequences having at least 95% identity to SEQ ID NOS. 1 and 3, 11 and 13, and 21 and 23, respectively. The antibody or antibody fragment may comprise heavy and light chain variable sequences comprising SEQ ID NOS. 2 and 4, 12 and 14, and 22 and 24, respectively, or may comprise light and heavy chain variable sequences having 95% identity to comprising SEQ ID NOS. 2 and 4, 12 and 14, and 22 and 24, respectively. The hybridoma or engineered cell may produce an antibody fragment that is a recombinant ScFv (single chain fragment variable) antibody, Fab fragment, F(ab')<sub>2</sub> fragment, or Fv fragment, may produce an antibody that is a chimeric antibody or a bispecific antibody, or may produce an antibody that is an IgG.

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 disclosure 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 disclosure, are given by way of illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

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### **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 disclosure. The disclosure may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

**FIG. 1. Identification of drivers of *GPC2* expression in neuroblastoma.** Prioritization pipeline for identification of highly and differentially expressed putative cell surface genes in high-risk neuroblastoma.

**FIG. 2. *GPC2* is differentially expressed in neuroblastoma vs. normal tissues.** Plot displaying prioritized candidate *GPC2* expression in high-risk neuroblastoma (n=126; TARGET, ocg.cancer.gov/programs/target) to normal tissue RNA sequencing data profiled via the GTEx consortium (n=7859 samples across 31 unique normal tissues, n=5-1152 samples per tissue; GTEx, gtexportal.org).

**FIG. 3. *GPC2* is a predicted cell surface heparan sulfate proteoglycan.** *GPC2* is a heparan sulfate proteoglycan signaling co-receptor with predicted glycosylphosphatidylinositol (GPI) linkage to the extracellular cell surface. Glypicans are integral facilitators of several pro-growth signaling pathways and also play critical roles in cancer cell migration, invasion, and metastasis. *GPC2* is known to bind midkine and sonic hedgehog. GAGs = Glycosaminoglycans.

**FIGS. 4A-H. *GPC2* is a cell surface molecule expressed in most neuroblastomas.** (FIG. 4A-D) *GPC2* exists in both its native form (62 kDa) and with heparan sulfate modifications (~80 kDa). *GPC2* Western blots showing expression in neuroblastoma primary tumors (FIG. 4A), patient derived xenografts (PDXs; FIG. 4B), and cell lines (FIG. 4C). *GPC2* is plasma membrane associated shown by Western blot after membrane extraction in cell lines (FIG. 4D), in primary tumors, PDXs (FIG. 4E) and cell lines (FIG. 4F) by IHC and in cell lines by immunofluorescence (FIG. 4G). (FIG. 4H) Medulloblastomas also express high levels of *GPC2* by IHC. IR = intermediate-risk, HR = high-risk, S = soluble fraction, M = membrane fraction, Na/K = sodium/potassium transporter = membrane control, TMA = tumor microarray.

**FIG. 5. *GPC2* co-localizes with a known neuroblastoma extracellular cell surface protein.** *GPC2* co-localizes with the known neuroblastoma cell surface protein, cadherin.



**FIG. 6.** GPC2 is significantly expressed in medulloblastoma. Greater than 50% of pediatric medulloblastomas also stain positively for GPC2 by IHC, suggesting high level expression in other pediatric cancers.

**FIG. 7.** GPC2 is significantly expressed in other pediatric malignancies. *GPC2* mRNA is found at high levels in multiple pediatric cancers as shown.

**FIGS. 8A-B.** GPC2 has restricted normal tissue expression and there is differential GPC2 exon expression between neuroblastomas and normal tissues. TMA of 37 pediatric normal tissues shows limited GPC2 expression in pediatric normal tissues via IHC. The pediatric esophagus is the only tissue with any significant plasma membrane associated GPC2 expression. 2 = weak IHC staining, 3 = intense IHC staining. Sashimi plots from 5 pediatric esophageal specimens and 5 representative primary neuroblastomas showing differential GPC2 exon 3 expression.

**FIGS. 9A-F.** GPC2 depletion results in neuroblastoma cell apoptosis. (FIGS. 9A-E) RNAi mediated knockdown of GPC2 (GPC2-2, GPC2-4) induces apoptosis and decreases neuroblastoma cell proliferation. Apoptosis induction indicated by elevation of cleaved PARP or cleaved caspase-3 by Western blot (FIG. 9A, FIG. 9D) or elevation of caspase 3/7 by luminescence assay (FIG. 9B). Representative neuroblastoma cell line example Nb-ebc1 from cell line panel (n=12) shown in FIGS. 9A-C and additional examples shown in FIGS. 9D-E. (FIG. 9F) GPC2 over-expression in the neuroblastoma cell line Kelly (heterozygous deletion at the *GPC2* locus) induces increased cell proliferation. NTC = non-targeting control shRNA, \* $p < 0.001$ , \*\* $p < 0.0001$ .

**FIGS. 10A-B.** Progress with GPC2 CAR engineering: binder development. Multiple fragment-antigen binding (Fab) proteins have been identified that bind specifically to GPC2 on neuroblastoma cells.

**FIG. 11.** There is differential GPC2 mRNA variant expression in neuroblastomas vs. normal tissues. Not only do normal tissues have low GPC2 expression in general they also have differential *GPC2* mRNA variant (and thus) epitope expression. Testes are the only normal tissue that predominantly expresses the same *GPC2* variant as expressed in neuroblastomas. Thus, targeting N-terminal derived GPC2 epitopes with immunotherapy may provide an even greater therapeutic index versus targeting the common GPC2 epitopes between neuroblastomas and normal tissues.

**FIG. 12.** GPC2 depletion induces apoptosis in most neuroblastomas. GPC2 loss of function *in vitro* assays were expanded to a panel of 12 neuroblastoma cell lines and revealed that GPC2 drives cell growth broadly in neuroblastoma cells.

**FIG. 13.** GPC2 overexpression induces increased neuroblastoma cell proliferation. Forced GPC2 overexpression in a low GPC2 expressing neuroblastoma cell line (Kelly; with a heterozygous deletion at the GPC2 locus) induces these cells to proliferate significantly faster than empty vector transfected cells. These results further support GPC2 playing a critical role in neuroblastoma cell proliferation.

**FIG. 14.** An integrated RNA sequencing based screen identifies GPC2 as a differentially expressed cell surface molecule and putative immunotherapeutic target in high-risk neuroblastoma. Plot displaying identification of 296 differentially expressed genes in high-risk neuroblastoma.

**FIGS. 15A-E.** Identification of drivers of GPC2 expression in neuroblastoma. (FIG. 15A) GPC2 expression in Huex (left) and NRC (right) neuroblastoma tumors stratified by both chromosome 7q/GPC2 locus gain and MYCN amplification. Neuroblastoma datasets in A obtained from the TARGET consortium (n = seq and mRNA array). P values were derived via unpaired t tests using GraphPad Prism version 5.01. (FIG. 15B) MYCN ChIP plot showing MYCN binds an Ebox motif upstream of the GPC2 promoter in the MYCN amplified neuroblastoma cell lines Kelly and NGP. Arrows represent Ebox (CACGTG motif). (FIG. 15C) GPC2 reporter assay with and without forced overexpression of MYCN in SHEP neuroblastoma and 293T cells. Inset with Western blot displaying MYCN overexpression in SHEP and 293T cells. (FIG. 15D) MYCN/GPC2 quantitative PCR in the MYCN amplified neuroblastoma cell line Kelly after MYCN depletion with 2 unique shRNAs. (FIG. 15E) Western blot of MYCN and GPC2 in the MYCN amplified neuroblastoma cell lines Kelly and NGP after MYCN depletion with an expanded set of 4 unique shRNAs. shNTC, non-targeting shRNA control. See also FIGS. 24A-E.

**FIGS. 16A-I.** GPC2 is expressed in most neuroblastomas and is plasma membrane localized. (FIGS. 16A-C) Western blots of GPC2 in a panel of neuroblastoma primary tumors (n=11; FIG. 16A), PDXs (n=12; FIG. 16B) and cell lines (n=24; FIG. 16C). See also FIG. 25A. (FIG. 16D) GPC2 IHC staining of neuroblastoma cell lines (high GPC2 expression - SMS-SAN, moderate - NBLS, and low - RPE1). See also FIG. 25B. (FIG. 16E) GPC2 flow cytometry analysis of a neuroblastoma cell lines with varied GPC2 expression. (FIG. 16F) Western blot of GPC2 following differential membrane extraction experiments in a panel of neuroblastoma cell lines (n=7). NaK represents a positive Western blot plasma membrane protein control. (FIG. 16G) GPC2 immunofluorescence staining in the neuroblastoma cell lines NB-Ebc1 and SMS-SAN. (FIG. 16H) Summary of membranous staining H-score of GPC2 IHC of PDX and primary tumor TMAs (n=32 and 98 tumors, respectively). (FIG. 16I) Representative membrane

staining H-score examples from PDX and primary tumor TMAs (H-score displayed in lower right corner). Scale bars represent 30  $\mu$ M (FIG. 16D), 10  $\mu$ M (FIG. 16G), 60  $\mu$ M (FIG. 16I) HR, high-risk; IR, intermediate risk; S, soluble (non-membrane) protein extract; M, membrane protein extract. See also Table S1 for PDX (FIG. 16B) and cell line (FIG. 16C and FIG. 16F) identification.

**FIGS. 17A-B. GPC2 expression is restricted in normal tissues.** (FIG. 17A) Summary of membranous H-scores from GPC2 IHC staining of a large pediatric normal tissue array (n=36 unique normal tissues). GPC2 IHC staining membranous H-scores from neuroblastoma primary tumors and PDXs from Figure 3H and I shown for comparison. *P* values were derived via unpaired t tests using GraphPad Prism version 5.01. (FIG. 17B) mRNA transcript specific analysis of *GPC2* expression in primary neuroblastomas and the low-level GPC2 expressing normal tissues skin and esophagus (n=126 HR neuroblastomas, TARGET; n=201 esophagus samples and 684 skin samples, GTEEx). See also FIGS. 26A-C and 27A-C and Table S2.

**FIGS. 18A-I. GPC2 is required for neuroblastoma cell growth.** (FIGS. 18A-B, top) *GPC2* quantitative PCR and GPC2 Western blot analysis following lentiviral transduction of 2 unique shRNA constructs targeting *GPC2* exon 4 and the *GPC2* 3' UTR in the neuroblastoma cell line NB-EBC1. (FIG. 18B, bottom) Western blot of cleaved PARP and caspase 3 after this GPC2 depletion in NB-EBC1. Positive GPC2 Western blot control shown in B was run on the same blot as 120 and 168 hour NB-EBC1 time points. (FIG. 18C) Caspase 3/7 activity measured after GPC2 depletion in NB-EBC1. (FIGS. 18D-F) NB-EBC1 cell growth following shRNA induced GPC2 depletion shown by (FIG. 18D) CellTiter-Glo luminescent assay, (FIG. 18E) RT-CES and (FIG. 18F) colony formation assay. (FIG. 18G) Plot of cell growth measured by CellTiter-Glo luminescent assay and caspase 3/7 elevation following GPC2 depletion with 2 unique shRNA constructs targeting *GPC2* exon 4 (FIG. 18G, top) and the *GPC2* 3' UTR (FIG. 18G, bottom) across an extended panel of neuroblastoma cell lines (n=11). *r*, Pearson correlation coefficient and *p* values by student's t-test shown for each GPC2 shRNA. (FIG. 18H) Neuroblastoma cell growth after forced GPC2 overexpression in Kelly and FIG. 18I) SKNDZ (right). Kelly has a heterozygous deletion of chromosome arm 7q including the *GPC2* locus. \* = *p* < 0.0001, \*\* = *p* < 0.001 by student's t-test as calculated by GraphPad Prism version 5.01. NTC, non-targeting shRNA control. Empty, empty pLenti CMV puro vector control.

**FIGS. 19A-E. GPC2 is expressed in other high-risk pediatric cancers.** (FIG. 19A) *GPC2* RNA sequencing data of additional medulloblastoma tumors (n=91) stratified by clinical grouping and amplification status at chromosome 7q/*GPC2* locus and the *MYC* and *MYCN* loci. (FIG. 19B) Summary of membranous H-scores from GPC2 IHC staining of a

medulloblastoma TMA (n=63). (FIG. 19C) Representative membranous staining H-score examples from medulloblastoma TMA (H-score displayed in lower right). (FIG. 19D and FIG. 19E) GPC2 IHC staining in human metastatic medulloblastoma xenograft murine models with GPC2 including evaluation of central nervous system metastasis (FIG. 19D and FIG. 19E), spinal metastasis (FIG. 19D) and liver metastasis (FIG. 19D). Scale bars in FIG. 19C, FIG. 19D (top and right), and FIG. 19E (right) represent 60  $\mu$ M, FIG. 19D (left) represent 5  $\mu$ M, FIG. 19E (left) 4  $\mu$ M, and FIG. 19D (top middle; spinal cord) 500  $\mu$ M and FIG. 19D (bottom middle; liver) 300  $\mu$ M. (FIG. 19F) mRNA transcript specific analysis of *GPC2* expression in primary medulloblastomas (n=91). See also FIGS. 29A-C and Table S3.

10 **FIG. 20.** A GPC2 targeting ADC, D3-GPC2-PBD, is cytotoxic to GPC2 expressing neuroblastoma cells. IC<sub>50</sub> curves.

**FIG. 21.** GPC2 is the only differentially expressed glypican between neuroblastomas and normal tissues. Plots displaying *GPC1-6* FPKM in high-risk neuroblastomas (n=126; TARGET, ocg.cancer.gov/programs/target) versus paired normal tissue *GPC1-6* FPKM from RNA sequencing data profiled via the GTEx consortium (n=7859 samples across 31 unique normal tissues, n=5-1152 samples per tissue; GTEx, gtxportal.org). See also FIGS. 1-2 and 14.

**FIGS. 22A-C.** GPC2 is the predominant glypican expressed in neuroblastoma and GPC2 expression inversely correlates with GPC3 expression and neuroblastoma tumor stromal and immune cell content. (FIG. 22A) *GPC1-6* FPKM plotted from neuroblastoma primary tumors (left, n=126 high-risk tumors, TARGET; right, n=498 tumors across all risk groups, seqC). (FIG. 22B) *GPC2* FPKM plotted versus *GPC3* FPKM (left, n=126, TARGET; right, n=498, seqC). r, Pearson correlation coefficient and p values shown for each data set. (FIG. 22C) *GPC2* FPKM plotted versus stromal and immune cell content (left, n=126, TARGET; right, n=498, seqC). r, Pearson correlation coefficient and p values shown for each data set.

**FIGS. 23A-C.** High GPC2 expression is associated with worse overall survival in neuroblastoma. (FIGS. 23A-C, left) Overall survival curves for 3 neuroblastoma data sets analyzed via the Genomics Analysis and Visualization Platform (R2; r2.amc.nl; (FIG. 23A) Kocak; n=649, (FIG. 23B) seqC; n=498, and (FIG. 23C) Versteeg; n=88).<sup>58-60</sup> (FIGS. 23A-C, right) Overall survival curves for 3 same neuroblastoma data sets limited to patients with tumors without MYCN amplification.

**FIGS. 24A-C.** MYCN does not significantly bind to GPC3-6. (FIGS. 24A-C) MYCN ChIP sequencing from the MYCN amplified neuroblastoma cell lines NGP and Kelly for *GPC1*

(FIG. 24A), *GPC3* and *GPC4* (FIG. 24B), *GPC5* and *GPC6* (FIG. 24C). See also FIGS. 15A-E.

**FIGS. 25A-C. GPC2 is cell surface localized in neuroblastoma.** (FIG. 25A) Western blot for GPC2 with the GPC2 monoclonal mouse antibody (sc-393824) for antibody validation. 5 CHP134, NBSD, and SMS-SAN are 3 representative high GPC2 expressing wild-type neuroblastoma cell lines. Also shown are lentiviral shRNA transduced cells (NBSD and SMS-SAN) and GPC2 plenti puro CMV overexpressed cells (Kelly) to complete antibody validation. (FIG. 25B) GPC2 IHC from a panel of neuroblastoma cell lines (n=8). (FIG. 25C) GPC2 immunofluorescence studies from the neuroblastoma cell lines NBEB1 and SMS-SAN. Ex 4 and UTR represented shRNA constructs targeting *GPC2* exon 4 and the *GPC2* 3' UTR, 10 respectively. NTC, non-targeting shRNA control. Empty, empty pLenti CMV puro vector control. Scale bars, 60  $\mu$ M (B), 10  $\mu$ M (FIG. 25C). See also FIGS. 16A-I.

**FIGS. 26A-C. GPC2 has restricted normal tissue expression by IHC.** (FIG. 26A and FIG. 26B) Representative GPC2 IHC in the esophagus (FIG. 26A) and skin (FIG. 26B). 15 Membranous staining H-scores indicated. (FIG. 26C) Representative GPC2 IHC from major human organs. Scale bar, 60  $\mu$ M.

**FIGS. 27A-C. GPC2 has restricted normal tissue expression by high-resolution mass spectrometry.** (FIGS. 27A-C) Spectral counts for GPC2 (FIG. 27A), L1CAM (FIG. 27B) and CD19 (FIG. 27C) shown across a panel of normal tissues (n=30).

**FIGS. 28A-J. GPC2 is required for cell growth in most neuroblastomas.** (FIGS. 28A-G) Lentiviral shRNA induced depletion of GPC2 with 2 unique shRNA constructs targeting *GPC2* exon 4 (Ex 4) and the *GPC2* 3' UTR (UTR) in a panel of cell lines (n=10). Each panel shows GPC2 Wb with shRNA indicated (left), RT-CES cell proliferation plot (top right) and colony formation assay (bottom right; not available for NLF, E). (FIGS. 28H-J) For those 20 neuroblastoma cell lines that did not grow solely as a monolayer prohibiting utilization of the RT-CES growth assay, only colony formation assays were done. Colony formation assay shown on left, GPC2 Western blot shown on right. \* =  $p < 0.0001$ , \*\* =  $p < 0.001$ , \*\*\* =  $p < 0.01$ . NTC, non-targeting control shRNA. See also FIGS. 18A-I.

**FIGS. 29A-C. GPC2 expression profiling across other pediatric malignancies identifies high GPC2 levels in medulloblastomas and retinoblastomas.** (FIG. 29A) GPC2 RNA 30 sequencing data across an array of pediatric malignancies (total n=1608, individual n indicated on figure x axis) including data from the Therapeutically Applicable Research to Generate Effective Treatments project (TARGET; [ocg.cancer.gov/programs/target](http://ocg.cancer.gov/programs/target)) project and the St. Jude Children's Research Hospital Pediatric Cancer Data Portal (PeCan; [pecan.stjude.org](http://pecan.stjude.org)).

\*Indicates RNA sequencing data from the TARGET project. \*\*Normal tissue from the GTEx portal included for comparison (average FPMKs for each tissue shown, total n=7859 samples across 31 unique normal tissues, n=5-1152 samples per tissue; GTEx, [gtexportal.org](http://gtexportal.org)). (FIG. 29B) Confirmatory *GPC2* mRNA array data for neuroblastomas, medulloblastomas, and retinoblastomas from the Genomics Analysis and Visualization Platform (R2; <http://r2.amc.nl>; individual n indicated on figure x axis). (FIG. 29C) Log2 *GPC2* expression from human primary and metastatic paired medulloblastoma samples. *P* value derived via unpaired t test. NB, neuroblastoma; RB, retinoblastoma; MB, medulloblastoma; ALL, acute lymphocytic leukemia; HGG, high-grade glioma; RHB, rhabdomyosarcoma; MLL, mixed-lineage leukemia; CPC, choroid plexus carcinoma; WT, Wilms tumor; LGG, low grade glioma; AML, acute myelogenous leukemia; OS osteosarcoma; EPD, ependymoma; MEL, melanoma; ACT, adrenocortical carcinoma; malignant rhaboid tumor; normal, normal tissues. See also FIGS. 19A-E.

**FIG. 30.** Heavy and light chain amino acid and nucleic acid sequences for human antibody m201. CDRs shown in bold italics.

**FIG. 31.** Heavy and light chain amino acid and nucleic acid sequences for human antibody m202. CDRs shown in bold italics.

**FIG. 32.** Heavy and light chain amino acid and nucleic acid sequences for human antibody m203. CDRs shown in bold italics.

20

## **DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS**

The inventors have determined that Glypican 2 (GPC2) is a candidate cell surface immunotherapeutic target and putative oncogene in high-risk neuroblastoma and possibly other pediatric cancers. More globally, the data presented here show that genome-wide transcriptome analysis integrated with genomic and functional validation can identify differentially expressed cell surface oncogenes that may be attractive immunotherapeutic targets. These and other aspects of the disclosure are described in greater detail below.

### **I. Glypican 2**

Glypican 2 (GPC2), also known cerebroglycan, is a protein which in humans is encoded by the *GPC2* gene. Cerebroglycan is a glycosphosphatidylinositol-linked integral membrane heparan sulfate proteoglycan found in the developing nervous system. Cerebroglycan participates in cell adhesion and is thought to regulate the growth and guidance of axons. Cerebroglycan has especially high affinity for laminin-1. The accession nos. for human Glypican 2 mRNA and protein sequences are NM\_152742 and NP\_689955, respectively, which are hereby incorporated by reference.

