

(51) International Patent Classification:
C07K 16/46 (2006.01)(21) International Application Number:
PCT/CA2017/051128(22) International Filing Date:
25 September 2017 (25.09.2017)

(25) Filing Language: English

(26) Publication Language: English

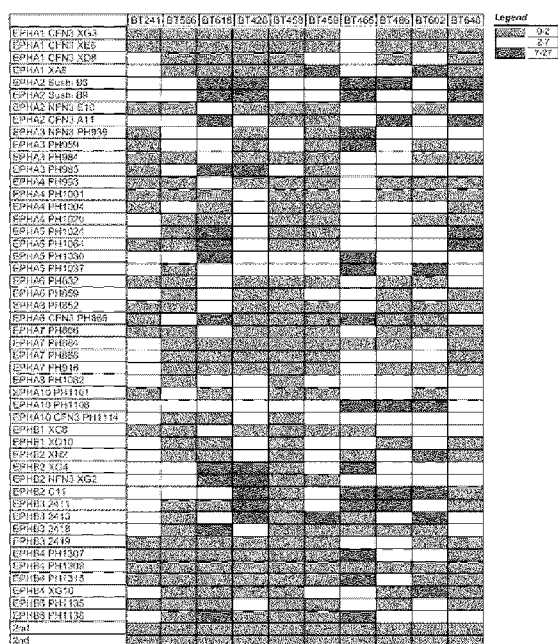
(30) Priority Data:
62/398,825 23 September 2016 (23.09.2016) US(71) Applicant: MCMaster UNIVERSITY [CA/CA]; Mc-
Master Industry Liaison Office, Suite 305, 175 Longwood
Road South, Hamilton, Ontario L8P 0A1 (CA).

(72) Inventors; and

(71) Applicants: SIDHU, Sachdev; 135 Brunswick Ave,
Toronto, Ontario M5S 2M3 (CA). PAN, Guohua; 182
Trelawn Ave, Oakville, Ontario L6J 4R2 (CA). ADAMS,
Jarrett; 1502-45 Balliol St., Toronto, Ontario M4S 1C3
(CA). MOFFAT, Jason; 361 Greer Road, Toronto, Ont-
ario M5M 3P4 (CA). LONDON, Max Eliot; 3104-25 Carl-
ton Street, Toronto, Ontario M5B 3P4 (CA). GORELIK,Maryna; 887 Bay St., Apt. 606, Toronto, Ontario M5S 3K4
(CA).(72) Inventors: VORA, Parvez; 1549 Upper Wellington St.,
Hamilton, Ontario L9B 2H4 (CA). SINGH, Sheila; c/o
MDCL 5027, McMaster University, 1280 Main Street W.,
Hamilton, Ontario L8S 4K1 (CA). VENUGOPAL, Chitra;
710 Spring Gardens Road, Unit 101, Burlington, L7T 4K7
(CA). QAZI, Maleeha A.; 5010 Corporate Drive, Suite
326, Burlington, Ontario L7L 0H6 (CA).(74) Agent: BERESKIN & PARR LLP/S.E.N.C.R.L., S.R.L.;
Scotia Plaza, 40 King Street West, 40th Floor, Toronto, On-
tario M5H 3Y2 (CA).(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,
KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(54) Title: EPHA2 AND EPHA3-BINDING AGENTS AND USES THEREOF

FIGURE 1A

(57) Abstract: Provided is an erythropoietin-producing hepatocellular
carcinoma receptor (EphR)-binding agent comprising: a first binding
agent which specifically binds a first EphR and a second binding agent
which binds a second EphR, optionally EphA2 and EphA3. Also pro-
vided are uses of the EphR-binding agent for treating or preventing
glioblastoma multiforme (GBM).

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

Published:

- *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*
- *with sequence listing part of description (Rule 5.2(a))*

TITLE: EPHA2 AND EPHA3-BINDING AGENTS AND USES THEREOF**FIELD**

[0001] This disclosure relates generally to EphA2 and EphA3-binding agents, and to methods and uses of these binding agents.

5 BACKGROUND

[0002] Glioblastoma (GBM) is the most malignant primary brain tumor in adults (DeAngelis, 2001; Louis et al., 2007). Despite aggressive standard therapy consisting of surgical resection followed by radiation and chemotherapy, tumor re-growth and patient relapse remain inevitable. On
10 average, patients face disease relapse at 7-9 months post-diagnosis and succumb to disease progression with a median survival of only 15 months (Stupp et al., 2005). The dismal prognosis of GBM has been increasingly attributed to extensive genetic, epigenetic, cellular and functional heterogeneity (Brennan et al., 2013; McLendon et al., 2008; Meyer et al.,
15 2015; Patel et al., 2014; Verhaak et al., 2010), allowing for redundancy in signalling pathways and rendering single-agent therapy obsolete for long-term disease remission and cure. Moreover, the genomic landscape of recurrent GBM has been shown to diverge significantly from the primary GBM, as actionable targets identified in primary, treatment naïve GBM are not present
20 at recurrence. Rather, recurrent disease is instead driven by a different mutational and signalling profile (Johnson et al., 2014; J. Kim et al., 2015; Wang et al., 2016). There is also accumulating evidence suggesting that GBM cells may be instigated by stem cell like populations termed glioblastoma stem cells (GSCs) (Lathia et al., 2010; Singh et al., 2003; Son et al., 2009; Suva et al., 2014). Moreover, GSCs are thought to account for GBM recurrence after
25 therapy as cells with GSC properties are resistant to radiation and chemotherapeutic agents (Bao et al., 2006; Chen et al., 2012; Liu et al., 2006; Qazi et al., 2016). Together, this evidence implies that treatment of recurrent GBM should be informed by the identification of molecular targets specific to
30 its evolved molecular landscape, and a poly-targeting approach could better address the advanced clonal heterogeneity that generates cellular escape from therapy, resulting in treatment resistance.

[0003] The EphR tyrosine kinase family, with 14 members, coordinates cell positioning, tissue and organ patterning during development, and is expressed in most adult stem cell niches and many cancers (Genander and Frisé, 2010; Nakada et al., 2011; Pasquale, 2008). Various members of the EphA/Ephrin-A and EphB/Ephrin-B subfamilies have been shown to play a role in GBM cell migration, invasion and angiogenesis (Binda et al., 2012; Day et al., 2013; Nakada et al., 2009; Wykosky, 2005). The expression of EphA2, EphA3, EphA4, EphA7 and EphB2 correlates with poor patient outcome in GBM, and each has a distinct role in GBM tumorigenicity, invasiveness, or maintenance of the GSC pool. In particular, EphA2 has been shown to drive tumorigenicity in GSCs, and infusion of EphrinA1-Fc into intracranial xenografts elicited strong tumor-suppressing effects (Binda et al., 2012). EphA2 overexpression has also been shown to promote invasiveness of GSCs in vivo in cooperation with the Akt signaling pathway (Miao et al., 2014; Wykosky, 2005). Similarly, EphA3 has also emerged as a GSC marker, which is overexpressed in GBM and maintains GBM cells in a stem-like state (Day et al., 2013). While these data validate EphA2 and EphA3 as therapeutic targets in brain tumors, the literature to date has only profiled or targeted single EphRs in treatment-naïve GBM and suggests single targeting of an EphR would leave other putative EphR driven GSC populations to seed tumor recurrence. Additionally, what has not been explored is the complex putative effects of multiple EphR family members dynamically activated or suppressed through therapy delivery and tumor progression.

SUMMARY

[0004] Using a highly specific antibody panel for all Eph receptor tyrosine kinases, the present inventors identified that EphA2 and EphA3 co-expression marks a highly tumorigenic cell population in recurrent glioblastoma multiforme (rGBM) that is enriched in cancer stem cell marker expression. The inventors showed that knockdown of EphA2 and EphA3 together blocks this tumorigenicity, and is marked by an increase in the expression of differentiation marker GFAP. The inventors also showed that treatment with a bispecific antibody (BsAb) that co-targets EphA2 and EphA3

reduces the tumorigenic potential of rGBM by down regulating Akt and Erk signaling pathways and increasing differentiation.

[0005] Accordingly, the present disclosure provides an erythropoietin-producing hepatocellular carcinoma receptor (EphR)-binding agent
5 comprising: a first binding portion which specifically binds a first EphR and a second binding portion which binds a second EphR, wherein the first EphR and the second EphR are different.

[0006] In one embodiment, the first binding portion and/or the second binding portion is an antibody or a binding fragment thereof.

10 [0007] In another embodiment, the first EphR is EphA2.

[0008] In another embodiment, the second EphR is EphA3.

[0009] In another embodiment, the first EphR is EphA2 and the second EphR is EphA3.

[0010] In another embodiment, the EphR binding agent is a bispecific
15 antibody.

[0011] In another embodiment, the first binding portion of the bispecific antibody comprises a first heavy chain variable region-derived segment and a first antibody heavy chain constant region-derived segment lacking a CH1 domain and the second binding portion of the bispecific antibody comprises a
20 second heavy chain variable region-derived segment and a second antibody heavy chain constant region-derived segment lacking a CH1 domain.

[0012] In another embodiment, the first binding portion binds an EphA2 epitope bound by an EphA2-binding subunit comprising a heavy chain having the amino acid sequence of SEQ ID NO: 3.

25 [0013] In another embodiment, the first binding portion comprises a heavy chain complementarity-determining region (CDR)1 comprising the amino acid sequence of SEQ ID NO: 6, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 7, and a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 8.

[0014] In another embodiment, the first binding portion comprises the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 3.

[0015] In another embodiment, the first binding portion comprises an R at amino acid position 16 of SEQ ID NO: 3, a G at amino acid position 35 of SEQ ID NO: 3, an R at amino acid position 39 of SEQ ID NO: 3, an E at amino acid position 45 of SEQ ID NO: 3, and/or an L at amino acid position 47 of SEQ ID NO: 3.

[0016] In another embodiment, the second binding portion comprises a heavy chain complementarity-determining region (CDR)1 comprising the amino acid sequence of SEQ ID NO: 6, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 16, and a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 17.

[0017] In another embodiment, the second binding portion comprises the amino acid sequence of SEQ ID NO: 4 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 4.

[0018] In another embodiment, the second binding portion comprises an R at amino acid position 16 of SEQ ID NO: 4, a G at amino acid position 35 of SEQ ID NO: 4, an R at amino acid position 39 of SEQ ID NO: 4, an E at amino acid position 45 of SEQ ID NO: 4, and/or an L at amino acid position 47 of SEQ ID NO: 4.

[0019] The disclosure also provides a pharmaceutical composition comprising an EphR-binding agent as described above, and a carrier.

[0020] The disclosure also provides use of an EphR-binding agent as described above or a pharmaceutical composition as described above for treating or preventing glioblastoma multiforme (GBM).

[0021] In one embodiment, the glioblastoma multiforme (GBM) is recurrent GBM.

[0022] The disclosure further provides a use of a first agent targeting a first EphR and a second agent targeting a second EphR for treating or preventing glioblastoma multiforme (GBM), wherein the first EphR and the

second EphR are different, and wherein the first and second agent reduce the expression and/or activity of the first EphR and the second EphR, respectively.

[0023] In one embodiment, the first agent specifically binds the first
5 EphR and a second agent specifically binds the second EphR.

[0024] In another embodiment, first EphR is EphA2.

[0025] In another embodiment, the second EphR is EphA3.

[0026] In another embodiment, the first EphR is EphA2 and the second EphR is EphA3.

10 [0027] In another embodiment, the first agent and/or the second agent is an antibody or fragment thereof.

[0028] In another embodiment, the antibody or fragment thereof is selected from the group consisting of a bispecific antibody, a Fab, a single-chain Fv (scFv), an IgG, a phage-Fab and a phage-scFv.

15 [0029] In another embodiment, the first agent and the second agent are for use simultaneously or sequentially.

[0030] In another embodiment, the first agent and the second agent form a bispecific antibody.

[0031] In another embodiment, the first agent comprises a heavy chain
20 complementarity-determining region (CDR)1 comprising the amino acid sequence of SEQ ID NO: 6, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 7, and a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 8 and/or the second agent comprises a heavy chain complementarity-determining region (CDR)1 comprising the amino acid
25 sequence of SEQ ID NO: 6, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 16, and a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 17.

[0032] In another embodiment, the glioblastoma multiforme (GBM) is recurrent GBM.

[0033] The disclosure also provides a method of treating cancer in a subject in need thereof comprising administering to the subject two or more different antibodies targeting different receptors belonging to the erythropoietin-producing hepatocellular carcinoma receptor (EphR) family.

5 [0034] In one embodiment, the cancer is glioblastoma multiforme (GBM).

[0035] In another embodiment, cancer is relapsed glioblastoma multiforme (GBM).

[0036] In another embodiment, the antibody is a bispecific antibody
10 targeting different EphR proteins.

[0037] In another embodiment, the antibody is a bispecific antibody targeting EphR proteins that are highly expressed in the subject.

[0038] In another embodiment, the antibody is a bispecific antibody targeting the EphA2 and EphA3 proteins.

15 **DRAWINGS**

[0039] Embodiments are described below in relation to the drawings in which:

[0040] Figure 1 shows that EphA2 and EphA3 have higher expression in recurrent GBM and co-express with glioblastoma stem cell (GSC) markers.

20 (A) Using the EphR profiler, the expression of all 14 Eph receptors across ten primary and recurrent GSC lines was identified. (B) Using flow cytometry, the surface expression of EphA2 and EphA3 in human neural stem/progenitor cells (NSPC) (n=2), pGBM (n=5) and rGBM (n=3) was determined. All samples are biological replicates. (C) Bar-chart showing increased expression
25 of both EphA2 and EphA3 in pGBM BT602 treated with an in vitro chemoradiotherapy protocol. (D) CyTOF-based expression of GSC markers CD133, CD15, Sox2, Bmi1, ITGA6 and FoxG1 in BT241. (E) Co-expression of EphA2 (top) and EphA3 (bottom) with GSC markers in rGBM (BT241) and pGBMs (BT459 and BT602). The black circle represents the cellular
30 population that co-expresses EphA2, EphA3 and all six GSC markers, with the percentage of cells listed at the bottom of each panel. Data is represented as mean±SD.

[0041] Figure 2 shows that EphA2 and EphA3 are highly expressed in recurrent GBMs. (A) EphA2 and EphA3 have higher expression in GBM (grade IV) compared to low grade gliomas, oligodendroglioma and astrocytoma in Rembrandt glioma database. (B) EphA2 and EphA3 have higher expression in classical and mesenchymal subgroups of GBM compared to pro-neural (Rembrandt). (C and D) Higher expression of EphA2 and EphA3 is associated with poor survival in GBM patients (Rembrandt). EphA2 and EphA3 have higher expression in rGBMs as compared to pGBM (E) and in matched primary-recurrent GBM samples as well (F) (TCGA). Data in panels A-D was generated using GlioVis data portal (Bowman et al., 2017).

[0042] Figure 3 shows that co-expression of EphA2 and EphA3 marks a highly clonogenic and tumorigenic cell population in recurrent GBM. (A) Flow profile of EphA2 and EphA3 in two rGBM samples, BT241 and BT618. (B) Secondary sphere formation assay of rGBM cells sorted based on expression of EphA2 and EphA3, where EphA2+/A3+ exhibits the highest clonogenic capacity in three rGBM cells. (C) Proliferation assay of rGBM cells sorted based on the expression of EphA2 and EphA2, where EphA2+/EphA3+ has the highest proliferation capacity. (D) Gene expression of GSC markers Bmi1 and Sox2 is higher in sorted EphA2+/EphA3+ compared to EphA2-/EphA3- rGBM cell populations. (E) H&E staining of mice brains engrafted with rGBM cells sorted based on EphA2 and EphA3 expression, with total tumor area presented in bar graph on the right (n=2). (F) In vivo limiting dilution assay of mice engrafted with different numbers of EphA2/EphA3- and EphA2+/EphA3+ cell, showing EphA2+/A3+ can form tumors at lower cell number. Bar graphs at the bottom show total tumor area of each panel (n=2). Tumor area is presented in the bottom panel for each cell dose. Data is represented as mean±SD. (ns – not significant, *p<0.05, **p<0.01, ***p<0.001). Scale bar represents 5mm.

[0043] Figure 4 shows that EphA2 and EphA3 knockdown in rGBM inhibits clonogenicity, decreases GSC marker expression and prevents tumorigenesis. (A) Western blot showing the expression of EphA2 and EphA3 after shRNA mediated knockdown (KD) of either EphA2 or EphA3 or both EphA2/A3 as compared to shGFP control in two rGBMs. (B) Secondary

sphere formation assay of rGBM with KD against EphA2 and EphA3 shows decrease sphere formation capacity of shEphA2/A3 cells. (C) Proliferation assay of rGBM cells with KD against EphA2 and EphA3 shows decreased proliferation capacity of shEphA2/A3 cells. (D) Gene expression of GSC markers Bmi1 and Sox2 is significantly decreased in rGBM cells with KD against EphA2 and EphA3, while no change is observed in the expression of CD133 and CD15. (E) Knockdown of EphA2 and EphA3 increased levels of GFAP in rGBM. (F) H&E staining of mouse brains engrafted with BT241 shGFP control, shEphA2, shEphA3 or shEphA2/A3 cells. Total tumor area is presented in the bar graph below (n=4). Data is represented as mean±SD. (*p<0.05, **p<0.01, ***p<0.001). See also Figure 7.

[0044] Figure 5 shows that treatment of rGBM with EphA2/A3 BsAb decreases EphA2 and EphA3 expression and decreases activation of Akt and Erk1/2. (A) CyTOF analysis showing binding of BsAb to EphA2+ and EphA3+ cells in BT241. Treatment with EphA2/A3 BsAb for three consecutive days (200nM) decreases EphA2 (B) and EphA3 (C) surface expression as shown by CyTOF in BT241. Bar graphs represent mean intensity of EphA2 or EphA3 expression in control IgG or EphA2/A3 BsAb treated BT241 cells. (D) After 3-day treatment with EphA2/A3 BsAb (200nM), the binding of the BsAb to BT241 also decreases. (E) Western blot showing protein levels of total and phosphorylated EphA2 and EphA3 and multiple proteins involved in downstream signalling when treated with 200nM of EphA2/A3 BsAb for 5 or 15 minutes. (F) Western blot showing protein levels after 60 minutes of treatment with 200nM of EphA2/A3 BsAb, showing a decrease in EphA2 total protein level. See also Figures 8, and 10-12.

[0045] Figure 6 shows that treatment of rGBM with EphA2/A3 BsAb inhibits in vitro clonogenicity, increases differentiation and reduces tumor burden. (A) Secondary sphere formation assay of rGBM treated with 200nM EphA2/A3 BsAb show a decrease as compared control IgG treated cells (scale bar represents 400µm). (B) rGBM treated with 200nM EphA2/A3 BsAb have fewer and smaller spheres. (C) Proliferation assay of rGBM cells treated with EphA2/A3 BsAb shows a decrease compared to control IgG-treated cells. (D) Limiting dilution assay of rGBM pre-treated with 200nM of EphA2/A3 BsAb

for 3-days. (E and F) Protein expression of GFAP and MAP2 using CyTOF in BT241 after 3-day treatment with 200nM of EphA2/A3 BsAb. Bar graph represents median intensity. (G) H&E staining of mouse brains engrafted with BT241 that were treated with 30µg intracranial dose of control IgG or EphA2/A3 BsAb, twice weekly for three weeks. Total tumor area is presented in the bottom bar graph (n=6). (H) EphA2 (top) and EphA3 (bottom) staining on BT241, control IgG or EphA2/A3 BsAb treated tumors. Bar graph represents average positive staining per mm² of tumor area for both EphA2 and EphA3 (n=3). (I) GFAP staining on BT241, control IgG or EphA2/A3 BsAb treated tumors. Bar graph represents average positive staining per mm² of tumor area. Data is represented as mean±SD. (*p<0.05, **p<0.01, ***p<0.001). See also Figure 13.

[0046] Figure 7 shows the effects of shRNA knockdown of EphA2 and EphA3 in rGBM cells. (A) Flow cytometry histogram cell cycle analysis depicting the anti-proliferative effect of shRNA knockdown of EphA2 and EphA3 in rGBM cells. (B) Flow cytometry dot plot analysis depicting increased apoptosis of rGBM cells subjected to shRNA knockdown of EphA2 and EphA3.

[0047] Figure 8 is a table depicting high affinity binding of EphA2/A3 BsAb to EphA2 and EphA3.

[0048] Figure 9 is a schematic of the bispecific anti-EphA2/anti-EphA3 antibody EphA2/A3 BsAb. The variable heavy (VH) portion of the antibody is shaded grey and the CDRs selected by EphA2 and EphA3 are shaded white and light grey respectively. The amino acid substitutions that function to promote autonomous behavior and the FLAG-containing linker are indicated. The CH2-CH3 (knob) of the EphA2 targeting component is shaded dark grey and the CH2-CH3 (hole) of the EphA3 targeting component is shaded black.

[0049] Figure 10 depicts the mechanism by which EphA2/A3 BsAb may reduce EphA2 and EphA3 levels. (A) Western blot depicting phosphorylation of EphA2 and EphA3 induced in rGBM cells in the presence of ephrin ligands. (B) Flow cytometry dot-plot depicting minimal expression of ephrinA5 in rGBM cells. (C) Histogram depicting that ephrinA1 and ephrinA5, both of which

activate EphA2 and EphA3, are highly expressed in EphA2-/EphA3- cells as compared to tumorigenic EphA2+/EphA3+ cells.

[0050] Figure 11 is a Western blot depicting that commercially available antibody against pEphA3 cross-reacts to pEphA2.

- 5 [0051] Figure 12 is a histogram depicting the effect of treating rGBM cells with EphA2/A3 BsAb on expression levels of different EphRs.

[0052] Figure 13 shows various studies assessing the functional effects of EphA2/A3 BsAb on rGBM. (A) and (B) Histograms depicting that EphA2/A3 BsAb targets EphA2+/EphA3- and EphA2-/EphA3+ cells as well as
10 EphA2+/EphA3+ cells. (C) Histogram depicting that loss of clonogenicity of rGBM cells is not caused by changes in cell cycle after treatment with EphA2/A3 BsAb. (D) Flow cytometry dot-plot depicting that loss of clonogenicity of rGBM cells is not caused by apoptosis after treatment with EphA2/A3 BsAb.

- 15 [0053] Figure 14 shows the expression patterns of the EphR receptors from patients with primary and recurrent GBM (A to C).

[0054] Figure 15 shows co-expression of the EphA2 and EphA3 receptors in GBM cells are associated with increased self-renewal and tumorigenicity and with recurrent GBM (A to D).

- 20 [0055] Figure 16 shows the changes in Eph receptor expression and increased self-renewal and proliferative properties in GBM cells after chemoradiotherapy treatment (A to F).

[0056] Figure 17 describes the production and validation of some of the Ephrin family antibodies used in these studies (A to E).

- 25 [0057] Figure 18 shows the effects of using a bispecific EphA2/A3 antibody on cellular signaling and tumor size in vivo of GBM cells (A to C).

DESCRIPTION OF VARIOUS EMBODIMENTS

- [0058] Unless otherwise defined, scientific and technical terms used in connection with the present disclosure shall have the meanings that are
30 commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and

plural terms shall include the singular. For example, the term "a cell" includes a single cell as well as a plurality or population of cells. Generally, nomenclatures utilized in connection with, and techniques of, cell and tissue culture, molecular biology, and protein and oligonucleotide or polynucleotide chemistry and hybridization described herein are those well-known and commonly used in the art (see, e.g. Green and Sambrook, 2012).

[0059] Terms of degree such as "about", "substantially", and "approximately" as used herein mean a reasonable amount of deviation of the modified term such that the end result is not significantly changed. These terms of degree should be construed as including a deviation of at least $\pm 5\%$ of the modified term if this deviation would not negate the meaning of the word it modifies.

Compositions of Matter:

EphR-Binding Agents

15 [0060] The present inventors have provided a bispecific antibody which is capable of specifically binding both EphA2 and EphA3. Treatment with this antibody reduces the tumorigenic potential of rGBM (recurrent glioblastoma) cells.

[0061] Erythropoietin-producing human hepatocellular receptors (EphRs) are a group of receptors that are activated in response to binding with Eph receptor-interacting proteins (Ephrins). EphRs form the largest known subfamily of receptor tyrosine kinases (RTKs). The EphR family includes 14 members, including EphA2 and EphA3. A full list of the EphR tyrosine kinase family members is set out in Figure 1A.

25 [0062] As used herein, the term "binding agent" refers to an agent which specifically binds a protein, for example cells expressing the protein, as opposed to cells not expressing the protein (as determined, e.g. via flow cytometric analysis) with a minimum affinity. In particular, as used herein, the term "EphR-binding agent" refers to an agent which specifically binds at least one erythropoietin-producing human hepatocellular receptor (EphR), for example EphR-expressing cells as opposed to cells not expressing an EphR (as determined, e.g. via flow cytometric analysis) with a minimum affinity. The

30

term “anti-EphR” binding agent is also used herein for the same purpose. In particular embodiments, the EphR-binding agent binds two different EphRs, for example, EphA2 and EphA3.

[0063] The term “EphA2” as used herein refers to the ephrin type-A
5 receptor 2 (also referred to as EPH receptor A2) and includes all known and naturally occurring EphA2 molecules including full length EphA2 protein and fragments thereof, as well as nucleic acids encoding said protein and fragments, as determinable from the context used. EphA2 includes, but is not limited to, mammalian EphA2 such as human EphA2. In humans, EphA2 is
10 encoded by the *EPHA2* gene. The human EphA2 protein or *EPHA2* gene may have any of the known published sequences for EphA2/*EPHA2* which can be obtained from public sources such as GenBank (for example, GenBank Accession P29317). An example of such a protein sequence includes, but is not limited to, the sequence set out as SEQ ID NO: 1.

15 [0064] The term “EphA3” as used herein refers to the ephrin type-A receptor 3 (also referred to as EPH receptor A3) and includes all known and naturally occurring EphA3 molecules including full length EphA3 protein and fragments thereof, as well as nucleic acids encoding said protein and fragments, as determinable from the context used. EphA3 includes, but is not
20 limited to, mammalian EphA3 such as human EphA3. In humans, EphA3 is encoded by the *EPHA3* gene. The human EphA3 protein or *EPHA3* gene may have any of the known published sequences for EphA3/*EPHA3* which can be obtained from public sources such as GenBank (for example, GenBank Accession P29320). An example of such a protein sequence includes, but is
25 not limited to, the sequence set out as SEQ ID NO: 2.

[0065] In one embodiment, the binding agent is a polypeptide. In other embodiments, the binding agent is a non-polypeptidic agent, such as a protein binding nucleic acid or a protein binding organic compound. The binding agent may be monomeric or multimeric. The binding agent may be polymeric
30 or non-polymeric. Alternately, the binding agent may be an engineered polypeptide (e.g. a naturally occurring polypeptide engineered to have a modified amino acid sequence; or a chimeric polypeptide engineered to comprise two or more naturally occurring amino acid sequences; or an

engineered polypeptide selected from a library of engineered polypeptides having randomized amino acid sequences), or a chemically modified polypeptide. As described in more detail below, in one embodiment, the binding agent is a heterodimer.

5 [0066] As used herein, the term “binding agent” includes, but is not limited to, an antibody or binding fragment thereof.

[0067] As used herein, and unless otherwise specified, the term “antibody” refers to an immunoglobulin (Ig) molecule. The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of
10 two identical pairs of polypeptide chains, each pair having one light (“L”) (about 25 kDa) and one heavy (“H”) chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition, and described in more detail below. The carboxy-terminal portion of each chain
15 defines a constant region primarily responsible for effector function. The term “antigen-binding site” or “binding portion” refers to the part of the binding protein that participates in antigen binding. In an antibody, the antigen binding site is formed by amino acid residues of the N-terminal variable (“V”) regions of the heavy and light chains. Three highly divergent stretches within the V
20 regions of the heavy and light chains, referred to as “hypervariable regions”, are interposed between more conserved flanking stretches known as “framework regions”, or “FRs”. Thus, the term “FR” refers to amino acid sequences which are naturally found between, and adjacent to, hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable
25 regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as
30 “complementarity-determining regions,” or “CDRs”. All CDRs and framework regions (FRs) disclosed herein, amino acid sequences of CDRs and FRs disclosed herein, and CDR-encoding or FR-encoding nucleic acid sequences disclosed herein, are intended to be defined in accordance with IMGT

numbering (Lefranc et al., 2003). Another system alternately employed in the art for such definitions is that of Kabat numbering (Kabat et al., 1991).