### **II. Producing Monoclonal Antibodies**

#### **A. General Methods**

Antibodies to Glypican 2 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.

5 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  
10 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.  
15 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  
20 fusion efficiency, and enzyme deficiencies that render them 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  
25 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  
30 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 disclosure 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  $1 \times 10^{-6}$  to  $1 \times 10^{-8}$ . 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.

5           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.

20           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 disclosure 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 disclosure can be synthesized using an automated peptide synthesizer.

30           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 disclosure include U.S. Patent 5,565,332, which describes the production of chimeric antibodies using a combinatorial approach; U.S. Patent 4,816,567  
5 which describes recombinant immunoglobulin preparations; and U.S. Patent 4,867,973 which describes antibody-therapeutic agent conjugates.

### **B. Antibodies of the Present Disclosure**

10 Antibodies according to the present disclosure may be defined, in the first instance, by their binding specificity, which in this case is for Glypican 2. In one 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  
15 subclasses (IgG1, 2, 3, and 4) in humans, named in order of their abundance in serum (IgG1 being the most abundant). These 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  
20 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-  
25 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  
30 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.

Furthermore, the antibodies sequences may vary from the sequences provided above, optionally using methods discussed in greater detail below. For example, amino sequences  
5 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%  
10 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  
15 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 hydrophatic index of amino acids may be considered. The importance of the hydrophatic amino acid index in  
20 conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982). It is accepted that the relative hydrophatic 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  
25 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  
30 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

( $-0.5 \pm 1$ ), 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  $\pm 2$  is preferred, those that are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  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.

### 15           **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,  
5 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  
10 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')<sub>2</sub>) 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.  
15 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  
20 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  
25 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  
30 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 disclosure also contemplates isotype modification. By modifying the Fc region to have a different isotype, different functionalities can be achieved. For example, changing to IgG<sub>4</sub> can reduce immune effector functions associated with other isotypes.

5 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.

#### **D. Expression**

10 Nucleic acids according to the present disclosure will encode antibodies, optionally linked to other protein sequences. As used in this application, the term “a nucleic acid encoding a Glypican 2 antibody” refers to a nucleic acid molecule that has been isolated free of total cellular nucleic acid. In certain embodiments, the disclosure concerns antibodies that are encoded by any of the sequences set forth herein.

15

**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		

The DNA segments of the present disclosure include those encoding biologically  
5 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,  
10 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.

Throughout this application, the term “expression construct” is meant to include any  
15 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. 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).

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  
5 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

10 In certain embodiments of the disclosure, 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  
15 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  
20 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  
25 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  
30 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

5 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).

#### 10 5. Termination Signals

The vectors or constructs of the present disclosure 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  
15 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  
20 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  
25 sequences.

Terminators contemplated for use in the disclosure 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  
30 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  
35 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 disclosure, 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 disclosure, cells containing a nucleic acid construct of the present disclosure 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). 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 disclosure and may be selected according to the requisite properties of the target system.

15

## 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 disclosure 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 (Kaepler *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

30

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.

5                                   **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 disclosure to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely  
10 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<sup>®</sup> 2.0 from Invitrogen<sup>®</sup> and BacPack<sup>™</sup> Baculovirus Expression System From  
15 Clontech<sup>®</sup>.

Other examples of expression systems include Stratagene<sup>®</sup>'s Complete Control<sup>™</sup> 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<sup>®</sup>, which carries the T-Rex<sup>™</sup> (tetracycline-  
20 regulated expression) System, an inducible mammalian expression system that uses the full-length CMV promoter. Invitrogen<sup>®</sup> 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  
25 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  
30 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.

5 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  
10 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  
15 phosphoribosyltransferase genes, in *tk-*, *hgppt-* 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.

#### 20 **E. Purification**

In certain embodiments, the antibodies of the present disclosure 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  
25 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  
30 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  
5 other techniques.

In purifying an antibody of the present disclosure, 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  
10 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  
15 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  
20 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  
25 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  
30 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  
5 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  
10 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  
15 residues such as alanine, 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.  
20  $5 \times 10^6$  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  
25 the V<sub>H</sub> 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 disclosure 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.  
30 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).

5 Cross-linking reagents are used to form molecular bridges that tie functional groups of two different molecules, *e.g.*, a stabilizing 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.

10 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  
15 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  
20 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  
25 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  
30 (*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  
5 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  
10 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  
15 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 aminoxy-containing linkers useful in a variety of immunodiagnostic and separative techniques.

20

## **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  
25 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.

30 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.

5           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  
10 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  
15 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  
20 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  
25 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.

30           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<sub>2</sub>CH<sub>3</sub> 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  $\zeta$ - 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.

5                                   **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 Glypican 2). 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  $\alpha$  emitting immunoconjugates and antibody-conjugated nanoparticles.

### 25                    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.

30                    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 Glypican 2.



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  
5 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  
10 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  
15 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**

20 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  
25 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 disclosure can be applied include generally any cell that expresses Glypican 2, and more particularly, that overexpresses Glypican 2. Cancer cells that may be treated according to the present disclosure include but  
30 are not limited to cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestinal, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, pancreas, testis, tongue, cervix, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell

carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular  
5 adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular  
10 adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometrioid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma;  
15 infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; Paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; androblastoma, malignant; sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-  
20 mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malignant melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal  
25 sarcoma; mixed tumor, malignant; Mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; Brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; Kaposi's sarcoma;  
30 hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; Ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma;

fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; Hodgkin's disease; paraganuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia. In certain aspects, the tumor may comprise an osteosarcoma, angiosarcoma, rhabdosarcoma, leiomyosarcoma, Ewing sarcoma, glioblastoma, medulloblastoma, neuroblastoma, or leukemia.

In addition, the methods of the disclosure 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 disclosure 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 disclosure provides pharmaceutical compositions comprising anti-Glypican 2 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 disclosure may include classic pharmaceutical preparations. Administration of these compositions according to the present disclosure 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 administration 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 disclosure, it also is contemplated that anti-Glypican 2 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- Glypican 2 antibodies with other therapies that target different aspects of Glypican 2 function.

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 disclosure, one would generally contact a “target” cell with an anti-Glypican 2 antibody according to the present disclosure 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-Glypican 2 antibody according to the present disclosure 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-Glypican 2 antibody according to the present disclosure and the other includes the other agent.

Alternatively, the anti-Glypican 2 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-Glypican 2 antibody are applied separately to the cell, one would generally ensure that a significant period of time did not expire between 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-Glypican 2 antibody or the other agent will be desired. Various combinations may be employed, where an anti-Glypican 2 antibody according to the present disclosure 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 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. A combination therapy may also include surgery. Various modes of these therapies are discussed below.

### 1. Chemotherapy

5           The term “chemotherapy” refers to the use of drugs to treat cancer. A “chemotherapeutic agent” is used to connote a compound or composition that is administered in the treatment of cancer. These agents or drugs are categorized by their mode of activity within a cell, for example, whether and at what stage they affect the cell cycle. Alternatively, an agent may be characterized based on its ability to directly cross-link DNA, to intercalate  
10 into DNA, or to induce chromosomal and mitotic aberrations by affecting nucleic acid synthesis. Most chemotherapeutic agents fall into the following categories: alkylating agents, antimetabolites, antitumor antibiotics, mitotic inhibitors, and nitrosoureas.

          Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines  
15 such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramidate, triethylenethiophosphoramidate and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; calystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues);  
20 cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil  
25 mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammaII and calicheamicin omegaII; dynemicin, including dynemicin A uncialamyacin and derivatives thereof; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic  
30 chromophores, aclacinomysins, actinomycin, authrarnycin, azaserine, bleomycins, cactinomycin, carabycin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin),

epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalarnycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitio stanol, mepitio stanol, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as folinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.*, paclitaxel and docetaxel; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum coordination complexes such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (*e.g.*, CPT-11); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, raloxifene, estrogen receptor binding agents, taxol, paclitaxel, docetaxel, gemcitabien, navelbine, farnesyl-protein transferase inhibitors, transplatinum, 5-fluorouracil, vincristin, vinblastin and methotrexate and pharmaceutically acceptable salts, acids or derivatives of any of the above.

## 2. Radiotherapy

Radiotherapy, also called radiation therapy, is the treatment of cancer and other diseases with ionizing radiation. Ionizing radiation deposits energy that injures or destroys cells in the area being treated by damaging their genetic material, making it impossible for these cells to continue to grow. Although radiation damages both cancer cells and normal cells, the latter are able to repair themselves and function properly.

Radiation therapy used according to the present disclosure may include, but is not limited to, the use of  $\gamma$ -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 induce a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on 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 wk), 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.

Radiotherapy may comprise the use of radiolabeled antibodies to deliver doses of radiation directly to the cancer site (radioimmunotherapy). Antibodies are highly specific proteins that are made by the body in response to the presence of antigens (substances recognized as foreign by the immune system). Some tumor cells contain specific antigens that trigger the production of tumor-specific antibodies. Large quantities of these antibodies can be made in the laboratory and attached to radioactive substances (a process known as radiolabeling). Once injected into the body, the antibodies actively seek out the cancer cells, which are destroyed by the cell-killing (cytotoxic) action of the radiation. This approach can minimize the risk of radiation damage to healthy cells.

Conformal radiotherapy uses the same radiotherapy machine, a linear accelerator, as the normal radiotherapy treatment but metal blocks are placed in the path of the x-ray beam to alter its shape to match that of the cancer. This ensures that a higher radiation dose is given to the tumor. Healthy surrounding cells and nearby structures receive a lower dose of radiation, so the possibility of side effects is reduced. A device called a multi-leaf collimator has been developed and may be used as an alternative to the metal blocks. The multi-leaf collimator consists of a number of metal sheets which are fixed to the linear accelerator. Each layer can be adjusted so that the radiotherapy beams can be shaped to the treatment area without the need for metal blocks. Precise positioning of the radiotherapy machine is very important for



conformal radiotherapy treatment and a special scanning machine may be used to check the position of internal organs at the beginning of each treatment.

High-resolution intensity modulated radiotherapy also uses a multi-leaf collimator. During this treatment the layers of the multi-leaf collimator are moved while the treatment is being given. This method is likely to achieve even more precise shaping of the treatment beams and allows the dose of radiotherapy to be constant over the whole treatment area.

Although research studies have shown that conformal radiotherapy and intensity modulated radiotherapy may reduce the side effects of radiotherapy treatment, it is possible that shaping the treatment area so precisely could stop microscopic cancer cells just outside the treatment area being destroyed. This means that the risk of the cancer coming back in the future may be higher with these specialized radiotherapy techniques.

Scientists also are looking for ways to increase the effectiveness of radiation therapy. Two types of investigational drugs are being studied for their effect on cells undergoing radiation. Radiosensitizers make the tumor cells more likely to be damaged, and radioprotectors protect normal tissues from the effects of radiation. Hyperthermia, the use of heat, is also being studied for its effectiveness in sensitizing tissue to radiation.

### 3. Immunotherapy

In the context of cancer treatment, immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. Trastuzumab (Herceptin™) is such an example. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually affect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, *etc.*) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells. The combination of therapeutic modalities, *i.e.*, direct cytotoxic activity and inhibition or reduction of ErbB2 would provide therapeutic benefit in the treatment of ErbB2 overexpressing cancers.

In one aspect of immunotherapy, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present disclosure. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis

Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, erb B and p155. An alternative aspect of immunotherapy is to combine anticancer effects with immune stimulatory effects. Immune stimulating molecules also exist including: cytokines such as IL-2, IL-4, IL-12, GM-CSF,  $\gamma$ -IFN, chemokines such as MIP-1, MCP-1, IL-8 and growth factors such as  
5 FLT3 ligand. Combining immune stimulating molecules, either as proteins or using gene delivery in combination with a tumor suppressor has been shown to enhance anti-tumor effects (Ju *et al.*, 2000). Moreover, antibodies against any of these compounds may be used to target the anti-cancer agents discussed herein.

Examples of immunotherapies currently under investigation or in use are immune  
10 adjuvants *e.g.*, *Mycobacterium bovis*, *Plasmodium falciparum*, dinitrochlorobenzene and aromatic compounds (U.S. Patents 5,801,005 and 5,739,169; Hui and Hashimoto, 1998; Christodoulides *et al.*, 1998), cytokine therapy, *e.g.*, interferons  $\alpha$ ,  $\beta$ , and  $\gamma$ ; IL-1, GM-CSF and TNF (Bukowski *et al.*, 1998; Davidson *et al.*, 1998; Hellstrand *et al.*, 1998) gene therapy, *e.g.*, TNF, IL-1, IL-2, p53 (Qin *et al.*, 1998; Austin-Ward and Villaseca, 1998; U.S. Patents  
15 5,830,880 and 5,846,945) and monoclonal antibodies, *e.g.*, anti-ganglioside GM2, anti-HER-2, anti-p185 (Pietras *et al.*, 1998; Hanibuchi *et al.*, 1998; U.S. Patent 5,824,311). It is contemplated that one or more anti-cancer therapies may be employed with the gene silencing therapies described herein.

In active immunotherapy, an antigenic peptide, polypeptide or protein, or an autologous  
20 or allogenic tumor cell composition or “vaccine” is administered, generally with a distinct bacterial adjuvant (Ravindranath and Morton, 1991; Morton *et al.*, 1992; Mitchell *et al.*, 1990; Mitchell *et al.*, 1993).

In adoptive immunotherapy, the patient’s circulating lymphocytes, or tumor infiltrated lymphocytes, are isolated *in vitro*, activated by lymphokines such as IL-2 or transduced with  
25 genes for tumor necrosis, and readministered (Rosenberg *et al.*, 1988; 1989).

#### 4. Surgery

Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative, and palliative surgery. Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment  
30 of the present disclosure, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part

of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and microscopically controlled surgery (Mohs' surgery). It is further contemplated that the present disclosure may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

5           Upon excision of part or all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

10           In some particular embodiments, after removal of the tumor, an adjuvant treatment with a compound of the present disclosure is believed to be particularly efficacious in reducing the reoccurrence of the tumor. Additionally, the compounds of the present disclosure can also be used in a neoadjuvant setting.

          It also should be pointed out that any of the foregoing therapies may prove useful by themselves in treating cancer. 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.

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#### **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.*, anti-cancer/general cell toxicity. 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.

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          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<sup>211</sup>, <sup>14</sup>carbon, <sup>51</sup>chromium, <sup>36</sup>chlorine, <sup>57</sup>cobalt, <sup>58</sup>cobalt, copper<sup>67</sup>, <sup>152</sup>Eu, gallium<sup>67</sup>, <sup>3</sup>hydrogen, iodine<sup>123</sup>, iodine<sup>125</sup>, iodine<sup>131</sup>, indium<sup>111</sup>, <sup>59</sup>iron, <sup>32</sup>phosphorus, rhenium<sup>186</sup>, rhenium<sup>188</sup>, <sup>75</sup>selenium, <sup>35</sup>sulphur, technetium<sup>99m</sup> and/or yttrium<sup>90</sup>. <sup>125</sup>I is often being preferred for use in certain embodiments, and technetium<sup>99m</sup> and/or indium<sup>111</sup> 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<sup>99m</sup> by ligand exchange process, for example, by reducing pertechnetate 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 pertechnetate, a reducing agent such as  $\text{SnCl}_2$ , 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.

5 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  
10 streptavidin compounds. The use of such labels is well known to those of skill in the art and is described, for example, in U.S. 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-  
15 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  
20 (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.

25 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 as diethylenetriaminepentaacetic acid anhydride (DTPA); ethylenetriaminetetraacetic acid; N-chloro-p-toluenesulfonamide; and/or tetrachloro-3 $\alpha$ -6 $\alpha$ -diphenylglycouril-3 attached to the antibody (U.S. Patents 4,472,509 and  
30 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 Glypican 2. 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 Glypican 2 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 Glypican 2 present in the sample. 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  
5 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  
10 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  
15 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  
20 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  
25 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  
30 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  
5 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  
10 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  
15 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  
20 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 disclosure are immobilized onto a  
25 selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition suspected of containing the Glypican 2 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-Glypican 2 antibody that is linked to a detectable label. This type of ELISA is a simple “sandwich ELISA.”  
30 Detection may also be achieved by the addition of a second anti-Glypican 2 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 Glypican 2 antigen are immobilized onto the well surface and then contacted with anti-Glypican 2 antibody.



After binding and washing to remove non-specifically bound immune complexes, the bound anti- Glypican 2 antibodies are detected. Where the initial anti-Glypican 2 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  
5 anti- Glypican 2 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  
10 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  
15 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,  
20 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”  
25 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  
30 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  
5 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.*,  
10 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<sub>2</sub>O<sub>2</sub>, in the case of peroxidase as the enzyme label. Quantification  
15 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  
20 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.

25 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  
30 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.

#### 15           **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 Glypican 2 antigen, and optionally an immunodetection reagent.

20           In certain embodiments, the Glypican 2 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.

25           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.

30           The kits may further comprise a suitably aliquoted composition of the Glypican 2 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  
5 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

10 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  
15 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 disclosure.

### EXAMPLE 1

20 The initial transcriptome-based discovery effort identified 649 significantly differentially expressed genes (log-fold change tumor vs. normal >1 for each tissue; adjusted  $p < 0.05$ ), 86 (13%) of which were predicted to be potential cell surface molecules. Through our analytic pipeline, we prioritized the extracellular glycosylphosphatidylinositol (GPI) anchored, signaling co-receptor Glypican-2 (GPC2) for validation given robust differential RNA  
25 expression (log-fold change tumor vs. normal tissue = 2.1-8.2;  $p < 3 \times 10^{-10}$ ), high-level absolute RNA expression (median FPKM=57; 85% of tumors with FPKM >25) and consistent DNA copy number gain (31% of primary neuroblastomas; N=177) associated with significantly higher *GPC2* expression ( $p < 0.0001$ ). Immunoblot analysis confirmed ubiquitous GPC2 protein expression (N=8 high-risk neuroblastomas and 23 cell lines) and membrane extraction, IF, and  
30 IHC confirmed dense plasma membrane associated GPC2 protein expression in neuroblastoma cell lines. IHC analysis of primary neuroblastoma tumors (N=83) compared to a parallel array of pediatric normal tissues (N=37) further confirmed GPC2 protein expression to be membrane associated and tumor specific with very limited normal tissue expression. Lentiviral mediated RNAi induced GPC2 depletion in a panel of 12 neuroblastoma cell lines resulted in significant

apoptosis and growth inhibition both in transient CellTiter-Glo and Caspase-Glo assays (20-87% decreased growth and 1.5-18.4-fold increased caspase 3/7 level vs. control) and with longer term real-time monitoring of cell growth (RT-CES). GPC2 overexpression resulted in significantly increased cellular proliferation (2.7-fold growth increase vs. control,  $p < 0.0001$ ).

5 Finally, GPC2 was also found to be significantly differentially overexpressed in other embryonal cancers, most notably medulloblastoma.

A panel of three fully human antibodies (m201, m202 and m203) specifically targeting cancer cell-associated GPC2 were isolated from a phage display antibody library and affinity matured. *In vitro* characterization demonstrates that these antibodies possess promising

10 therapeutic activity for use in CAR-T, antibody drug conjugate (ADC) and bispecific antibody development for cancer therapy. The sequences of the antibodies are shown in FIGS. 30-32.

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All of the compositions and methods disclosed and claimed herein can be made and

15 executed without undue experimentation in light of the present disclosure. While the compositions and methods of this disclosure 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 disclosure. More

20 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 disclosure 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

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U.S. Patent 4,867,973

U.S. Patent 4,879,236

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U.S. Patent 5,196,066

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U.S. Patent 5,322,783

U.S. Patent 5,384,253

U.S. Patent 5,464,765

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 5,780,448  
U.S. Patent 5,789,215  
U.S. Patent 5,824,544  
U.S. Patent 5,856,456  
U.S. Patent 5,858,744  
U.S. Patent 5,871,982  
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**WHAT IS CLAIMED IS:**

1. A method of treating a Glypican 2 (GPC2)-positive cancer comprising contacting a Glypican 2-positive cancer cell in a subject with an antibody or antibody fragment that binds selectively to a cancer cell-associated Glypican 2, wherein the antibody or antibody fragment comprises a heavy chain variable domain including a CDR1 comprising the amino acid sequence of SEQ ID NO: 5, a CDR2 comprising the amino acid sequence of SEQ ID NO: 6, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 7, and a light chain variable domain including a CDR1 comprising the amino acid sequence of SEQ ID NO: 8, a CDR2 comprising the amino acid sequence of SEQ ID NO: 9, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 10.
2. The method of claim 1, wherein said Glypican 2-positive cancer cell is a solid tumor cancer cell.
3. The method of claim 2, 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, kidney cancer cell, rectal cancer cell, uterine cancer cell, cervical cancer cell, ovarian cancer cell, testicular cancer cell, skin cancer cell, or esophageal cancer cell.
4. The method of claim 1, wherein said Glypican 2-positive cancer cell is an embryonal cancer cell.
5. The method of claim 1, wherein said cancer cell is a sarcoma cell, a neuroblastoma cell, a rhabdoid cancer cell, medulloblastoma cell or neuroblastoma cell.
6. The method of any one of claims 1-5, further comprising contacting said Glypican 2-positive cancer cell with a second anti-cancer agent or treatment.
7. The method of claim 6, wherein said second anti-cancer agent or treatment is selected from chemotherapy, radiotherapy, immunotherapy, hormonal therapy, or toxin therapy.
8. The method of claim 6, wherein said Glypican 2 antibody is (i) given before said second agent or treatment, (ii) given at the same time as the first agent, or (iii) given before and/or after the first agent.

9. The method of any one of claims 1-8, wherein said Glypican 2-positive cancer cell is a metastatic cancer cell, a multiply drug resistant cancer cell or a recurrent cancer cell.
10. The method of any one of claims 1-9, wherein said antibody or antibody fragment is (i) a single chain antibody, (ii) a single domain antibody, (iii) a chimeric antibody, or (iv) a Fab fragment.
11. The method of claim 9 or 10, wherein said antibody is a recombinant antibody having specificity for the Glypican 2 and a distinct cancer cell surface antigen.
12. The method of any one of claims 1-11, wherein said antibody is (i) a murine antibody, such as an IgG, (ii) a human antibody, or (iii) a humanized antibody.
13. The method of claim 12, wherein said humanized antibody is an IgG.
14. The method of any one of claims 1-13, wherein said antibody further comprises an antitumor drug linked thereto.
15. The method of claim 14, wherein said antitumor drug is (i) linked to said antibody through a photolabile linker or an enzymatically-cleaved linker, and/or (ii) a toxin, a radioisotope, a cytokine, or an enzyme.
16. The method of any one of claims 1-15, wherein said antibody further comprises a label.
17. The method of claim 16, wherein said label is a peptide tag, an enzyme, a magnetic particle, a chromophore, a fluorescent molecule, a chemiluminescent molecule, or a dye.
18. The method of any one of claims 1-17, wherein said antibody (i) is conjugated to a liposome or nanoparticle and/or (ii) results in the induction of cell death, such as by antibody-dependent cell cytotoxicity or complement-mediated cytotoxicity.
19. The method of claim 1, wherein said antibody fragment is a chimeric antigen receptor (CAR) or a bispecific antibody.
20. A fusion protein comprising:
  - (i) a first single chain antibody or antibody fragment that binds selectively to a cancer cell-associated Glypican 2, wherein the first single chain antibody:

(a) is an IgG antibody;

(b) inhibits cancer cell growth;

(c) induces cancer cell death; and

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

wherein the first antibody or antibody fragment comprises a heavy chain variable domain including a CDR1 comprising the amino acid sequence of SEQ ID NO: 5, a CDR2 comprising the amino acid sequence of SEQ ID NO: 6, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 7, and a light chain variable domain including a CDR1 comprising the amino acid sequence of SEQ ID NO: 8, a CDR2 comprising the amino acid sequence of SEQ ID NO: 9, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 10.

21. The fusion protein of claim 20, wherein said second single chain antibody (i) binds to a T cell, such as through CD3 or (ii) binds to a B cell.

22. The fusion protein of claim 20, wherein said fusion protein further comprises a label or a therapeutic moiety.

23. A chimeric antigen receptor (CAR) comprising:

(a) an ectodomain comprising a single chain antibody variable region that binds selectively to a cancer cell-associated Glypican 2, wherein said single chain antibody is derived from a monoclonal antibody and said monoclonal antibody:

- (i) is an IgG antibody;
- (ii) inhibits cancer cell growth;
- (iii) induces cancer cell death, and has a flexible hinge attached at the C-terminus of said single chain antibody variable region; and

(b) a transmembrane domain; and

(c) an endodomain,

wherein said endodomain comprises a signal transduction function when said single-chain antibody variable region is engaged with Glypican 2, and

wherein the single chain antibody comprises a heavy chain variable domain including a CDR1 comprising the amino acid sequence of SEQ ID NO: 5, a CDR2 comprising the amino acid sequence of SEQ ID NO: 6, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 7, and a light chain variable domain including a CDR1 comprising the amino

acid sequence of SEQ ID NO: 8, a CDR2 comprising the amino acid sequence of SEQ ID NO: 9, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 10.

24. The CAR of claim 23, wherein said transmembrane and endodomains are derived from the same molecule.
25. The CAR of claim 23, wherein:
  - (a) said endodomain comprises a CD3-zeta domain or a high affinity FcERI; and/or
  - (b) said endodomain further comprises an intracellular domain of a costimulatory protein selected from the group consisting of CD28, 41BB, OX40, and ICOS.
26. The CAR of claim 23, wherein the flexible hinge is from CD8a or an immunoglobulin (Ig).
27. A cell expressing the CAR of any one of claims 23-26.
28. A cell expressing the fusion protein of claim 20.
29. The cell of claim 28, further expressing a single chain antibody that binds to CD3, a T cell, or a B cell.
30. A monoclonal antibody or fragment thereof that binds selectively to a cancer cell-associated Glypican 2 comprising a heavy chain variable domain including a CDR1 comprising the amino acid sequence of SEQ ID NO: 5, a CDR2 comprising the amino acid sequence of SEQ ID NO: 6, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 7, and a light chain variable domain including a CDR1 comprising the amino acid sequence of SEQ ID NO: 8, a CDR2 comprising the amino acid sequence of SEQ ID NO: 9, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 10.
31. The monoclonal antibody or fragment thereof of claim 30, wherein the heavy chain variable domain and the light chain variable domain:
  - (a) are encoded by the nucleotide sequences set forth in SEQ ID NOs: 1 and 3;
  - (b) are encoded by nucleotide sequences having at least 70%, 80%, or 90% sequence identity to SEQ ID NOs: 1 and 3;
  - (c) are encoded by nucleotide sequences having at least 95% sequence identity to SEQ ID NOs: 1 and 3;

- (d) comprise the amino acid sequence of SEQ ID NOs: 2 and 4; or
  - (e) comprise amino acid sequences having 95% sequence identity to SEQ ID NOs: 2 and 4.
32. The monoclonal antibody or fragment thereof of claim 30, wherein:
- (a) the antibody fragment is a recombinant ScFv (single chain fragment variable) antibody, Fab fragment, F(ab')<sub>2</sub> fragment, or Fv fragment; and/or
  - (b) the antibody is a chimeric antibody or a bispecific antibody; and/or
  - (c) the antibody is an IgG, and/or is conjugated to a label, and/or is conjugated to a therapeutic agent; and/or
  - (d) the antibody or antibody fragment further comprises a label.
33. A hybridoma or engineered cell comprising an antibody or antibody fragment, wherein the antibody or antibody fragment thereof that binds selectively to a cancer cell-associated Glypican 2 and comprises a heavy chain variable domain including a CDR1 comprising the amino acid sequence of SEQ ID NO: 5, a CDR2 comprising the amino acid sequence of SEQ ID NO: 6, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 7, and a light chain variable domain including a CDR1 comprising the amino acid sequence of SEQ ID NO: 8, a CDR2 comprising the amino acid sequence of SEQ ID NO: 9, and a CDR3 comprising the amino acid sequence of SEQ ID NO: 10.
34. The hybridoma or engineered cell of claim 33, wherein the heavy chain variable domain and the light chain variable domain:
- (a) are encoded by nucleotide sequences of SEQ ID NOs: 1 and 3;
  - (b) are encoded by nucleotide sequences having at least 70%, 80%, or 90% sequence identity to SEQ ID NOs: 1 and 3;
  - (c) are encoded by nucleotide sequences having 95% identity to SEQ ID NOs: 1 and 3;
  - (d) comprise the amino acid sequences of SEQ ID NOs: 2 and 4;
  - (e) comprise the amino acid sequences having at least 70%, 80%, or 90% sequence identity to SEQ ID NOs: 2 and 4; or
  - (f) comprise the amino acid sequences having 95% sequence identity to SEQ ID NOs: 2 and 4.



35. The hybridoma or engineered cell of claim 33 or 34, wherein the antibody fragment is a recombinant ScFv (single chain fragment variable) antibody, Fab fragment, F(ab')<sub>2</sub> fragment, or Fv fragment.
36. The hybridoma or engineered cell of any one of claims 33-35, wherein:
- (a) said antibody is a chimeric antibody or a bispecific antibody; and/or
  - (b) said antibody is an IgG.
37. A pharmaceutical composition comprising a monoclonal antibody or fragment thereof according to any one of claims 30-32.
38. A chimeric antigen receptor (CAR) comprising:
- (a) an ectodomain comprising a single chain antibody variable region that binds selectively to a cancer cell-associated Glypican 2,
    - wherein said single chain antibody variable region comprises a heavy chain variable domain including a CDR1 comprising the amino acid sequence of SEQ ID NO: 5, a CDR2 comprising the amino acid sequence of SEQ ID NO: 6, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 7; and a light chain variable domain including a CDR1 comprising the amino acid sequence of SEQ ID NO: 8, a CDR2 comprising the amino acid sequence of SEQ ID NO: 9, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 10;
    - wherein the single chain antibody has a flexible hinge attached at the C-terminus of the single chain antibody variable region, and
    - wherein single chain antibody variable region is derived from a monoclonal antibody and said monoclonal antibody:
      - (i) is an IgG antibody;
      - (ii) inhibits cancer cell growth; and
      - (iii) induces cancer cell death,
  - (b) a transmembrane domain; and
  - (c) an endodomain;
    - wherein the endodomain comprises a signal transduction function when the single chain antibody variable region is engaged with cancer cell-associated Glypican 2; and

wherein the single chain antibody comprises a C-terminus of a light chain variable domain fused to an N-terminus of a heavy chain variable domain by a flexible linker.

39. The CAR of claim 38, wherein the light chain variable domain comprises the amino acid sequence of SEQ ID NO: 4, and the heavy chain variable domain comprises the amino acid sequence of SEQ ID NO: 2.
40. The CAR of claim 38 or 39, wherein the flexible linker is selected from the group consisting of a peptide linker, a non-peptide linker, a chemical unit, a hindered cross-linker, and a non-hindered cross-linker.
41. The CAR of any one of claims 38-40, wherein the linker is a peptide linker, and optionally, wherein the peptide linker is at least 15 amino acids in length.
42. The CAR of claim 41, wherein the peptide linker is a glycine-serine linker.
43. The CAR of claim 38, wherein the transmembrane domain comprises a CD28 domain.
44. The CAR of claim 38, where the endodomain comprises a CD3-zeta domain or a high affinity FcεRI.
45. The CAR of claim 44, wherein the endodomain further comprises an intracellular domain of a costimulatory protein selected from the group consisting of CD28, 41BB, OX40, and ICOS.
46. The CAR of claim 38, wherein the flexible hinge is from CD8a or an immunoglobulin (Ig).
47. Use of the fusion protein of any one of claims 20-22 in the manufacture of a medicament for the treatment of a GPC2-positive cancer.
48. Use of the CAR of any one of claims 23-26 or 38-46 in the manufacture of a medicament for the treatment of a GPC2-positive cancer.
49. Use of the monoclonal antibody of any one of claims 30-32 in the manufacture of a medicament for the treatment of a GPC2-positive cancer.

50. The use of any one of claims 47-49, wherein the cancer is lung cancer, brain cancer, head & neck cancer, breast cancer, skin cancer, liver cancer, pancreatic cancer, stomach cancer, colon cancer, kidney cancer, rectal cancer, uterine cancer, cervical cancer, ovarian cancer, testicular cancer, skin cancer, or esophageal cancer.

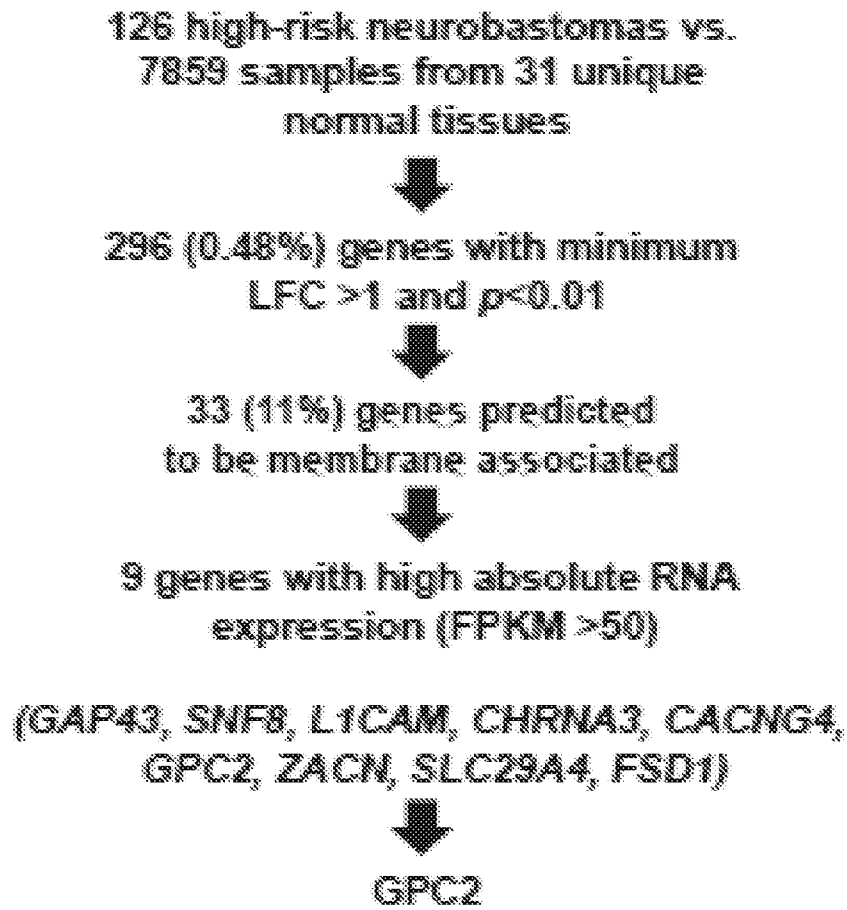


FIG. 1

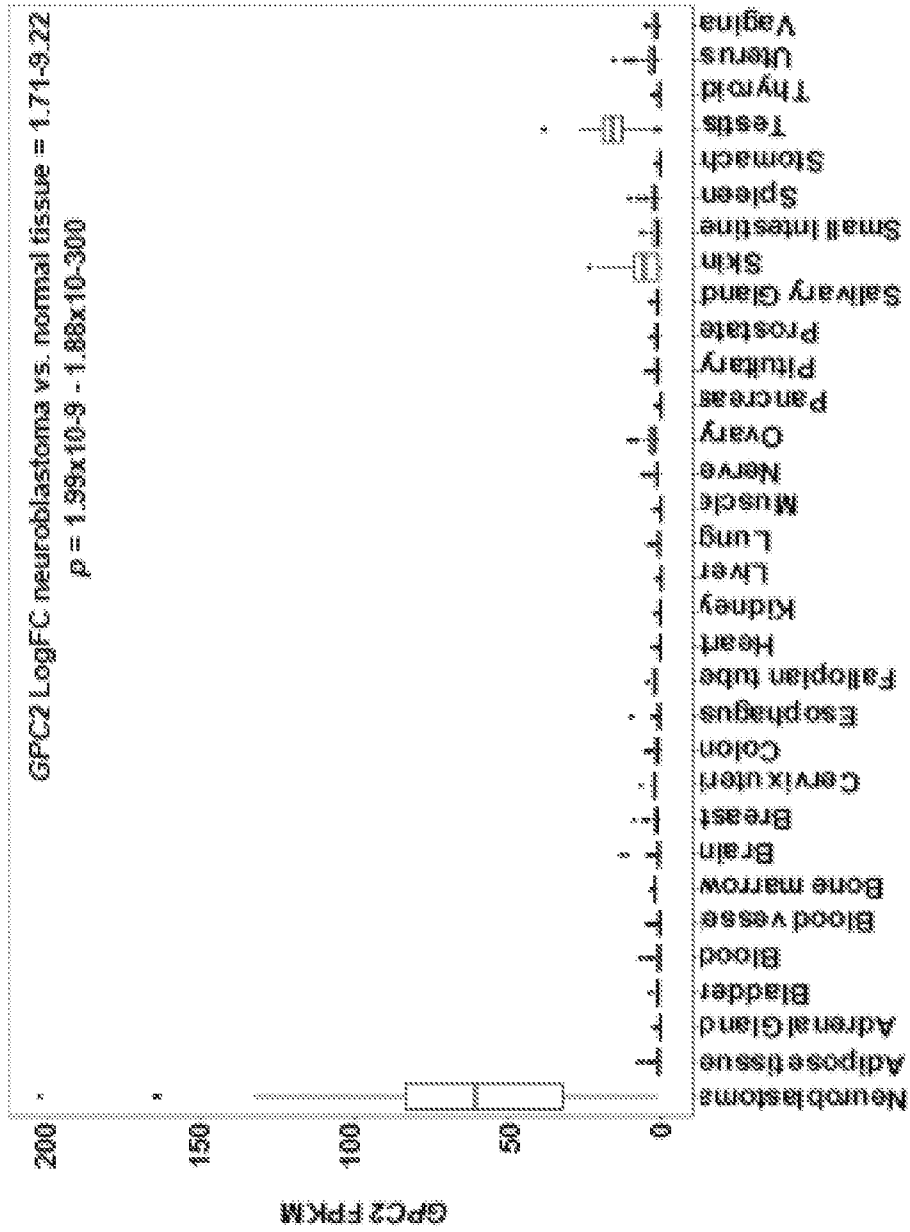


FIG. 2

- Glypicans (1 - 6) are cell surface signalling co-receptors
- GPC2 binds midkine in neuroblastoma - integral in cell migration
- GPC2 on chromosome 7q
- 7q locus gained in ~1/3 of primary neuroblastomas, associated with increased GPC2 expression
- Other glypicans are validated immunotherapy targets/oncogenes