[0068] In one embodiment, the “antibody or binding fragment” is selected from a fragment antigen-binding (Fab), single-chain Fv (scFv),
 5 single-chain Fab (scFab), Fab', Fv, chemically linked F(ab')₂, dsFv, dsFv', sc(Fv)₂, ds-scFv, (dsFv)₂, scFv-Fc, scFv-based chimeric antigen receptors (CARs), Fab-based CARs, scFab-based CARs, IgG, single-chain immunoglobulin (e.g. scIgG), single-domain antibody (sdAb, nanobody), scFv-Fc, minibody (scFv-CH3), diabody, tribody, tetrabody, multimeric antibody
 10 (e.g. scFv dimer, bivalent diabody), multispecific antibody (e.g. bispecific antibody, trispecific antibody, di-scFv, tri-scFv, bispecific Fab₂, trispecific Fab₂, trispecific triabody, trispecific Fab₃), multimeric/multispecific antibody (e.g. scFv dimer, bispecific diabody, dsFv-dsFv'), heavy-chain antibody, Fab₃, divalent VHH, pentavalent VHH (pentabody), (scFv-SA)₄ or [sc(Fv)2]₂.

15 [0069] Embodiments of binding agents of the present disclosure still further include protein-binding nucleic acid aptamers (e.g. RNA aptamers or DNA aptamers; see, e.g. Lipi et al., 2016), peptide aptamers (see, e.g. Parashar, 2016), and chemically synthesized agents (e.g. synthetic antibody mimics; see, e.g. McEnaney et al., 2014).

20 [0070] In another embodiment, the binding agent is a peptide analog. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed “peptide mimetics” or ‘peptidomimetics” (see, e.g. Fauchere, 1986); Veber and Freidinger, 1985;
 25 and Evans et al., 1987). Such compounds are often developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to biologically useful peptides may be used to produce an equivalent biological effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biochemical property or
 30 pharmacological activity), such as human antibody, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: -CH₂NH-, -CH₂S-, -CH₂-CH₂-, -CH=CH- (cis and trans), -COCH₂-, CH(OH)CH₂- and -CH₂SO-, by methods well known in the art.

Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g. D-lysine in place of L-lysine) may be used to generate more stable peptides. In addition, constrained peptides comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (see, e.g. Rizo and Gierasch, 1992), for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

[0071] The EphR-binding agent may be an antibody, such as a human antibody, containing engineered variable regions or a chimeric antibody comprising human constant regions and an antibody variable region of a non-human mammal. The EphR-binding agent may be a humanized antibody, e.g. an antibody comprising human constant regions, human variable region framework regions, and EphR-binding CDRs generated in a non-human mammal. The non-human mammal may be a rodent, such as a mouse, rat, rabbit, guinea pig or hamster. Alternately, the non-human mammal may be an ungulate, such as a camelid or a bovid. The EphR-binding agent may be an antibody comprising heavy chain constant regions belonging to any type of class, or subclass. The EphR-binding agent may comprise any type of light chain.

[0072] In one embodiment, the EphR-binding agent is a bispecific antibody. As used herein, the term "bispecific antibody" refers to a monomeric or multimeric protein comprising two different binding portions which confer binding specificities for at least two different antigens, where one binding portion is an antibody variable region (formed by a VH domain and a VL domain) or is an antibody variable domain (VH domain or VL domain) which confers binding specificity to one EphR, and where the other binding portion is a different antibody variable region or antibody variable domain which confers binding specificity to another EphR.

[0073] Accordingly, in one embodiment, the EphR-binding agent comprises a first binding portion which specifically binds a first EphR and a second binding portion which specifically binds a second EphR. In one embodiment, the first EphR and the second EphR are different EphR proteins. For example, the first EphR is optionally an EphR selected from the group

consisting of EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4 and EphB6 and the second EphR is optionally an EphR selected from the group consisting EphA1, EphA2, EphA3, EphA4EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4 and EphB6, where the first EphR and the second EphR are different proteins. In another embodiment, the first EphR is EphA2 and the second EphR is EphA3.

[0074] Various techniques for making and isolating bispecific antibodies directly from recombinant cell culture have been described. For example, bispecific antibodies have been produced using leucine zippers (see, e.g. Kostelny et al., 1992), using “diabody” technology (see, e.g. Hollinger et al., 1993), and using single-chain Fv (scFv) dimers (see, e.g. Gruber et al., 1994). In addition, bispecific antibodies can be generated by chemical conjugation of two different, purified monoclonal antibodies (mAbs) or by fusing two hybridomas resulting in a quadroma cell line producing, among others, bispecific IgG molecules.

[0075] In one embodiment, as experimentally disclosed herein, the EphR-binding agent is a bispecific antibody having a binding portion which specifically binds EphA2 and a binding portion which specifically binds EphA3 (also referred to herein as “EphA2/A3 BsAb”). EphA2/A3 BsAb is a heterodimer composed of an anti-EphA2 VH-CH2-CH3 single chain (also referred to herein as the “EphA2-binding subunit”) and an anti-EphA3 VH-CH2-CH3 single chain (also referred to herein as the “EphA3-binding subunit”). EphA2/A3 BsAb includes the amino acid and nucleic sequences set out in Tables 3-9. In one embodiment, the bispecific antibody comprises (a) a first heavy chain variable region-derived segment and a first truncated antibody heavy chain constant region-derived segment and (b) a second heavy chain variable region-derived segment and a second truncated antibody heavy chain constant region-derived segment, wherein the first heavy chain variable region-derived segment targets EphA2 and the second heavy chain variable region-derived segment targets EphA3.

[0076] Accordingly, in an embodiment, the EphR-binding agent comprises a EphA2 binding portion comprising a heavy chain

complementarity-determining region (CDR)1 comprising the amino acid sequence of SEQ ID NO: 6, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 7, and/or a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 8. In another embodiment, the EphR-binding agent comprises a heavy chain complementarity-determining region (CDR)1 consisting of the amino acid sequence of SEQ ID NO: 6, a heavy chain CDR2 consisting of the amino acid sequence of SEQ ID NO: 7, and/or a heavy chain CDR3 consisting of the amino acid sequence of SEQ ID NO: 8.

[0077] In another embodiment, the EphA2 binding portion comprises the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence having at least 70%, 75%, 80%, 85%, 90%, 95% or 99% sequence identity to the framework regions of SEQ ID NO: 3.

[0078] As described in Example 1, various point mutations were introduced into the framework regions of the VH-A2 and VH-A3 segments to promote functionality in the absence of an antibody light chain. Accordingly, in a further embodiment, the EphA2 binding portion comprises an R at amino acid position 16 of SEQ ID NO: 3, a G at amino acid position 35 of SEQ ID NO: 3, an R at amino acid position 39 of SEQ ID NO: 3, an E at amino acid position 45 of SEQ ID NO: 3, and/or an L at amino acid position 47 of SEQ ID NO: 3.

[0079] In another embodiment, the EphR-binding agent comprises a EphA3 binding portion comprising a heavy chain complementarity-determining region (CDR)1 comprising the amino acid sequence of SEQ ID NO: 6, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 16, and/or a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 17. In another embodiment, the EphR-binding agent comprises a heavy chain complementarity-determining region (CDR)1 consisting of the amino acid sequence of SEQ ID NO: 6, a heavy chain CDR2 consisting of the amino acid sequence of SEQ ID NO: 16, and/or a heavy chain CDR3 consisting of the amino acid sequence of SEQ ID NO: 17.

[0080] In another embodiment, the EphA3 binding portion comprises the amino acid sequence of SEQ ID NO: 4 or an amino acid sequence having

at least 70%, 75%, 80%, 85%, 90%, 95% or 99% sequence identity to the framework regions of SEQ ID NO: 4.

[0081] In further embodiments, the EphA3 binding portion comprises an R at amino acid position 16 of SEQ ID NO: 4, a G at amino acid position 35 of
 5 SEQ ID NO: 4, an R at amino acid position 39 of SEQ ID NO: 4, an E at amino acid position 45 of SEQ ID NO: 4, and/or an L at amino acid position 47 of SEQ ID NO: 4.

[0082] Functional variants of the antibodies and binding fragments described herein are also encompassed by the present disclosure. The term
 10 "functional variant" as used herein includes one or more amino acid and/or nucleotide modifications in a sequence (polypeptide or nucleic acid respectively) for example, one or more modifications of a light chain or a heavy chain complementarity determining region (CDR) disclosed herein that perform substantially the same function as the light chain and heavy chain
 15 CDRs disclosed herein in substantially the same way. For instance, variants of the CDRs disclosed herein have the same function of being able to specifically bind to the same epitopes on EphA2 and EphA3 as EphA2/A3 BsAb. In one embodiment, variants of CDRs disclosed herein include, without limitation, conservative amino acid substitutions. Variants of the CDRs also
 20 include additions and deletions to the CDR sequences disclosed herein. In addition, variant nucleotide sequences and polypeptide sequences include analogs and derivatives thereof.

[0083] A "conservative amino acid substitution" as used herein, is one in which one amino acid residue is replaced with another amino acid residue
 25 without abolishing the protein's desired properties. Suitable conservative amino acid substitutions can be made by substituting amino acids with similar hydrophobicity, polarity, and R-chain length for one another. Examples of conservative amino acid substitutions include:

Conservative Substitutions	
<u>Type of Amino Acid</u>	<u>Substitutable Amino Acids</u>
Hydrophilic	Ala, Pro, Gly, Glu, Asp, Gln, Asn, Ser, Thr
Sulphydryl	Cys

Conservative Substitutions	
Aliphatic	Val, Ile, Leu, Met
Basic	Lys, Arg, His
Aromatic	Phe, Tyr, Trp

[0084] Thus, in one embodiment, the present disclosure includes functional variants to the amino acid sequences disclosed herein.

[0085] In particular, the disclosure provides functional variants of the CDR sequences disclosed herein. In one embodiment, functional variants of the CDR sequences of the light and heavy chains disclosed herein have at least 70%, 75%, 80%, 85%, 90%, 95% or 99% sequence identity with the CDR sequences disclosed herein. In another embodiment, functional variants of the CDR sequences disclosed herein comprise at least 1, 2, 3 or 4 amino acid substitutions, optionally conservative substitutions, in the CDR sequences disclosed herein.

[0086] The disclosure also provides functional variants of the amino acid sequences of EphA2- and EphA3-binding subunits described herein. In one embodiment, the variant amino acid sequences of the amino acid sequences disclosed herein comprise sequences having at least 70%, 75%, 80%, 85%, 90%, 95% or 99% sequence identity to SEQ ID NOS: 3 and 4 or sequences having at least 70%, 75%, 80%, 85%, 90%, 95% or 99% to the framework regions of SEQ ID NOS: 3 or 4.

[0087] The term "sequence identity" as used herein refers to the percentage of sequence identity between two amino acid sequences or two nucleic acid sequences. To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g. gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino acid or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the

second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity=number of identical overlapping positions/total number of positions.times.100%). In one
5 embodiment, the two sequences are the same length. The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. One non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul, 1993. Such an
10 algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990. BLAST nucleotide searches can be performed with the NBLAST nucleotide program parameters set, e.g. for score=100, wordlength=12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the present disclosure. BLAST protein searches can be performed with the
15 XBLAST program parameters set, e.g. to score=50, wordlength=3 to obtain amino acid sequences homologous to a protein molecule of the present invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., 1997. Alternatively, PSI-BLAST can be used to perform an iterated search which detects distant
20 relationships between molecules. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g. of XBLAST and NBLAST) can be used (see, e.g. the NCBI website). Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988. Such an algorithm is
25 incorporated in the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. The percent identity between two sequences can be determined using techniques similar to those
30 described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

Nucleic Acids and Vectors

[0088] Also provided in the present disclosure are nucleic acids encoding the antibodies, binding fragment, variable regions and CDRs described herein. As used herein, the term “nucleic acids” includes isolated
5 nucleic acids as well as single stranded nucleic acid sequences, double stranded nucleic acid sequences and cDNA.

[0089] In particular the present disclosure provides nucleic acids encoding the CDR regions of disclosure herein (for example, nucleic acids corresponding to SEQ ID NOS 23, 24, 25, 33 and 34), and functional variants
10 thereof; and nucleic acids encoding the EphA2-binding and EphA3-binding subunit of EphA2/A3 BsAb as set out in SEQ ID NOs: 20 and 21, respectively, and functional variants thereof.

[0090] In another embodiment, the present disclosure includes functional variants to the nucleic acid sequences that encode the amino acid
15 sequences disclosed herein. In addition, the functional variants include nucleotide sequences that hybridize to the nucleic acids encoding the amino acid sequences of the present disclosure, or the complement thereof, under at least moderately stringent hybridization conditions.

[0091] By “at least moderately stringent hybridization conditions” it is
20 meant that conditions are selected which promote selective hybridization between two complementary nucleic acid molecules in solution. Hybridization may occur to all or a portion of a nucleic acid sequence molecule. The hybridizing portion is typically at least 15 (e.g. 20, 25, 30, 40 or 50) nucleotides in length. Those skilled in the art will recognize that the stability of
25 a nucleic acid duplex, or hybrids, is determined by the T_m , which in sodium containing buffers is a function of the sodium ion concentration and temperature ($T_m = 81.5^{\circ}\text{C} - 16.6 (\text{Log}_{10} [\text{Na}^+]) + 0.41(\%(\text{G}+\text{C}) - 600/\text{l})$, or similar equation). Accordingly, the parameters in the wash conditions that determine hybrid stability are sodium ion concentration and temperature. In
30 order to identify molecules that are similar, but not identical, to a known nucleic acid molecule a 1% mismatch may be assumed to result in about a 1°C decrease in T_m , for example if nucleic acid molecules are sought that

- have a >95% identity, the final wash temperature will be reduced by about 5°C. Based on these considerations those skilled in the art will be able to readily select appropriate hybridization conditions. In some embodiments, stringent hybridization conditions are selected. By way of example the following conditions may be employed to achieve stringent hybridization: hybridization at 5x sodium chloride/sodium citrate (SSC)/5x Denhardt's solution/1.0% SDS at $T_m - 5^\circ\text{C}$ based on the above equation, followed by a wash of 0.2x SSC/0.1% SDS at 60°C. Moderately stringent hybridization conditions include a washing step in 3x SSC at 42°C. It is understood, however, that equivalent stringencies may be achieved using alternative buffers, salts and temperatures. Additional guidance regarding hybridization conditions may be found in: Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 2002, and in: Sambrook et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, 2001.
- [0092] Polypeptidic binding agents disclosed herein can be expressed by a vector containing a nucleic acid encoding the polypeptide of interest using methods which are well known and routinely practiced in the art. Accordingly, the present disclosure also provides a vector expressing any of the nucleic acids described herein.
- [0093] The polypeptidic binding agents can be prepared by constructing a nucleic acid encoding a polypeptidic binding agent, inserting the construct into an expression vector, and then expressing it in appropriate host cells. Vectors useful for expressing the polypeptidic binding agents disclosed herein are well known in the art. In one embodiment, the vector includes suitable translation initiation and termination signals in operable reading phase with a functional promoter and can comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and, if desirable, to provide amplification within the host. In addition to vectors, the nucleic acids of the present disclosure can be delivered to a cell or a subject via any other method known in the art including, but not limited to, liposomes, naked DNA, adjuvant-assisted DNA, gene gun, catheters, etc.

Affinity

[0094] Non-covalent interactions occur between binding agent and an antigen for which the binding agent is specific. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation constant (K_D) of the interaction, wherein a smaller K_D represents a greater affinity. Immunological binding properties of specific polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and geometric parameters that equally influence the rate in both directions. Thus, both the “on rate constant” (K_{on}) and the “off rate constant” (K_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation (see, e.g. Malmqvist, 1993). The ratio of K_{off}/K_{on} enables the cancellation of all parameters not related to affinity, and is equal to the dissociation constant K_D (see, e.g. Davies et al., 1990).

[0095] In various embodiments, the EphR-binding agent binds EphA2 with a dissociation constant (K_D) of ≤ 1 micromolar, ≤ 900 nM, ≤ 800 nM, ≤ 700 nM, ≤ 600 nM, ≤ 500 nM, ≤ 400 nM, ≤ 300 nM, ≤ 200 nM, ≤ 100 nM, ≤ 90 nM, ≤ 80 nM, ≤ 70 nM, ≤ 60 nM, ≤ 50 nM, ≤ 40 nM, ≤ 30 nM, ≤ 20 nM, ≤ 10 nM, ≤ 9 nM, ≤ 8 nM, ≤ 7 nM, ≤ 6 nM, ≤ 5 nM, ≤ 4 nM, ≤ 2 nM, ≤ 1 nM, ≤ 0.9 nM, ≤ 0.8 nM, ≤ 0.7 nM, ≤ 0.6 nM, ≤ 0.5 nM, ≤ 0.4 nM to 0.3 nM, ≤ 0.2 nM, or ≤ 100 pM to about 1 pM.

[0096] In additional various embodiments, the EphR-binding agent binds EphA2 with a dissociation constant (K_D) of 1 nM to 50 nM, 5 nM to 20 nM, 8 to 12 nM or 10 nM or about 10 nM.

[0097] In various embodiments, the EphR-binding agent binds EphA3 with a dissociation constant (K_D) of ≤ 1 micromolar, ≤ 900 nM, ≤ 800 nM, ≤ 700 nM, ≤ 600 nM, ≤ 500 nM, ≤ 400 nM, ≤ 300 nM, ≤ 200 nM, ≤ 100 nM, ≤ 90 nM, ≤ 80 nM, ≤ 70 nM, ≤ 60 nM, ≤ 50 nM, ≤ 40 nM, ≤ 30 nM, ≤ 20 nM, ≤ 10 nM, ≤ 9 nM, ≤ 8 nM, ≤ 7 nM, ≤ 6 nM, ≤ 5 nM, ≤ 4 nM, ≤ 2 nM, ≤ 1 nM, ≤ 0.9

nM, ≤ 0.8 nM, ≤ 0.7 nM, ≤ 0.6 nM, ≤ 0.5 nM, ≤ 0.4 nM to 0.3 nM, ≤ 0.2 nM, or ≤ 100 pM to about 1 pM.

[0098] In additional various embodiments, the EphR-binding agent binds EphA3 with a dissociation constant (K_D) of 1 nM to 50 nM, 5 nM to 20 nM, 8 to 12 nM or 9 nM or about 9 nM.

[0099] A bivalent EphA2-binding agent or binding portion disclosed herein, such as a EphA2-binding agent comprising two EphA2-binding antibody variable regions (e.g. an antibody or $F(ab')_2$), is considered to specifically bind EphA2 when the dissociation constant (K_D) of the binding is ≤ 1 micromolar. A monovalent EphA2-binding agent disclosed herein (i.e. which has single EphA2-binding site, such as a single EphA2-binding antibody variable region, e.g. a scFv or a Fab) is said to specifically bind EphA2 when the dissociation constant (K_D) of the binding of the EphA2-binding agent in bivalent form is ≤ 1 micromolar. Similarly, a bivalent EphA3-binding agent or binding portion disclosed herein, such as a EphA3-binding agent comprising two EphA3-binding antibody variable regions (e.g. an antibody or $F(ab')_2$), is considered to specifically bind EphA3 when the dissociation constant (K_D) of the binding is ≤ 1 micromolar. A monovalent EphA3-binding agent disclosed herein (i.e. which has single EphA3-binding site, such as a single EphA3-binding antibody variable region, e.g. a scFv or a Fab) is said to specifically bind EphA3 when the dissociation constant (K_D) of the binding of the EphA3-binding agent in bivalent form is ≤ 1 micromolar. Methods for joining monovalent binding agents of the disclosure for generating suitable bivalent forms thereof are well known in the art (e.g. where the monovalent agent comprises a single antibody variable region, production of bivalent antibodies/ $F(ab')_2$ comprising two copies of the antibody variable region; or e.g. using suitable linkers, such as polypeptide linkers, nucleic acid linkers or chemically synthesized linkers).

[00100] In addition, the disclosure provides an EphR-binding agent which specifically binds a EphA2 epitope bound by an EphA2-binding subunit comprising a heavy chain having the amino acid sequence of SEQ ID NO: 3 and/or a EphA3 epitope bound by EphA3-binding subunit comprising a heavy chain having the amino acid sequence of SEQ ID NO: 4.

[00101] Any one of various methods known in the art can be used to identify an EphR-binding agent which specifically binds a EphA2 epitope bound by the EphA2-binding subunit and/or a EphA3 epitope bound by the EphA3-binding subunit. A person skilled in the art will appreciate that binding assays such as a competition binding assay can be used for this purpose. Those skilled in the art will recognize that it is possible to determine, without undue experimentation, if a binding agent specifically binds a EphA2 epitope bound by the EphA2-binding subunit and/or a EphA3 epitope bound by the EphA3-binding subunit by ascertaining whether the binding agent prevents EphA2-binding subunit and/or EphA3-binding subunit from binding to human EphA2 or EphA3, respectively. If the binding agent being tested competes with EphA2-binding subunit and/or EphA3-binding subunit, as shown by a decrease in binding to human EphA2 or EphA3, respectively by EphA2-binding subunit and/or EphA3-binding subunit,, then the binding agent binds to the same epitope as EphA2-binding subunit and/or EphA3-binding subunit. Methods for the testing the specificity of binding agents include, but are not limited to, enzyme linked immunosorbent assay (ELISA) and other immunologically mediated techniques known within the art.

Detection Agents

[00102] The EphR-binding agents described herein are optionally labeled with a detection agent. As used herein, the term "detection agent" refers to any agent that allows the presence of the binding agent to be detected and/or quantified. Examples of detection agents include, but are not limited to, peptide tags, enzymes (for example, HRP or alkaline phosphatase), proteins (for example phycoerythrin or biotin/streptavidin), magnetic particles, chromophores, fluorescent molecules, chemiluminescent molecules, radioactive labels and dyes. The binding agent may be labeled directly or indirectly with the detection agent.

Immunoconjugates

[00103] The present disclosure also includes an immunoconjugate comprising (1) a EphR-binding agent that has been attached to (2) an effector agent.

[00104] In one embodiment, the effector agent is a label, which can generate a detectable signal, directly or indirect. Examples of labels include radioactive isotopes (i.e., a radioconjugate).

5 [00105] In another embodiment, the effector agent is a therapeutic agent. Therapeutic agents include, but are not limited to, cancer therapeutic agents/antineoplastic agents. In yet another embodiment, the therapeutic agent is a toxin.

[00106] The toxin may be an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or a fragment thereof. Toxins and fragments
10 thereof that can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), Momordica charantia inhibitor, curcin, crotin,
15 Saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

[00107] Radioconjugated EphR-binding agents of the disclosure, such as antibodies of the disclosure, may be employed to bind radionuclides to EphR-expressing cells, for example to visualize the cells or as a cytotoxic
20 treatment of the cells. A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include ²¹²Bi, ¹³¹I, ¹³¹In, ⁹⁰Y, and ¹⁸⁶Re.

[00108] Those of ordinary skill in the art will recognize that a large variety of possible moieties can be coupled to the polypeptidic EphR-binding
25 agents of the disclosure (see, for example, Cruse and Lewis, 1989, the entire contents of which are incorporated herein by reference). Coupling may be accomplished by any chemical reaction that will bind a moiety and an EphR-binding agent of the disclosure, so long as these retain their respective activities/characteristics for the intended use thereof. This linkage can include
30 many chemical mechanisms, for instance covalent binding, affinity binding, intercalation, coordinate binding and complexation.

[00109] For example, conjugates of a polypeptidic EphR-binding agent of the disclosure, such as an antibody and an effector agent can be made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutarelddehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science 238:1098 (1987).

[00110] Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody (see, e.g. W094/11026).

Pharmaceutical compositions

[00111] The disclosure also provides pharmaceutical compositions comprising an EphR-binding agent or immunoconjugate or radioconjugate described herein as an active ingredient and a pharmaceutically acceptable carrier.

[00112] As used herein, the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Optional examples of such carriers or diluents include, but are not limited to, water, saline, ringer's solutions, dextrose solution, and 5% human serum albumin.

[00113] A pharmaceutical composition is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g. intravenous, intradermal, subcutaneous, oral (e.g. inhalation), transdermal (i.e., topical), transmucosal, and rectal administration.

[00114] In one embodiment, the active ingredient is prepared with a carrier that will protect it against rapid elimination from the body, such as a sustained/controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers
5 can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art.

[00115] In one embodiment, oral or parenteral compositions are formulated in dosage unit form for ease of administration and uniformity of
10 dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms are dictated by and directly dependent
15 on the unique characteristics of the active ingredient and the particular therapeutic effect to be achieved, and the limitations inherent in the art of preparing such an active ingredient for the treatment of individuals.

[00116] The formulation can also contain more than one active ingredient as necessary for the particular indication being treated, optionally
20 those with complementary activities that do not adversely affect each other. Alternatively, or in addition, the pharmaceutical composition can comprise an agent that enhances its function, such as, for example, a cytotoxic agent, cytokine, chemotherapeutic agent, or growth-inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the
25 purpose intended.

Methods and uses

[00117] As described herein, the present inventors identified that EphA2 and EphA3 co-expression marks a highly tumorigenic cell population in rGBM that is enriched in cancer stem cell marker expression. The inventors showed
30 that knockdown of EphA2 and EphA3 together blocks this tumorigenicity, and is marked by an increase in the expression of differentiation marker GFAP. The inventors also showed that treatment with a bispecific antibody (BsAb)

that co-targets EphA2 and EphA3 reduces the tumorigenic potential of rGBM by down regulating Akt and Erk signaling pathways and increasing differentiation.

[00118] Accordingly, the EphR-binding agents and pharmaceutical
5 compositions of the present disclosure are useful for treating a cancer, for example glioblastoma multiforme, optionally recurrent glioblastoma multiforme. As used herein, the term “recurrent glioblastoma” (also referred to as “relapsed glioblastoma”) refers to glioblastoma that has returned after initial
10 treatment (for example, surgery, chemotherapy, radiation, or a combination thereof).

[00119] In another embodiment, the cancer is an EphA2-positive and EphA3-positive cancer (also referred to as an EphA2- and EphA3-expressing cancer). In one embodiment, an EphA2-positive and EphA3-positive cancer is defined as a cancer with greater than 80%, 85%, 90%, 95% or 99% EphA2-
15 positive and EphA3-positive cells (i.e., EphA2-expressing and EphA3-expressing cells). The percentage of cells expressing EphA2 and EphA3 may be determined, for example, in a tumor cell culture. Accordingly, in particular embodiments, the cancer is an EphA2-positive and EphA3-positive glioblastoma. In other embodiments, the cancer is an EphA2-positive and
20 EphA3-positive ovarian cancer or an EphA2-positive and EphA3-positive pancreatic cancer.

[00120] In one embodiment, the EphR-binding agents and pharmaceutical compositions described herein are used in a method for treating cancer, the method comprising administering an effective amount of a
25 EphR-binding agent or pharmaceutical composition disclosed herein to an animal or cell in need thereof, optionally wherein the cancer is glioblastoma multiforme, optionally recurrent glioblastoma multiforme.

[00121] In another embodiment, an effective amount of an EphR-binding agent or pharmaceutical composition disclosed herein is used for treating or
30 preventing a cancer, optionally wherein the cancer is glioblastoma multiforme, optionally recurrent glioblastoma multiforme. In another embodiment, a EphR-binding agent or pharmaceutical composition disclosed herein is used in the

preparation of a medicament for treating or preventing a cancer, optionally wherein the cancer is glioblastoma multiforme, optionally recurrent glioblastoma multiforme.

[00122] In yet another embodiment, an effective amount of an EphR-
5 binding agent or pharmaceutical composition disclosed herein is used for treating or preventing a cancer, optionally wherein the cancer is glioblastoma multiforme, optionally recurrent glioblastoma multiforme.

[00123] As described above, the present disclosure provides immunoconjugates comprising (1) a EphR-binding agent and (2) an effector
10 agent, where the effector agent is optionally a toxin or an anti-neoplastic agent.

[00124] Accordingly, the present disclosure provides a method of using an immunoconjugate disclosed herein for treating or preventing a cancer, the method comprising administering an effective amount of an immunoconjugate
15 disclosed herein to an animal or cell in need thereof, optionally wherein the cancer is glioblastoma multiforme, optionally recurrent glioblastoma multiforme.

[00125] In one embodiment, an effective amount of an immunoconjugate disclosed herein is used for treating or preventing a cancer, optionally wherein
20 the cancer is glioblastoma multiforme, optionally recurrent glioblastoma multiforme. In another embodiment, an immunoconjugate disclosed herein is used in the preparation of a medicament for treating or preventing a cancer, optionally wherein the cancer is glioblastoma multiforme, optionally recurrent glioblastoma multiforme.