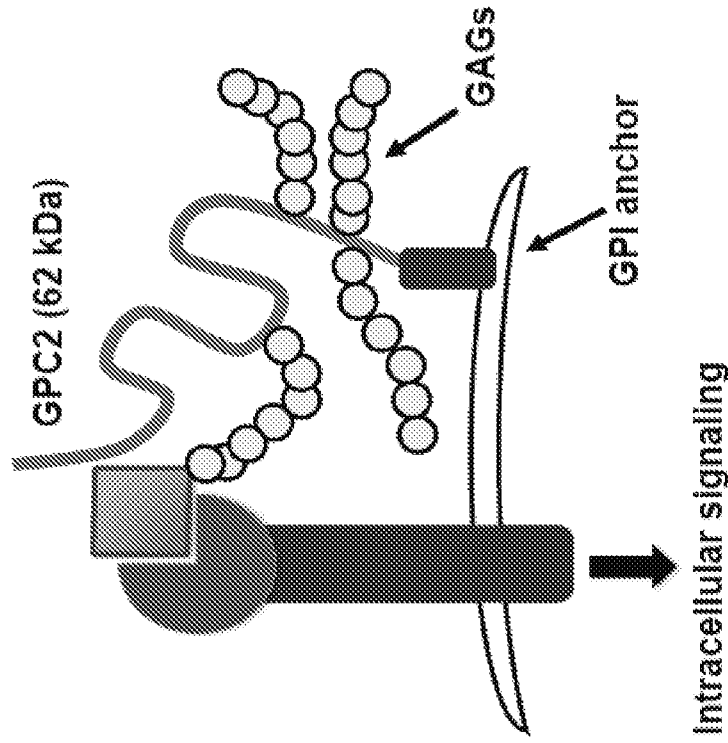
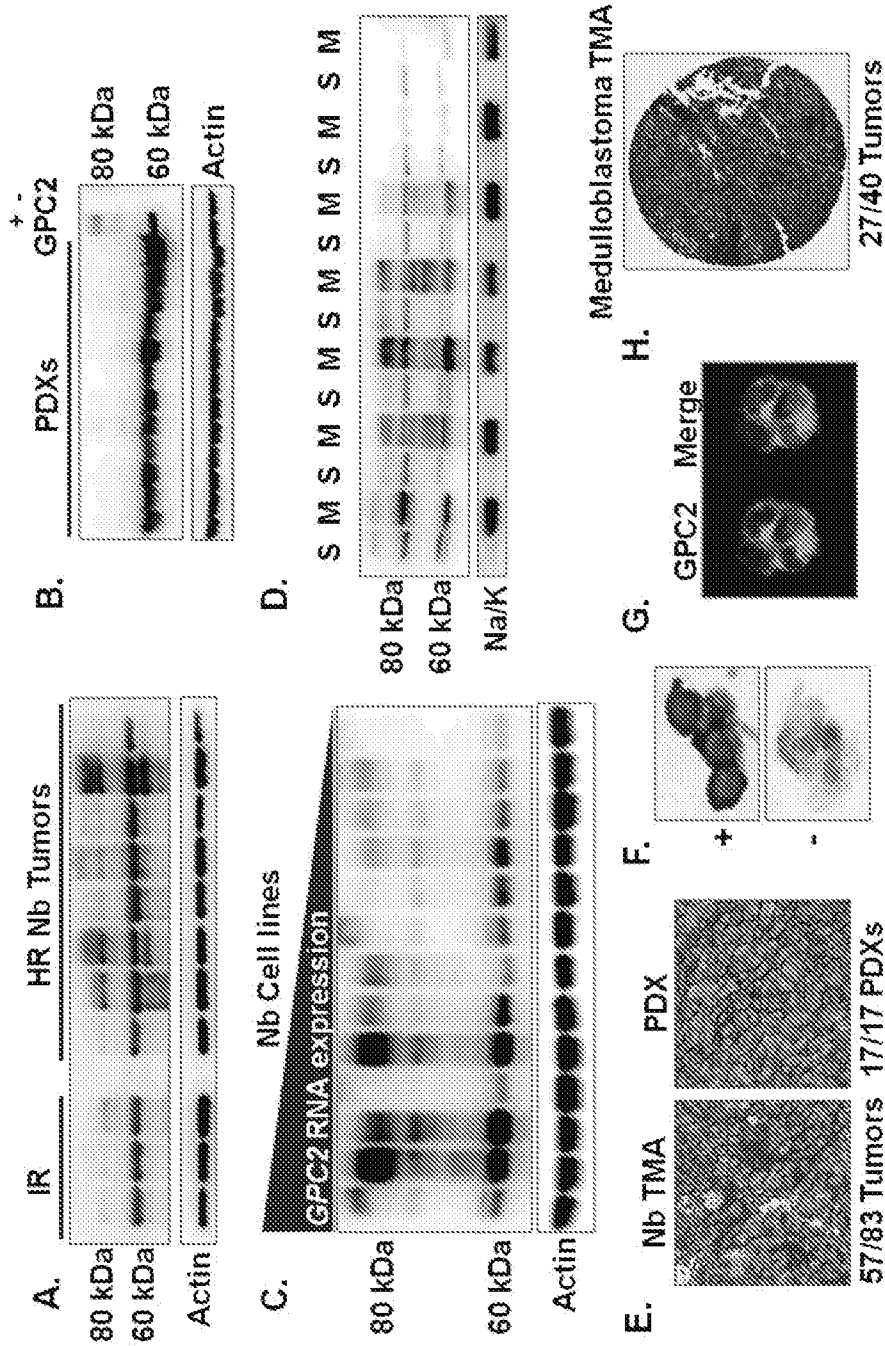
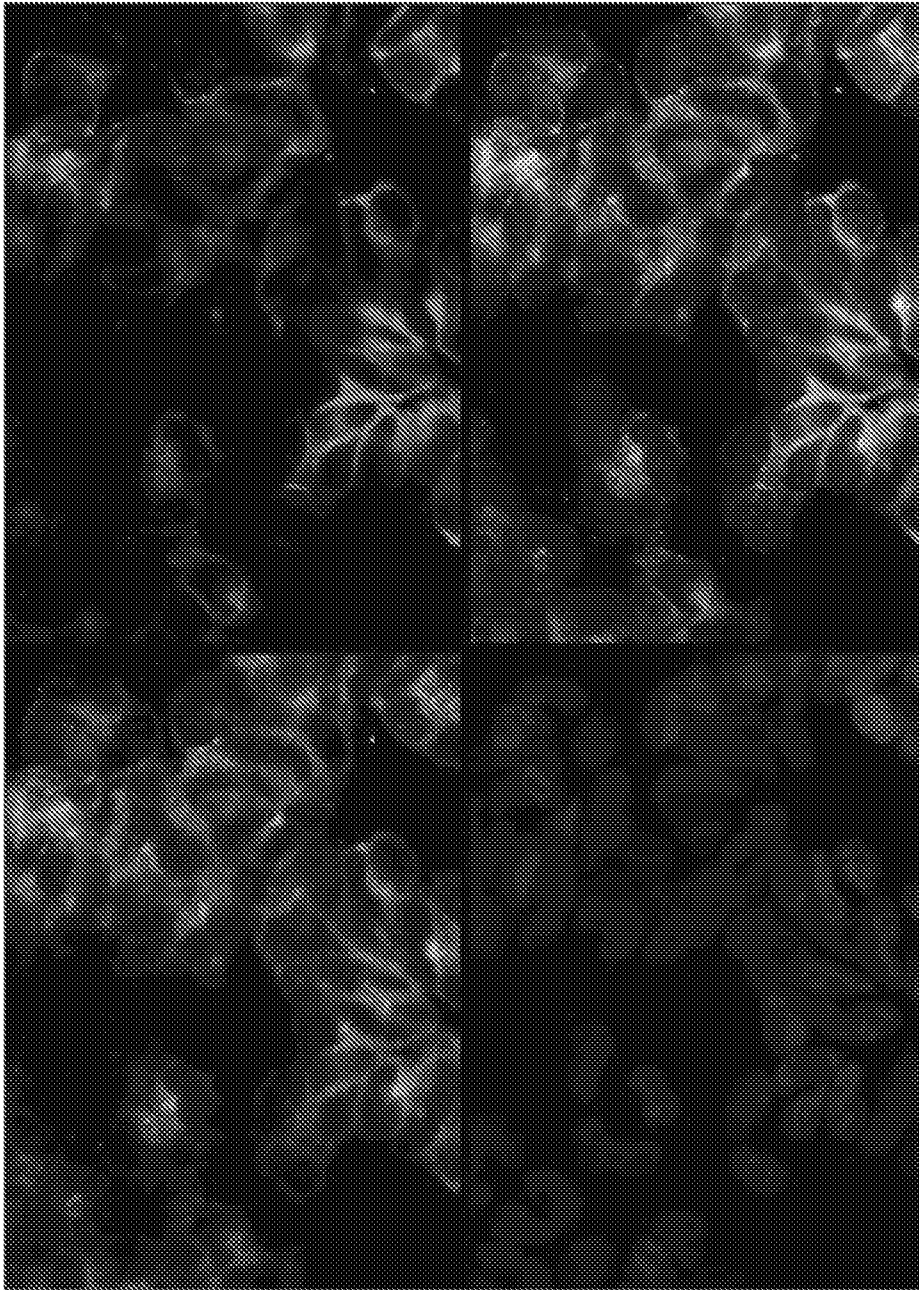


FIG. 3



FIGS. 4A-H



Green- GPC2, Red- Cadherin, Blue- Hoechst

FIG. 5



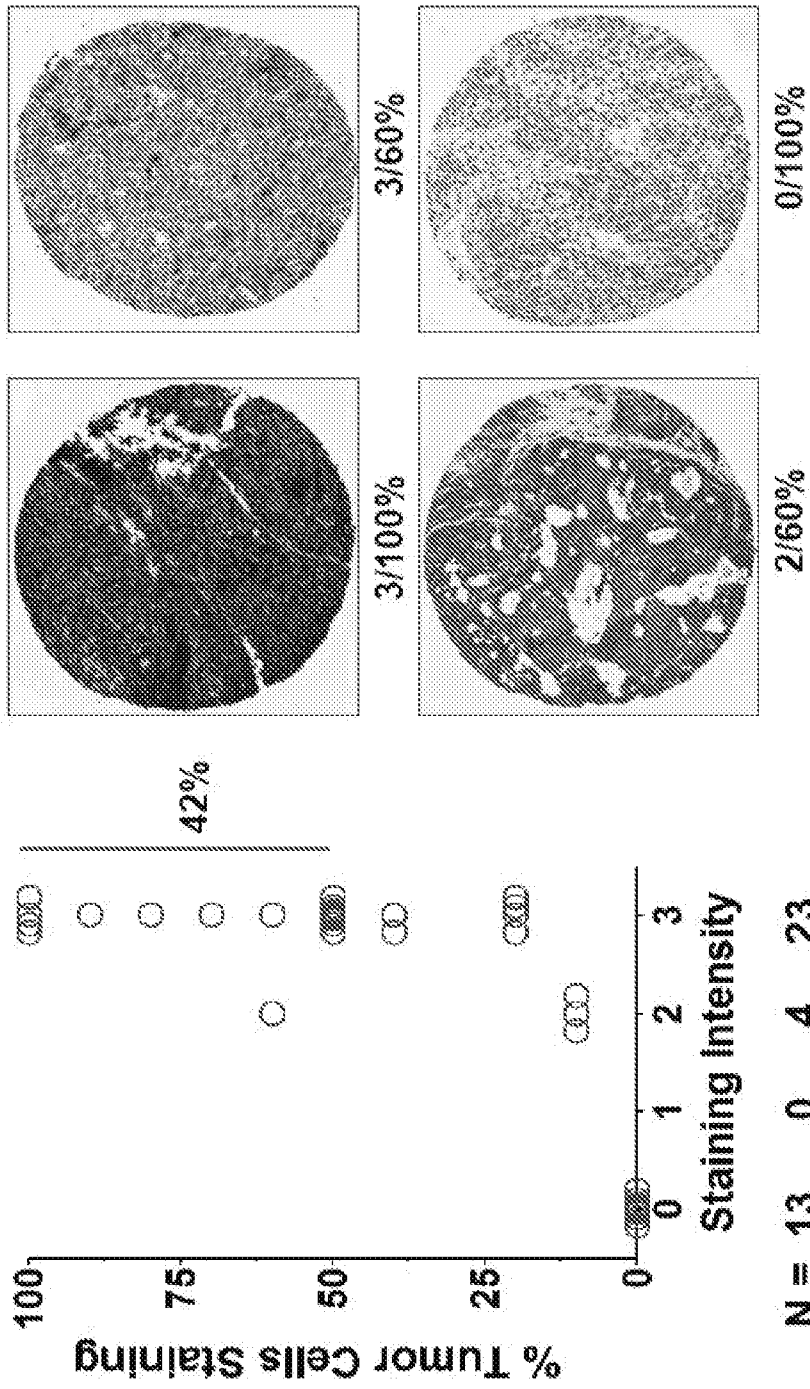


FIG. 6

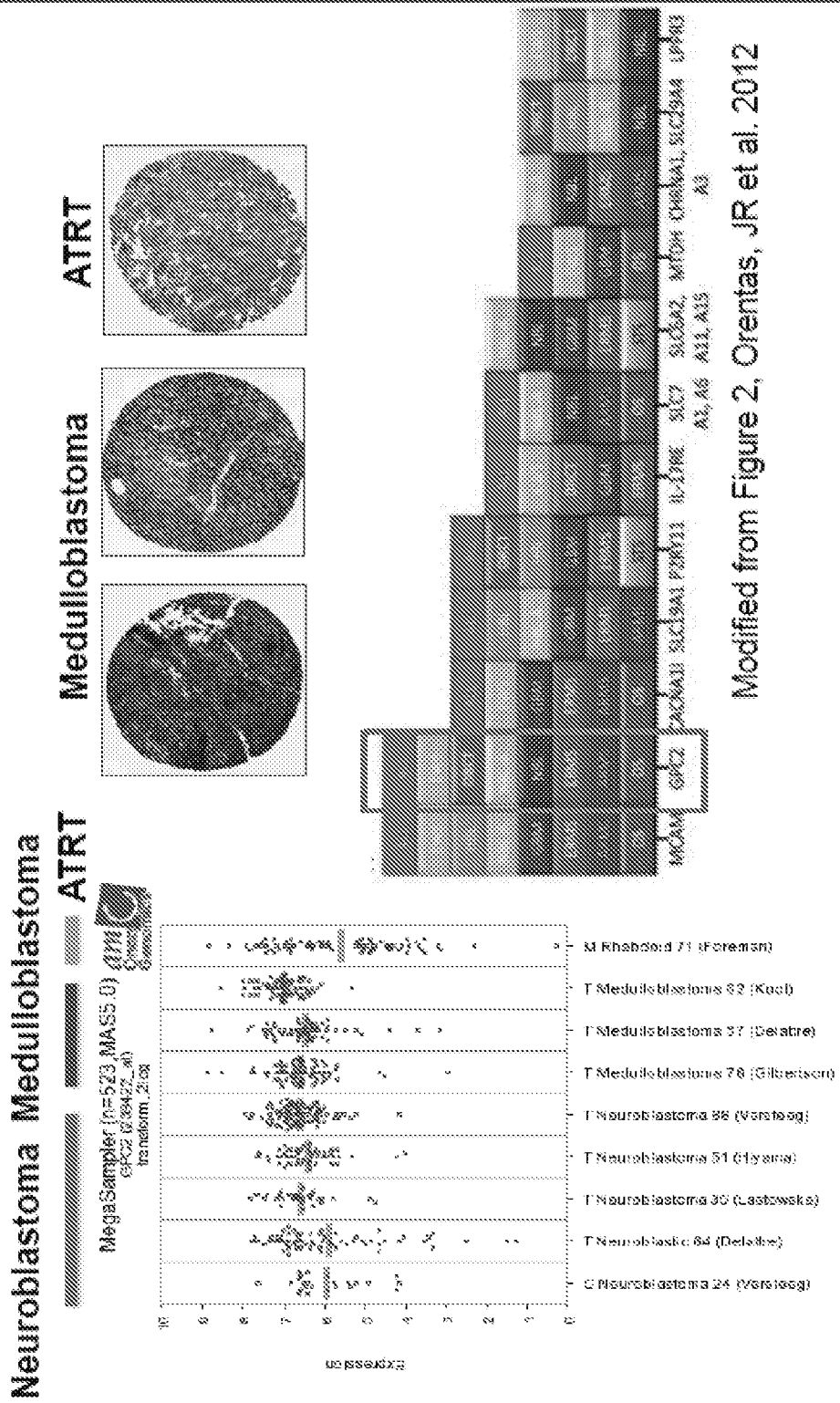
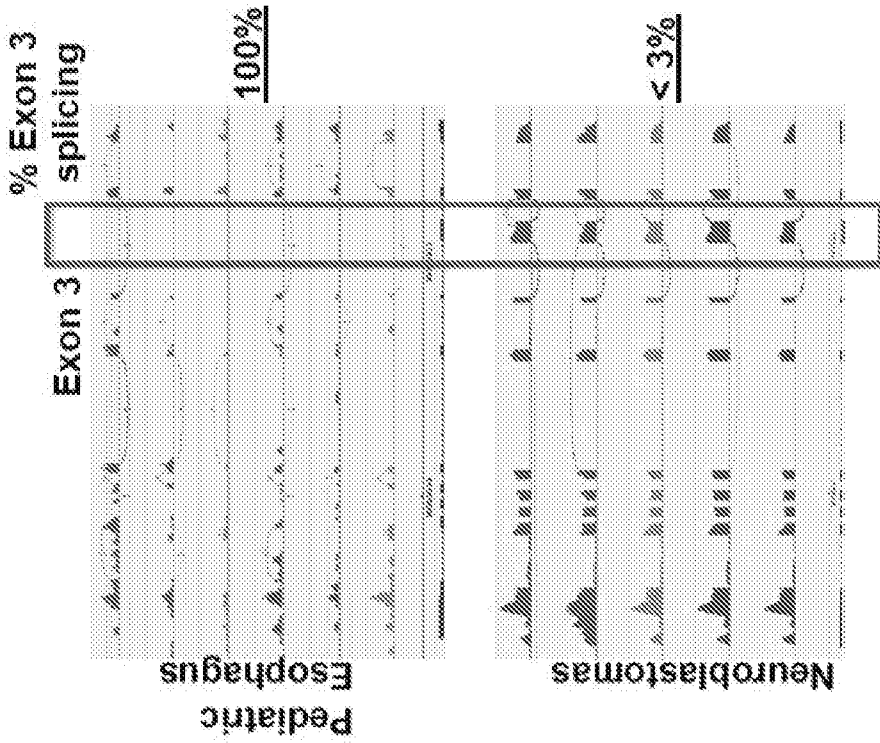


FIG. 7



Normal Tissue	Core 1	Core 2	Membrane Staining
aorta, appendix, bladder, brain cerebellum, bone marrow, breast, colon, choroid plexus, heart, gallbladder, fat, fallopian tube, ileocecum, kidney, pancreas, ovary, lymph node, lung, parathyroid, parotid, skeletal muscle, prostate, small intestine, spinal cord, tonsil, thymus, ureter	no significant staining	no significant staining	n/a
	<u>28 Tissues</u>	<u>28 Tissues</u>	
Adrenal gland	80% 2	80% 2	0
Brain cortex	10% 2	10% 2	0
Esophagus	80% 2	80% 2	Yes
Liver	100% 2	100% 2	0
Pituitary gland	50% 2	75% 2	0
Skin	10% 2	10% 2	0
Stomach	90% 2	80% 2	0
Thyroid	50% 3	75% 3	0
Testis	30% 2	30% 2	0

2 = Weak, 3 = Intense

FIG. 8A

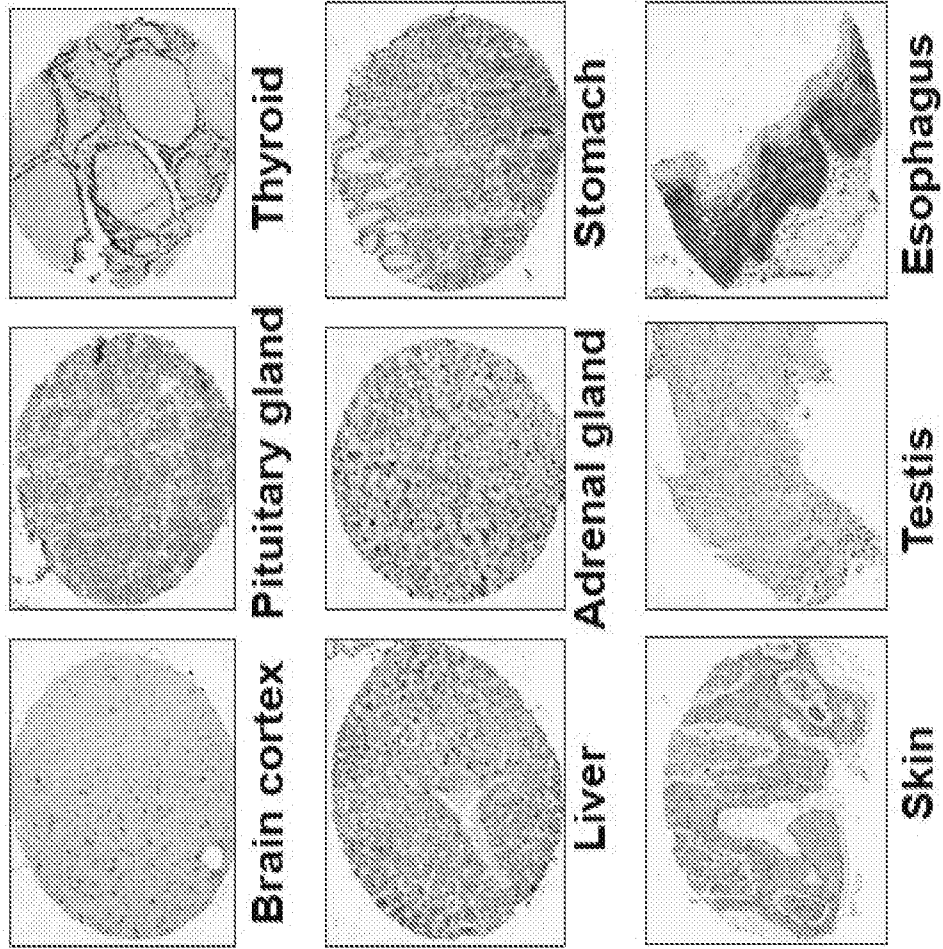
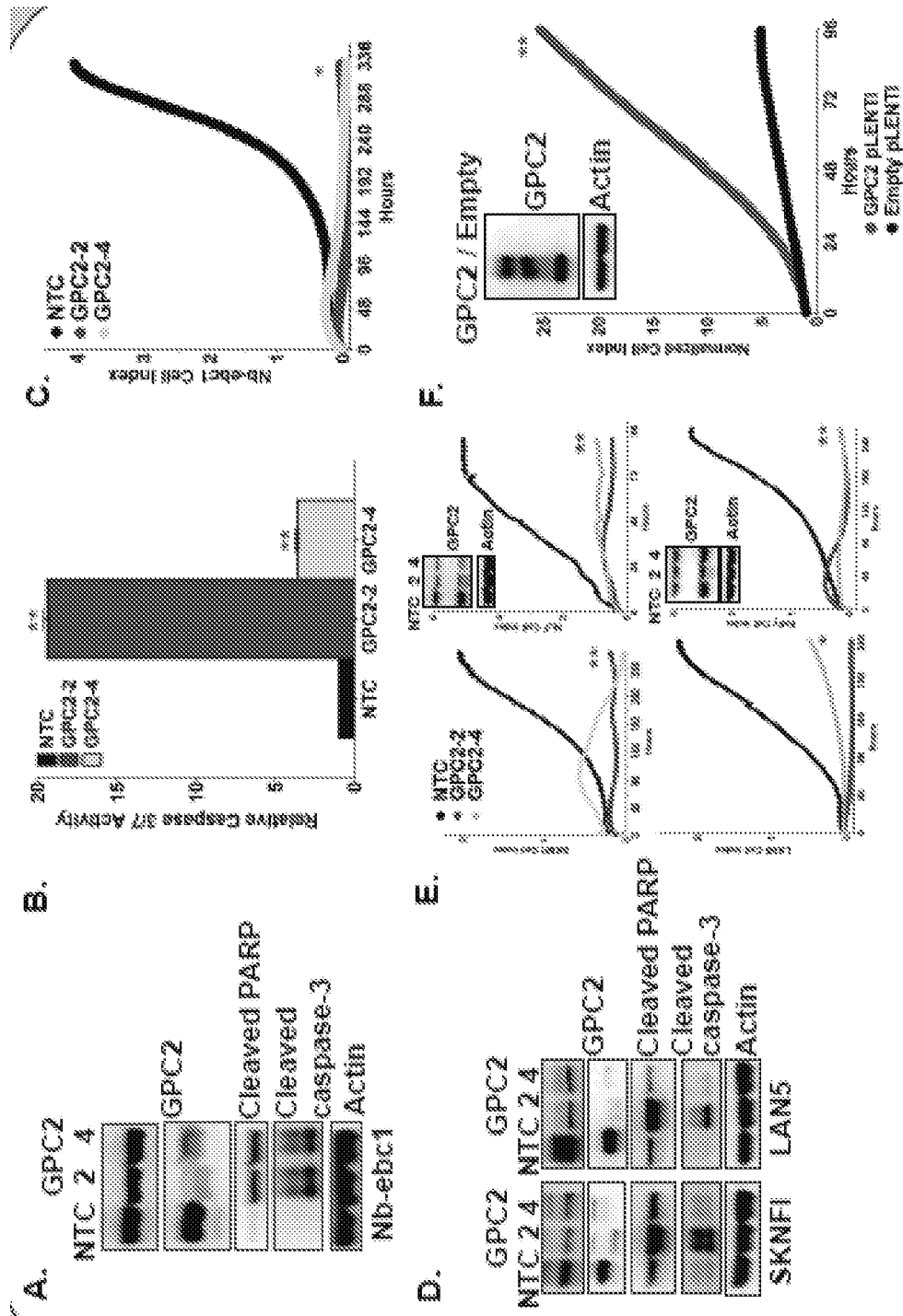


FIG. 8B



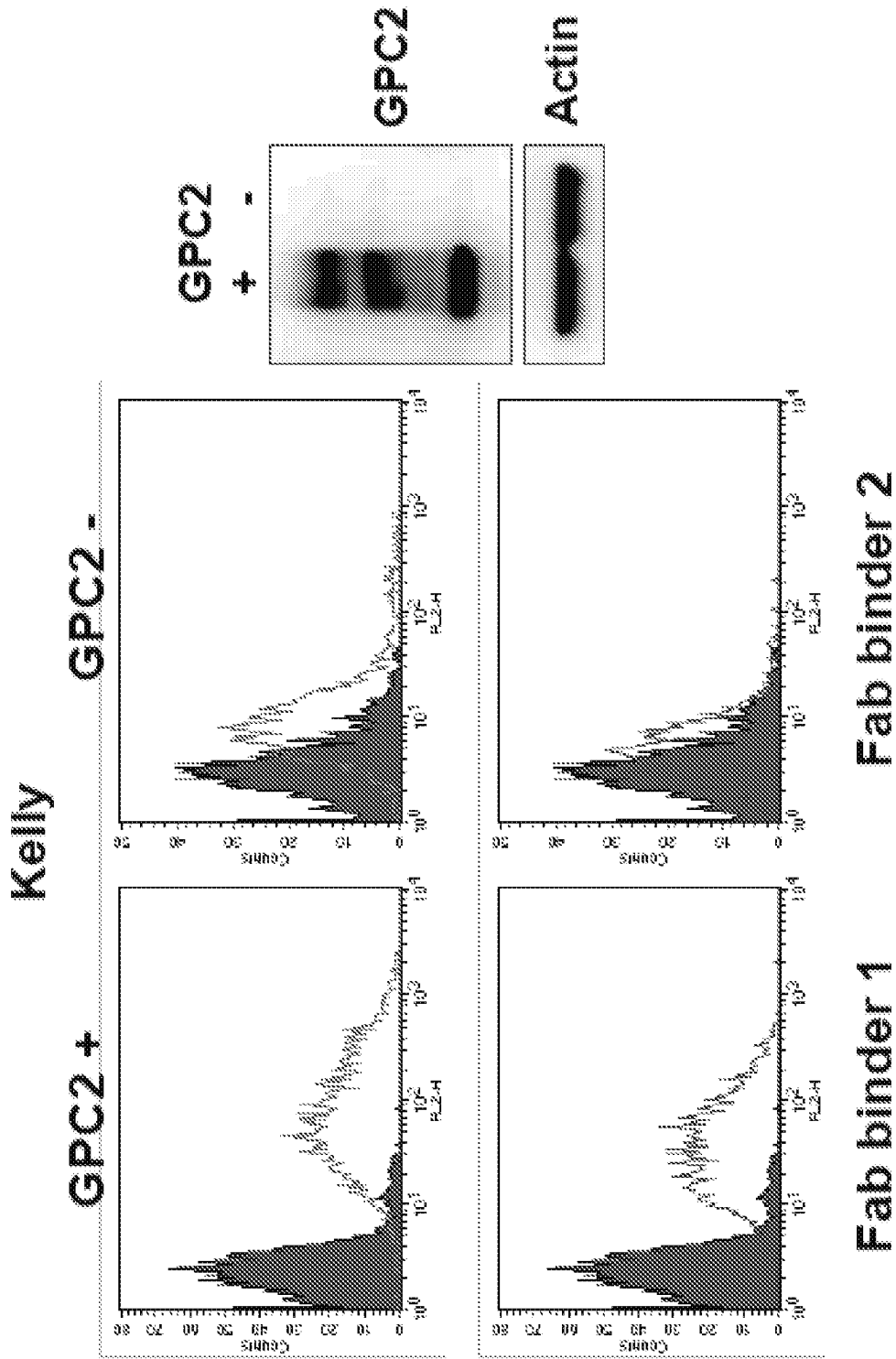


FIG. 10A

FAB Binder 3

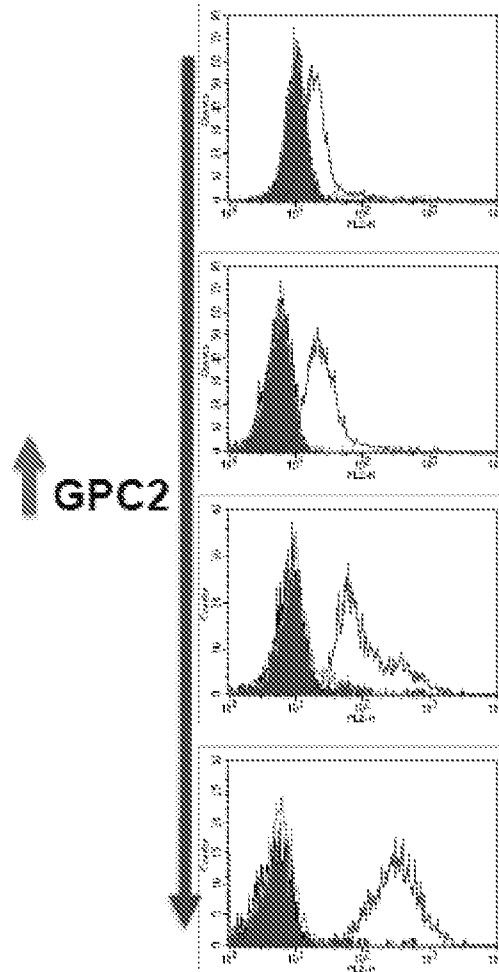
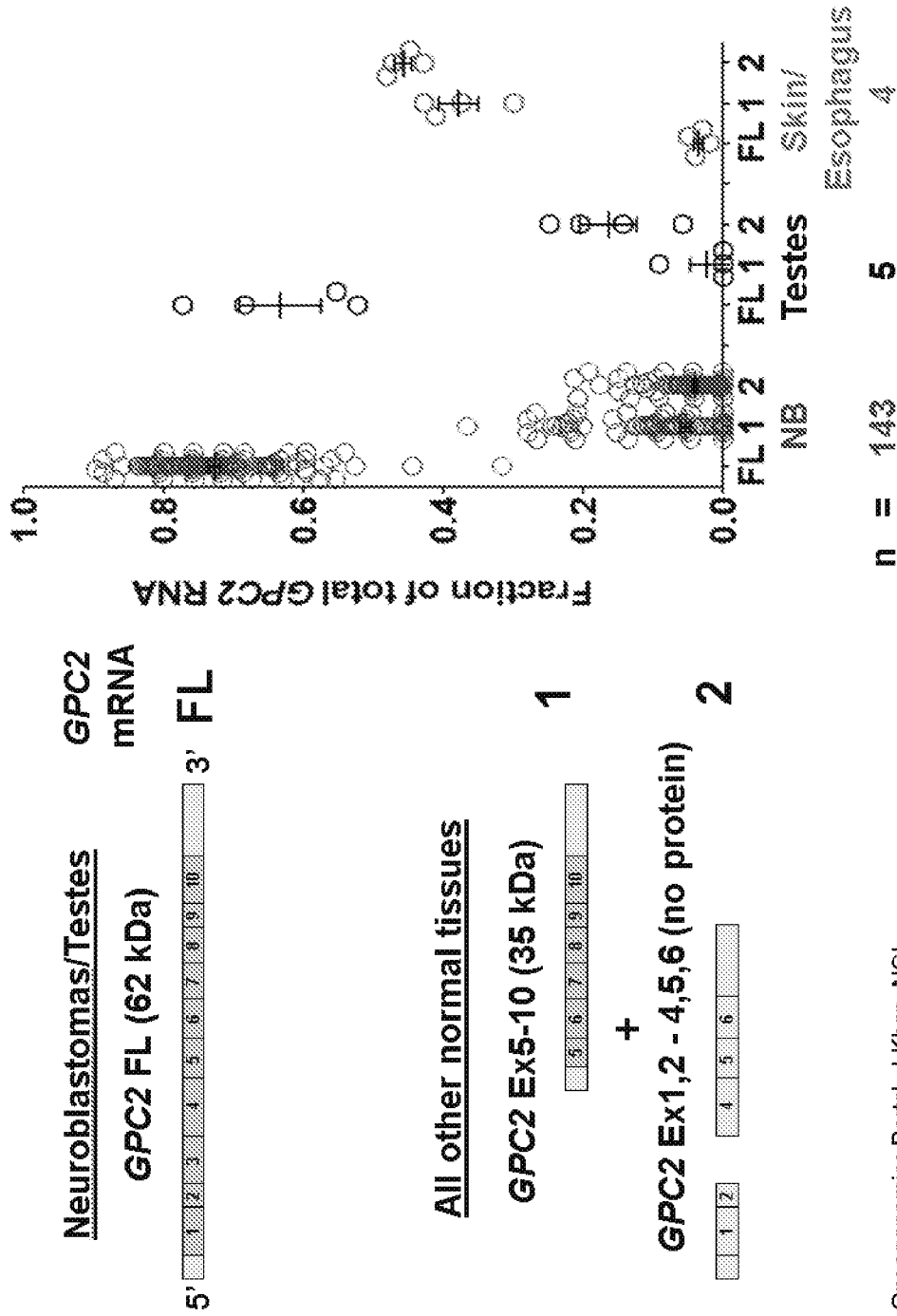


FIG. 10B



Oncogenomics Portal, J.Khan, NCI

FIG. 11



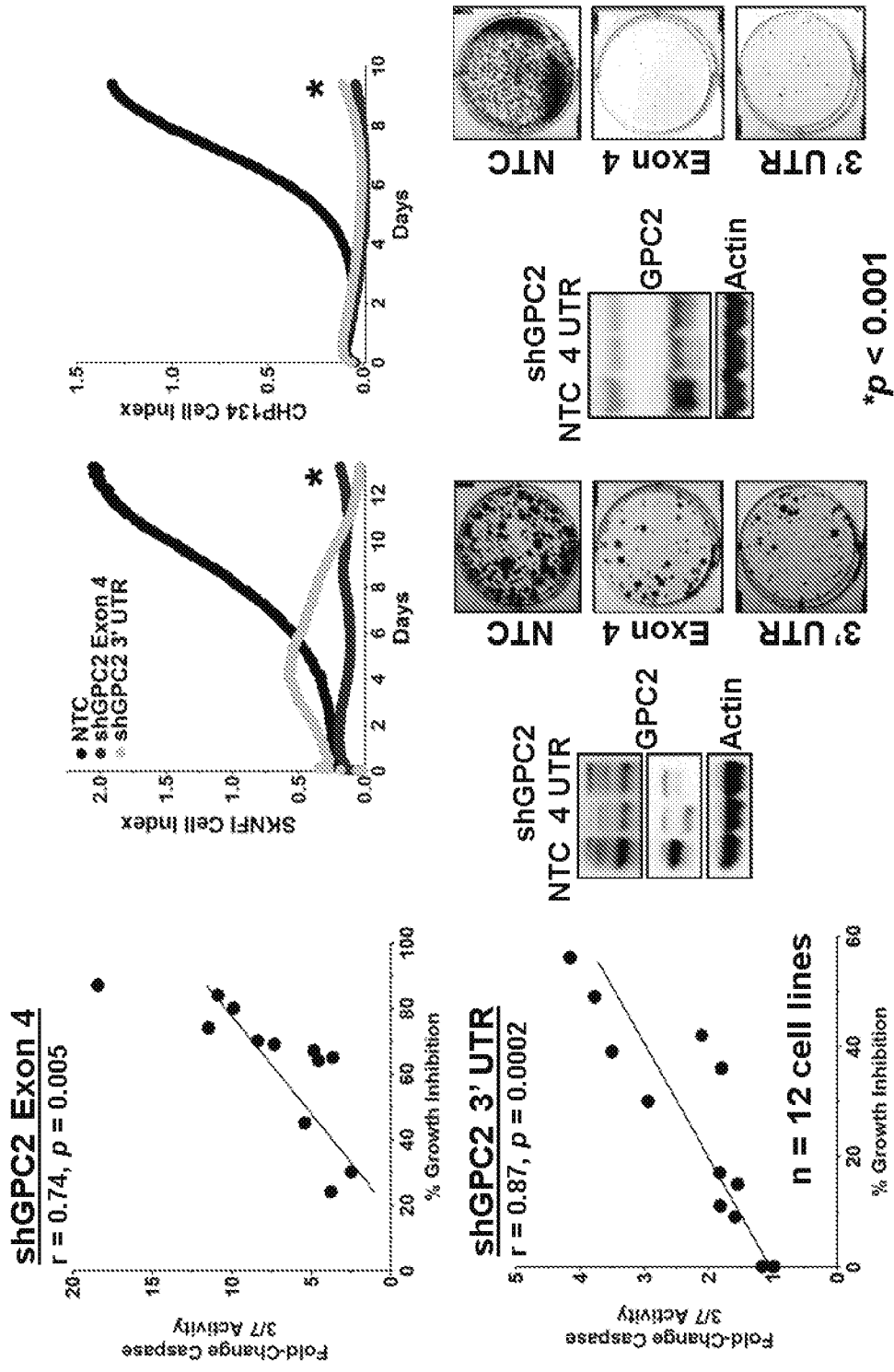
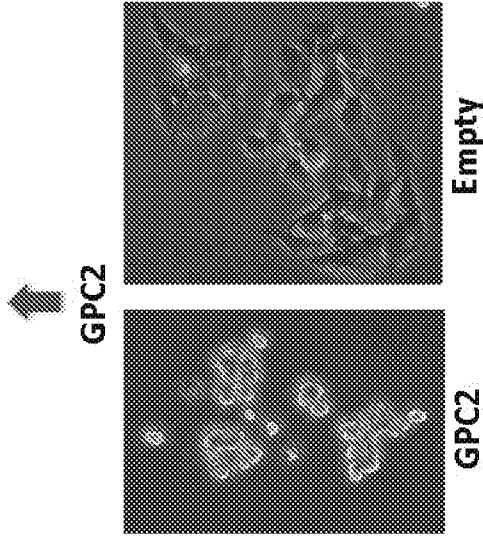
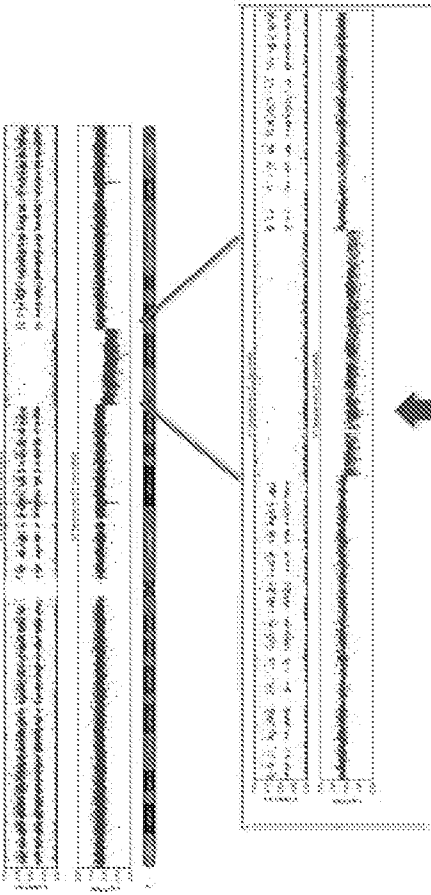


FIG. 12

### Kelly has a chromosome 7q/GPC2 deletion



\*  $p < 0.001$

### Kelly GPC2 / Empty

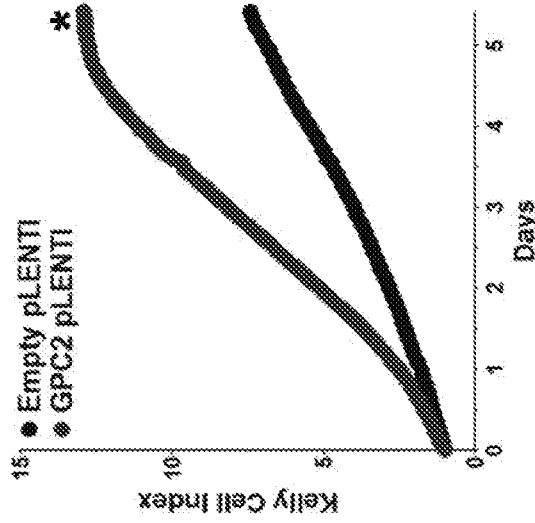
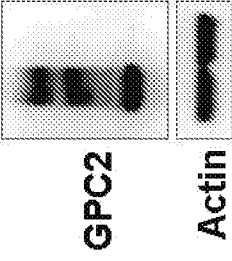
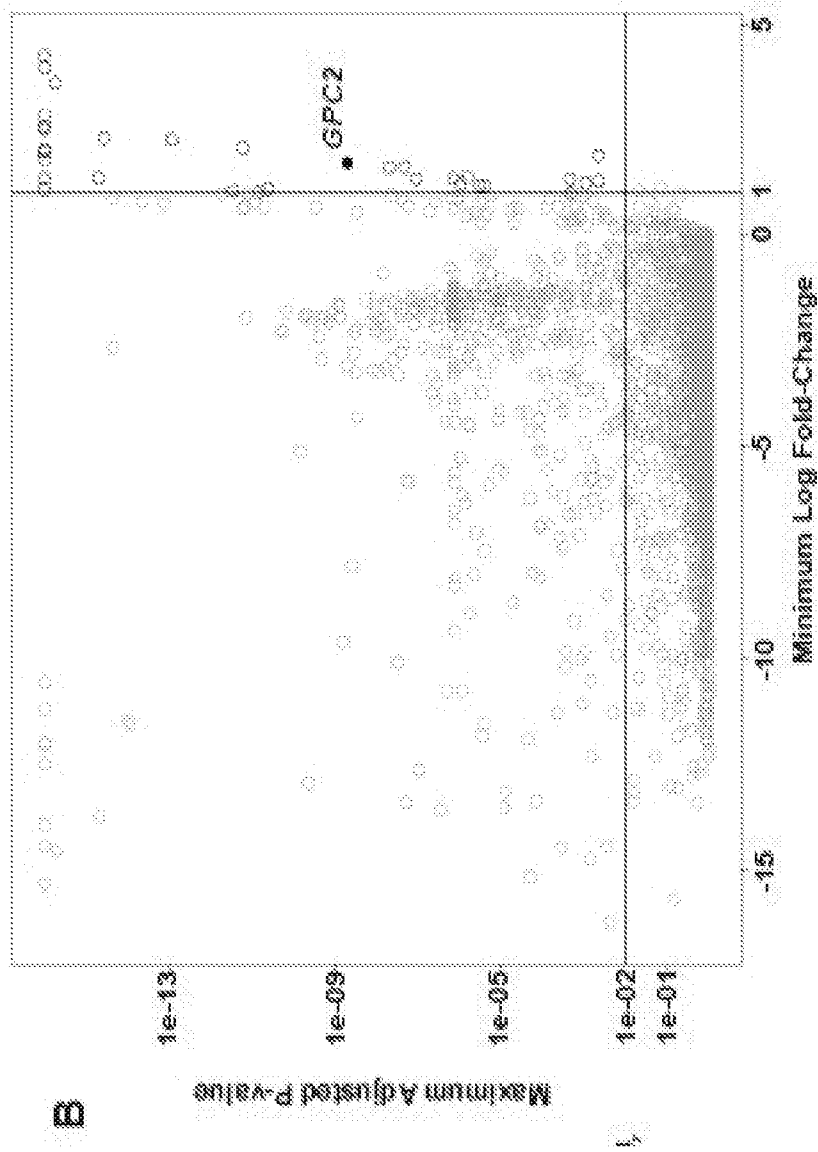
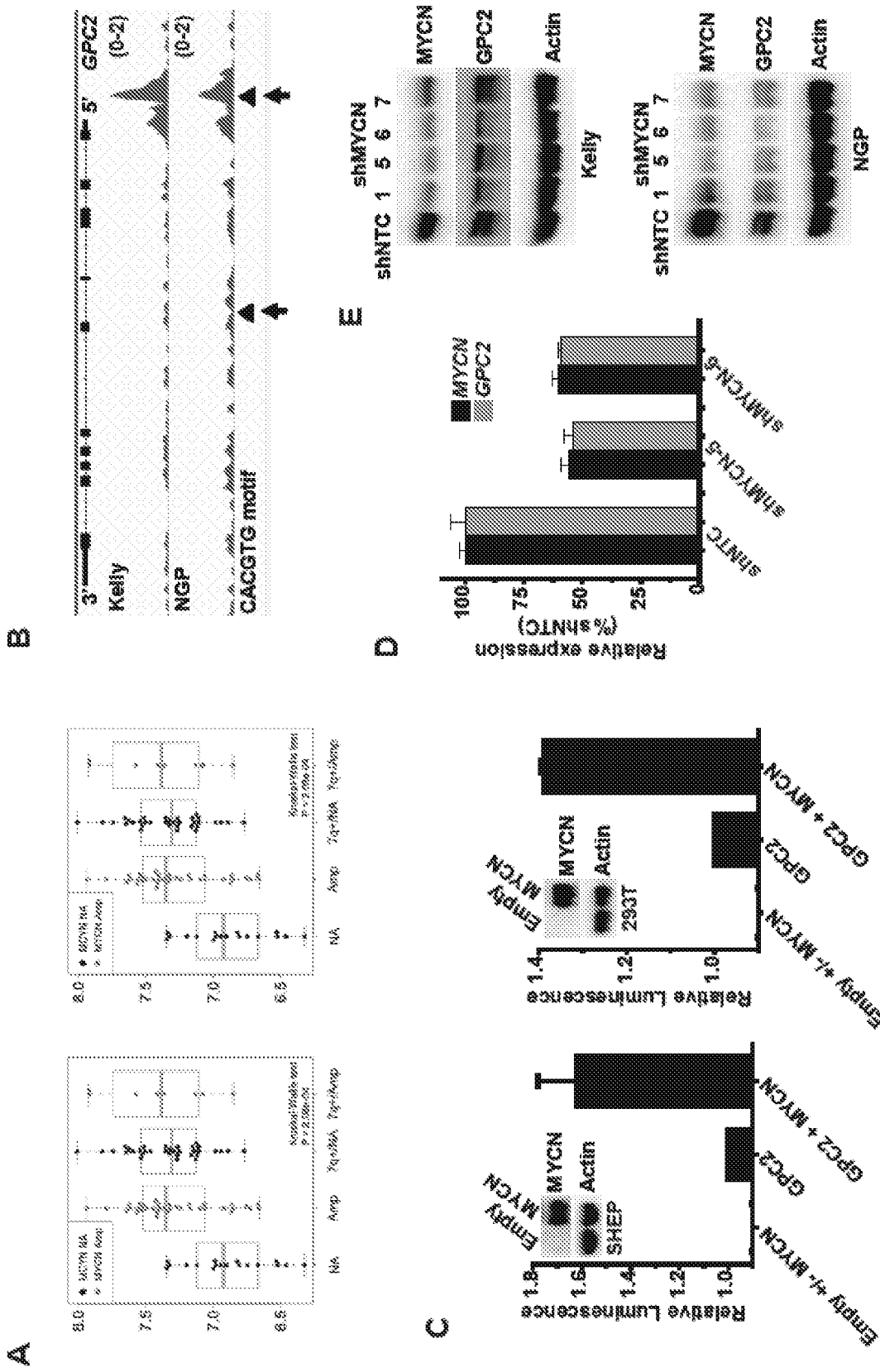