[00126] The present disclosure also provides a use of a first agent targeting a first EphR and a second agent targeting a second EphR for treating or preventing cancer, wherein the first EphR and the second EphR are different, and wherein the first and second agent reduce the expression and/or activity of the first EphR and the second EphR, respectively. Also
25 provided is a method of for treating or preventing a cancer, the method comprising administering an effective amount of a first agent targeting a first EphR and a second agent targeting a second EphR, to an animal or cell in
30

need thereof. In another embodiment, a first agent targeting a first EphR and a second agent targeting a second EphR is used in the preparation of a medicament for treating or preventing a cancer. In one embodiment, the cancer is glioblastoma multiforme, optionally recurrent glioblastoma multiforme.

[00127] As used herein, the term "reducing the expression and/or activity" of a protein refers to any direct or indirect means, wherein the protein or gene expression or functional activity of the protein is decreased compared to a protein which has not be exposed to the recited agent. In one embodiment, the expression and/or activity is reduced by at least 5, 10, 15, 25, 50 75, 95, 99 or 100%.

[00128] In one embodiment, the first agent and the second agent are antibodies or binding fragments thereof. In another embodiment, the first agent and the second agent are shRNAs targeting a first and a second EphR, respectively.

[00129] The first agent may be administered prior to, overlapping with, concurrently, and/or after administration of the second agent. When administered concurrently, the first and the second agent be administered in a single formulation or in separate formulations, and if administered separately, then optionally, by different modes of administration.

[00130] As used herein, the terms "subject" and "animal" include all members of the animal kingdom, in one embodiment the subject is a mammal. In a further embodiment the subject is a human being. In one embodiment, the subject is a patient having a disease, such as a cancer, associated with EphA2 and EphA3-expressing cells.

[00131] The term "a cell" includes a single cell as well as a plurality or population of cells.

[00132] An effective amount of an EphR-binding agent, immunoconjugate or pharmaceutical composition of the disclosure relates generally to the amount needed to achieve a therapeutic objective.

[00133] The amount required to be administered will furthermore depend on the binding affinity of the EphR-binding agent for EphR, and will also

depend on the rate at which an administered EphR-binding agent is depleted from the free volume of the subject to which it is administered. Common ranges for therapeutically effective dosing of an EphR-binding agent, immunoconjugate or pharmaceutical composition of the disclosure may be, by way of non-limiting example, from about 0.1 mg/kg body weight to about 50 mg/kg body weight. Common dosing frequencies may range, for example, from twice daily to once a week.

[00134] Efficaciousness of treatment is determined in association with any known method for diagnosing or treating the particular cancer. Alleviation of one or more symptoms of the cancer indicates that the antibody confers a clinical benefit.

[00135] As used herein, "treating a cancer" includes, but is not limited to, reversing, alleviating or inhibiting the progression of the cancer or symptoms or conditions associated with the cancer. Preventing includes preventing occurrence of the cancer or symptoms or conditions associated with the cancer or preventing worsening of the severity of the cancer or symptoms or conditions associated with the cancer. "Treating the cancer" also includes extending survival in a subject. Survival is optionally extended by at least 1, 2, 3, 6 or 12 months, or at least 2, 3, 4, 5 or 10 years over the survival that would be expected without treatment with an EphR-binding agent, immunoconjugate or pharmaceutical composition as described herein. "Treating the cancer" also includes reducing tumour mass and/or reducing tumour burden (for example, brain tumour mass and/or brain tumour burden). Optionally, tumour mass and/or tumour burden is reduced by at least 5, 10, 25, 50, 75 or 100% following treatment with an EphR-binding agent, immunoconjugate or pharmaceutical composition as described herein. In other embodiments, "treating the cancer" includes reducing the aggressiveness, grade and/or invasiveness of a tumour. The tumour is optionally a newly formed tumour or a tumour already present at the time of treatment.

[00136] In one embodiment, the active ingredient may be used in combination with at least one additional therapeutic agent. Accordingly, the application provides a method of preventing or treating a cancer using the EphR-binding agents, immunoconjugates or pharmaceutical compositions

disclosed herein in combination with at least one additional therapeutic agent. An additional therapeutic agent may be administered prior to, overlapping with, concurrently, and/or after administration of the active ingredients. When administered concurrently, the EphR-binding agents, immunoconjugates or pharmaceutical compositions and an additional therapeutic agent may be administered in a single formulation or in separate formulations, and if administered separately, then optionally, by different modes of administration. The combination of one or more E-binding agents, immunoconjugates or pharmaceutical compositions and one or more other therapeutic agents may synergistically act to combat the cancer.

[00137] Embodiments of the additional therapeutic agent include additional EphR-binding agents, additional EphR-binding immunoconjugates, additional EphR-binding pharmaceutical compositions, cytokines, growth factor inhibitors, immunosuppressants, anti-inflammatory agents, metabolic inhibitors, enzyme inhibitors, anti-neoplastic agents, cytotoxic agents and/or cytostatic agents. Such combination therapies may advantageously utilize lower dosages of an administered active ingredient, thus avoiding possible toxicities or complications associated with monotherapy.

Examples

Example 1

[00138] The present disclosure discloses experiments in which an ephrin receptor (EphR) profiler was used to simultaneously assess the protein expression of all EphRs in cells comprising primary and recurrent glioblastoma (GBM), to identify the putative cooperative role of multiple EphRs in driving GBM tumorigenesis. The inventors showed that EphA2 and EphA3 together mark a more potent tumorigenic cancer stem cell population in recurrent GBM. The inventors also showed that co-targeting of EphA2 and EphA3 using a bispecific antibody approach impacts the functional GSC pool more effectively than monotherapies.

MATERIALS AND METHODS

[00139] **Patient Tumors:** Human GBM brain tumors were obtained from consenting patients, as approved by the Hamilton Health Sciences/McMaster
5 Health Sciences Research Ethics Board.

[00140] **Dissociation and culture of GBM tissue:** Human GBM tissue was dissociated and cells were maintained in NeuroCult complete media (StemCell Technologies; 10ng/mL bFGF, 20ng/mL EGF, and 2µg/mL Heparin) either as tumorspheres or grown adherently on poly-L-
10 ornithine/laminin.

[00141] **Eph Profiler:** Receptor-selective Abs for all 14 Eph homologs were used to profile the expression of EphRs in primary and recurrent GBM cells.

[00142] **In vitro Chemoradiotherapy:** Primary GBM BT602 were
15 treated with radiation and temozolomide as described in Qazi et al. (2015).

[00143] **Flow cytometry analysis:** The percentage expression of EphA2, EphA3 and ephrin A5 was determined on a MoFlo XDP flow cytometer (Beckman Coulter) along with Summit 5.4 software using in-house anti-EphA2 Fab conjugated to Alexa Fluor 488 as secondary antibody
20 (1:1000), in-house anti-EphA3 Fab conjugated to APC as secondary antibody, and in-house anti-EphrinA5 Fab conjugated to APC as secondary antibody. Data was analyzed with Kaluza® Flow Analysis software.

[00144] **CyTOF and viSNE analysis:** "Cytometry time-of-flight" (CyTOF), a mass spectrometry based flow assay, in which antibodies are
25 labeled with lanthanide metals rather than fluorochromes, allows a great degree of multiplexing without spectral overlap of the antibodies. Expression of EphRs along with a panel of stem cell markers implicated in GBM tumorigenesis including CD133, CD15, Bmi1, Sox2, Integrin-α6 and FoxG1 was determined. The CyTOF system, HELIOS was used along with analysis
30 platform, Cytobank and computational analysis software, viSNE to map the high-dimensional cytometry data onto two dimensions for co-expression analysis. Commercial lanthanide metal tags were selected using Fluidigm's

Maxpar Panel Designer and conjugated to commercial and synthetically-raised monoclonal IgGs using Fluidigm's MAXPAR antibody labeling kit. Synthetically-raised monoclonal Fabs used for analysis were pre-clustered to anti-Fab IgGs conjugated with metal isotope tags.

5 [00145] **Sphere formation and proliferation assay:** After primary sphere formation was noted, spheres were dissociated to single cells and re-plated in 0.2 mL Neurocult complete media as previously published (Singh et al., 2003; 2004). Briefly, neurospheres were treated with Liberase Blendzyme® 3 and plated at 200 cells/well density for sphere formation assay
10 and 1000 cells/well for proliferation assay in a 96 well microwell plate in 0.2 mL volume of Neurocult complete media. The spheres were counted 3 days later. Proliferation was measured using PrestoBlue® cell viability reagent (Thermo Fisher Scientific).

[00146] **Limiting dilution assay:** Cells were plated at limiting dilution
15 from 150 cells to 1 cell per well in 200 µL of Neurocult complete media in a 96-well plate and 0.37 intercepts were calculated to determine the sphere-forming frequency (Singh et al., 2003).

[00147] **Real-time quantitative PCR:** Total RNA was isolated using NorgenTotal RNA Purification kit. cDNA was synthesized by iScript
20 cDNAsupermix (Quanta Biosciences) followed by real-time quantitative PCR using SsoAdvanced™ Universal SYBR®GreenSupermix (Bio-Rad). Samples were quantified using CFX Manager™ software. Data is presented as the ratio of the gene of interest to GAPDH or bActin.

[00148] **Cell cycle and apoptosis analysis:** Cells were stained for DNA
25 cell cycle using DNA Prep Reagent Kit (Beckman Coulter) and analyzed via flow cytometry (MoFlo XDP, Beckman Coulter). Annexin V conjugated to APC was used along with 7-AAD viability for analysis of apoptosis in cells of interest using flow cytometry.

[00149] **Orthotopic Xenografts:** Animal studies were performed
30 according to guidelines under Animal Use Protocols of McMaster University Central Animal Facility. rGBM BT241 cells were sorted based on expression of EphA2 and EphA3 and intracranially injected into right frontal lobes of 5-8

- week old NOD-SCID mice. Animals were sacrificed when control mice from the experiment showed signs of tumor formation (head swelling, hunching, rough coat, weight loss). For EphA2 and EphA3 knockdown in BT241, 100,000 live cells were intracranially injected in 5-8 week old NOD-SCID mice. For EphA2/A3 BsAb treatment, 100,000 BT241 cells were intracranially injected in the right frontal lobe of 6-8 week old NSG mice. Intracranial treatment with BsAb (in-house) or control IgG (Jacksons AffiniPure Goat Anti-Human IgG, F(ab')₂ fragment specific) was started 10-14 days later and continued for twice a week until control mice succumbed to disease burden. Mice were perfused with 10% formalin and collected brains were sliced at 2mm thickness using brain-slicing matrix. Sections were paraffin-embedded and multiple immunohistochemical tests were performed (H&E, EphA2, EphA3 and GFAP). For in vitro experiments, GBM cells were treated with 200nM of BsAb or control IgG.
- [00150] **Western Blot:** For western blotting, Santa-Cruz antibodies EphA2 (sc-924, 1:500) and EphA3 (sc-919, 1:1,000), and Cell Signaling antibodies pEphA2 (12677S, 1:1,000), pEphA3 (8862S, 1:1,000), Erk1/2 (4695S, 1:1,000), pERK1/2 (4377S, 1:1,000), Akt (4691S, 1:1,000), pAkt (4051S, 1:1,000) were used. GAPDH was used as a loading control.
- [00151] **Statistical Analysis:** All quantitative data presented are the mean±SEM. Samples used and respective n values are listed in the figure legends. The level of significance was determined by Student's two-tailed t-test or ANOVA using GraphPad Prism 5 software.

RESULTS

EPHA2 AND EPHA3 ARE OVEREXPRESSED IN GLIOBLASTOMA AND LEVELS THEREOF CORRELATE WITH DISEASE SEVERITY

EphRs are expressed heterogeneously in human GBM cells and co-express with stem cell markers

- [00152] The surface protein expression of all 14 members of the Eph receptor family in primary, treatment-naïve (pGBM cells: BT428, BT458, BT459, BT465, BT486, BT602 and BT648) and recurrent (rGBM cells: BT241, BT566 and BT618) GSCs was profiled using the Eph profiler. Without being

bound by theory, the heterogeneous expression of all EphRs in human GSCs (Fig. 1a) suggest a variety of signaling paradigms that might drive oncogenesis in these patients. It was observed that EphA2 and EphA3 were expressed at moderate to high levels across all GSC lines, and accordingly,
5 their expression was characterized in human neural stem and progenitor cells (NSPCs), pGBM cells and rGBM cells by flow cytometry.

[00153] The amino acid sequences of human EphA2 and human EphA3 are shown in Table 1 and Table 2, respectively.

Table 1. Amino acid sequence of human EphA2 (GenBank Accession P29317).

```

MELQAARACFALLWGCALAAAAAQQGKEVVLLDFAAAGGELGWLTHPYGKGWDLMQNIMNDMPIYMYSCVN
VMSGDQDNWLRNWNVYRGEAERIFIELKFTVRDCNSFPGGASSCKETFNLYYAESDLDYGTNFQKRLFTKI
DTIAPDEITVSSDFEARHVKLNVEERSVGPLTRKGFYLAQDQIGACVALLSVRVYKKCPPELLQGLAHFPE
TIAGSDAPSLATVAGTCVDHAVVPPGGEEPRMHCAVDGEWLVPIGQCLCQAGYEKVEDACQACSPGFFKFE
ASESPCLECPEHTLPSPEGATSCCEEGFFRAPQDPASMPCTRPPSAPHYLTAVGMGAKVELRWTPPQDSG
GREDIVYSVTCEQCWPESGECGPCEASVRYSEPPHGLTRTSVTVSDLEPHMNYTFTVEARNGVSGLVTSRS
FRTASVSINQTEPPKVRLEGRSTTSLSVSWSIPEPPQQSRVWKYEVTRYKKGDSNSYNVRTEGFSVTLDDL
APDTTYLVQVQALTQEGQGAGSKVHEFQTLSPEGSGNLAVIGGVAVGVVLLLVLAGVGFFIHRRRKNQRRAR
QSPEDVYFSKSEQLKPLKTYVDPHTYEDPNQAVLKFTTEIHPSCVTRQKVI GAGEFGEVYKGMKLTSSGKK
EVPVAIKTLKAGYTEKQRVDFLGEAGIMGQFSHHNIIIRLEGVISKYKPMIITEYMENGALDKFLREKDGE
FSVLQLVGMRLGIAAGMKYLANMNYVHRDLAARNILVNSNLVCKVSDFGLSRVLEDDPEATYTTSGGKIPI
RWTAPAEISYRKFTSASDVWSFGIVMWVMTYGERPYWELSNHEVMKAINDGFRLLPTPMDCPSAIYQLMMQ
CWQQERARRPKFADIVSILDKLIRAPDSLKTLADFDPRVSI RLPSTSGSEGVPFRTVSEWLESIKMQQYTE
HFMAAGYTAIEKVVQMTNDDIKRIGVRLPGHQKRIAYSLGLKQVNTVGIP (SEQ ID NO: 1)

```

Table 2. Amino acid sequence of human EphA3 (GenBank Accession P29320).

5

```

MDCQLSILLLLSCSVLDSFGELIPQPSNEVNLLDSKTIQGELGWISYPHGWEEISGVDEHYTPIRTYQVC
NVMDHSQNNWLRNWNVPRNSAQKIYVELKFTLRDCNSIPLVLGTCKETFNLYYMESDDDHGVKFRHQFTK
IDTIAADESFTQMDLGDRILKLNTEIREVGVNKKGFYLAQDVGACVALVSVRVYFKCPTVKNLAMFP
DTVPMDSQSLVEVRGSCVNNSKEEDPPRMYCSTEGEWLVPIGKCSNAGYEERGFMCQACRPGFYKALDGN
MKCAKCPPHSTQEDGSMNCRCENNYFRADKDPSPMACTRPPSSPRNVISININETSIVLDWSWPLDTGGRK
DVTFNIICKKCGWNIKQCEPCSPNVRFLPRQFGLTNTTVTVDLLAHTNYTFEIDAVNGVSELSSPPRQFA
AVSITTNQAAPSPVLTIKKDRTSRNSISLSWQEPHEPNGIILDYEVKYYEKQEQTSTYILRARGTNVTIS
SLKPDITYVFQIRARTAAAGYGTNSRKFEFETSPDSFSSISGESSQVVMIAISAAVAIIILLTVVIYVLIGRFC
GYKSKHGADEKRLHFGNGHLKLPGLRTYVDPHTYEDPTQAVHEFAKELDATNISIDKVVGAGEFGEVCSGR
LKLPSKKEISVAIKTLKVGYTEKQRRDFLGEASIMGQFDHPNIIIRLEGVVTKSKPVMIVTEYMENGSLDSF
LRKHDAQFTVIQLVGMRLRGIASGMKYLSDMGVHRDLAARNILINSNLVCKVSDFGLSRVLEDDPEAAAYTT
RGGKIPIRWTSPEAIAIRKFTSASDVWSYGVVLWEVMSYGERPYWEMSNQDVIKAVDEGYRLPPPMDCPAA
LYQLMLDCWQKDRNNRPKEQIVSILDKLIRNPGSLKIITSAAARPSNLLLDQSNVDITTFRTTGDWLNQV
WTAHCKEIFTGVEYSSCDTIKISTDDMKKVGVTVVGPPQKKIISIKALETQSKNGPVPV (SEQ ID NO: 2)

```

[00154] Flow cytometry analysis indicated that EphA2 and EphA3 expression is enriched in rGBM compared to pGBM (Fig. 1b). A stem cell culture model of GBM recurrence, pGBM BT602 (Qazi et al., 2016) was subjected to an in vitro chemoradiotherapy protocol, and increased expression of both EphA2 and EphA3 post-treatment was noted (Fig. 1c). Primary GBM cells and rGBM cells were then profiled for all EphRs along with a panel of GSC markers including CD133, CD15, Bmi1, Sox2, Integrin- α 6 and FoxG1, using mass “cytometry time-of-flight” (CyTOF) assays. CyTOF, which employs antibodies labelled with lanthanide metals rather than fluorochromes, permits a greater degree of multiplexing than traditional flow cytometry and

the simultaneous quantification of numerous cell surface targets. It was found that although GBM cells display heterogeneous expression of these markers at the single cell level (Fig. 1d, plots shown for BT241), there is heightened intensity of EphA2 and EphA3 co-localizing with GSC marker expression (Fig. 1e, population in circle). In fact, BT241, a rGBM sample, co-expressed EphA2 and EphA3 with all GSC markers in a population twice as large as that of two pGBMs, BT459 and BT602 (1.30% in BT241 vs 0.52% and 0.43% in BT459 and BT602, respectively).

EphA2 and EphA3 are highly expressed in GBM, overrepresented in poor-outcome subgroups of GBM and have higher expression in rGBM

[00155] Since the data herein shows that EphA2 and EphA3 are expressed at higher levels at GBM recurrence, the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) database was interrogated for EphA2 and EphA3 expression. Both EphA2 and EphA3 are highly expressed in GBM compared to low-grade oligodendrogliomas and astrocytomas (Fig. 2a). In addition, EphA2 and EphA3 expression was higher in classical and mesenchymal subgroups of GBM, which have a slightly worse outcome (Verhaak et al., 2010) when compared to the better performing pro-neural subgroup (Fig. 2b). Higher expression of both EphA2 and EphA3 predicted poor survival in GBM patients (Fig. 2c and d). More importantly, EphA2 and EphA3 co-expression was higher in rGBM patients compared to primary GBM patients (Fig. 2e). Similar results were found when EphA2 and EphA3 expression was compared in six paired-pGBM and rGBM samples from the TCGA dataset (Fig. 2f). This data shows that EphA2 and EphA3 co-identify an even more potent GSC population in rGBM cells than expression of either EphR alone.

EphA2 and EphA3 co-expression marks a highly tumorigenic GSC population in rGBM

[00156] To reinforce the correlation between EphA2 and EphA3 co-expression in rGBM GSCs, rGBM cells were FACS-sorted into four pools, expressing either low EphA2 and EphA3 (EphA2-/EphA3-), high EphA2 only (EphA2+/EphA3-), high EphA3 only (EphA2-/EphA3+) and high EphA2 and

EphA3 (EphA2+/EphA3+) (Fig. 3a), and then their in vitro clonogenicity and intracranial tumorigenic capacity was assessed. The EphA2+/EphA3+ fraction contained the most clonogenic cells (Fig. 3b) compared to the EphA2-/EphA3- cells, with the EphA2+/EphA3- and EphA2-/EphA3+ cells presenting intermediate clonogenic capacity. The same trend was seen in the proliferation capacity of these fractionated cell populations (Fig. 3c). The expression of key GSC markers was assessed in EphA2-/EphA3- and EphA2+/EphA3+ rGBM fractions and no difference in expression of CD133 or CD15 was found but a significantly higher expression of Bmi1 and Sox2 in EphA2+/EphA3+ cells was found compared to EphA2-/EphA3- cells (Fig. 3d). Despite very low percentage of EphA2+/EphA3- cell population and low sorting efficiency of rGBM BT241, the cells were sorted for intracranial injections. Mice were intracranially implanted with the sorted cell populations. It was found that EphA2+/EphA3+ cells give rise to much larger tumors compared to EphA2-/EphA3- cells, with EphA2+/EphA3- and EphA2-/EphA3+ cells giving rise to intermediate-sized tumors, replicating the in vitro clonogenic data (Fig. 3e). In vivo limiting dilution intracranial transplantation assays using EphA2-/EphA3- and EphA2+/EphA3+ cells confirmed that high EphA2 and EphA3 are hallmarks of GSCs in rGBM and can be used for their enrichment (Fig. 3f). EphA2+/EphA3+ cells were able to give rise to tumors with as few as 4,000 cells compared to 40,000 cells when implanting EphA2-/EphA3- cells.

Loss of EphA2 and EphA3 inhibits clonogenicity and tumor formation capacity of rGBM cells

[00157] Next, the effect of EphA2 and EphA3 knockdown (KD) on in vitro clonogenicity and intracranial tumorigenic capacity of rGBM cells was investigated. Small-hairpin RNA (shRNA) to KD either EphA2 or EphA3 were used individually or in a combined fashion in two rGBM samples (Fig. 4a). It was found that that combined EphA2 and EphA3 KD led to greater loss in clonogenic capacity of rGBM cells as compared to single EphA2 or EphA3 KD (Fig. 4b). In addition, proliferative capacity was only significantly inhibited in cells with double-KD compared to single EphA2 or EphA3 KD (Fig. 4c). Furthermore, the combined EphA2 and EphA3 KD led to decreased

expression of all GSC markers in rGBM cells, suggesting loss of the undifferentiated, stem-like state (Fig. 4d). In fact, KD of EphA2 and EphA3 increased levels of GFAP in rGBM, further indicating that the decrease in EphA2 and EphA3 directs rGBM cells to a more differentiated, astrocytic lineage (Fig. 4e). Next, these cells were intracranially implanted in mice. It was found that combined KD of EphA2 and EphA3 completely prevented the cells from forming tumors in half of the transplanted mice (2/4 mice formed tumors with shEphA2/A3 cells), while EphA3 KD formed tumors as large as control shGFP and EphA2 KD formed intermediate-sized tumors (Fig. 4f). The KD of EphA2 and EphA3 also affects the cell cycle of rGBM, decreasing the percentage of cells in DNA replication S phase and increasing percentage of cells in quiescent G0G1 phase (Fig. 7a). An increase in apoptosis of rGBM with shEphA2/A3 was also noted, illustrating that EphA2 and EphA3 are integral to cell survival (Fig. 7b). These results show that EphA2 and EphA3 can be co-targeted to inhibit rGBM clonogenicity, proliferation and tumorigenic capacity through a differentiation mechanism to astrocytic cell type.

ANTI-EPHA2 / ANTI-EPHA3 BISPECIFIC ANTIBODY EPHA2/A3 BSAB DEMONSTRATES ANTI-TUMOR EFFECT AGAINST GLIOBLASTOMA IN VITRO

20 Co-targeting of EphA2 and EphA3 with bispecific antibody (BsAb) decreases EphA2 and EphA3 surface expression and limits Akt and Erk1/2 Pathway activation in rGBM

[00158] A bispecific antibody was designed that co-targets both EphA2 and EphA3 (EphA2/A3 BsAb; in-house) with high affinity (Fig. 5a and Fig. 8).

25 [00159] As shown in Fig. 9, EphA2/A3 BsAb is a heterodimer composed of a single-chain EphA2-binding subunit and a single-chain EphA3-binding subunit. The EphA2-binding subunit consists of an EphA2-binding antibody heavy chain variable region-derived segment ("VH-A2") fused via a FLAG tag-containing linker to a truncated antibody heavy chain constant region-derived segment ("CH-A2K") consisting of a carboxy-terminal segment of hinge region, CH2 domain and CH3 domain. Similarly, the EphA3-binding subunit consists of an EphA3-binding antibody heavy chain variable region-derived

30

segment ("VH-A3") fused via a FLAG tag-containing linker to a truncated antibody heavy chain constant region-derived segment ("CH-A3H") consisting of a carboxy-terminal segment of hinge region, CH2 domain and CH3 domain. Point mutations H35G, Q39R, L45E and W47L were introduced into
5 framework region 2 (FR2) of the VH-A2 and VH-A3 segments to promote functionality in the absence of antibody light chain. Point mutation G16R was introduced into framework region 1 (FR1) of the VH-A2 and VH-A3 segments to improve binding of the subunits to protein A. The sequences of the CH-A2K and CH-A3H segments were engineered to create a "knob" structure and a
10 complementary "hole" structure, respectively, to enable knobs-into-holes (KIH)-based heterodimerization of the EphA2-binding subunit and the EphA3-binding subunit.

[00160] The amino acid sequences of the EphA2-binding subunit and the EphA3-binding subunit are shown in Table 3. The amino acid sequences
15 of segments of the EphA2-binding subunit and of segments of the EphA3-binding subunit are shown in Table 4 and Table 5, respectively.

Table 3. Amino acid sequences of EphA2-binding subunit and EphA3-binding subunit.

EphA2-binding subunit	EVQLVESGGGLVQPGRSLRLSCAASGFNIKDTYIGWVRRAPGKGEELVARIYPSSGSTSYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARYPWWYSYYPYGGAMDYWGQGTLVTVSS ASTDYKDDDDKRS DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPMVFDLPPSREEMTKNQVSLWCMVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 3)
EphA3-binding subunit	EVQLVESGGGLVQPGRSLRLSCAASGFNIKDTYIGWVRRAPGKGEELVARIYPSSGYTSYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARPYYYAGGYWVGFYWGQGTLVTVSSASTDYKDDDDKRS DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPIRELMTSNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 4)

Table 4. Segments of EphA2-binding subunit and amino acid sequences thereof.

Segment of EphA2-binding subunit	Amino acid sequence coordinates relative to SEQ ID NO: 3	Amino acid sequence
VH-A2	1-125	EVQLVESGGGLVQPGRSLRLSCAASGFNIKDTYIGWVRRAPGKGEELVARIYPSSGSTSYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARYPWWYSYYPYGGAMDYWGQGTLVTVSS (SEQ ID NO: 5)
CDR1*	26-33	GFNIKDTY (SEQ ID NO: 6)
CDR2	51-58	IYPSSGST (SEQ ID NO: 7)
CDR3	97-114	ARYPWWYSYYPYGGAMDY (SEQ ID NO: 8)
FR1*	1-25	EVQLVESGGGLVQPGRSLRLSCAAS (SEQ ID NO: 9)
FR2*	34-50	IGWVRRAPGKGEELVAR (SEQ ID NO: 10)
FR3*	59-96	SYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC (SEQ ID NO: 11)
FR4*	115-125	WGQGTLVT (SEQ ID NO: 12)
FR1 mutation G16R*	16	R
FR2 mutation H35G*	35	G
FR2 mutation Q39R*	39	R
FR2 mutation L45E*	45	E
FR2 mutation W47L*	47	L
Linker*	126-138	ASTDYKDDDDKRS (SEQ ID NO: 13)

CH-A2K	139-365	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPMVFDLPISR EEMTKNQVSLWCMVKGFYPSDIAVEWESNGQPENNYKTTPVLDS DGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNYHTQKSLSLSP GK (SEQ ID NO: 14)
* identical sequence in EphA2-binding subunit and EphA3-binding subunit		

Table 5. Segments of EphA3-binding subunit and amino acid sequences thereof.