FIG. 13

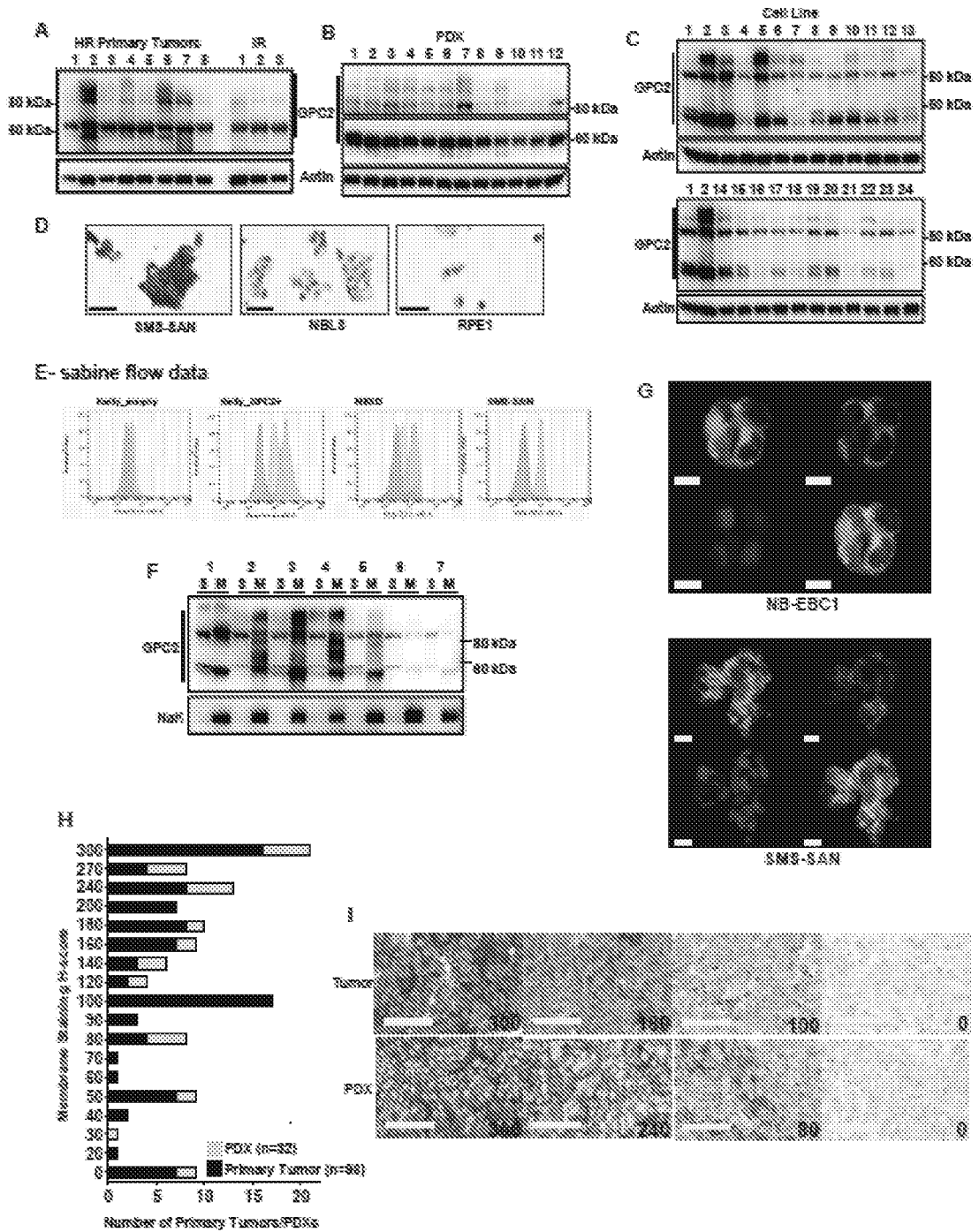


GPC2 LogFC neuroblastoma vs. normal tissue = 1.71-9.22  
 $p = 1.99 \times 10^{-9} - 1.88 \times 10^{-300}$

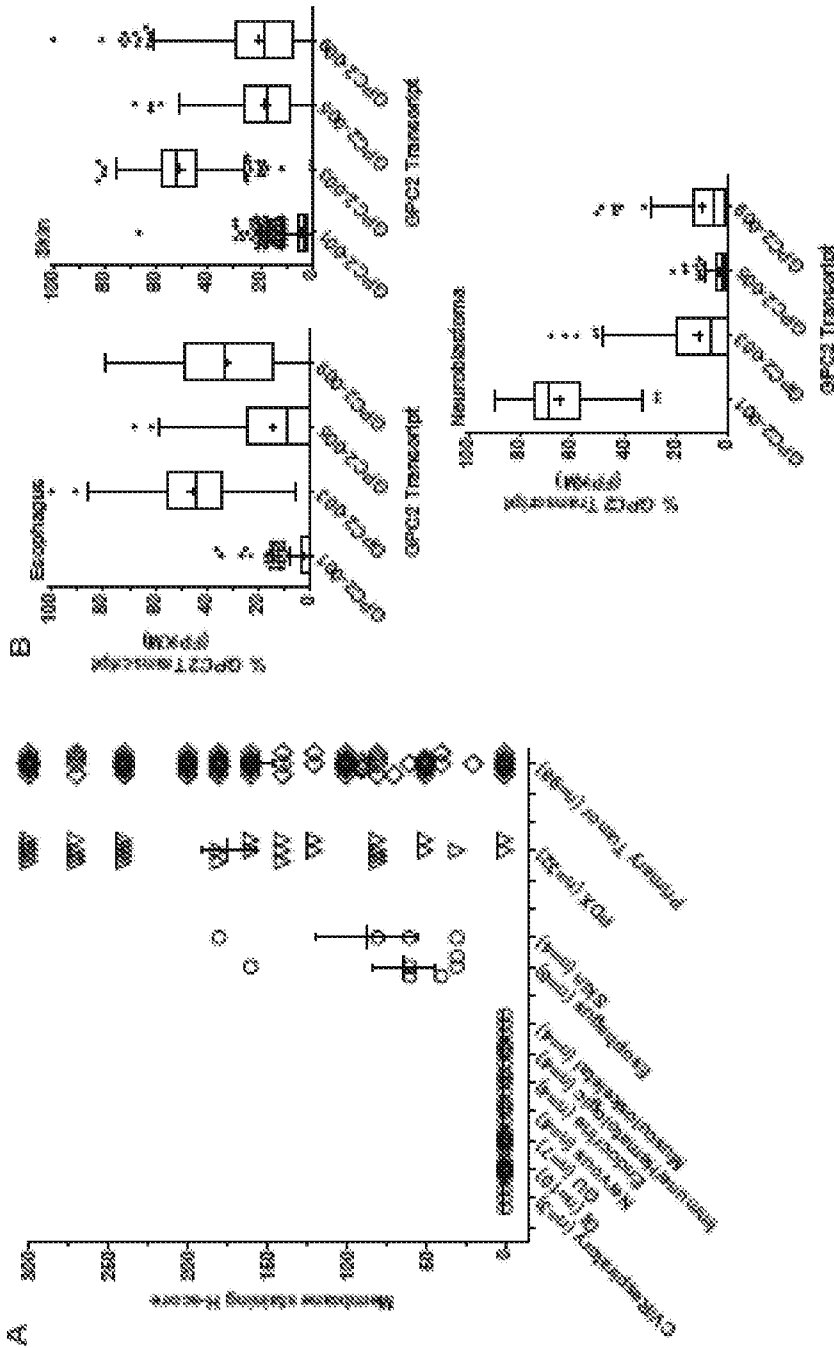
FIG. 14



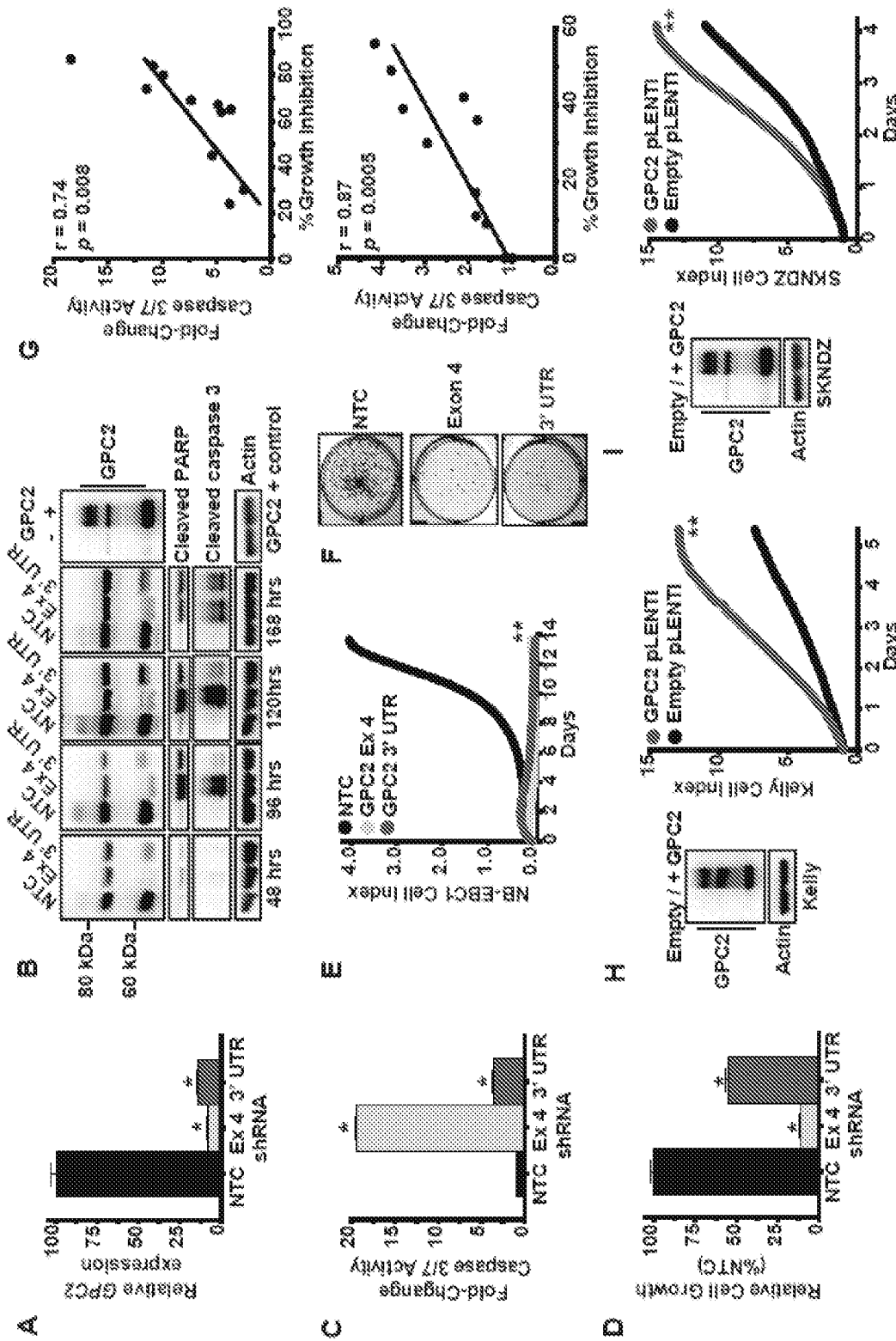
FIGS. 15A-E



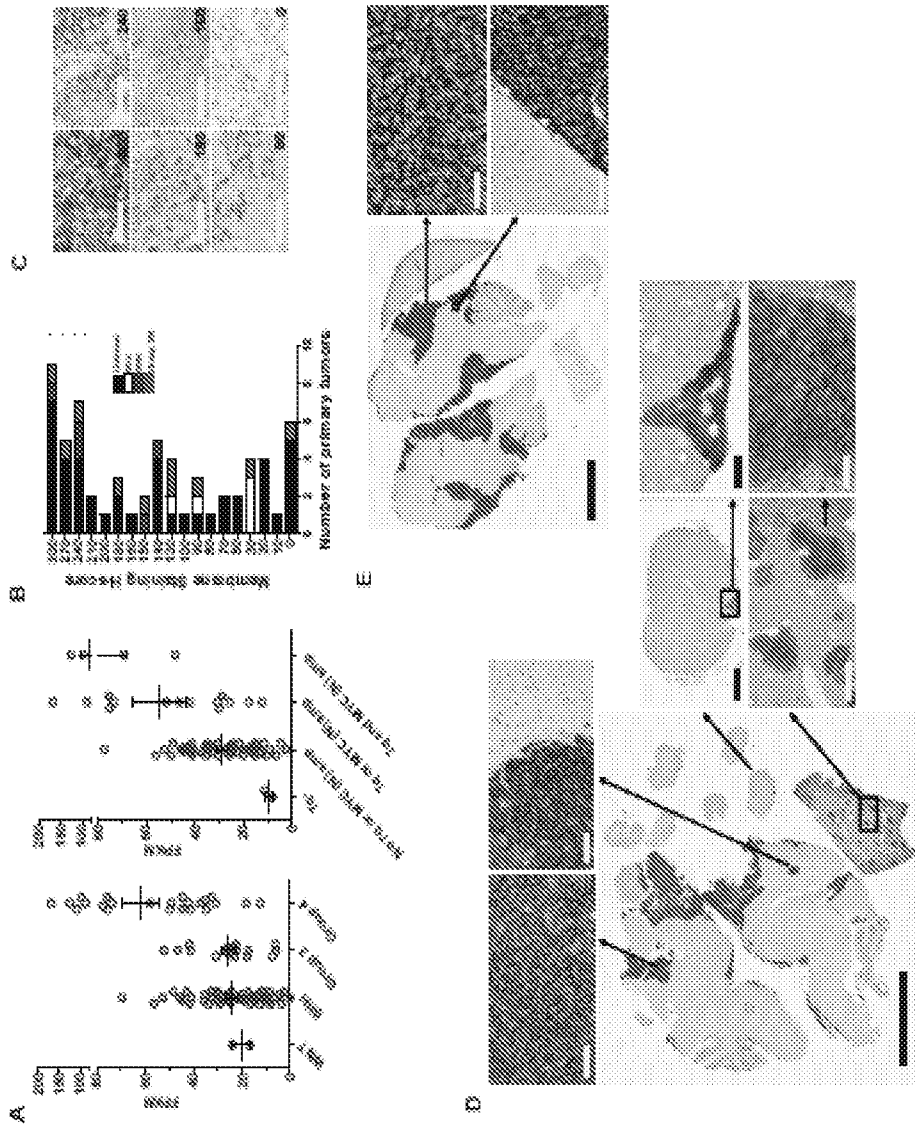
FIGS. 16A-I



FIGS. 17A-B



FIGS. 18A-I



FIGS. 19A-E



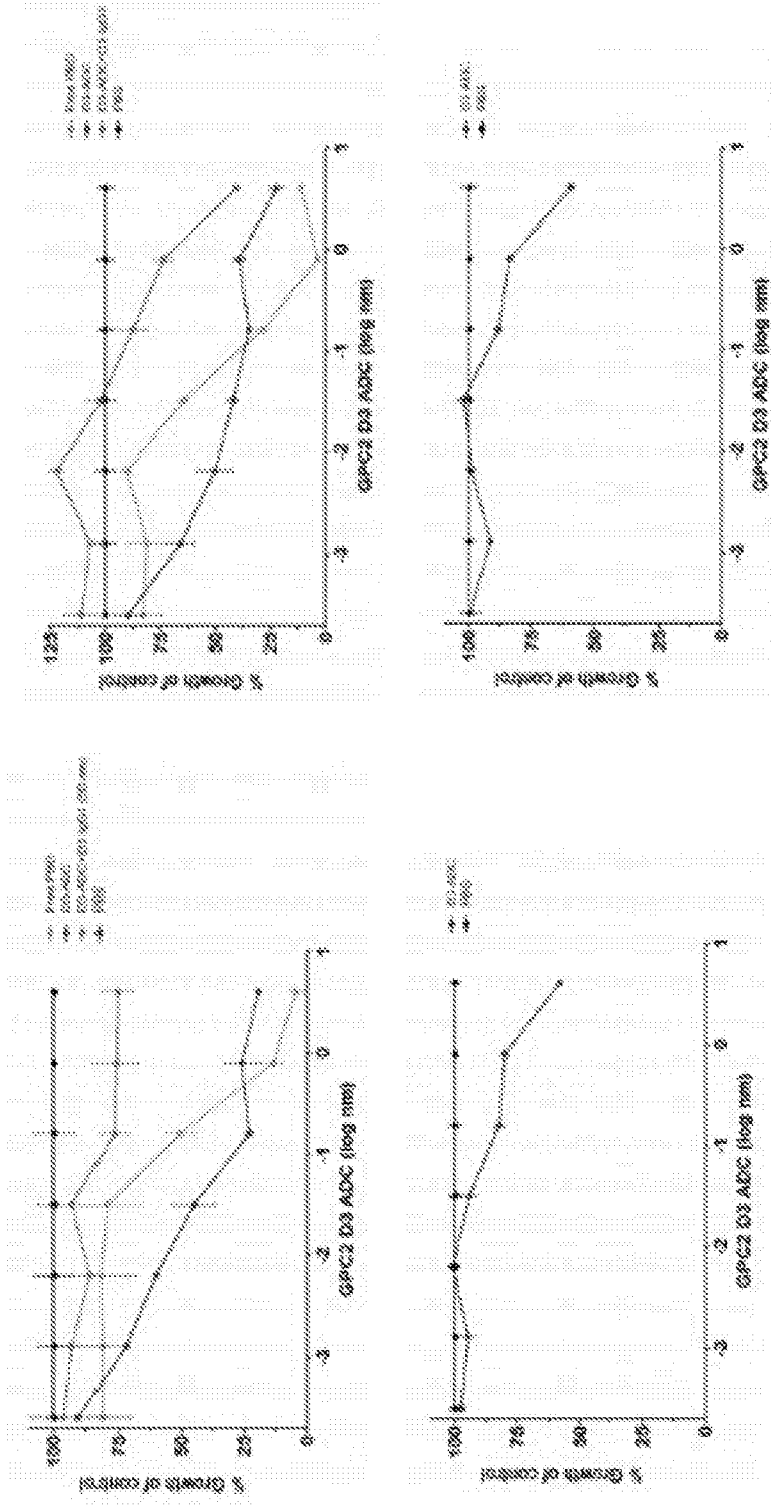


FIG. 20

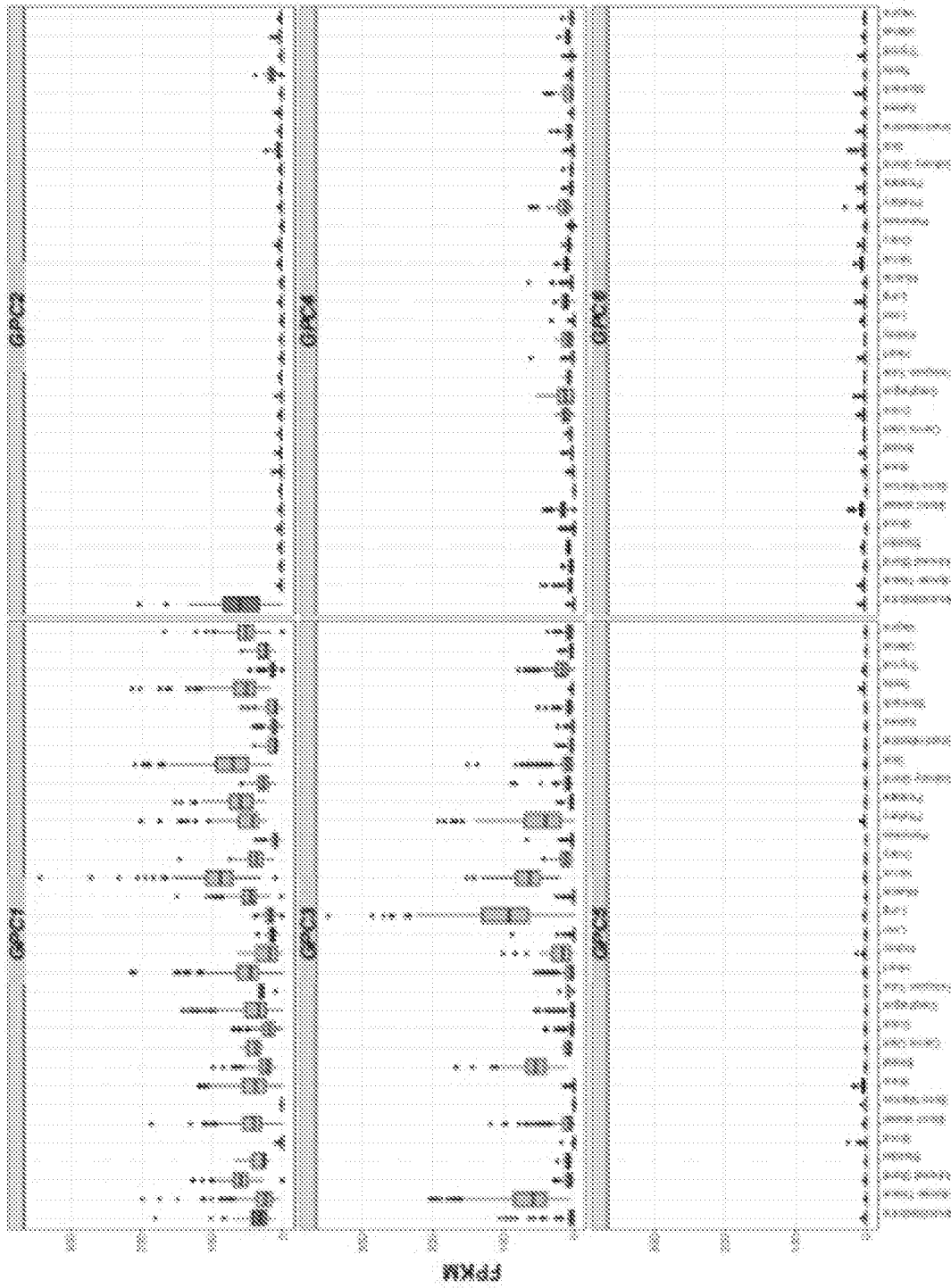
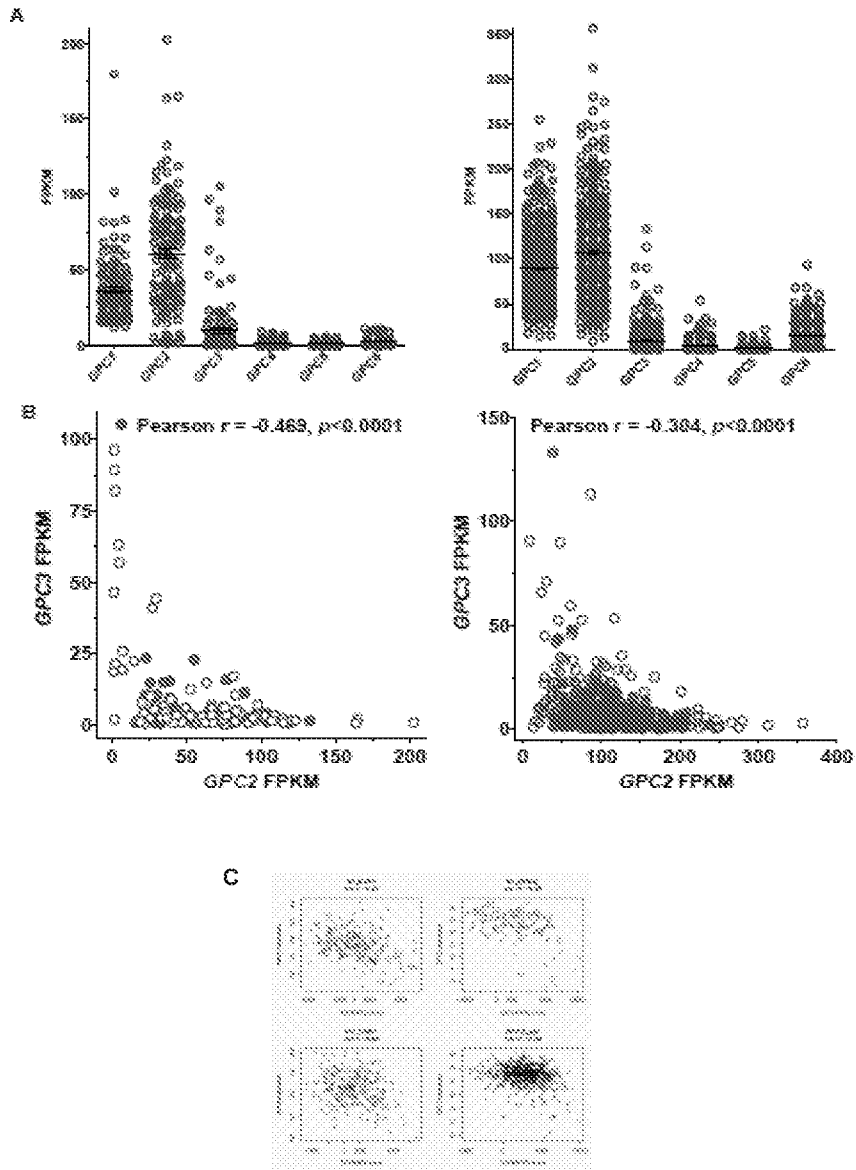
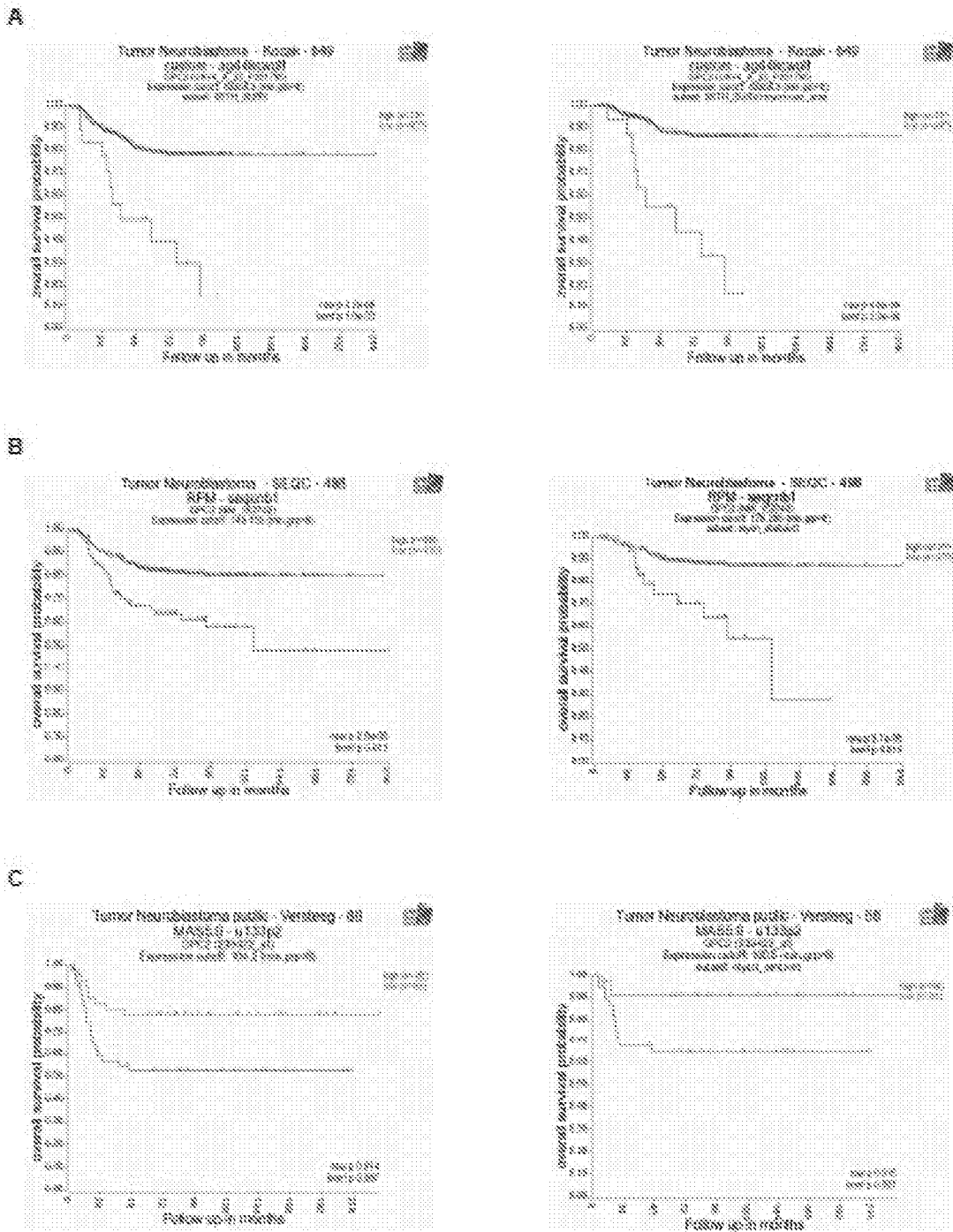