Segment of EphA3-binding subunit	Amino acid sequence coordinates relative to SEQ ID NO: 4	Amino acid sequence
VH-A3	1-122	EVQLVESGGGLVQPGRSRLRLSCAASGFNIKDTYIGWVRRAPGKGE ELVARIYPSSGYTSYADSVKGRFTISADTSKNTAYLQMNSLRAED TAVYYCARPYYYAGGYWVGFDYWGQGLTVTVSS (SEQ ID NO: 15)
CDR1*	26-33	GFNIKDTY (SEQ ID NO: 6)
CDR2	51-58	IYPSSGYT (SEQ ID NO: 16)
CDR3	97-112	ARPYYYAGGYWVGFDY (SEQ ID NO: 17)
FR1*	1-25	EVQLVESGGGLVQPGRSRLRLSCAAS (SEQ ID NO: 9)
FR2*	34-50	IGWVRRAPGKGEELVAR (SEQ ID NO: 10)
FR3*	59-96	SYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYY (SEQ ID NO: 11)
FR4*	113-122	WGQGLTVTVSS (SEQ ID NO: 12)
FR1 mutation G16R*	16	R
FR2 mutation H35G*	35	G
FR2 mutation Q39R*	39	R
FR2 mutation L45E*	45	E
FR2 mutation W47L*	47	L
Linker*	124-136	ASTDYKDDDDKRS (SEQ ID NO: 13)
CH-A3H	137-363	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPIR ELMTSNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPVLDS DGSFFLVSKLTVDKSRWQQGNVFCSCVMHEALHNYHTQKSLSLSP GK (SEQ ID NO: 18)
* identical sequence in EphA2-binding subunit and EphA3-binding subunit		

- [00161] To enable knobs-in-holes-mediated heterodimerization of the EphA2-binding subunit and the EphA3-binding subunit, point mutations Q127M, Y129F, T130D and S146W were inserted into segment CH-A2K at positions 127, 129, 130 and 146 (relative to SEQ ID NO: 14) to form the “knob” structure, and, complementarily, point mutations S134I, E137L, K140S, T146S, M148A and Y187V were inserted into segment CH-A3H at positions 134, 137, 140, 146, 148 and 187 (relative to SEQ ID NO: 18) to form the “hole” structure.
- 10 [00162] The CH-A2K and CH-A3H segments correspond to the segment spanning amino acid residues 104-330 of human IgG1 (GenBank Accession P01857.1, SEQ ID NO: 19). The amino acid sequence of human IgG1 (GenBank Accession P01857.1, SEQ ID NO: 19) is shown in Table 6.

15 **Table 6. Amino acid sequence of human IgG1 (GenBank Accession P01857.1).**

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT TPFVLDSDGSFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 19).
--

- [00163] Thus, segment CH-A2K containing KIH knob structure-creating point mutations Q127M, Y129F, T130D and S146W at positions 127, 129, 130 and 146 (relative to SEQ ID NO: 14), respectively, corresponds to the segment of a human Ig molecule [e.g. human IgG1 (SEQ ID NO: 19)] spanning amino acid positions 104-330 in which Glu, Tyr, Thr, and Ser residues have been inserted at positions 230, 232, 233 and 249, respectively, for creating a KIH knob structure. Similarly, segment CH-A3H containing KIH hole structure-creating point mutations S134I, E137L, K140S, T146S, M148A and Y187V at positions 134, 137, 140, 146, 148 and 187 (relative to SEQ ID NO: 18), respectively, corresponds to the segment of a human Ig molecule [e.g. human IgG1 (SEQ ID NO: 19)] spanning amino acid positions 104-330 in which Ile, Leu, Ser, Ser, Ala or Val residue have been inserted at positions

237, 240, 243, 249, 251 and 290, respectively, for creating a KIH hole structure.

[00164] The nucleotide sequences encoding the EphA2-binding subunit and the EphA3-binding subunit are shown in Table 7. Segments of the EphA2-binding subunit and of the EphA3-binding subunit, and nucleotide sequences encoding these are shown in Table 8 and Table 9, respectively.

Table 7. Nucleotide sequences encoding the EphA2-binding subunit and the EphA3-binding subunit.

EphA2-binding subunit	GAGGTTTCAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGGCGTTCACTCCGTTTGTCTGTGCAGCTTCTGGCTTCAACATAAAAGATACTTATATCGGCTGGGTGCGTCGTGCCCCGGGTAAGGGCGAGGAAC TGTTGCACGTATTTATCCTTCTTCTGGCTCTACTTCTTATGCCGATAGCGTCAAGGGCCGTTTCACTATAAGCGCAGACACATCCAAAAACACAGCTACCTACAAATGAACAGCTTAAGAGCTGAGGACACTGCCGTCTATTATTGTGCTCGCTACCCGTGGTGGTACTCTTACTACCCGTACGGTGGTGCATGGACTACTGGGGTCAAGGAACCCGTGGTCACCGTCTCCTCGGCCTCCACCGATTACAAAGATGACGATGACAAAAGATCTGACAAAACACACATGCCACCGTGCCAGCACCTGAACCTCTGGGGGGACCGTCAGTCTTCTCTTCCCCCAAAACCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGCGAGGAGCAGTACAACAGCAGTACCGTGTGGTCAGCGTCTCACCCTGCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCAATGGTGTGTTGACCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAAGCTGTGGTGCATGGTCAAGGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTGTACAGCAAGCTCACCGTGGACAAGAGCCGCTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGA (SEQ ID NO: 20)
EphA3-binding subunit	GAGGTTTCAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGGCGTTCACTCCGTTTGTCTGTGCAGCTTCTGGCTTCAACATAAAAGATACTTATATCGGCTGGGTGCGTCGTGCCCCGGGTAAGGGCGAGGAAC TGTTGCACGTATTTATCCTTCTTCTGGCTATACTTCTTATGCCGATAGCGTCAAGGGCCGTTTCACTATAAGCGCAGACACATCCAAAAACACAGCTACCTACAAATGAACAGCTTAAGAGCTGAGGACACTGCCGTCTATTATTGTGCTCGCCGTACTACTACGCTGGTGGTTACTGGGTTGGTTTTGACTACTGGGGTCAAGGAACCCTGTACCCGTCTCCTCGGCCTCCACCGATTACAAAGATGACGATGACAAAAGATCTGACAAACTCACACATGCCACCGTGCCAGCACCTGAACCTCTGGGGGACCGTCAGTCTTCTTCTCCCCCAAAACCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCTCACCCTGCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCAATCCGGGAGCTGATGACCAACAACAGGTCAAGCTGAGCTCGCCGTCAAAGGCTTCTATCCAGCGACATCGCCGTGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTCGTGAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGA (SEQ ID NO: 21)

Table 8. Segments of EphA2-binding subunit and nucleotide sequences encoding same.

Segment of EphA2-binding subunit	Nucleotide sequence coordinates relative to SEQ ID NO: 20	Nucleotide sequence
VH-A2	1-375	GAGGTTTCAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGGC GTTCACTCCGTTTGTCTGTGCAGCTTCTGGCTTCAACATAAAAGA TACTTATATCGGCTGGGTGCGTCGTGCCCCGGGTAAGGGCAGGAA CTGGTTGCACGTATTTATCCTTCTTCTGGCTCTACTTCTTATGCCG ATAGCGTCAAGGGCCGTTTCACTATAAGCGCAGACACATCCAAAAA CACAGCCTACCTACAAATGAACAGCTTAAGAGCTGAGGACACTGCC GTCTATTATTGTGCTCGCTACCCGTGGTGGTACTCTTACTACCCGT ACGGTGGTGCTATGGACTACTGGGGTCAAGGAACCTGGTCACCGT CTCCTCG (SEQ ID NO: 22)
CDR1*	76-99	GGCTTCAACATAAAAGATACTTAT (SEQ ID NO: 23)
CDR2	151-174	ATTTATCCTTCTTCTGGCTCTACT (SEQ ID NO: 24)
CDR3	289-342	GCTCGCTACCCGTGGTGGTACTCTTACTACCCGTACGGTGGTGCTA TGGACTAC (SEQ ID NO: 25)
FR1*	1-75	GAGGTTTCAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGGC GTTCACTCCGTTTGTCTGTGCAGCTTCT (SEQ ID NO: 26)
FR2*	100-150	ATCGGCTGGGTGCGTCGTGCCCCGGGTAAGGGCAGGAACTGGTTG CACGT (SEQ ID NO: 27)
FR3*	175-288	TCTTATGCCGATAGCGTCAAGGGCCGTTTCACTATAAGCGCAGACA CATCCAAAAACACAGCCTACCTACAAATGAACAGCTTAAGAGCTGA GGACACTGCCGTCTATTATTGT (SEQ ID NO: 28)
FR4*	343-37	TGGGGTCAAGGAACCTGGTCACCGTCTCCTCG (SEQ ID NO: 29)
FR1 mutation G16R*	46-48	CGT
FR2 mutation H35G*	103-105	GGC
FR2 mutation Q39R*	115-117	CGT
FR2 mutation L45E*	133-135	GAG
FR2 mutation W47L*	139-141	CTG
Linker*	376-414	GCCTCCACCGATTACAAAGATGACGATGACAAAAGATCT (SEQ ID NO: 30)
CH-A2K	415-1098	GACAAAACCTCACACATGCCACCGTGCCAGCACCTGAACTCCTGG GGGGACCGTCAGTCTTCTCTTCCCCCAAACCCAAGGACACCCCT CATGATCTCCCGGACCCCTGAGGTACATGCGTGGTGGTGGACGTG AGCCACGAAGACCCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCG TGGAGGTGCATAATGCCAAGACAAAGCCGCGCAGGAGCAGTACAA CAGCACGTACCGTGTGGTCAGCGTCCTACCGTCTGCACAGGAC TGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC TCCCAGCCCCCATCGAGAAAACCATCTCCAAGCCAAGGGCAGCC CCGAGAACCAATGGTGTGTTTGACCTGCCCCCATCCGGGAGGAGATG ACCAAGAACCAGGTCAGCCTGTGGTGCATGGTCAAGGGCTCTATC CCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAA CAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTC

		TTCTGTACAGCAAGCTCACCGTGGACAAGAGCCGCTGGCAGCAGG GGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCA CTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGA (SEQ ID NO: 31)
* identical sequence in EphA2-binding subunit and EphA3-binding subunit		

Table 9. Segments of EphA3-binding subunit and nucleotide sequences encoding same.

Segment of EphA3-binding subunit	Nucleotide sequence coordinates relative to SEQ ID NO: 21	Nucleotide sequence
VH-A3	1-369	GAGGTTTCAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGG CGTTCACCTCCGTTTGTCTGTGCAGCTTCTGGCTTCAACATAAAA GATACTTATATCGGCTGGGTGCGTCGTGCCCCGGGTAAGGGCGAG GAACTGGTTGCACGATTTATCCTTCTTCTGGCTATACTTCTTAT GCCGATAGCGTCAAGGGCCGTTTCACTATAAGCGCAGACACATCC AAAAACACAGCCTACCTACAAATGAACAGCTTAAGAGCTGAGGAC ACTGCCGTCTATTATTGTGCTCGCCCGTACTACTACGCTGGTGGT TACTGGGTTGGTTTTGACTACTGGGGTCAAGGAACCCTGGTCACC GTCTCCTCG (SEQ ID NO: 32)
CDR1*	76-99	GGCTTCAACATAAAAGATACTTAT (SEQ ID NO: 23)
CDR2	151-174	ATTTATCCTTCTTCTGGCTATACT (SEQ ID NO: 33)
CDR3	289-336	GCTCGCCCGTACTACTACGCTGGTGGTTACTGGGTTGGTTTTGAC TAC (SEQ ID NO: 34)
FR1*	1-75	GAGGTTTCAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGG CGTTCACCTCCGTTTGTCTGTGCAGCTTCT (SEQ ID NO: 26)
FR2*	100-150	ATCGGCTGGGTGCGTCGTGCCCCGGGTAAGGGCGAGGAAGTGGTT GCACGT (SEQ ID NO: 27)
FR3*	175-288	TCTTATGCCGATAGCGTCAAGGGCCGTTTCACTATAAGCGCAGAC ACATCCAAAAACACAGCCTACCTACAAATGAACAGCTTAAGAGCT GAGGACACTGCCGTCTATTATTGT (SEQ ID NO: 28)
FR4*	337-369	TGGGGTCAAGGAACCCTGGTCACCGTCTCCTCG (SEQ ID NO: 29)
FR1 mutation G16R*	46-48	CGT
FR2 mutation H35G*	103-105	GGC
FR2 mutation Q39R*	115-117	CGT
FR2 mutation L45E*	133-135	GAG
FR2 mutation W47L*	139-141	CTG
Linker*	370-408	GCCTCCACCGATTACAAAGATGACGATGACAAAAGATCT (SEQ ID NO: 30)
CH-A3H	409-1092	GACAAAACCTCACACATGCCACCGTGCCAGCACCTGAACTCCTG GGGGGACCGTCAGTCTTCTCTTCCCCCAAACCAAGGACACC CTCATGATCTCCCGACCCCTGAGGTACATGCGTGGTGGTGGAC GTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGAC

		GGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAG TACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCAC CAGGACTGGCTGAATGGCAAGGAGTACAAGTGAAGGTCTCCAAC AAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAA GGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCAATCCGG GAGCTGATGACCAGCAACCAGGTCAGCCTGAGCTGCGCCGTCAAA GGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGG CAGCCGGAGAACAAC TACAAGACCACGCCTCCCGTGCTGGACTCC GACGGCTCCTTCTTCTCGTGAGCAAGCTCACCGTGGACAAGAGC AGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAG GCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCG GGTAAATGA (SEQ ID NO: 35)
* identical sequence in EphA2-binding subunit and EphA3-binding subunit		

[00165] The EphA2-binding subunit and the EphA3-binding subunit were expressed in cells with an N-terminal signal peptide for directing secretion of the mature subunit from which the signal peptide is cleaved. The amino acid sequence of the signal peptide used has the amino acid sequence

MNLLLILTFVAAAVA (SEQ ID NO: 36) and is encoded by the nucleotide sequence

ATGAATCTCCTGCTAATTCTTACGTTCGTCGCGGCCCGCAGTCGCC (SEQ ID NO: 37).

[00166] Recurrent GBM cells were treated with EphA2/A3 BsAb for 3 days and CyTOF was performed to identify surface co-expression of EphA2 and EphA3. It was found that treatment with EphA2/A3 BsAb leads to a complete loss of surface EphA2 receptor expression and a ~50% decrease in EphA3 surface receptor levels (Fig. 5b and c). After three-day treatment with EphA2/A3 BsAb, the EphA2/A3 BsAb itself has much lower levels of binding to rGBM consistent with the loss of target EphA2+/EphA3+ cell population (Fig. 5d).

[00167] The mechanism by which EphA2/A3 BsAb reduces EphA2 and EphA3 levels was investigated next. At baseline, rGBM cells do not display any phosphorylation of EphA2 or EphA3, but it is induced in the presence of ephrin ligands (Fig. 10a). The expression of ephrinA5 in rGBM was profiled and very minimal expression in the cells was found (Fig. 10b). Upon further investigation, ephrinA1 and ephrinA5, both of which activate EphA2 and EphA3, were found to be highly expressed in the EphA2-/EphA3- cell fraction as compared to the tumorigenic EphA2+/EphA3+ cells (Fig. 10c). Without

being bound by theory, this may illustrate a bidirectional signaling mechanism between the non-GSC EphA2-/EphA3- cells and GSCC EphA2+/EphA3+ cells which co-exist in a regulatory cancer stem cell niche (Plaks et al., 2015).

[00168] Recurrent GBM cells were treated with EphA2/A3 BsAb and
5 checked for phosphorylation of EphA2 and EphA3 as well as known downstream targets of Eph signaling using western blot. While an apparent increase in both EphA2 and EphA3 phosphorylation upon BsAb treatment was seen (Fig. 5e), it was found that commercially available antibody against pEphA3 cross-reacts to pEphA2 (Figure 11), suggesting that the observed
10 increase in EphA3 phosphorylation could potentially be due to phosphorylation of EphA2 alone. Indeed, phospho-proteomics on phosphorylated tyrosines revealed high levels of phosphorylated EphA2 peptides, but no phosphorylated EphA3 peptides were identified (data not shown). Hence, the decrease in EphA2 levels by EphA2/A3 BsAb is mediated
15 through phosphorylation and consequent internalization and degradation of EphA2 receptor, as a significant decrease was observed in total EphA2 levels after 60-minute treatment with EphA2/A3 BsAb (Fig. 5f). Meanwhile, treatment with EphA2/A3 BsAb does decrease EphA3 surface levels (Fig. 5c) through a phosphorylation-independent mechanism.

20 [00169] To further explore the mechanistic regulation of EphA2/A3 BsAb on rGBM, the activation level of downstream targets such as Akt and Erk1/2 was assessed. Although 5 minutes of treatment with EphA2/A3 BsAb leads to phosphorylation of EphA2, no difference was found in the activation of Akt and a slight decrease in activated Erk1/2 (Fig. 5e). After 15 minutes of
25 treatment with EphA2/A3 BsAb, a decrease in the activation of both Akt and Erk1/2 was observed (Fig. 5e). Even after 60 minutes of treatment with EphA2/A3 BsAb, levels of pAkt and pERK1/2 remained lower (Fig. 5f). Interestingly, in vitro treatment of rGBM with EphA2/A3 BsAb does not lead to change in expression of other EphRs as shown by CyTOF profiling (Fig. 12),
30 demonstrating a lack of compensatory response in an otherwise redundant EphR family signaling. Hence, treatment of rGBM with EphA2/A3 BsAb rapidly clears the levels of tumorigenic EphA2 and slowly reduces the EphA3 receptors in rGBM.

EphA2/A3 BsAb inhibits clonogenicity, promotes differentiation and reduces tumorigenicity of rGBM cells

[00170] To assess the functional effects of EphA2/A3 BsAb on rGBM, secondary sphere formation and proliferation assays were performed. Upon in vitro treatment of rGBM cells with EphA2/A3 BsAb, a reduction in both the clonogenicity (Fig. 6a and b) and proliferation capacity (Fig. 6c) of rGBM cells was seen. In fact, the activity of the EphA2/A3 BsAb is not limited to EphA2+/EphA3+ cell fraction alone; rather the EphA2/A3 BsAb targets EphA2+/EphA3- and EphA2-/EphA3+ cell fractions as well (Fig. 13a and Fig. 13b), illustrating the efficacy of EphA2/A3 BsAb against three subpopulations in rGBM. An in vitro limiting dilution assay of rGBM cells pre-treated with EphA2/A3 BsAb was performed and a significant decrease in stem cell frequency of rGBM treated with EphA2/A3 BsAb as compared to control cells was found (Fig. 6d; stem cell frequencies: BT241 control IgG treated 1/9 cells vs EphA2/A3 BsAb treated 1/13 cells; BT618 control IgG treated 1/37 cells vs EphA2/A3 BsAb treated 1/78 cells). To understand the mechanism of action of the EphA2/A3 BsAb, cell cycle analysis and apoptosis assays on rGBM cells treated with EphA2/A3 BsAb as compared to control were performed. It was found that loss of clonogenicity was not caused by changes in cell cycle or apoptosis after treatment with EphA2/A3 BsAb (Fig. 13c and Fig. 13d). Hence, EphA2/A3 BsAb hinders clonogenicity in rGBM GSCs independent of cell cycle and perhaps in a non-cytotoxic way. To assess whether treatment with EphA2/A3 BsAb induced a differentiation-like phenotype in rGBM, rGBM were treated with EphA2/A3 BsAb for three consecutive days. It was found that treatment with EphA2/A3 BsAb leads to an increase in the protein levels of GFAP and MAP2. Without being bound by theory, this suggests that the EphA2/A3 BsAb acts in a similar way to EphA2/A3 KD by directing rGBM cells to cellular differentiation (Fig. 6e and f).

TREATMENT OF GLIOBLASTOMA IN VIVO WITH ANTI-EPHA2/ANTI-EPHA3 BISPECIFIC ANTIBODY RESULTS IN TUMOR SIZE REDUCTION

[00171] To test the efficacy of EphA2/A3 BsAb against rGBM cells, mice engrafted with rGBM were intracranially treated with twice-weekly doses of 30µg of EphA2/A3 BsAb until control mice succumbed to disease burden.

Although treatment delivery had not been optimized due to limited tolerance for repeated intracranial dosing, limitations on the maximal volume that can be safely injected into mouse brains and lack of knowledge of the half-life of the EphA2/A3 BsAb, a 30% decrease in tumor volume was still found in mice
5 treated with EphA2/A3 BsAb as compared to control IgG (Fig. 6g). Immunohistochemistry was performed on EphA2/A3 BsAb treated tumors to determine if any residual EphA2+ and EphA3+ population survives post-treatment (Fig. 6h). Despite a decrease in EphA2 and EphA3 levels in EphA2/A3 BsAb-treated tumors, some EphA2 and EphA3 positive cells
10 survive the therapy, which could be explained by the EphA2/A3 BsAb dosage limitations described above. Similar to the CyTOF results for in vitro treatment with EphA2/A3 BsAb, an increase in GFAP-positive cells in tumors treated with EphA2/A3 BsAb is seen, which, without being bound by theory, suggests that the EphA2/A3 BsAb does indeed drive the differentiation of rGBM toward
15 the astrocytic lineage (Fig. 6i). Thus, the novel EphA2/A3 BsAb shows initial efficacy against rGBM cells that are driven by EphA2+/EphA3+ GSCs.

Discussion

[00172] GBM is a lethal disease that is refractory to standard surgery and chemoradiotherapy, with the majority of patients facing tumor re-growth
20 and uniformly fatal outcomes upon disease progression post-therapy. Intratumoral heterogeneity (ITH) at the cellular, genetic and functional level is increasingly appreciated as a key determinant of treatment failure, and poor patient survival also correlates with increased frequency of GSCs, which are also implicated in the development of treatment resistance. Meta-analysis of
25 recent clinical trials for GBM patients has also predicted the failure of monotherapy to target the well- documented complexity of ITH in GBM, highlighting the need to develop innovative and informed polytherapeutic strategies for this highly complex disease.

[00173] In the present disclosure, the first effective application of a
30 bispecific antibody in a preclinical model of recurrent human GBM is demonstrated, thereby promoting the concept of poly-targeting of multiple GSC pools that may escape therapy to drive disease recurrence. The role of EphA2 and EphA3 receptors in cooperatively driving pathogenesis of

recurrent human GBM is shown. It is reported herein that rGBM cells have enhanced expression of both EphA2 and EphA3 and it is shown that co-expression of EphA2 and EphA3 is directly correlated to the highly tumorigenic in vitro and in vivo capacity of these GSCs. Furthermore, loss of

5 EphA2 and EphA3 expression in rGBM leads to drastic decrease in self-renewal capacity of these cells and the ability to establish intracranial rGBM cells. This decrease in tumorigenicity is mediated through a loss of expression of stem cell genes and a gain in expression of differentiation markers. A novel

10 BsAb against EphA2 and EphA3 for targeting of this potent GSC population in rGBM was developed. The mechanism of action of the EphA2/A3 BsAb was mediated through phosphorylation and subsequent internalization and degradation of EphA2 receptor and decrease in surface EphA3 levels, which together led to the down-regulation of both Akt and Erk pathways. Intracranial administration of EphA2/A3 BsAb led to a reduction in tumor burden of

15 established rGBMs.

[00174] Previous studies of Eph receptors in GBM had individually identified EphA2 and subsequently EphA3 as markers of cancer stem cells in human GBM(Binda et al., 2012; Day et al., 2013). However, similar to other studies in GBM, discovery of molecular targets such as EphRs has been

20 limited to characterization in primary, de novo GBMs, with little focus on recurrent GBM biology. Recent studies have shown that rGBM presents a different molecular landscape, with unique clonal events driving therapy-resistant populations (H. Kim et al., 2015; Wang et al., 2016). The lack of adequate models combined with limited strategies for target discovery in

25 rGBM could explain the failure of new therapies in prolonging GBM patient survival, which are largely derived from the study of primary GBM alone. The current disclosure is focused on the identification of EphRs that marked a GSC population in recurrent GBM. With the Eph profiler, the differential expression of all 14 EphR in recurrent and primary GSC lines was elucidated.

30 It was found that EphA2 and EphA3 were enriched in rGBM. Further characterization of EphR expression in GBM using CyTOF showed that EphA2 and EphA3 co-expressed with multiple known GSC markers, and that this co-expression was enhanced in rGBM, possibly identifying EphA2/A3 co-

expressing cells as a stem-cell like population in rGBM. Upon analyzing a large GBM dataset (Rembrandt), high expression of EphA2 and EphA3 was identified as being characteristic of the poor-performing classical and mesenchymal subgroups of GBM and also predicted lower survival in GBM.

5 Importantly, despite the few recurrent GBM samples present in the TCGA dataset, trends of higher expression of EphA2 and EphA3 in recurrent GBM were identified as compared to primary GBM (all samples and paired-samples). Altogether, this illustrated that EphA2 and EphA3 together mark a tumorigenic GSC population exclusive to recurrent GBM.

10 [00175] In addition, fractionating rGBM into EphA2-/EphA3-, EphA2+/EphA3-, EphA2-/EphA3+ and EphA2+/EphA3+ populations showed that the highest in vitro clonogenic potential and in vivo tumorigenic potential was associated with combined high EphA2/EphA3 expression (EphA2+/EphA3+). The EphA2/EphA3 co-expressing population also had the
15 highest expression of known GSC markers, Bmi1 and Sox2, validating the CyTOF data. The mutual cooperation of EphA2 and EphA3 in driving rGBM GSC was reinforced by the fact that knockdown of both EphA2 and EphA3 was required to significantly reduce in vitro tumorigenicity of rGBM. In fact, intracranial injection of rGBM with double EphA2/EphA3 knockdown
20 abrogated tumor initiation in half of the mice, while single EphA2 or EphA3 knockdown still lead to initiation of tumors in all mice. Additionally, knockdown of both EphA2 and EphA3 led to a significant increase in astrocytic differentiation marker GFAP, suggesting the decrease in tumorigenicity is driven by an increase in differentiation of GBM cells. For the first time, it is
25 shown that two EphR together mark and drive a highly potent GSC population in recurrent GBM, where loss of EphA2 and EphA3 together promotes differentiation of GBM.

[00176] A poly-targeting strategy through the development of a bispecific antibody against both EphA2 and EphA3 driven GSC population in recurrent
30 GBM is shown herein. The treatment of rGBM with EphA2/A3 BsAb reduced clonogenicity and proliferative capacity of the cells, mediated through a reduction in EphA2 and EphA3 levels which in turn down regulated Akt and Erk1/2, known oncogenic pathways in GBM. The attenuation of EphA2 and

EphA3 by EphA2/A3 BsAb also resulted in partial differentiation as evidenced by increase in GFAP and MAP2 levels, mimicking the effect of knockdown of these receptors.

[00177] The efficacy of EphA2/A3 BsAb in reducing established recurrent GBM xenografts was tested via twice weekly intracranial doses of the EphA2/A3 BsAb and a reduction in tumor growth, a decrease in EphA2 and EphA3 and an increase in GFAP levels was seen. The EphA2/A3 BsAb hence dually targets a highly tumorigenic, multi-target driven GSC population in recurrent GBM through the promotion of a differentiation phenotype. The mechanism of action of the BsAb is through phosphorylation and internalization of the EphA2 receptor, leading to its degradation, whereas the decrease in EphA3 at the cell surface appears to be phosphorylation-independent. The latter finding is not surprising, as anti-EphA3 antibodies in clinical trials for advanced hematologic malignancies induce reduction of EphA3 levels and subsequent apoptosis and activation of antibody-dependent cell mediated cytotoxicity in treated leukemia cells, with no evidence of phosphorylation of EphA3; thus the mechanism of EphA3 receptor reduction at the cell surface remains unknown (Swords et al., 2016).

[00178] Therapeutic monoclonal antibodies have several major limitations in their mode of action, including redundancy of molecular pathways leading to tumor cell survival, effects of the microenvironment, and activation of inhibitory receptors. To overcome the functional redundancy among pro-tumorigenic signaling pathways a bispecific antibody modality was empirically applied to target heterogeneous GSC populations, while also blocking the activity of the pro-tumorigenic non-GSC populations that comprise the tumor niche. Comprehensive profiling of the entire EphR family in recurrent human GBM and in-depth functional characterization of GSC populations that contribute to ITH have together generated a novel, empiric poly-targeted immunotherapy that offers a new and promising treatment paradigm for patients with recurrent GBM.

Example 2

[00179] Many of the 14 members of the erythropoietin-producing hepatocellular carcinoma receptor (EphR) family are expressed in GBM cells and constitute potential molecular targets for novel therapeutic agents.

- 5 However, the EphR-ephrin signaling mechanism is complex, and the observed 'multiplicity of representation' of EphRs in GBM contributes to functional redundancy and futility of single receptor targeting.

- [00180] Using a unique model for GBM recurrence combined with a highly specific, novel collection of synthetic mAbs to all Ephs, a
10 comprehensive expression profile of the EphR family in multiple GBM PDLs was generated. In the present disclosure, EphA2 and EphA3 were identified together as marks for tumorigenic brain tumor initiating cell (BTIC) populations from recurrent GBM, with higher self-renewal, proliferation and in vivo tumorigenic potential as compared to single positive or double negative cell
15 populations. Knock-down of EphA2 and EphA3 also decreases the self-renewal and proliferation capacity of recurrent GBM cells. Bispecific antibodies against both EphA2 and EphA3 were engineered and the dual targeting of these receptors decreased tumor volume in mice harbouring intracranial human recurrent GBM. Phospho-proteomic analysis shows that
20 the bispecific antibodies were able to modulate the downstream signalling partners of EphA2 and EphA3. Hence, dual targeting of EphA2 and EphA3 by bispecific antibody may serve as a new therapeutic for recurrent GBM.

Use of bispecific antibodies targeting Eph receptors for treatment of cancer

- 25 [00181] A growing body of evidence indicates that lack of effective therapies for GBM is due to intratumoral heterogeneity, which is further complicated by redundancy in multiple signaling pathways. To address the complexity of GBM heterogeneity and treatment failure, clinically relevant, novel models of human GBM were merged with a highly sensitive and
30 comprehensive technology platform, and phosphoproteomic profiling of the dynamic signaling events that characterize treatment-refractory GBM. Characterization of treatment with bsEphA2/A3 mAb in recurrent GBM models

offer a therapeutic strategy for employing polytherapy with mAbs directed against EphRs that are expressed at recurrence.

EphR heterogeneity in Primary GBM cells

[00182] Surface expression of all 14 EphR was detected by flow
5 cytometry using a set of 50 receptor selective Fabs (Figure 1A). A comparison between three primary and three recurrent GBM BTICs showed differential EphR gene expression patterns (Figure 14A). Visensy analysis on primary GBM cells shows protein expression of stem cells markers (Figure 14B, top). The percentage of cells co-expressing of stem cell markers with EphA2 in
10 three different GBM samples is shown in circle, with the percentage listed on bottom left of each panel (Figure 14B, bottom). SPADE analysis shows heterogeneous co-expression of stem cell markers with EphA2 in primary (left) versus recurrent (right) GBM samples (Figure 14C).

EphA2 and EphA3 receptors drive self-renewal and tumorigenicity in recurrent GBM

[00183] Self-renewal, as assessed by secondary sphere formation assay (Figure 15A, top), and proliferation (Figure 15A, bottom) capacity was determined for recurrent GBM cells sorted based on the expression of EphA2 and EphA3. EphA2+/EphA3+ had the highest self-renewal and proliferation
20 capacity, followed by intermediate self renewal and proliferation capacity of single EphA2+ and EphA3+ GBM cells as compared to EphA2-/EphA3- (double negative) population. EphA2+/EphA3+ GBM BTICs injected intracranially in NOD-SCID mice formed tumours at a low cell number (4,000 cells, Figure 15B, top panel), while EphA2-/EphA3- only formed small tumours
25 at higher cell number (40,000 cells, Figure 15B, bottom panel). Arrows represent the GBM tumours in NOD-SCIDs. Figure 15C shows lentiviral mediated knock down of EphA2 and EphA3 receptors in recurrent GBM decreased the self renewal (left panel) and proliferation (right panel) capacity of recurrent GBM cells. Recurrent GBMs in TCGA database have higher
30 expression of both EphA2 and EphA3 as compared to their matched primaries. *p<0.05, **p<0.01, ***p<0.001 (Figure 15D).

Ephrin profiling in GBM cells through chemoradiotherapy

[00184] In vitro chemoradiotherapy increases the sphere formation capacity of cells (Figure 16A). In vitro chemoradiotherapy leads to differential changes in gene expression of multiple EphR in GBM (Figure 16B). Flow cytometry shows change in protein expression of EphA and EphA3 following in vitro chemoradiotherapy (Figure 16C). Chemoradiotherapy of mice also leads to increased survival, where post-treatment cells showed increased sphere formation (Figure 16D) and proliferation (Figure 16E) capacity. Gene expression of EphA3 also increased after in vivo chemoradiotherapy. *p<0.05, **p<0.01, ***p<0.001 (Figure 16F).

Production and Validation of Ephrin family antibodies

[00185] An extremely functional (Fab')₂ library termed Library F was used to raise synthetic Abs to the entire family of human EphR receptors to produce 563 unique variants (Figure 17A). Figure 17B shows the therapeutic modalities derived from a single Ab framework using either mammalian or bacterial expression platforms. All modalities can be used as EphR signaling modulators while Fc-containing fragments can potentially elicit ADCC and CDC immune clearance. Ab fragments produced in bacteria serve as templates for phage displayed libraries. A recombinant specificity of EphA2.7 and EphA3.1 Ab modalities including monovalent Fab and monolIgG proteins as well as bivalent IgG and bsIgG proteins (Figure 17C). Figure 17D shows the effects on pEphA2 signal when targeting or co-targeting EphA2 and EphA3 receptor in A2780cis with distinct Ab modalities. Figure 17E shows the cross-species reactivity of EphA2.7 and EphA3.1 Fabs and EphA2/A2 bsIgG.

Pharmacological and functional assessment of EphR mAb and co-targeting of multiple EphA receptors

[00186] Co targeting of EphA2 and EphA3 receptors by bispecific EphA2/A3 mAb leads to pharmacological blockade of pEphA2 in recurrent GBM (Figure 18A). GBM cells were intracranially engrafted into NOD/SCID mice followed by intracranial treatment with bispecific EphA2/A3 mAb for in vivo experiment, which lead to decrease in tumour size (Figure 18B, bottom panel, arrows represent the GBM tumour in NOD/SCID mice). Figure 18C

shows the downstream effects after blocking with bispecific EphA2/3 mAb was evaluated using Western Blot analysis. Protein levels of pFAK, total FAK, pAKT, total AKT, pERK and total ERK were determined. Using densitometry it was found that co-targeting of EphA2 and EphA3 in both primary (BT602) and
5 recurrent (BT241) GBM sample leads to decreased level of pAKT and pERK in the presence of EphrinA5 ligand. In BT602, pFAK is also decreased in the presence of EphrinA5 ligand when both EphA2 and EphA3 were co-targeted. However, in BT241, co-targeting of EphA2 and EphA3 leads to increased expression of pFAK in the presence of EphrinA5 ligand.

10

References

- Bao, S., Wu, Q., McLendon, R.E., Hao, Y., Shi, Q., Hjelmeland, A.B., Dewhirst, M.W., Bigner, D., Rich, J.N., 2006. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 444, 756–760. doi:10.1038/nature05236
- Binda, E., Visioli, A., Giani, F., Lamorte, G., Copetti, M., Pitter, K.L., Huse, J.T., Cajola, L., Zanetti, N., DiMeco, F., De Filippis, L., Mangiola, A., Maira, G., Anile, C., De Bonis, P., Reynolds, B.A., Pasquale, E.B., Vescovi, A.L., 2012. The EphA2 Receptor Drives Self-Renewal and Tumorigenicity in Stem-like Tumor-Propagating Cells from Human Glioblastomas. *Cancer Cell* 22, 765–780. doi:10.1016/j.ccr.2012.11.005
- Bowman, R.L., Wang, Q., Carro, A., Verhaak, R.G.W., Squatrito, M., 2017. GliOVis data portal for visualization and analysis of brain tumor expression datasets. *Neuro-Oncology* 19, 139–141. doi:10.1093/neuonc/now247
- Brennan, C.W., Verhaak, R.G.W., McKenna, A., Campos, B., Nounshmehr, H., Salama, S.R., Zheng, S., Chakravarty, D., Sanborn, J.Z., Berman, S.H., Beroukhi, R., Bernard, B., Wu, C.-J., Genovese, G., Shmulevich, I., Barnholtz-Sloan, J., Zou, L., Vegesna, R., Shukla, S.A., Ciriello, G., Yung, W.K., Zhang, W., Sougnez, C., Mikkelsen, T., Aldape, K., Bigner, D., Van Meir, E.G., Prados, M., Sloan, A., Black, K.L., Eschbacher, J., Finocchiaro, G., Friedman, W., Andrews, D.W., Guha, A., Iacocca, M., O'Neill, B.P., Foltz, G., Myers, J., Weisenberger, D.J., Penny, R., Kucherlapati, R., Perou, C.M., Hayes, D.N., Gibbs, R., Marra, M., Mills, G.B., Lander, E., Spellman, P., Wilson, R., Sander, C., Weinstein, J., Meyerson, M., Gabriel, S., Laird, P.W., Haussler, D., Getz, G., Chin, L., TCGA Research Network, 2013. The somatic genomic landscape of glioblastoma. *Cell* 155, 462–477. doi:10.1016/j.cell.2013.09.034
- Chen, J., Li, Y., Yu, T.-S., McKay, R.M., Burns, D.K., Kernie, S.G., Parada, L.F., 2012. A restricted cell population propagates glioblastoma growth after chemotherapy. *Nature* 1–6. doi:10.1038/nature11287
- Day, B.W., Stringer, B.W., Al-Ejeh, F., Ting, M.J., Wilson, J., Ensbe, K.S., Jamieson, P.R., Bruce, Z.C., Lim, Y.C., Offenhäuser, C., Charmsaz, S.,

- Cooper, L.T., Ellacott, J.K., Harding, A., Leveque, L., Inglis, P., Allan, S., Walker, D.G., Lackmann, M., Osborne, G., Khanna, K.K., Reynolds, B.A., Lickliter, J.D., Boyd, A.W., 2013. EphA3 Maintains Tumorigenicity and Is a Therapeutic Target in Glioblastoma Multiforme. *Cancer Cell* 23, 238–248. doi:10.1016/j.ccr.2013.01.007
- 5 DeAngelis, L.M., 2001. Brain tumors. *N Engl J Med* 344, 114–123. doi:10.1056/NEJM200101113440207
- Ferluga, S., Tomé, C.M.L., Herpai, D.M., D'Agostino, R., Debinski, W., 2016. Simultaneous targeting of Eph receptors in glioblastoma. *Oncotarget* 7, 59860–59876. doi:10.18632/oncotarget.10978
- 10 Genander, M., Frisén, J., 2010. Ephrins and Eph receptors in stem cells and cancer. *Current Opinion in Cell Biology* 22, 611–616. doi:10.1016/j.ceb.2010.08.005
- Johnson, B.E., Mazar, T., Hong, C., Barnes, M., Aihara, K., McLean, C.Y., Fouse, S.D., Yamamoto, S., Ueda, H., Tatsuno, K., Asthana, S., Jalbert, L.E., Nelson, S.J., Bollen, A.W., Gustafson, W.C., Charron, E., Weiss, W.A., Smirnov, I.V., Song, J.S., Olshen, A.B., Cha, S., Zhao, Y., Moore, R.A., Mungall, A.J., Jones, S.J.M., Hirst, M., Marra, M.A., Saito, N., Aburatani, H., Mukasa, A., Berger, M.S., Chang, S.M., Taylor, B.S., Costello, J.F., 2014. Mutational analysis reveals the origin and therapy-driven evolution of recurrent glioma. *Science* 343, 189–193. doi:10.1126/science.1239947
- 15 Kim, H., Zheng, S., Amini, S.S., Virk, S.M., Mikkelsen, T., Brat, D.J., Grimsby, J., Sougnez, C., Muller, F., Hu, J., Sloan, A.E., Cohen, M.L., Van Meir, E.G., Scarpace, L., Laird, P.W., Weinstein, J.N., Lander, E.S., Gabriel, S., Getz, G., Meyerson, M., Chin, L., Barnholtz-Sloan, J.S., Verhaak, R.G.W., 2015. Whole-genome and multisector exome sequencing of primary and post-treatment glioblastoma reveals patterns of tumor evolution. *Genome Res.* gr.180612.114. doi:10.1101/gr.180612.114
- 25 Kim, J., Lee, I.-H., Cho, H.J., Park, C.-K., Jung, Y.-S., Kim, Y., Nam, S.H., Kim, B.S., Johnson, M.D., Kong, D.-S., Seol, H.J., Lee, J.-I., Joo, K.M., Yoon, Y., Park, W.-Y., Lee, J., Park, P.J., Nam, D.-H., 2015. Spatiotemporal
- 30

- Evolution of the Primary Glioblastoma Genome. *Cancer Cell* 28, 318–328. doi:10.1016/j.ccell.2015.07.013
- Lathia, J.D., Gallagher, J., Heddleston, J.M., Wang, J., Eyler, C.E., Macsworlds, J., Wu, Q., Vasanji, A., McLendon, R.E., Hjelmeland, A.B., Rich, J.N., 2010. Integrin alpha 6 regulates glioblastoma stem cells. *Cell Stem Cell* 6, 421–432. doi:10.1016/j.stem.2010.02.018
- Liu, G., Yuan, X., Zeng, Z., Tunici, P., Ng, H., Abdulkadir, I.R., Lu, L., Irvin, D., Black, K.L., Yu, J.S., 2006. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma . *Mol Cancer* 5, 67. doi:10.1186/1476-4598-5-67
- Louis, D.N., Ohgaki, H., Wiestler, O.D., Cavenee, W.K., Burger, P.C., Jouvet, A., Scheithauer, B.W., Kleihues, P., 2007. The 2007 WHO Classification of Tumours of the Central Nervous System. *Acta Neuropathol* 114, 97–109. doi:10.1007/s00401-007-0243-4
- McLendon, R., Friedman, A., Bigner, D., Van Meir, E.G., Brat, D.J., M Mastrogiannis, G., Olson, J.J., Mikkelsen, T., Lehman, N., Aldape, K., Alfred Yung, W.K., Bogler, O., VandenBerg, S., Berger, M., Prados, M., Muzny, D., Morgan, M., Scherer, S., Sabo, A., Nazareth, L., Lewis, L., Hall, O., Zhu, Y., Ren, Y., Alvi, O., Yao, J., Hawes, A., Jhangiani, S., Fowler, G., San Lucas, A., Kovar, C., Cree, A., Dinh, H., Santibanez, J., Joshi, V., Gonzalez-Garay, M.L., Miller, C.A., Milosavljevic, A., Donehower, L., Wheeler, D.A., Gibbs, R.A., Cibulskis, K., Sougnez, C., Fennell, T., Mahan, S., Wilkinson, J., Ziaugra, L., Onofrio, R., Bloom, T., Nicol, R., Ardlie, K., Baldwin, J., Gabriel, S., Lander, E.S., Ding, L., Fulton, R.S., McLellan, M.D., Wallis, J., Larson, D.E., Shi, X., Abbott, R., Fulton, L., Chen, K., Koboldt, D.C., Wendl, M.C., Meyer, R., Tang, Y., Lin, L., Osborne, J.R., Dunford-Shore, B.H., Miner, T.L., Delehaunty, K., Markovic, C., Swift, G., Courtney, W., Pohl, C., Abbott, S., Hawkins, A., Leong, S., Haipok, C., Schmidt, H., Wiechert, M., Vickery, T., Scott, S., Dooling, D.J., Chinwalla, A., Weinstock, G.M., Mardis, E.R., Wilson, R.K., Getz, G., Winckler, W., Verhaak, R.G.W., Lawrence, M.S., O'Kelly, M., Robinson, J., Alexe, G., Beroukhir, R., Carter, S., Chiang, D., Gould, J., Gupta, S., Korn, J., Mermel, C., Mesirov, J., Monti, S., Nguyen, H., Parkin, M.,

- Reich, M., Stransky, N., Weir, B.A., Garraway, L., Golub, T., Meyerson, M., Chin, L., Protopopov, A., Zhang, J., Perna, I., Aronson, S., Sathiamoorthy, N., Ren, G., Yao, J., Wiedemeyer, W.R., Kim, H., Won Kong, S., Xiao, Y., Kohane, I.S., Seidman, J., Park, P.J., Kucherlapati, R., Laird, P.W., Cope, L.,
- 5 Herman, J.G., Weisenberger, D.J., Pan, F., Van Den Berg, D., Van Neste, L., Mi Yi, J., Schuebel, K.E., Baylin, S.B., Absher, D.M., Li, J.Z., Southwick, A., Brady, S., Aggarwal, A., Chung, T., Sherlock, G., Brooks, J.D., Myers, R.M., Spellman, P.T., Purdom, E., Jakkula, L.R., Lapuk, A.V., Marr, H., Dorton, S., Gi Choi, Y., Han, J., Ray, A., Wang, V., Durinck, S., Robinson, M., Wang,
- 10 N.J., Vranizan, K., Peng, V., Van Name, E., Fontenay, G.V., Ngai, J., Conboy, J.G., Parvin, B., Feiler, H.S., Speed, T.P., Gray, J.W., Brennan, C., Socci, N.D., Olshen, A., Taylor, B.S., Lash, A., Schultz, N., Reva, B., Antipin, Y., Stukalov, A., Gross, B., Cerami, E., Qing Wang, W., Qin, L.-X., Seshan, V.E., Villafania, L., Cavatore, M., Borsu, L., Viale, A., Gerald, W., Sander, C.,
- 15 Ladanyi, M., Perou, C.M., Neil Hayes, D., Topal, M.D., Hoadley, K.A., Qi, Y., Balu, S., Shi, Y., Wu, J., Penny, R., Bittner, M., Shelton, T., Lenkiewicz, E., Morris, S., Beasley, D., Sanders, S., Kahn, A., Sfeir, R., Chen, J., Nassau, D., Feng, L., Hickey, E., Zhang, J., Weinstein, J.N., Barker, A., Gerhard, D.S., Vockley, J., Compton, C., Vaught, J., Fielding, P., Ferguson, M.L., Schaefer,
- 20 C., Madhavan, S., Buetow, K.H., Collins, F., Good, P., Guyer, M., Ozenberger, B., Peterson, J., Thomson, E., 2008. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 455, 1061–1068. doi:10.1038/nature07385
- Meyer, M., Reimand, J., Lan, X., Head, R., Zhu, X., Kushida, M., Bayani, J.,
- 25 Pressey, J.C., Lionel, A.C., Clarke, I.D., Cusimano, M., Squire, J.A., Scherer, S.W., Bernstein, M., Woodin, M.A., Bader, G.D., Dirks, P.B., 2015. Single cell-derived clonal analysis of human glioblastoma links functional and genomic heterogeneity. *Proc. Natl. Acad. Sci. U.S.A.* 201320611. doi:10.1073/pnas.1320611111
- 30 Miao, H., Gale, N.W., Guo, H., Qian, J., Petty, A., Kaspar, J., Murphy, A.J., Valenzuela, D.M., Yancopoulos, G., Hambardzumyan, D., Lathia, J.D., Rich, J.N., Lee, J., Wang, B., 2014. EphA2 promotes infiltrative invasion of glioma

- stem cells in vivo through cross-talk with Akt and regulates stem cell properties. *Oncogene*. doi:10.1038/onc.2013.590
- Nakada, M., Anderson, E.M., Demuth, T., Nakada, S., Reavie, L.B., Drake, K.L., Hoelzinger, D.B., Berens, M.E., 2009. The phosphorylation of ephrin-B2
 5 ligand promotes glioma cell migration and invasion. *Int. J. Cancer NA-NA*. doi:10.1002/ijc.24849
- Nakada, M., Hayashi, Y., Hamada, J.I., 2011. Role of Eph/ephrin tyrosine kinase in malignant glioma. *Neuro-Oncology* 13, 1163–1170. doi:10.1093/neuonc/nor102
- 10 Pasquale, E.B., 2008. Eph-Ephrin Bidirectional Signaling in Physiology and Disease. *Cell* 133, 38–52. doi:10.1016/j.cell.2008.03.011
- Patel, A.P., Tirosh, I., Trombetta, J.J., Shalek, A.K., Gillespie, S.M., Wakimoto, H., Cahill, D.P., Nahed, B.V., Curry, W.T., Martuza, R.L., Louis, D.N., Rozenblatt-Rosen, O., Suva, M.L., Regev, A., Bernstein, B.E., 2014.
 15 Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* 344, 1396–1401. doi:10.1126/science.1254257
- Plaks, V., Kong, N., Werb, Z., 2015. The cancer stem cell niche: how essential is the niche in regulating stemness of tumor cells? *Cell Stem Cell* 16, 225–238. doi:10.1016/j.stem.2015.02.015
- 20 Qazi, M.A., Vora, P., Venugopal, C., McFarlane, N., Subapanditha, M.K., Murty, N.K., Hassell, J.A., Hallett, R.M., Singh, S.K., 2016. A novel stem cell culture model of recurrent glioblastoma. *J Neurooncol* 126, 57–67. doi:10.1007/s11060-015-1951-6
- Singh, S.K., Clarke, I.D., Terasaki, M., Bonn, V.E., Hawkins, C., Squire, J.,
 25 Dirks, P.B., 2003. Identification of a cancer stem cell in human brain tumors. *Cancer Research* 63, 5821–5828.
- Singh, S.K., Hawkins, C., Clarke, I.D., Squire, J.A., Bayani, J., Hide, T., Henkelman, R.M., Cusimano, M.D., Dirks, P.B., 2004. Identification of human brain tumour initiating cells. *Nature* 432, 396–401. doi:10.1038/nature03128

- Son, M.J., Woolard, K., Nam, D.-H., Lee, J., Fine, H.A., 2009. SSEA-1 is an enrichment marker for tumor-initiating cells in human glioblastoma. *Cell Stem Cell* 4, 440–452. doi:10.1016/j.stem.2009.03.003
- Stupp, R., Mason, W.P., Van Den Bent, M.J., 2005. Radiotherapy plus
5 concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 352, 987–996. doi:10.1056/NEJMoa043330
- Suva, M.L., Rheinbay, E., Gillespie, S.M., Patel, A.P., Wakimoto, H., Rabkin, S.D., Riggi, N., Chi, A.S., Cahill, D.P., Nahed, B.V., Curry, W.T., Martuza, R.L., Rivera, M.N., Rossetti, N., Kasif, S., Beik, S., Kadri, S., Tirosh, I.,
10 Wortman, I., Shalek, A.K., Rozenblatt-Rosen, O., Regev, A., Louis, D.N., Bernstein, B.E., 2014. Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. *Cell* 157, 580–594. doi:10.1016/j.cell.2014.02.030
- Swords, R.T., Greenberg, P.L., Wei, A.H., Durrant, S., Advani, A.S.,
15 Hertzberg, M.S., Lewis, I.D., Rivera, G., Gratzinger, D., Fan, A.C., Felsher, D.W., Cortes, J.E., Watts, J.M., Yarranton, G.T., Walling, J.M., Lancet, J.E., 2016. KB004, a first in class monoclonal antibody targeting the receptor tyrosine kinase EphA3, in patients with advanced hematologic malignancies: Results from a phase 1 study. *Leuk. Res.* 50, 123–131. doi:10.1016/j.leukres.2016.09.012
20
- Verhaak, R.G.W., Hoadley, K.A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M.D., Miller, C.R., Ding, L., Golub, T., Mesirov, J.P., Alexe, G., Lawrence, M., O’Kelly, M., Tamayo, P., Weir, B.A., Gabriel, S., Winckler, W., Gupta, S., Jakkula, L., Feiler, H.S., Hodgson, J.G., James, C.D., Sarkaria, J.N., Brennan, C.,
25 Kahn, A., Spellman, P.T., Wilson, R.K., Speed, T.P., Gray, J.W., Meyerson, M., Getz, G., Perou, C.M., Hayes, D.N., Cancer Genome Atlas Research Network, 2010. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 17, 98–110. doi:10.1016/j.ccr.2009.12.020
30
- Wang, J., Cazzato, E., Ladewig, E., Frattini, V., Rosenbloom, D.I.S., Zairis, S., Abate, F., Liu, Z., Elliott, O., Shin, Y.-J., Lee, J.-K., Lee, I.-H., Park, W.-Y.,

Eoli, M., Blumberg, A.J., Lasorella, A., Nam, D.-H., Finocchiaro, G., Iavarone, A., Rabadan, R., 2016. Clonal evolution of glioblastoma under therapy. *Nat. Genet.* 48, 768–776. doi:10.1038/ng.3590

Wykosky, J., 2005. EphA2 as a Novel Molecular Marker and Target in
5 Glioblastoma Multiforme. *Molecular Cancer Research* 3, 541–551.
doi:10.1158/1541-7786.MCR-05-0056

CLAIMS:

1. An erythropoietin-producing hepatocellular carcinoma receptor (EphR)-binding agent comprising: a first binding portion which specifically binds a first EphR and a second binding portion which binds a second EphR,
5 wherein the first EphR and the second EphR are different.
2. The EphR-binding agent of claim 1, wherein the first binding portion and/or the second binding portion is an antibody or a binding fragment thereof.
3. The EphR-binding agent of claim 1 or 2, wherein the first EphR
10 is EphA2.
4. The EphR-binding agent of any one of claims 1-3, wherein the second EphR is EphA3.
5. The EphR-binding agent of any one of claims 1-4, wherein the first EphR is EphA2 and the second EphR is EphA3.
- 15 6. The EphR-binding agent of any one of claims 1-5, wherein the EphR binding agent is a bispecific antibody.
7. The EphR-binding agent of claim 6, wherein the first binding portion of the bispecific antibody comprises a first heavy chain variable region-derived segment and a first antibody heavy chain constant region-derived segment lacking a CH1 domain and the second binding portion of the
20 bispecific antibody comprises a second heavy chain variable region-derived segment and a second antibody heavy chain constant region-derived segment lacking a CH1 domain.
8. The EphR-binding agent of any one of claims 1-7, wherein the
25 first binding portion binds an EphA2 epitope bound by an EphA2-binding subunit comprising a heavy chain having the amino acid sequence of SEQ ID NO: 3.

9. The EphR-binding agent of any one of claims 1-8, wherein the first binding portion comprises a heavy chain complementarity-determining region (CDR)1 comprising the amino acid sequence of SEQ ID NO: 6, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 7, and a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 8.

10. The EphR-binding agent of any one of claims 1-9, wherein the first binding portion comprises the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 3.

11. The EphR-binding agent of claim 10, wherein the first binding portion comprises an R at amino acid position 16 of SEQ ID NO: 3, a G at amino acid position 35 of SEQ ID NO: 3, an R at amino acid position 39 of SEQ ID NO: 3, an E at amino acid position 45 of SEQ ID NO: 3, and/or an L at amino acid position 47 of SEQ ID NO: 3.

12. The EphR-binding agent of any one of claims 1-11, wherein the second binding portion comprises a heavy chain complementarity-determining region (CDR)1 comprising the amino acid sequence of SEQ ID NO: 6, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 16, and a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 17.

13. The EphR-binding agent of any one of claims 1-12, wherein the second binding portion comprises the amino acid sequence of SEQ ID NO: 4 or an amino acid sequence having at least 70% sequence identity to the framework regions of SEQ ID NO: 4.

14. The EphR-binding agent of claim 13, wherein the second binding portion comprises an R at amino acid position 16 of SEQ ID NO: 4, a G at amino acid position 35 of SEQ ID NO: 4, an R at amino acid position 39 of SEQ ID NO: 4, an E at amino acid position 45 of SEQ ID NO: 4, and/or an L at amino acid position 47 of SEQ ID NO: 4.

15. A pharmaceutical composition comprising the EphR-binding agent of any one of claims 1-14, and a carrier.

16. A use of the EphR-binding agent of any one of claims 1-14 or the pharmaceutical composition of claim 15 for treating or preventing
5 glioblastoma multiforme (GBM).

17. The use of claim 16, wherein the glioblastoma multiforme (GBM) is recurrent GBM.

18. A use of a first agent targeting a first EphR and a second agent targeting a second EphR for treating or preventing glioblastoma multiforme
10 (GBM), wherein the first EphR and the second EphR are different, and wherein the first and second agent reduce the expression and/or activity of the first EphR and the second EphR, respectively.

19. The use of claim 18, wherein the first agent specifically binds the first EphR and a second agent specifically binds the second EphR.

15 20. The use of claim 18 or 19, wherein the first EphR is EphA2.

21. The use of any one of claims 18-20, wherein the second EphR is EphA3.

22. The use of any one of claims 18-21, wherein the first EphR is EphA2 and the second EphR is EphA3.

20 23. The use of any one of claims 18-21, wherein the first agent and/or the second agent is an antibody or fragment thereof.

24. The use of claim 23, wherein the antibody or fragment thereof is selected from the group consisting of a bispecific antibody, a Fab, a single-chain Fv (scFv), an IgG, a phage-Fab and a phage-scFv.

25 25. The use of any one of claims 18-24, wherein the first agent and the second agent are for use simultaneously or sequentially.