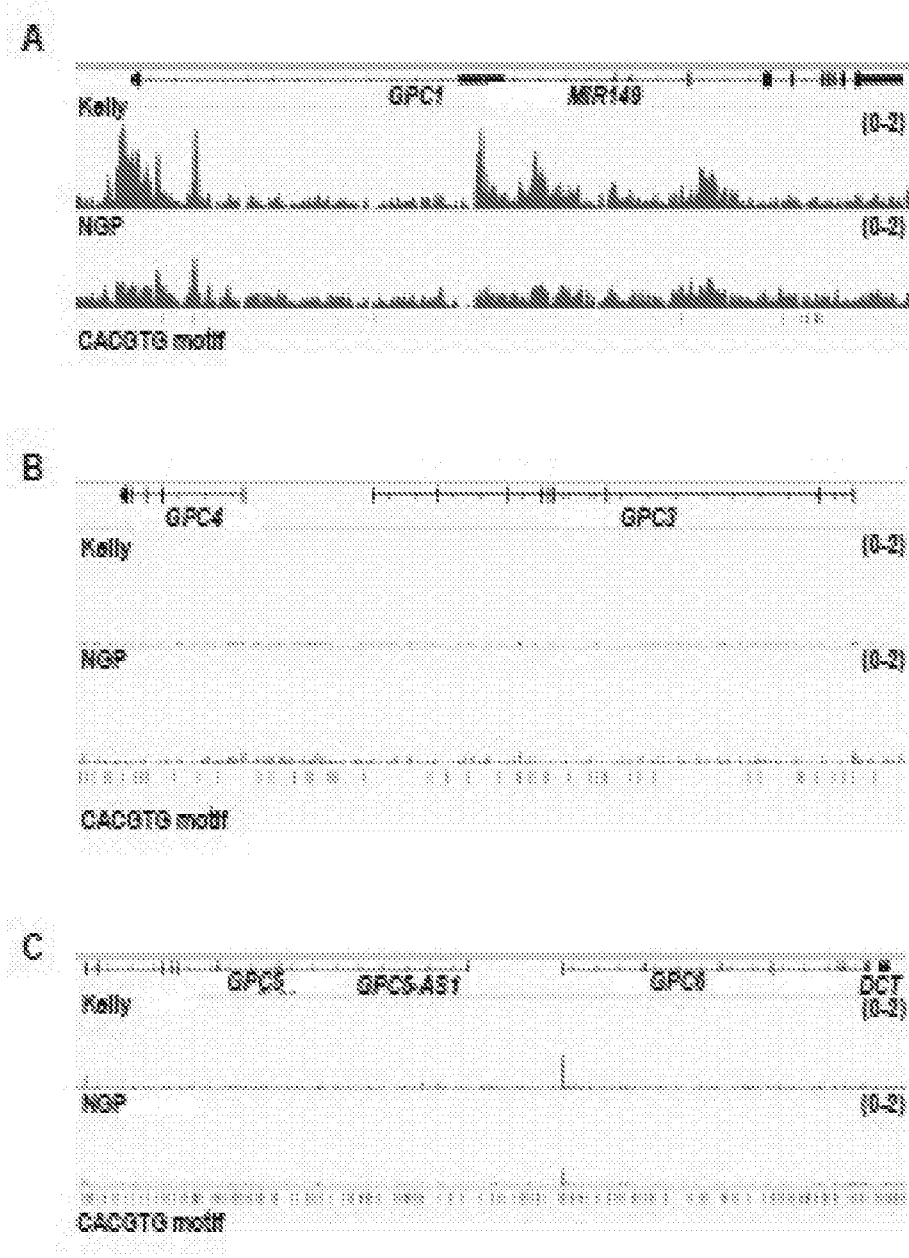
FIG. 21



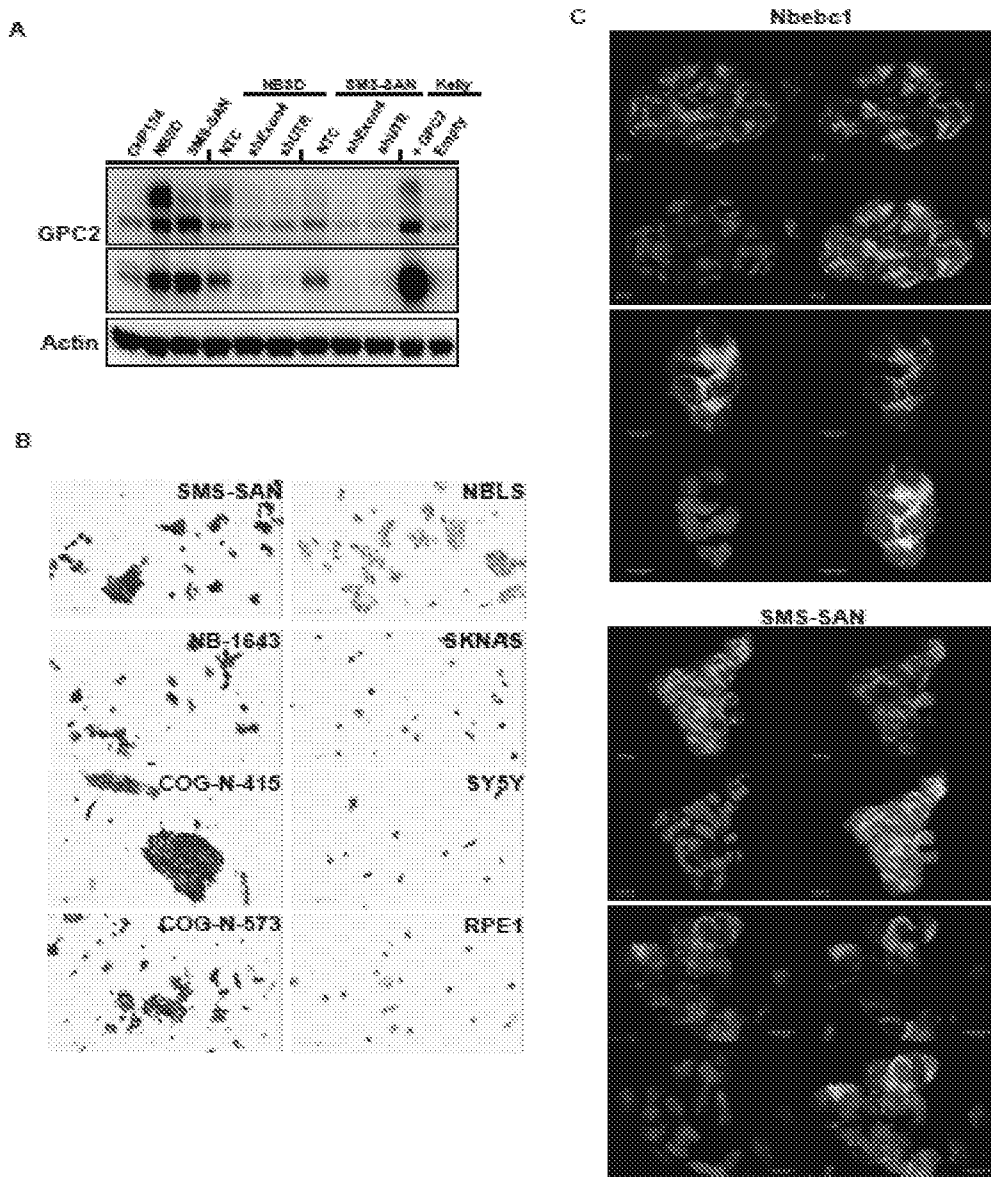
FIGS. 22A-C



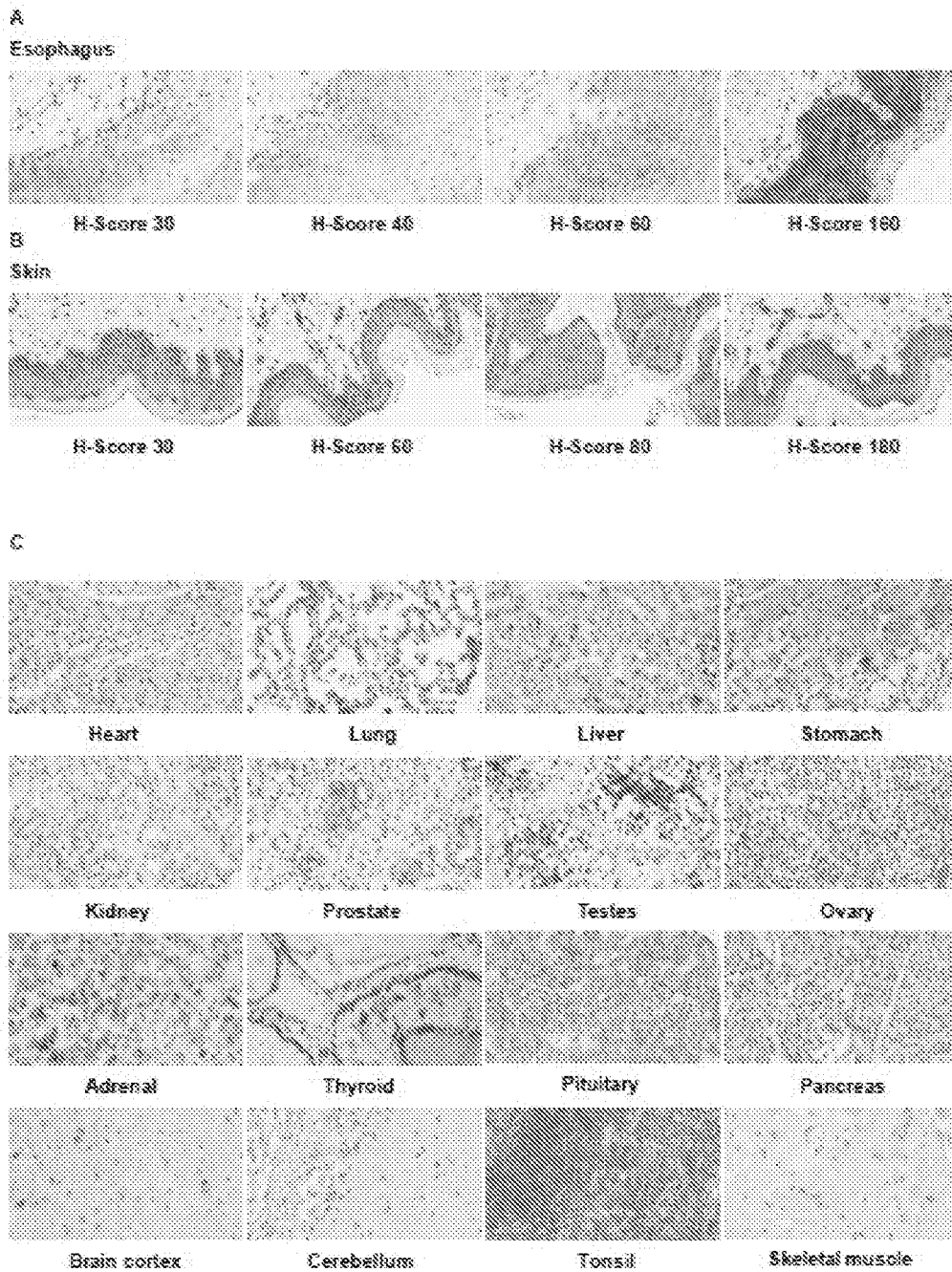
FIGS. 23A-C



FIGS. 24A-C



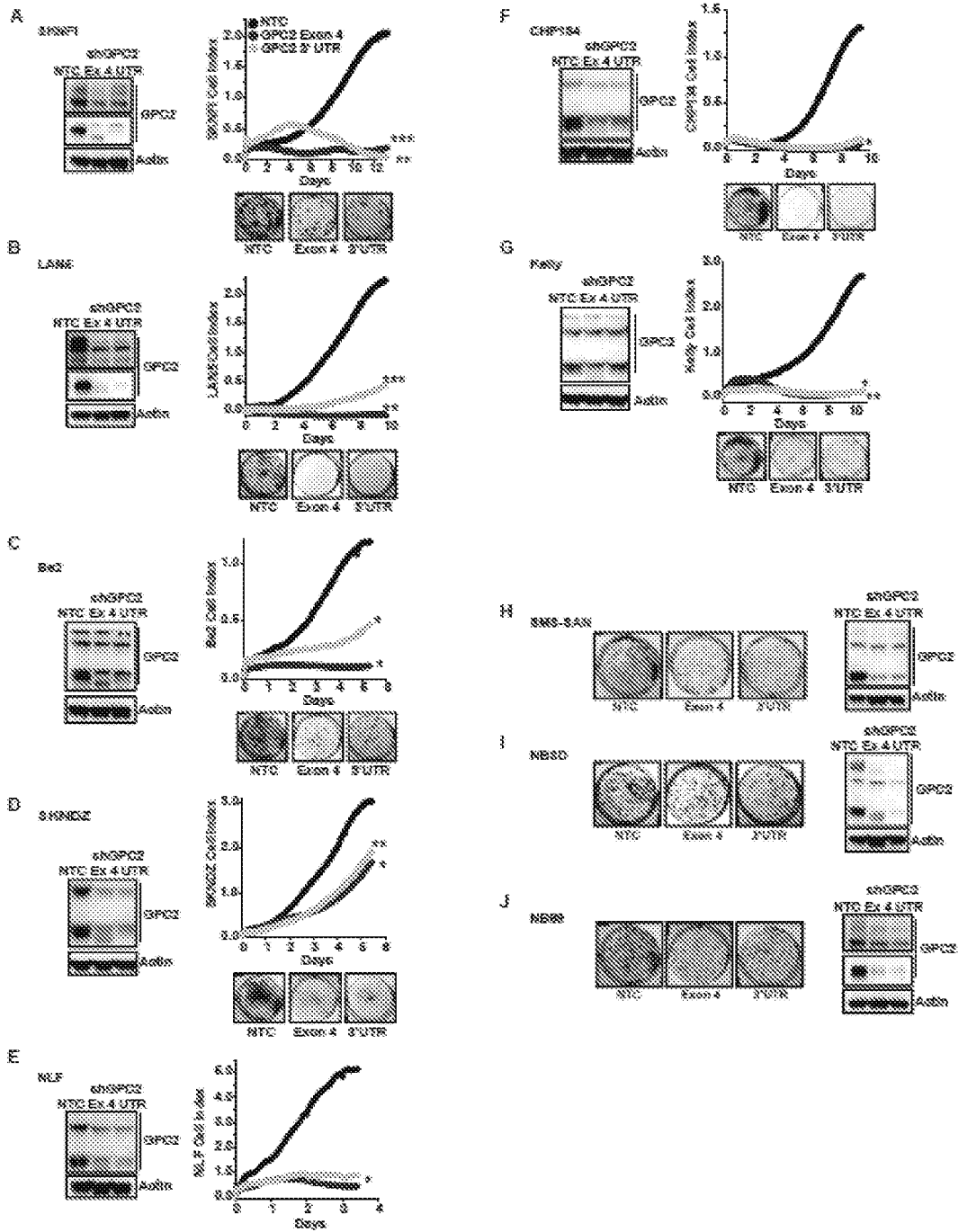
FIGS. 25A-C



FIGS. 26A-C







FIGS. 28A-J



M201VH  
 SEQ ID NO: 1  
 GAGGTGCAGCTGGTGGAGACTGGGGGAGGGGTGGTCAAGCCTGGAGGGTCCCTGAGACTCTCTGTGCAGCCTCTGGATTCACTTCAGTGA  
 CTACATGAGCTGGATCCGCCAGGCTCCAGGGAAGGGCTGGAGTGGGTTTCATACATTAGTAGTAGTGGTAGTACCATATACTACGCAGACTCCG  
 TGAAGGGCCGATTCACCATCTCCAGAGACAAATCCAAAGAACACGCTGTATCTGCAAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGTATTACT  
 GTGGAGAGAGAGTGGCTACGATTACGTGTTTGACTACTGGGGCCAGGGAACCTGGTCCGCCGTCCTCTCA

SEQ ID NO: 2  
 EVQLVETGGVVKPGGSLRLSCAASGFFTSFYIMSWIRQAPGKGLEWVSYISSSGSHYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAREZ  
GYDYVFDVWGGQTLVAVSS

M201VL  
 SEQ ID NO: 3  
 GACATCCAGATGACCCAGTCTCCTCCACCCTGTCTGCATTTGTAGGAGACAGAGTCAACCATCACTGCCGGGCCAGTCAAGATATTAGTAGCTGGT  
 TGGCCTGGTATCAGCAAAAACCAGGGAAGCCCTAAGCTCCTGATCTATGCTGCATCCACTTTGCAAAGTGGGTCCCATCAAGGTTCAAGCGGCA  
 GTGGATCTGGGACAGAAATCACTCTCACAAATCAGCAGCCTGCAGCCTGAAGATTTGCAACTTATTACTGTCAACAGCTTAATAGTTACCCCTATCACC  
 TTCGGCCAAGGACACGACTGGAGATTAAACGA

SEQ ID NO: 4  
 DIQMTQSPSTLSAFVGDRTTTCRASQSSMFLAWYQQKPKAPKLLYAASTLOSQVPSRFSGSGGTEFTLTISLQPEDFATYYCQQLMSYPIITFGGQT  
 RLEIKR

FIG. 30

M202VH  
 SEQ ID NO: 11  
 CAGGTGCAGCTGGTGCAGTCTGGAGGAGGCTTGATCCAGCCTGGGGGCTCCTGAGACTCTCCTGTGCAGCCTCTGGGTTACCGTCAGTAGCAA  
 CTACATGAGCTGGTCCGCCAGGCTCCAGGGAAGGGCTGGAGTGGGCTCAGTTATTTATAGCGGTGGTAGCACATACTACGCAGACTCCGTGA  
 AGGGCGGATTCACCATCTCCAGAGACAATCCAAAGAACCGCTGATCTTCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTTACTGTG  
 CGAGAGATTCGAATGCTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCTCA

SEQ ID NO: 12  
 QVQLVQSGGGLIQPGGSLRLSCAASGFTVSSWYMSWVRQAPGKLEWVSIVYSGGTYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCA#D  
SNAFDHWGQGTMTVSS

M202VL  
 SEQ ID NO: 13  
 GAAATTGTGCTGACTCAGTCTCCACTCTCCCTGCCCGTCAACCCTGGAGAGCCGCCCTCCATCTCCTGCAGGTCTAGTCAGAGCCTCCTGTATAGTA  
 ATGGATACAACATAITGGATTGGTACCTGCAGAAAGCCAGGAAAGTCTCCACAGGTCTGATCTATTTGGGTTCTAATCGGGCCTCCGGGGTCCCGG  
 ACAGGTTCAGTGGCAGTGGATCAGGCACAGATTCACACTGAAAATCAGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCATGCAAGCT  
 CTACAACTCCGATCACCTTCGGCCAAGGGACACGACTGGAGATTAACCGA

SEQ ID NO: 14  
 EIVLTQSPLSLPTPGEPAISCRSSQSLIYSNGYNYLDWYLQKPKSPQVLIYLGSNRASGVPRFSGSGSDFTLKISRVEAEDVGVVYCMQALQIPIIF  
 GQGTREIKR

FIG. 31

M203VH  
 SEQ ID NO: 21  
 GAGGTGCAGCTGGTGCAGTCTGGGGAGGCTTGGTACAGCCTGGGGGTCCTGAGACTCTCCTGTGCAGCCTCTGGATTACACCTTTAGCAGCTA  
 TGCCATGAGCTGGGTCGGCCAGGCTCCAGGAAGGGCTGGAGTGGGTCAGCTATTAGTGGTAGTGGTAGCACATACTACGCAGACTCCG  
 TGAAGGGCCGCTTACCATCTCCAGAGACAAATCCAAAGAACACCGTCTCTGCAAAATGGACAGCCTGAGACCCGAGACACGGCCGTATATTACT  
 GTCGAAAAGTCGAGATAGTGGAACTACCTTGATCTTGGGGCCAAAGGACAATGGTCACCGTCTCTTCA

SEQ ID NO: 22  
 EVQLVQSGGGLVQPGGSLRLSCAASGFFTFSSYAMSWVRQAPGKGLEWVSAISGGGSTYYADSVKGRFTISRDNKNTLSLQMDSLRPEDTAVYYCAK\$  
RDSENYLDAPDFEWGGTMTVSS

M203VL  
 SEQ ID NO: 23  
 GACATCCAGTTGACCCAGTCTCCTCCACCCTGTCTGCATCTGTAGGAGACAGAGTACCATCACCCTGCCGGGCCAGTCAAGATATTAGTAGCTGGT  
 TGGCCTGGTATCAGCAGAAAGCAGGAAAGCTCCTAGGCTCCTGATCTATGATGCCCTCCACTTTGGAAAGTGGAGTCCCATCAAGGTTCAAGCGGCA  
 CTGGATCTGGGACATATTCACTCTCACCATCAGCAGCCTGCAGCCTGAAGATTTGCCAACHATTACTGTCAACAGTTAATAGTTCCCGCTCACTT  
 TCGGCGGAGGGACCAAGGTGGAGATCAAACGA

SEQ ID NO: 24  
 DIQLTQSPSTLSASVGDRTTTCRASQSSSWLAWYQQKAGKAPRLLIYDASTLESGVPSRFSGTSGTYFTLTSSLPEDFATYYCQQQMFPITFGGGTK  
 VEIKR

FIG. 32

SEQUENCE LISTING

<110> The Children's Hospital of Philadelphia  
The Government of the United States of America as  
represented by the Secretary of the Department of Health and  
Human Services

<120> GLYPICAN 2 AS A CANCER MARKER AND THERAPEUTIC TARGET

<130> CHOP.P0005W0

<140> Unknown

<141> 2016-11-08

<150> US 62/253,000

<151> 2015-11-09

<150> US 62/350,976

<151> 2016-06-16

<160> 30

<170> PatentIn version 3.5

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<211> 357

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<223> Synthetic oligonucleotide

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tcctgtgcag cctctggatt caccttcagt gactactaca tgagctggat ccgccaggct 120

ccaggaagg ggctggagtg ggtttcatac attagtagta gtggtagtac catatactac 180

gcagactccg tgaagggccg attcaccatc tccagagaca attccaagaa cacgctgtat 240

ctgcaaatga acagcctgag agctgaggac acggctgtgt attactgtgc gagagagagt 300

ggctacgatt acgtgtttga ctactggggc caggaaccc tggtcgccgt ctctca 357

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<212> PRT

<213> Artificial sequence

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<400> 2

Glu Val Gln Leu Val Glu Thr Gly Gly Gly Val Val Lys Pro Gly Gly  
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asp Tyr  
20 25 30

Tyr Met Ser Trp Ile Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val  
35 40 45

Ser Tyr Ile Ser Ser Ser Gly Ser Thr Ile Tyr Tyr Ala Asp Ser Val  
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr  
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys  
85 90 95

Ala Arg Glu Ser Gly Tyr Asp Tyr Val Phe Asp Tyr Trp Gly Gln Gly  
100 105 110

Thr Leu Val Ala Val Ser Ser  
115

<210> 3  
<211> 324  
<212> DNA  
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atcacttgcc gggccagtca gagtattagt agctgggttg cctggatatca gcaaaaacca 120  
gggaaagccc ctaagctcct gatctatgct gcatccactt tgcaaagtgg ggtcccatca 180  
aggttcagcg gcagtggatc tgggacagaa ttcactctca caatcagcag cctgcagcct 240  
gaagatthttg caacttatta ctgtcaacag cttaatagtt accctatcac cttcggccaa 300  
gggacacgac tggagattaa acga 324

<210> 4  
<211> 108  
<212> PRT  
<213> Artificial sequence

<220>  
<223> Synthetic amino acid

<400> 4

Asp Ile Gln Met Thr Gln Ser Pro Ser Thr Leu Ser Ala Phe Val Gly  
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Ser Trp  
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile

35

40

45

Tyr Ala Ala Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Leu Asn Ser Tyr Pro Ile  
85 90 95

Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys Arg  
100 105

<210> 5  
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<400> 5

Gly Phe Thr Phe Ser Asp Tyr Tyr  
1 5

<210> 6  
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<400> 6

Ile Ser Ser Ser Gly Ser Thr Ile  
1 5

<210> 7  
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Ala Arg Glu Ser Gly Tyr Asp Tyr Val Phe Asp Tyr  
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<211> 6  
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<220>  
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<400> 8

Gln Ser Ile Ser Ser Trp  
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<210> 9  
<211> 3  
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<220>  
<223> Synthetic amino acid

<400> 9

Ala Ala Ser  
1

<210> 10  
<211> 9  
<212> PRT  
<213> Artificial sequence

<220>  
<223> Synthetic amino acid

<400> 10

Gln Gln Leu Asn Ser Tyr Pro Ile Thr  
1 5

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<211> 345  
<212> DNA  
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tcctgtgcag cctctggggtt caccgtcagt agcaactaca tgagctgggt ccgccaggct 120  
ccaggggaagg ggctggagtg ggtctcagtt atttatagcg gtggtagcac atactacgca 180  
gactccgtga agggccgatt caccatctcc agagacaatt ccaagaacac gctgtatctt 240  
caaatgaaca gcctgagagc cgaggacacg gccgtgtatt actgtgagag agattcgaat 300  
gcttttgata tctggggcca agggacaatg gtcaccgtct ctca 345

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<220>

<223> Synthetic amino acid

<400> 12

Gln Val Gln Leu Val Gln Ser Gly Gly Gly Leu Ile Gln Pro Gly Gly  
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Val Ser Ser Asn  
20 25 30

Tyr Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val  
35 40 45

Ser Val Ile Tyr Ser Gly Gly Ser Thr Tyr Tyr Ala Asp Ser Val Lys  
50 55 60

Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu  
65 70 75 80

Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala  
85 90 95

Arg Asp Ser Asn Ala Phe Asp Ile Trp Gly Gln Gly Thr Met Val Thr  
100 105 110

Val Ser Ser  
115

<210> 13

<211> 339

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligonucleotide

<400> 13

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atctcctgca ggtctagtca ggcctcctg tatagtaatg gatacaacta tttggattgg 120

tacctgcaga agccagggaa gtctccacag gtcctgatct atttgggttc taatcgggcc 180

tccggggctc ccgacaggtt cagtggcagt ggatcaggca cagatttcac actgaaaatc 240

agcagagtgg aggctgagga tgttgggggtt tattactgca tgcaagctct acaaactccg 300

atcaccttcg gccaaggac acgactggag attaaacga 339

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<211> 113

<212> PRT

<213> Artificial sequence

<220>

<223> Synthetic amino acid

<400> 14

Glu Ile Val Leu Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly  
1 5 10 15

Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu Tyr Ser  
20 25 30

Asn Gly Tyr Asn Tyr Leu Asp Trp Tyr Leu Gln Lys Pro Gly Lys Ser  
35 40 45

Pro Gln Val Leu Ile Tyr Leu Gly Ser Asn Arg Ala Ser Gly Val Pro  
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile  
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala  
85 90 95

Leu Gln Thr Pro Ile Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys  
100 105 110

Arg

<210> 15

<211> 8

<212> PRT

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Gly Phe Thr Val Ser Ser Asn Tyr  
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<210> 16

<211> 8

<212> PRT

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<400> 16

Val Ile Tyr Ser Gly Gly Ser Thr  
1 5

<210> 17

<211> 9  
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<400> 17

Ala Arg Asp Ser Asn Ala Phe Asp Ile  
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<210> 18  
<211> 11  
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<400> 18

Gln Ser Leu Leu Tyr Ser Asn Gly Tyr Asn Tyr  
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<210> 19  
<211> 3  
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<400> 19

Leu Gly Ser  
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<210> 20  
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<400> 20

Met Gln Ala Leu Gln Thr Pro Ile Thr  
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tcctgtgcag cctctggatt cacctttagc agctatgccca tgagctgggt cgcaggct 120  
 ccaggaagg ggctggagt ggtctcagct attagtggta gtggtgtag cacatactac 180  
 gcagactccg tgaagggccg cttcaccatc tccagagaca attccaagaa cacgctgtct 240  
 ctgcaaatgg acagcctgag acccgaggac acggccgtat attactgtgc gaaaagtcga 300  
 gatagtggga actaccttga tgcttttgat ttctggggcc aaggacaat ggtcaccgtc 360  
 tcttca 366

<210> 22  
 <211> 122  
 <212> PRT  
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<220>  
 <223> Synthetic amino acid

<400> 22

Glu Val Gln Leu Val Gln Ser Gly Gly Gly Leu Val Gln Pro Gly Gly  
 1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr  
 20 25 30

Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val  
 35 40 45

Ser Ala Ile Ser Gly Ser Gly Gly Ser Thr Tyr Tyr Ala Asp Ser Val  
 50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Ser  
 65 70 75 80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95

Ala Lys Ser Arg Asp Ser Gly Asn Tyr Leu Asp Ala Phe Asp Phe Trp  
 100 105 110

Gly Gln Gly Thr Met Val Thr Val Ser Ser  
 115 120

<210> 23  
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<400> 23

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atcacttgcc gggccagtca gaggattagt agctgggttg cctggtatca gcagaaagca 120  
gggaaagctc ctaggctcct gatctatgat gcctccactt tggaaagtgg agtcccatca 180  
aggttcagcg gcactggatc tgggacatat ttcactctca ccatcagcag cctgcagcct 240  
gaagattttg caacttatta ctgtcaacag tttaatagtt tcccgtcac ttcggcgga 300  
gggaccaagg tggagatcaa acga 324

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<400> 24

Asp Ile Gln Leu Thr Gln Ser Pro Ser Thr Leu Ser Ala Ser Val Gly  
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Ser Trp  
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Ala Gly Lys Ala Pro Arg Leu Leu Ile  
35 40 45

Tyr Asp Ala Ser Thr Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly  
50 55 60

Thr Gly Ser Gly Thr Tyr Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Phe Asn Ser Phe Pro Leu  
85 90 95

Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys Arg  
100 105

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<210> 26  
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<400> 26

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<210> 27  
<211> 15  
<212> PRT  
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<400> 27

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

<210> 28  
<211> 6  
<212> PRT  
<213> Artificial sequence

<220>  
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<400> 28

Gln Ser Ile Ser Ser Trp  
1 5

<210> 29  
<211> 3  
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<220>  
<223> Synthetic amino acid

<400> 29

Asp Ala Ser  
1

<210> 30  
<211> 9  
<212> PRT  
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<400> 30

Gln Gln Phe Asn Ser Phe Pro Leu Thr  
1 5