26. The use of any one of claims 18-24, wherein the first agent and the second agent form a bispecific antibody.

27. The use of claim 26, wherein the first agent comprises a heavy chain complementarity-determining region (CDR)1 comprising the amino acid
5 sequence of SEQ ID NO: 6, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 7, and a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 8 and/or the second agent comprises a heavy chain complementarity-determining region (CDR)1 comprising the amino acid
10 sequence of SEQ ID NO: 6, a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO: 16, and a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO: 17.

28. The use of any one of claims 18-27, wherein the glioblastoma multiforme (GBM) is recurrent GBM.

FIGURE 1A

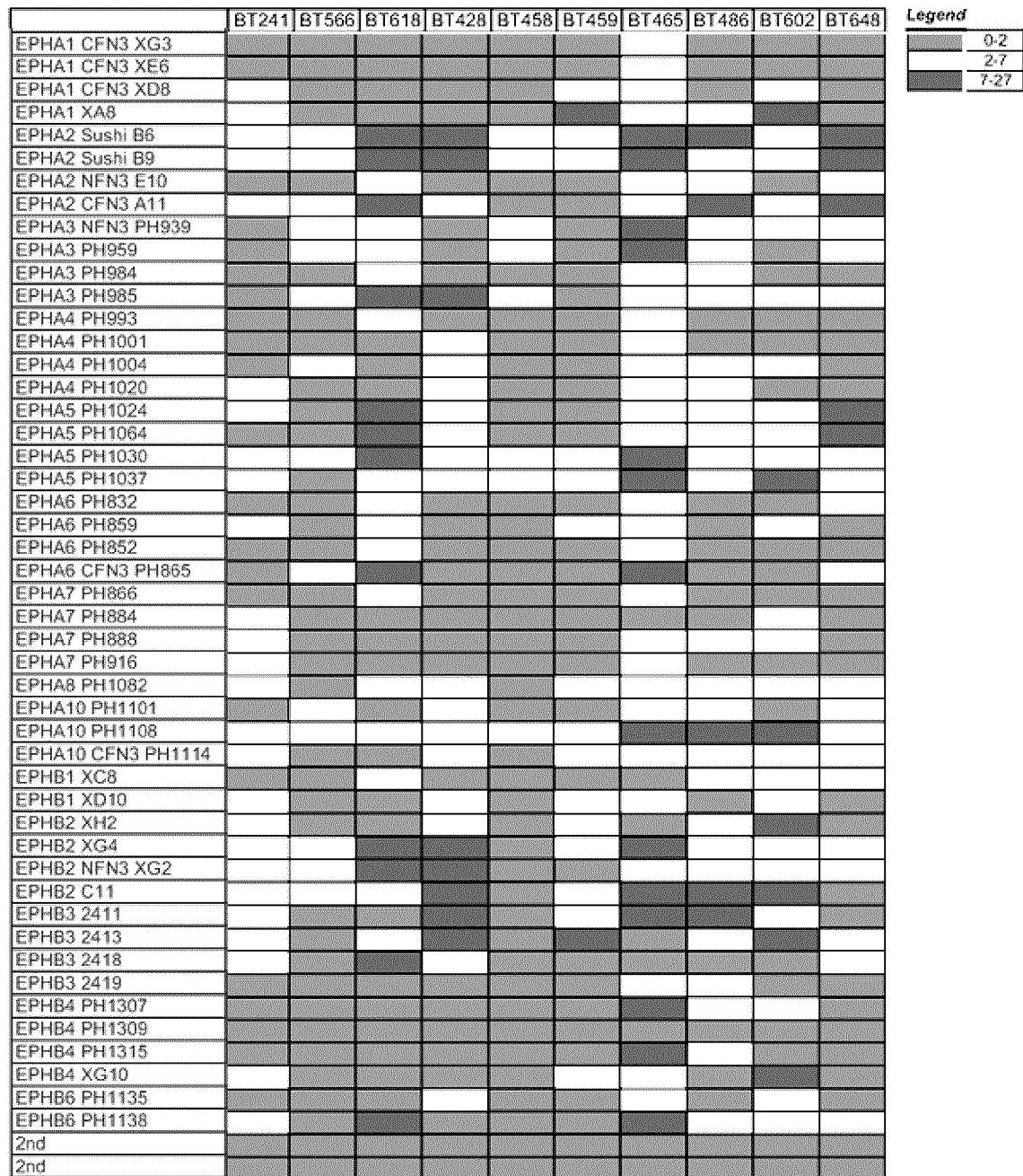


FIGURE 1B

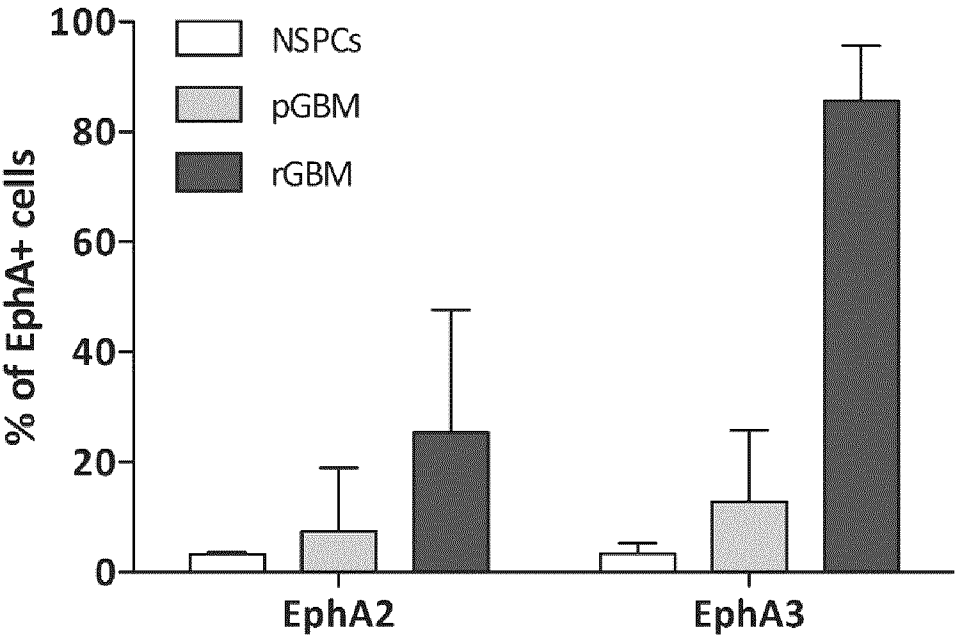


FIGURE 1C

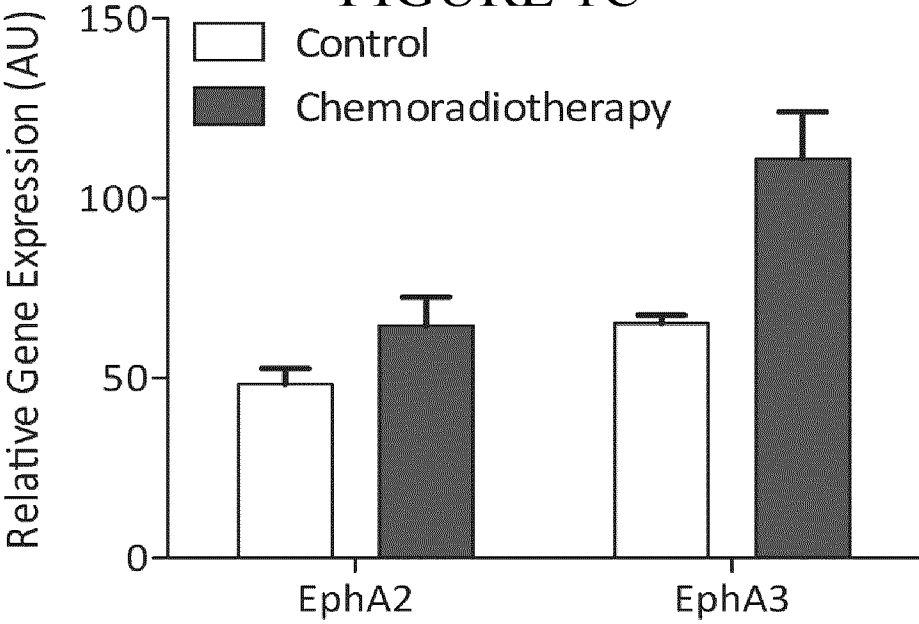


FIGURE 1D

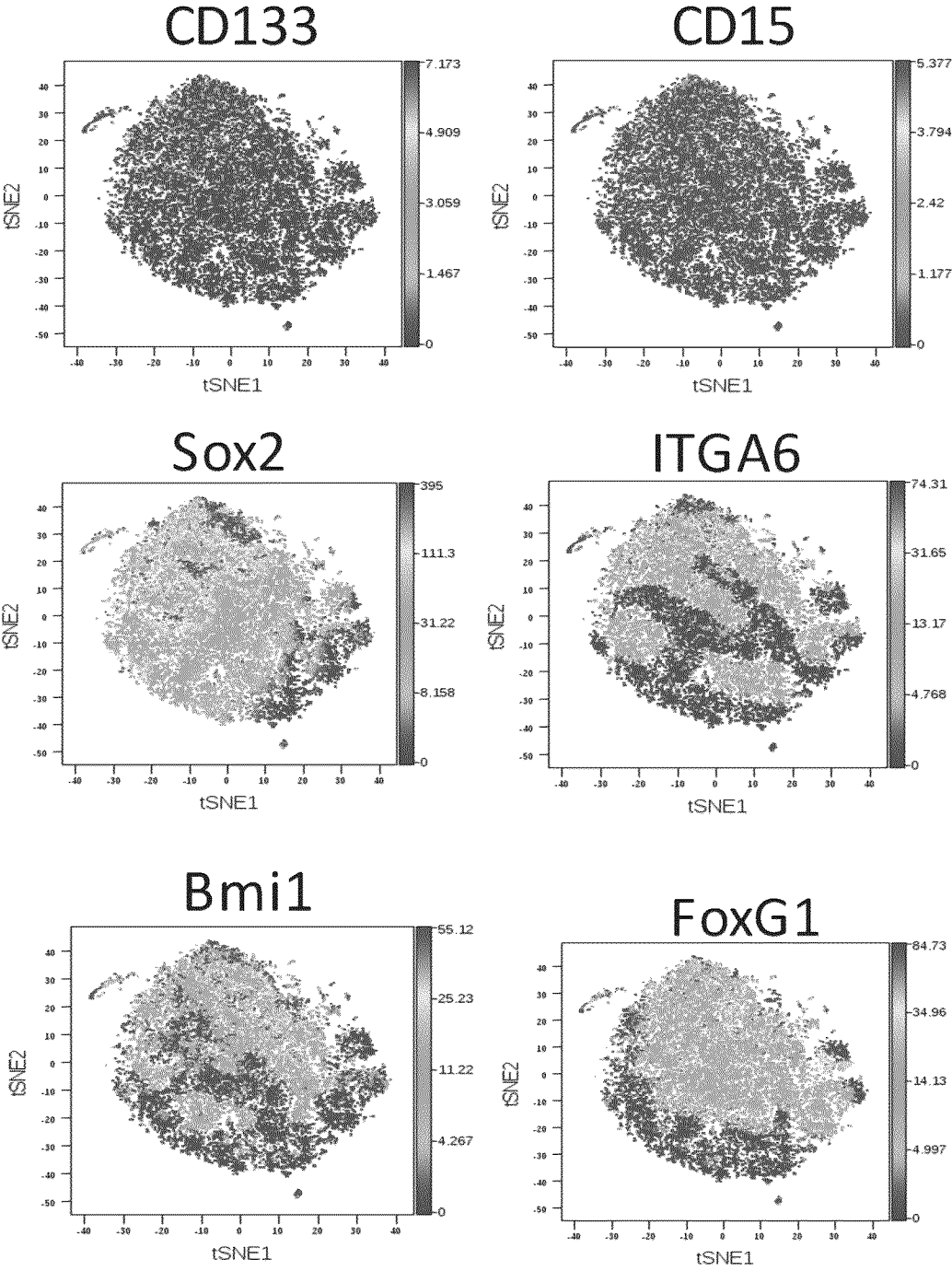
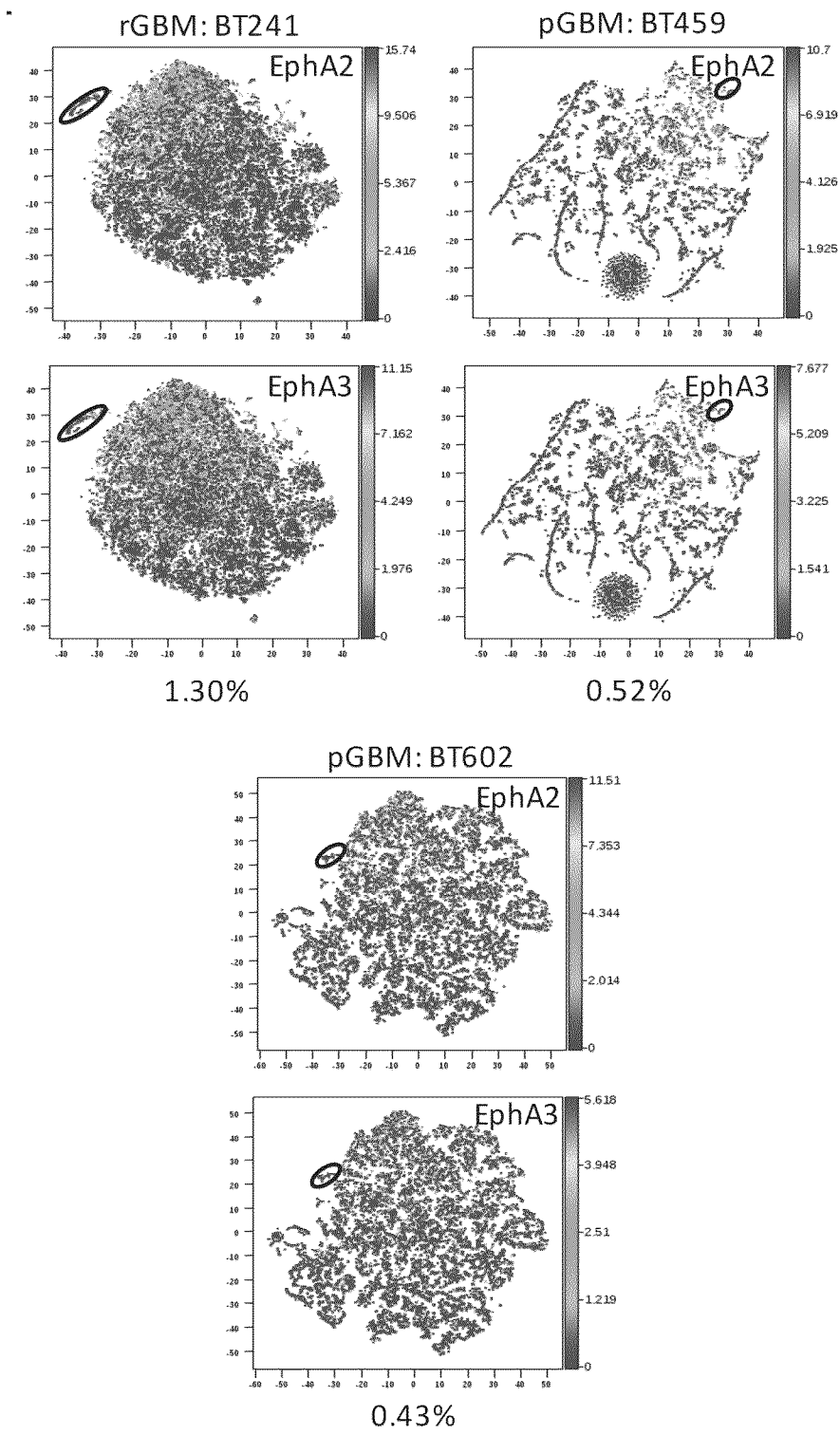
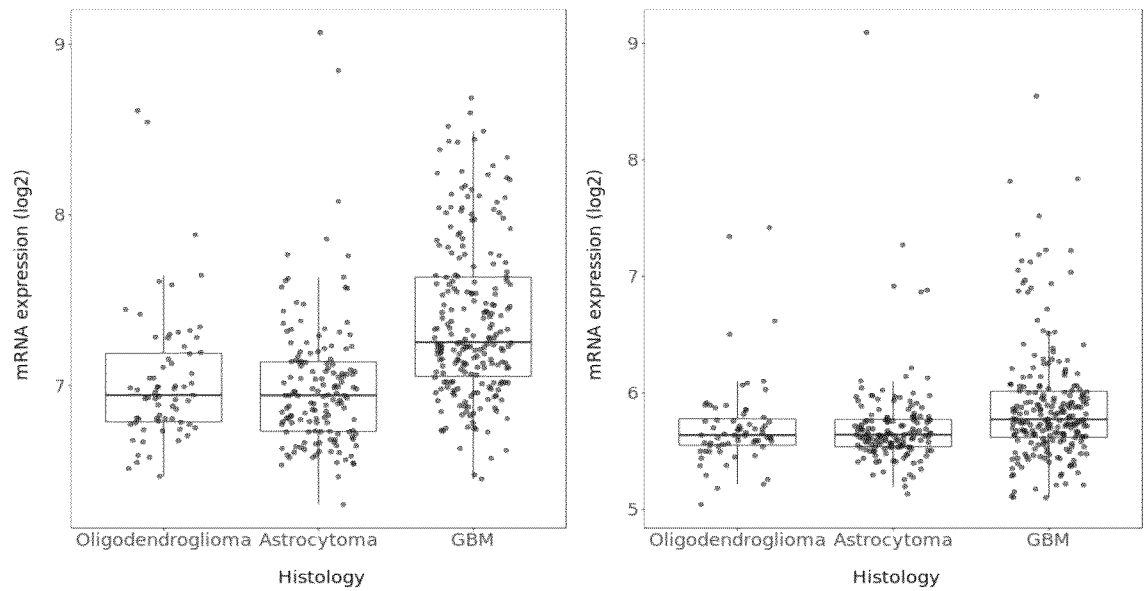


FIGURE 1E



EphA2 **FIGURE 2A** EphA3



EphA2 **FIGURE 2B** EphA3

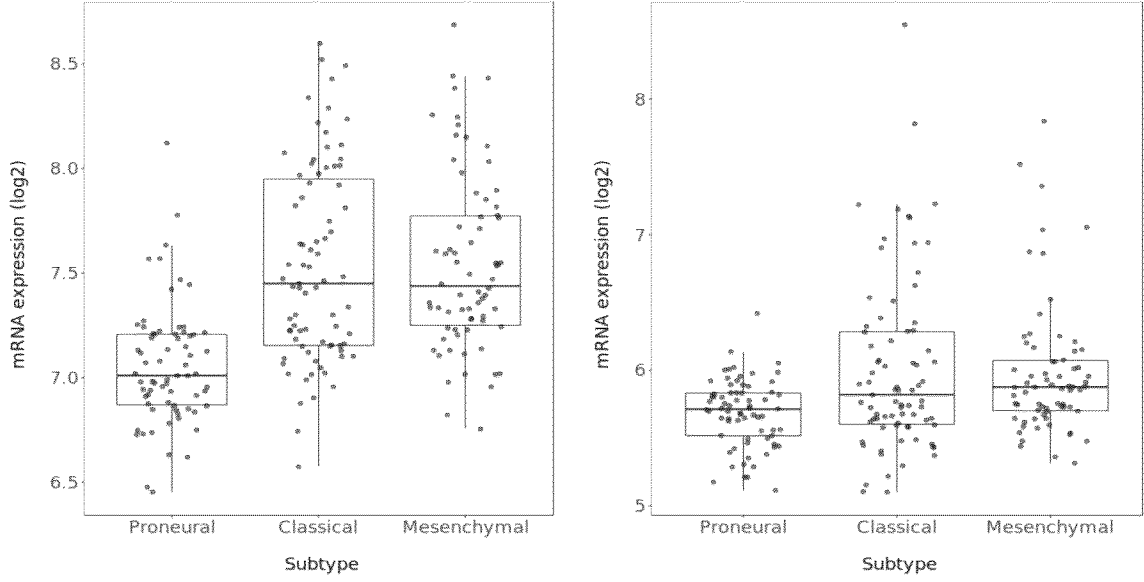


FIGURE 2C

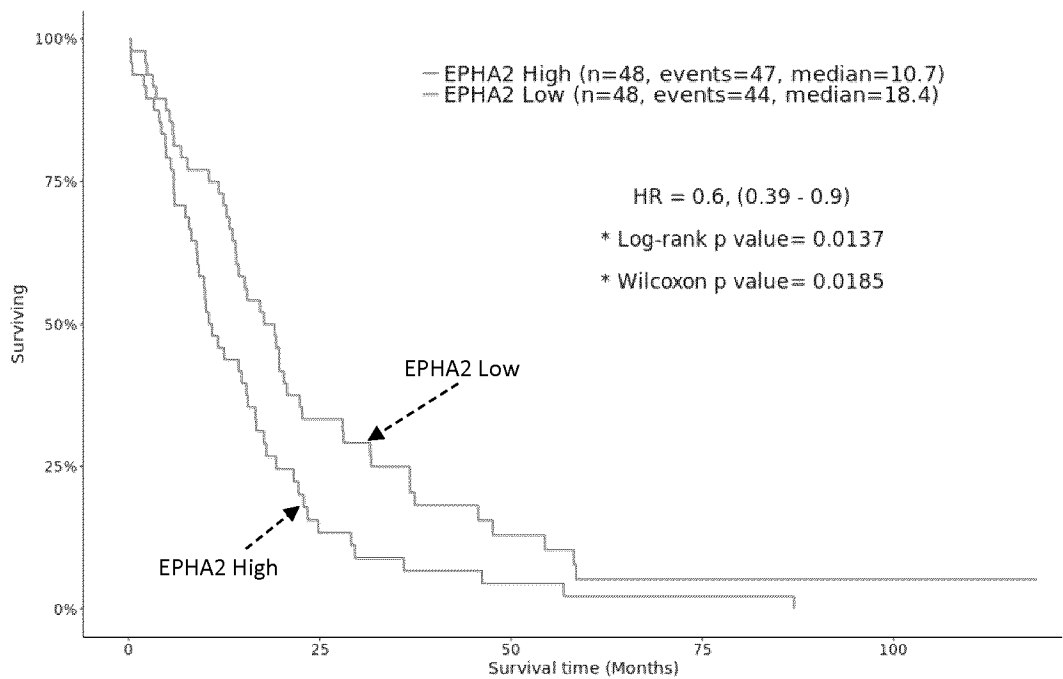


FIGURE 2D

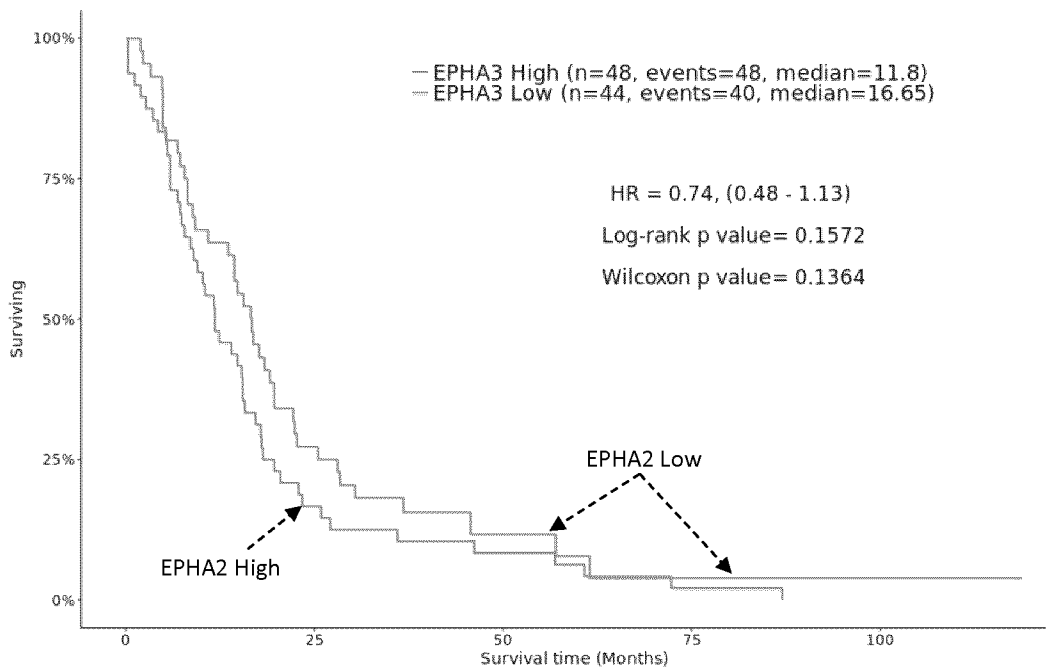


FIGURE 2E

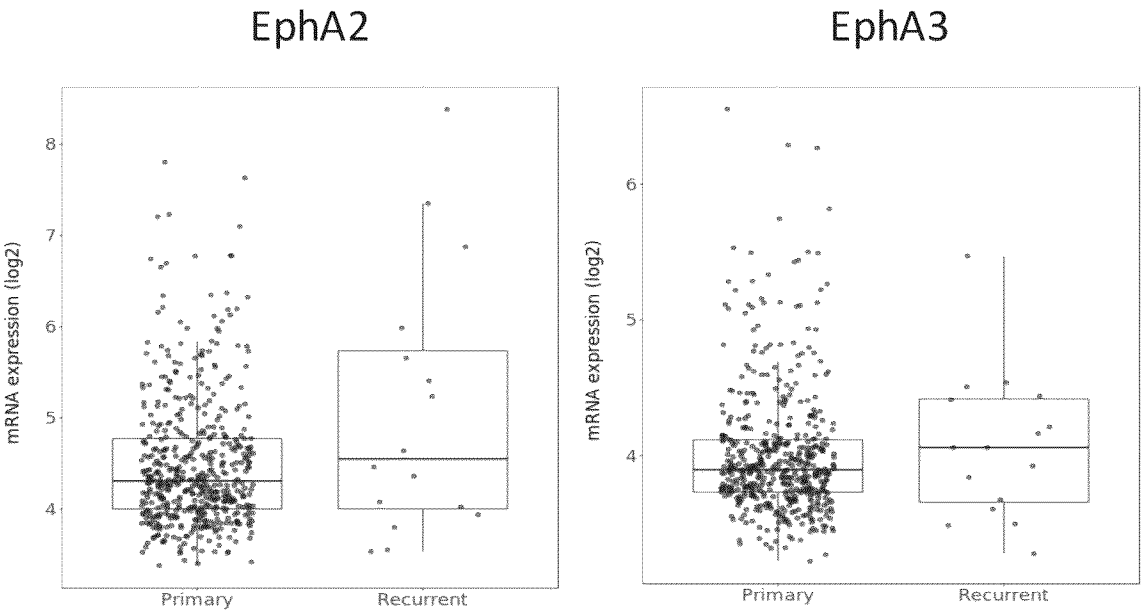


FIGURE 2F

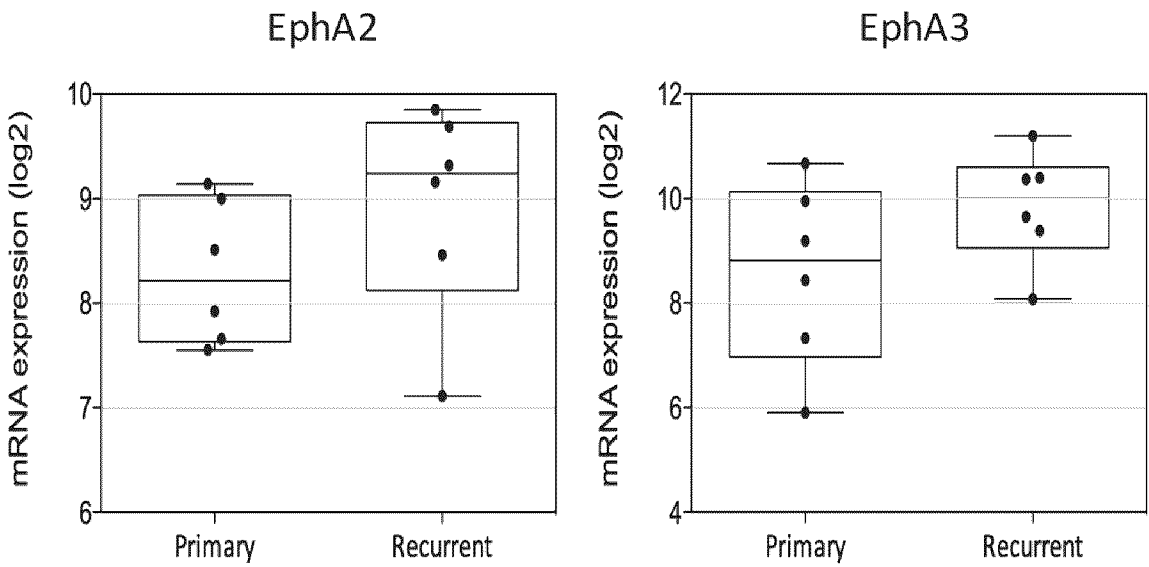
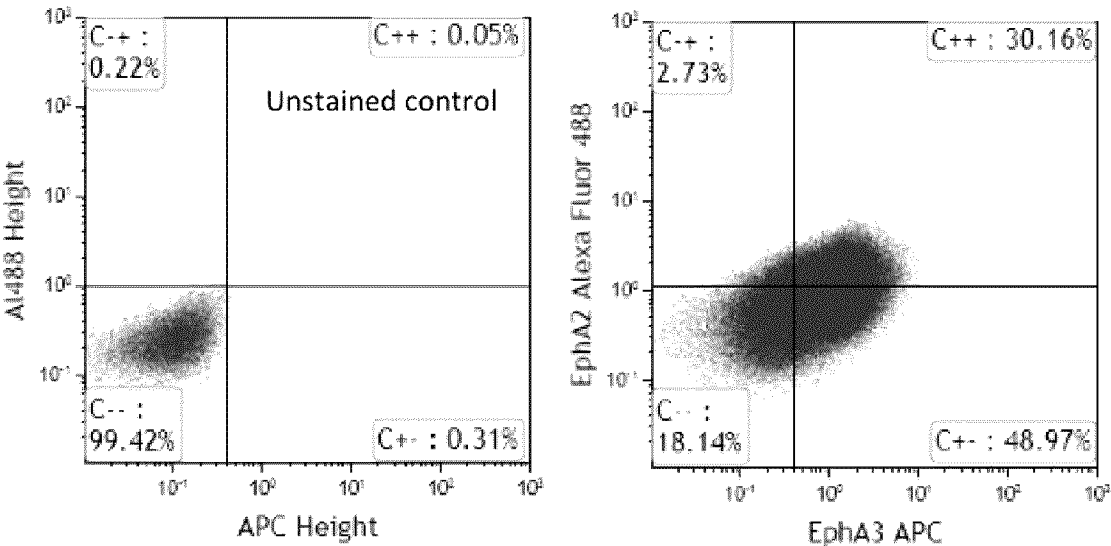
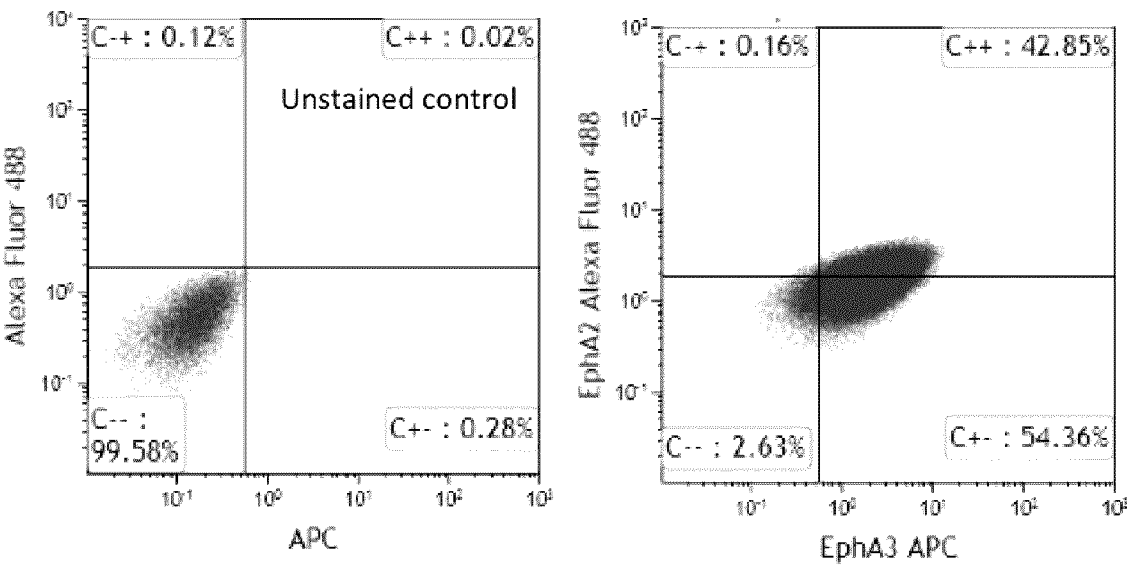


FIGURE 3A

BT241

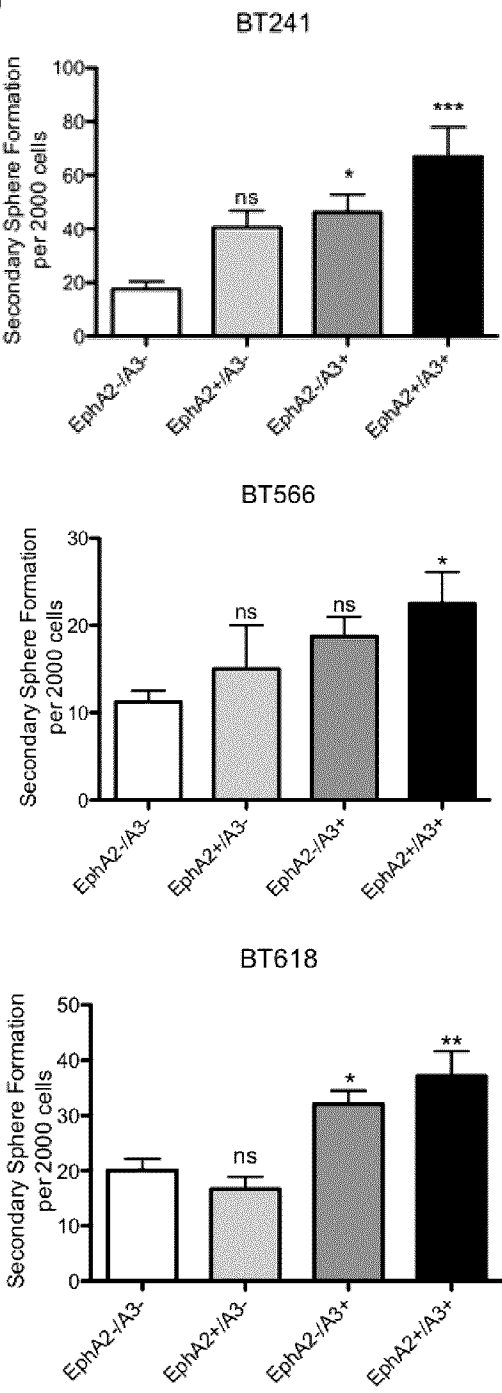


BT618



9/47

FIGURE 3B



10/47

FIGURE 3C

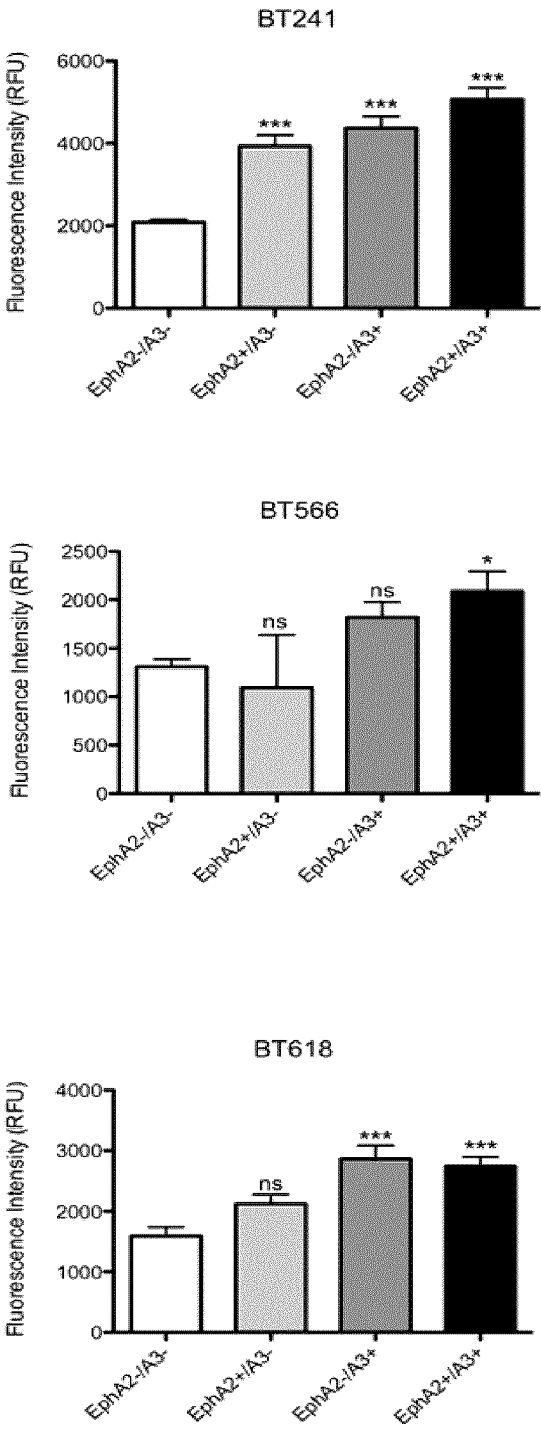


FIGURE 3D

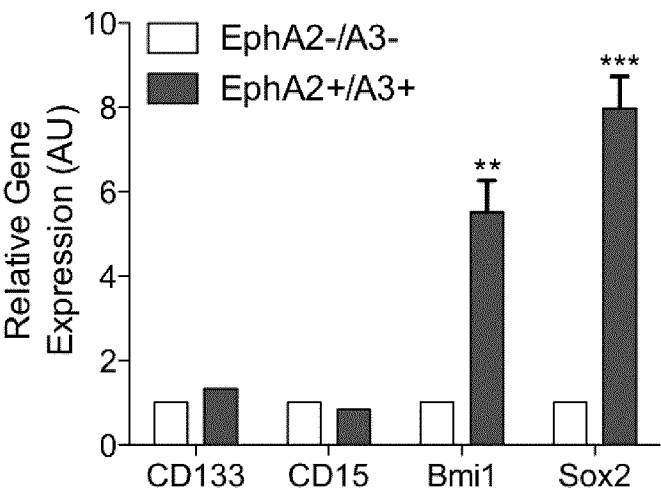


FIGURE 3E

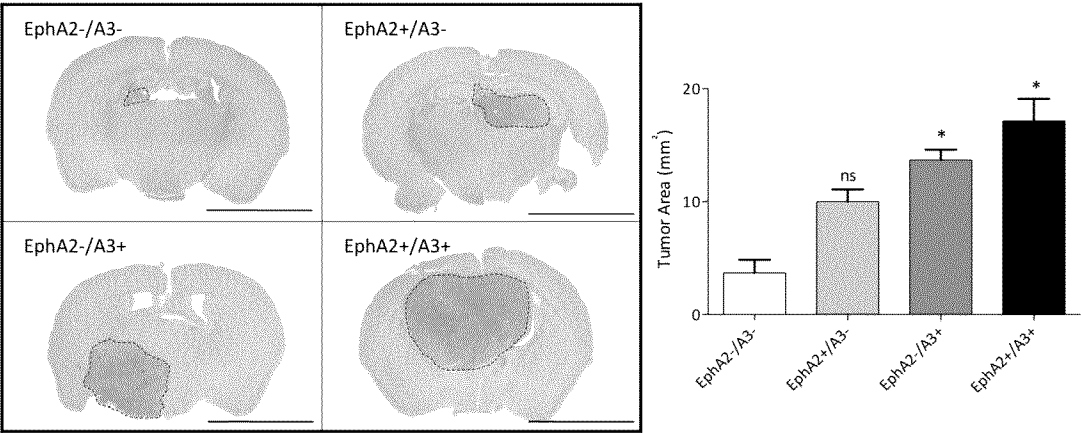


FIGURE 3F

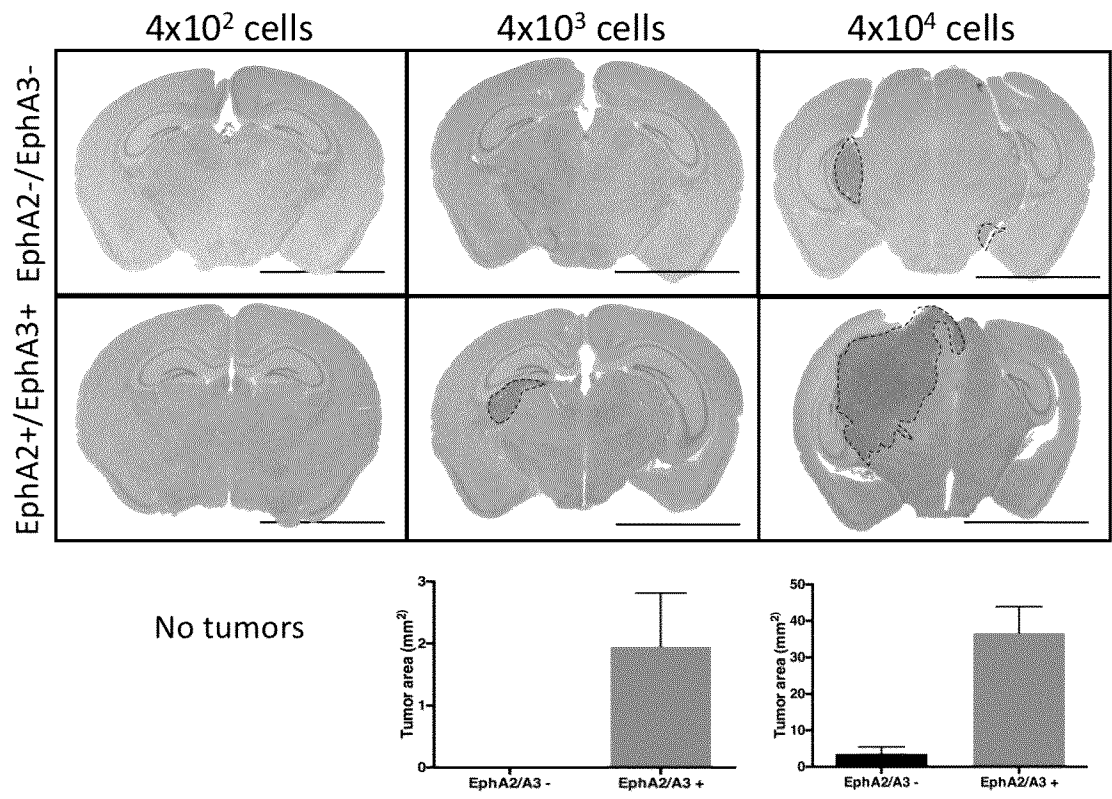


FIGURE 4A

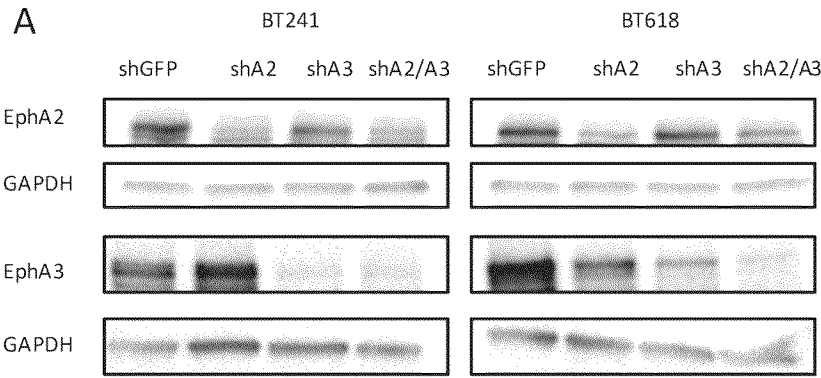


FIGURE 4B

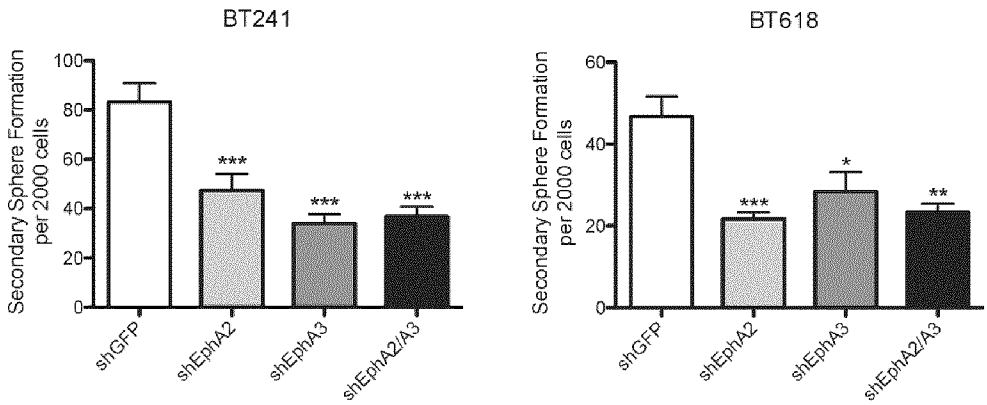


FIGURE 4C

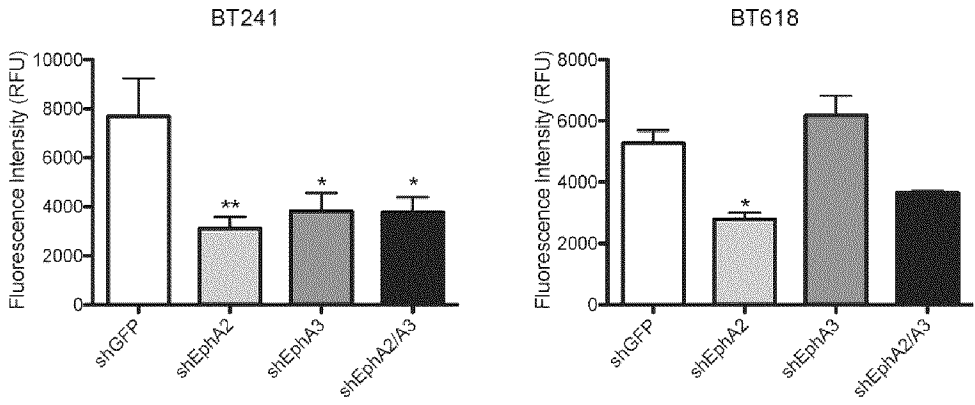


FIGURE 4D

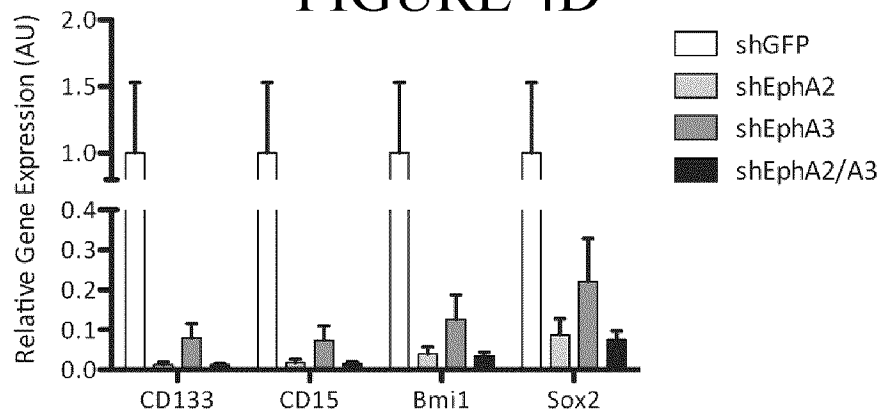


FIGURE 4E

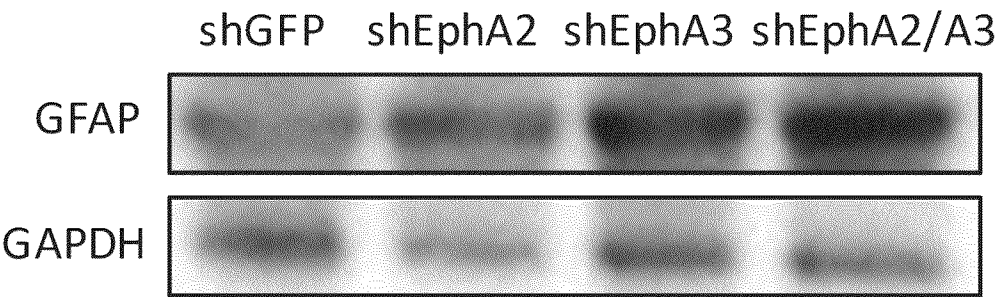


FIGURE 4F

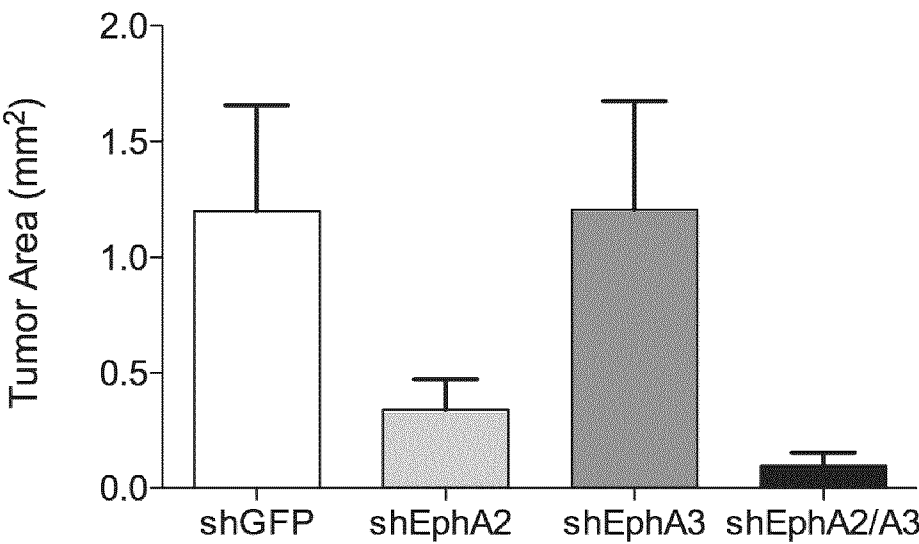
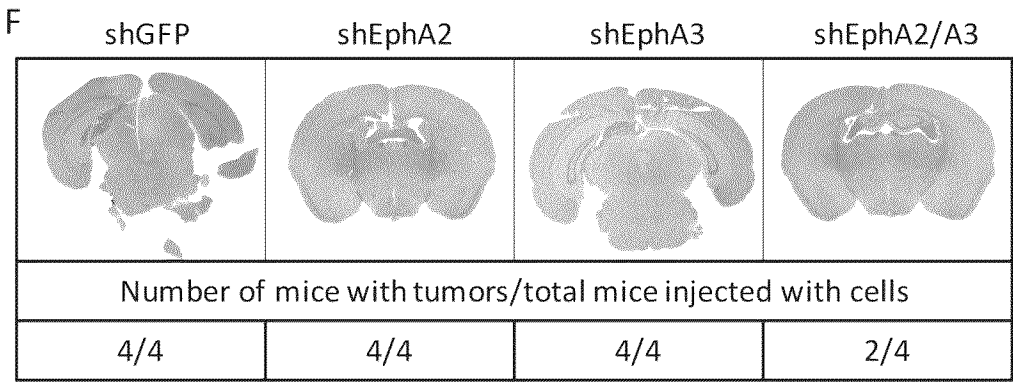


FIGURE 5A

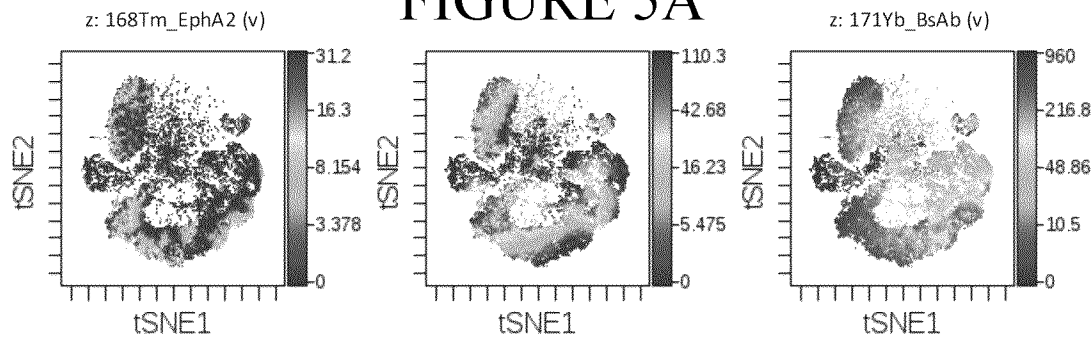


FIGURE 5B

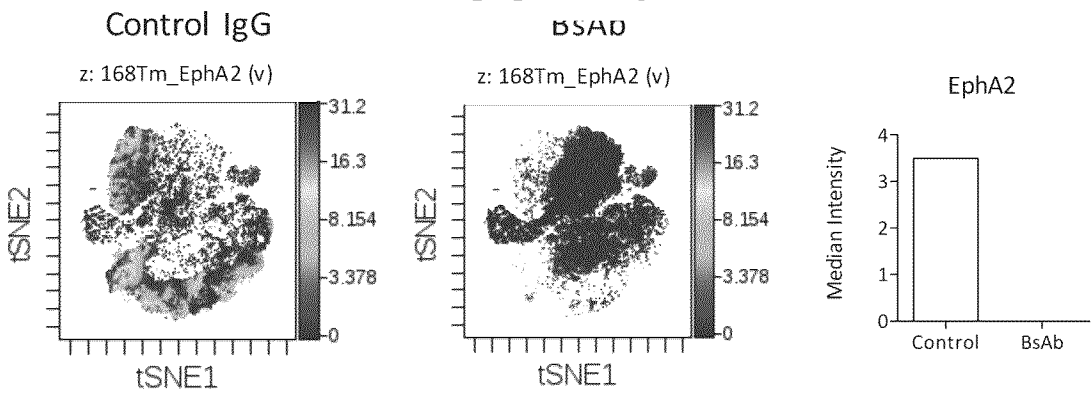


FIGURE 5C

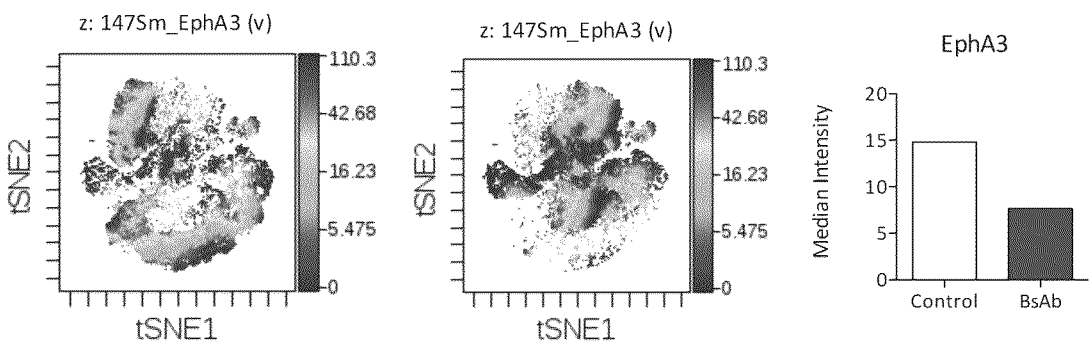


FIGURE 5D

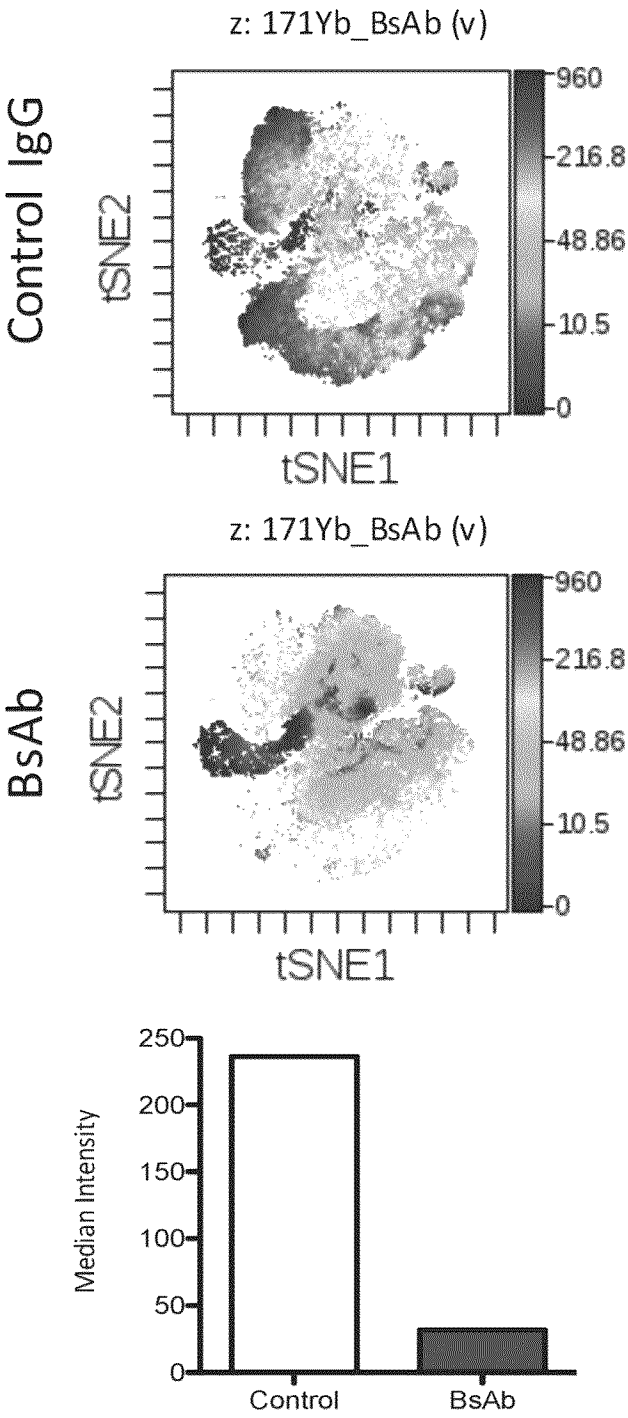


FIGURE 5E

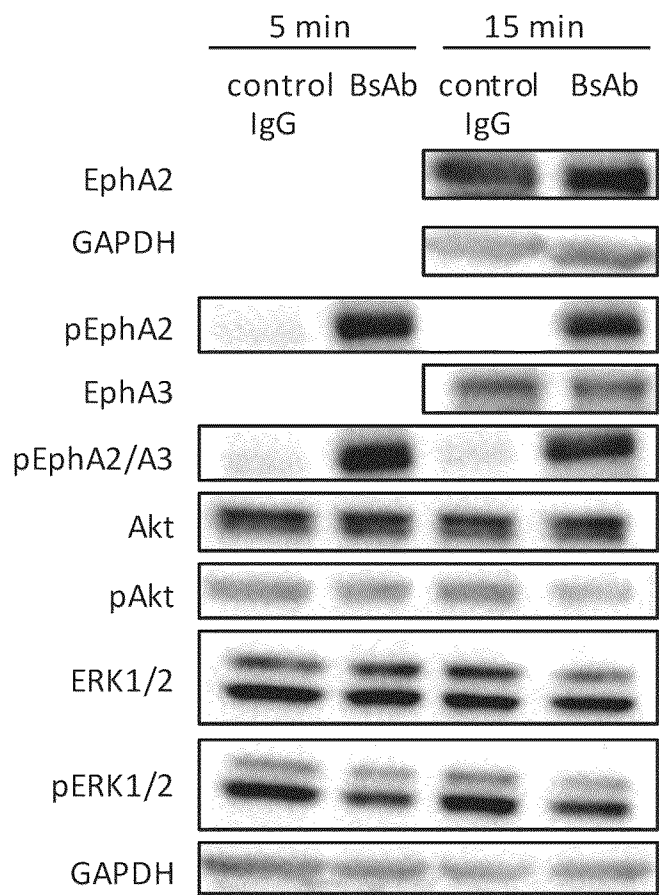


FIGURE 5F

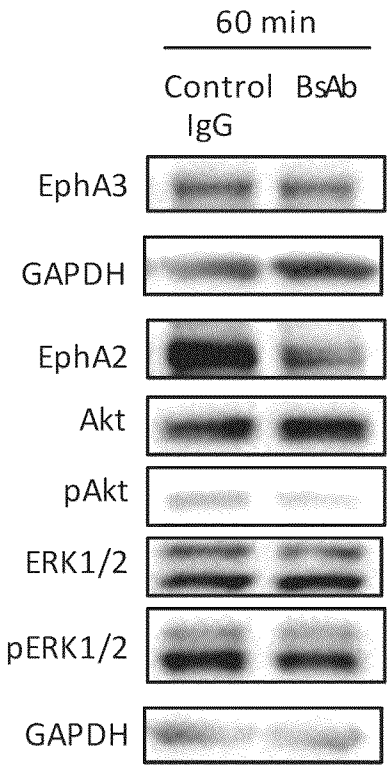


FIGURE 6A

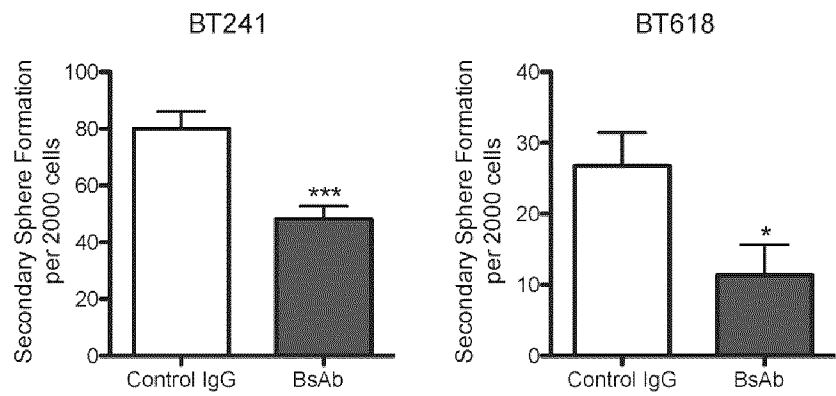


FIGURE 6B

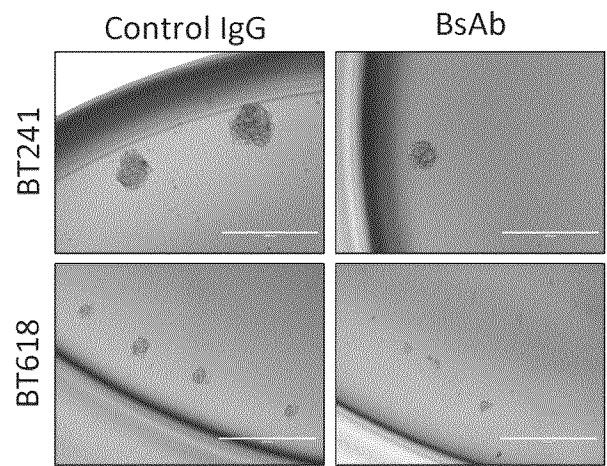


FIGURE 6C

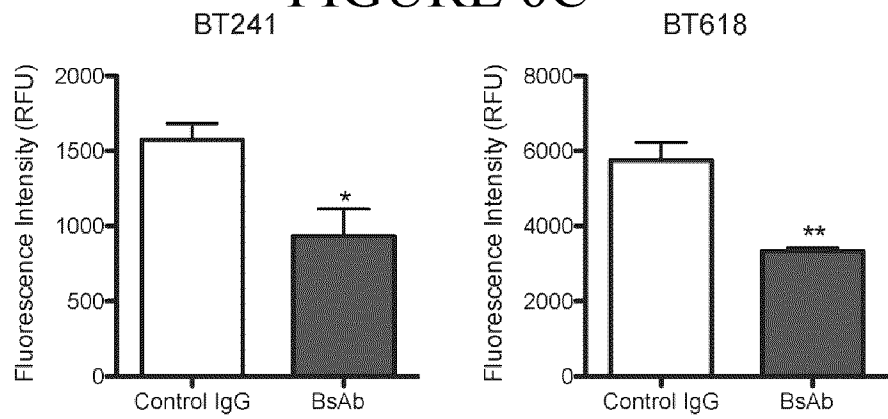


FIGURE 6D

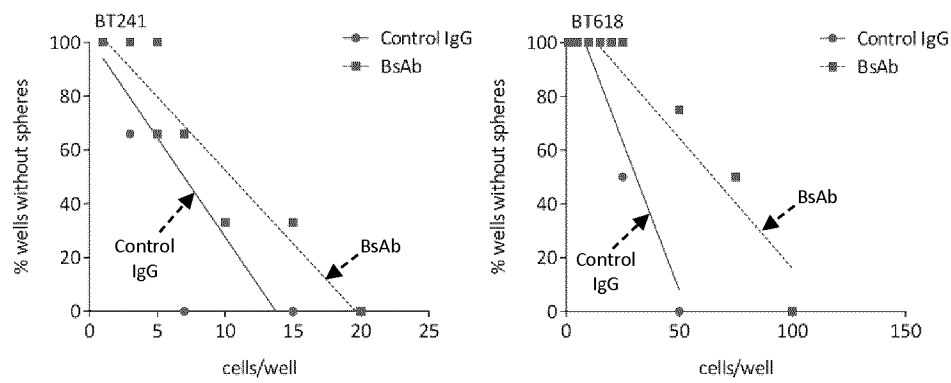


FIGURE 6E

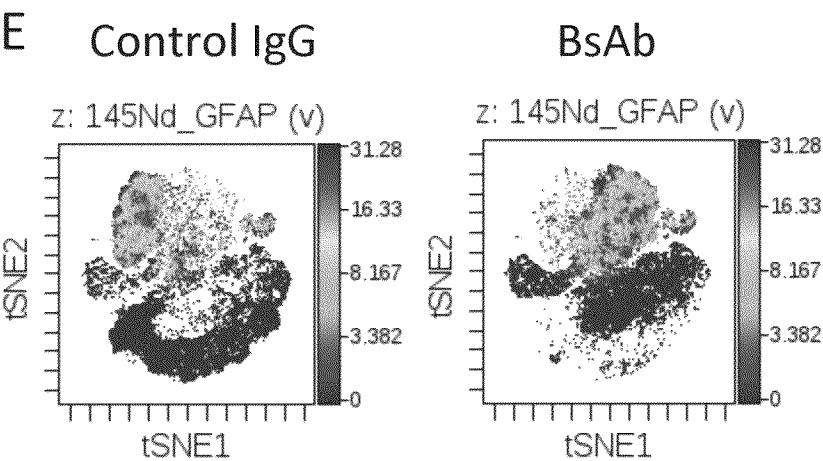


FIGURE 6F

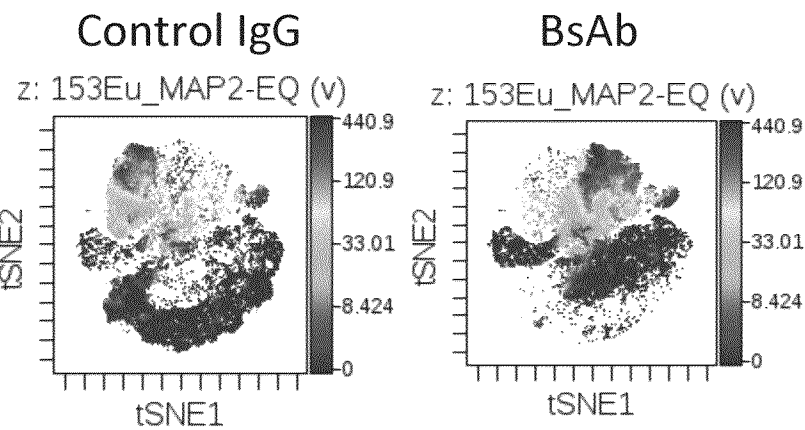


FIGURE 6G

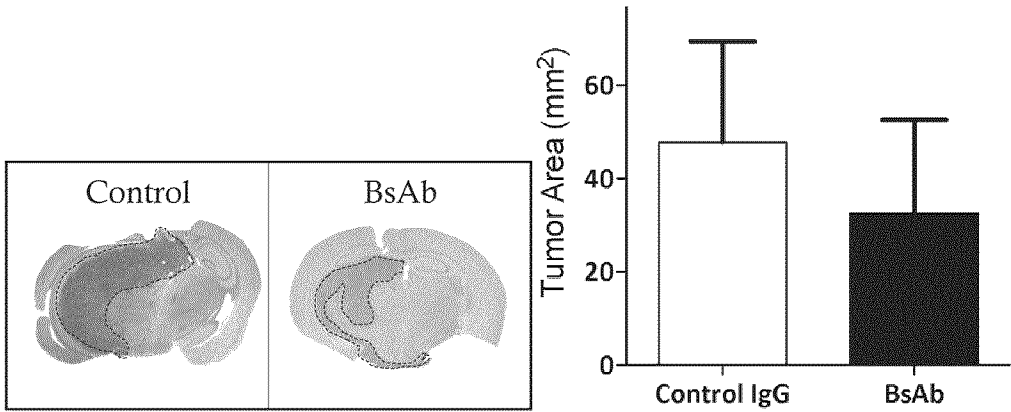


FIGURE 6H

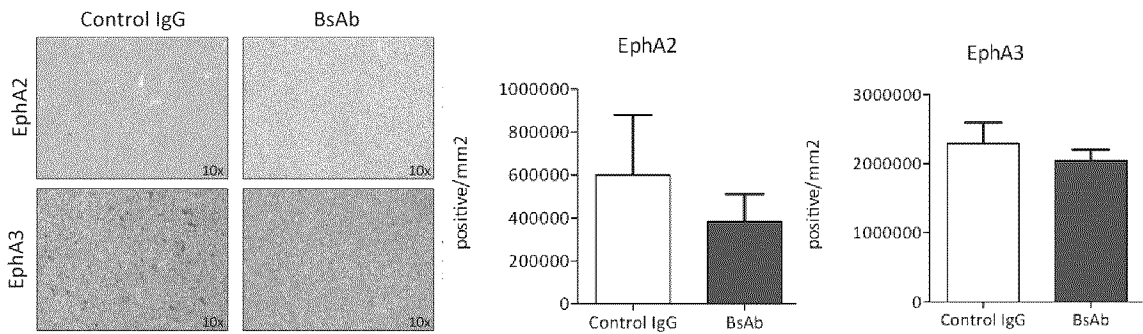


FIGURE 6I

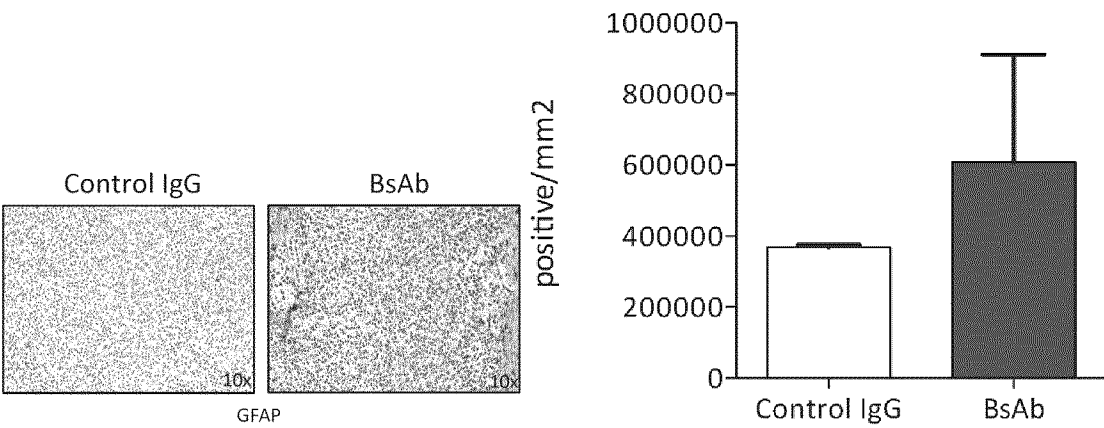


FIGURE 7A

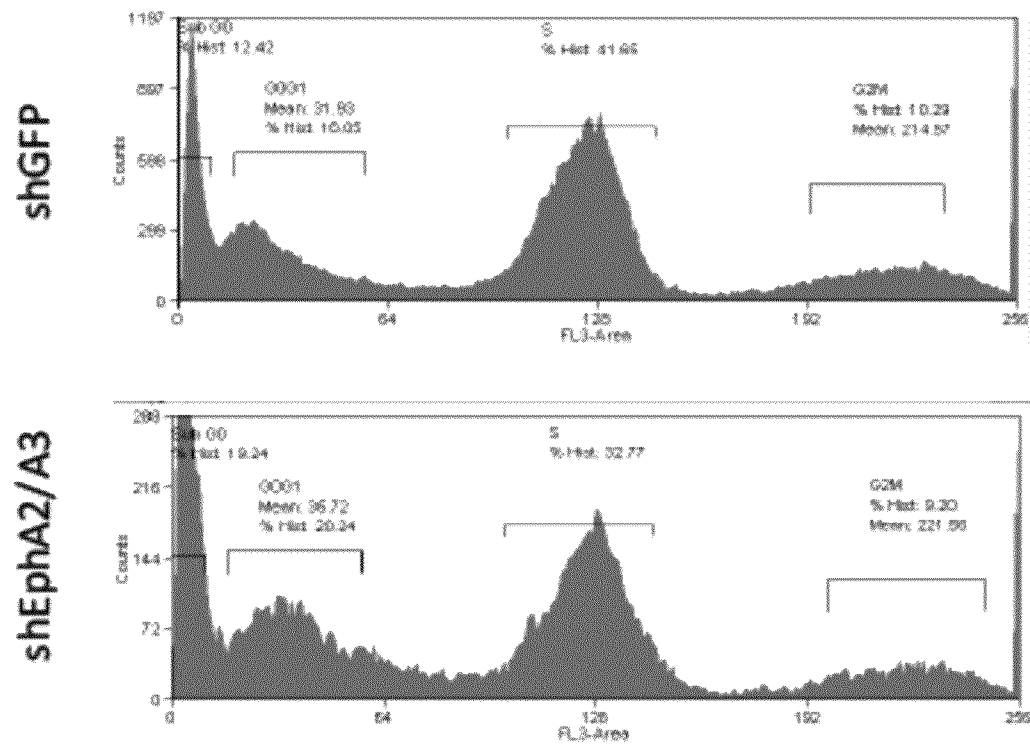


FIGURE 7B

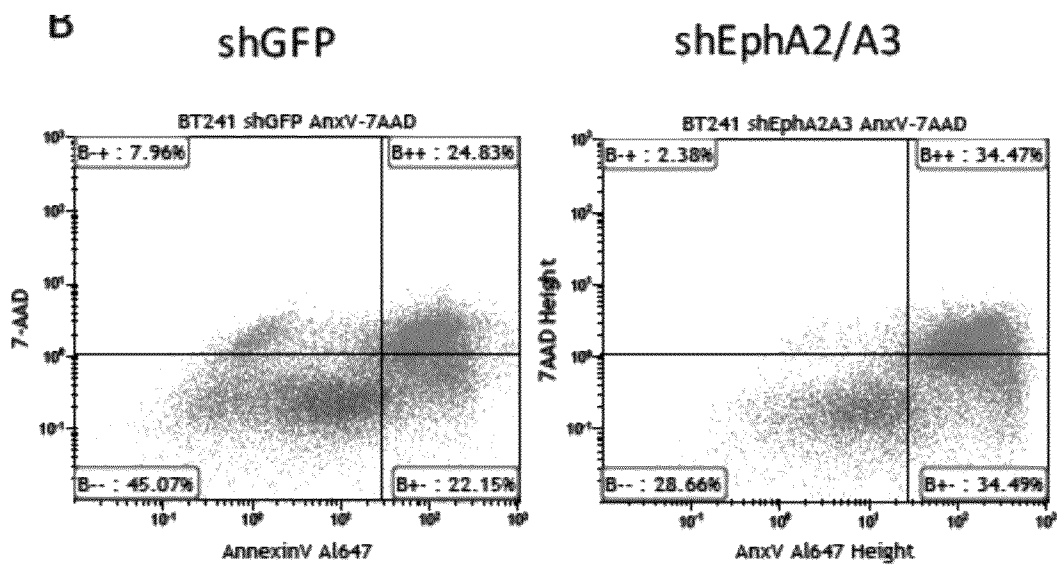


FIGURE 8

	EPHA2				EPHA3			
	K _{on} (s/M)	K _{off} (1/s)	K _D (M)	R _{max} (RU)	K _{on} (s/M)	K _{off} (1/s)	K _D (M)	R _{max} (RU)
BsAb	1.03x10 ⁵	1.11x10 ⁻³	1.08x10 ⁻⁸	98.09	5.62x10 ⁴	5.56x10 ⁻⁴	9.90x10 ⁻⁹	142.65

FIGURE 9

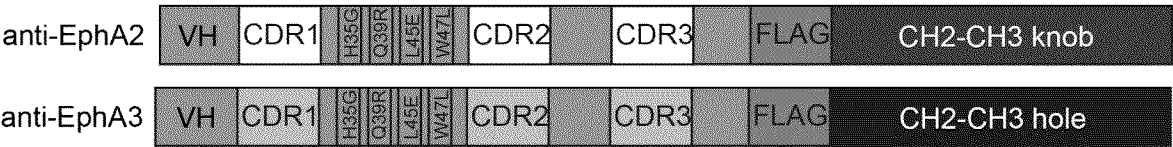


FIGURE 10A

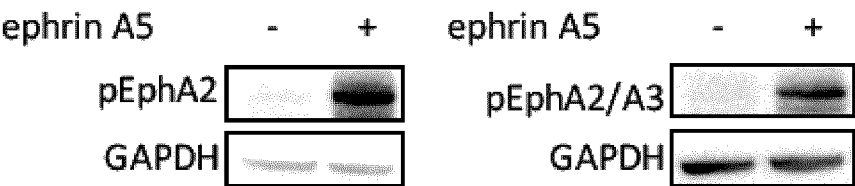


FIGURE 10B

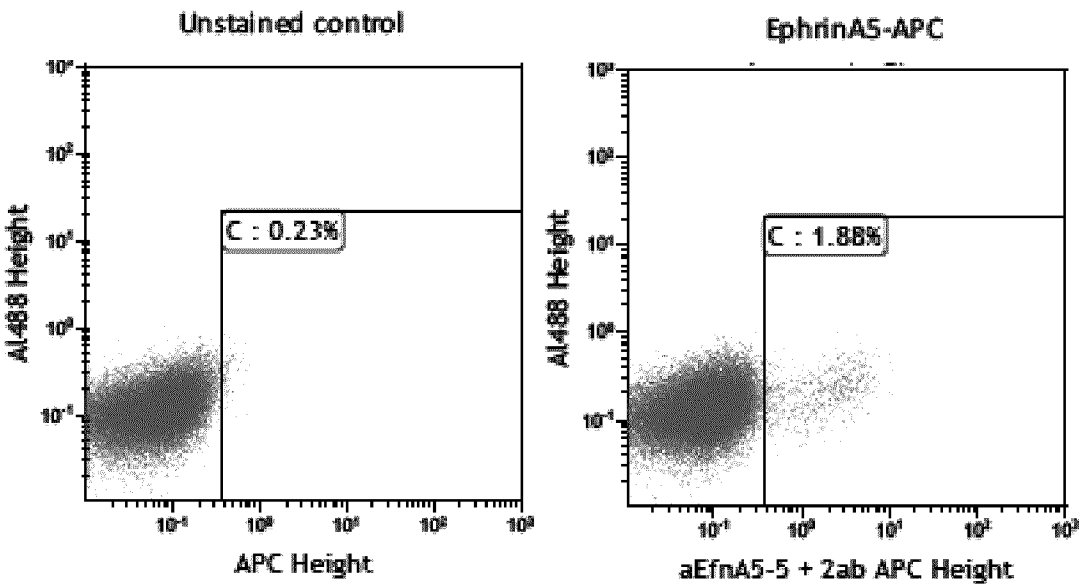


FIGURE 10C

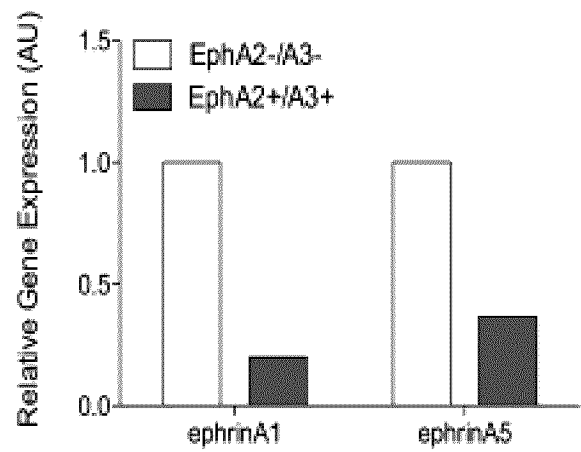


FIGURE 11

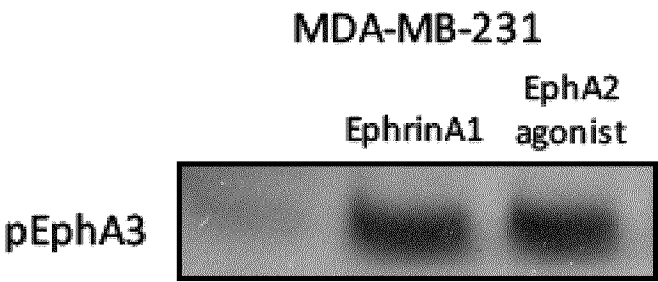


FIGURE 12

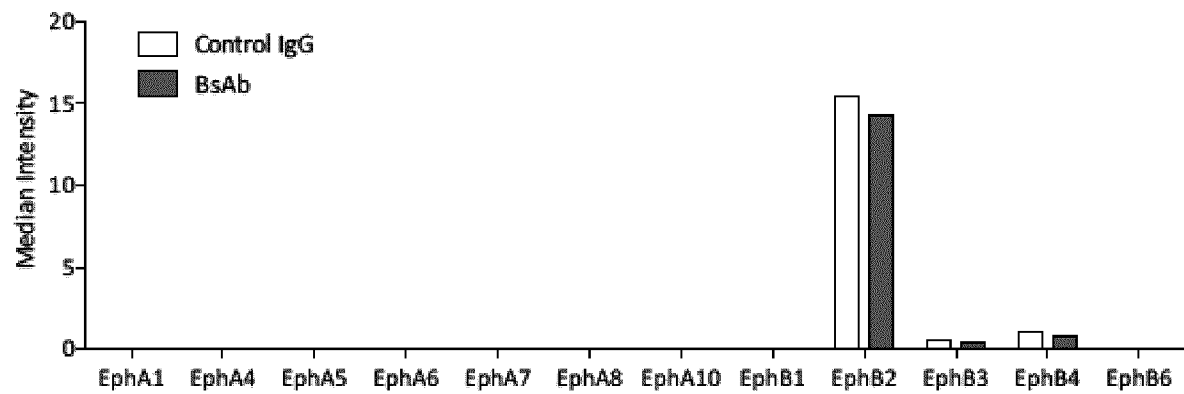


FIGURE 13A

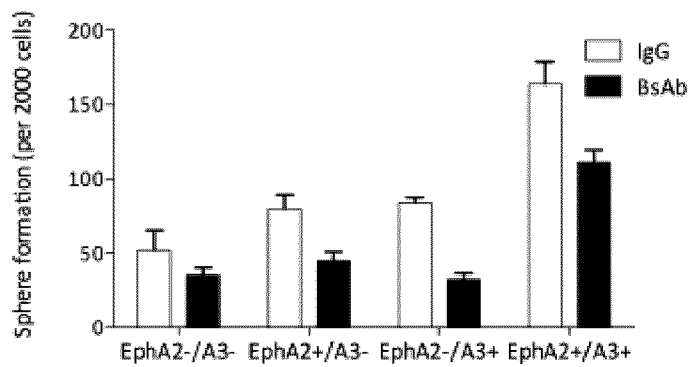


FIGURE 13B

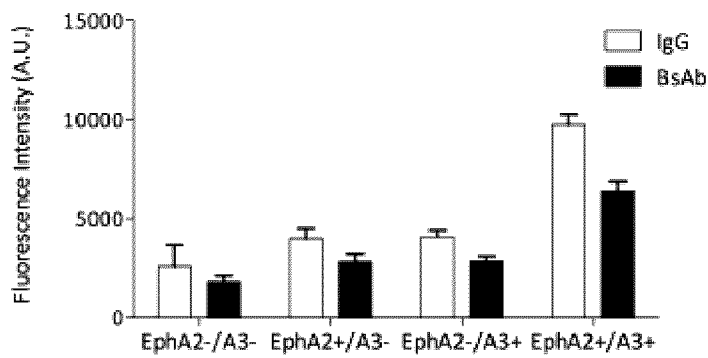


FIGURE 13C

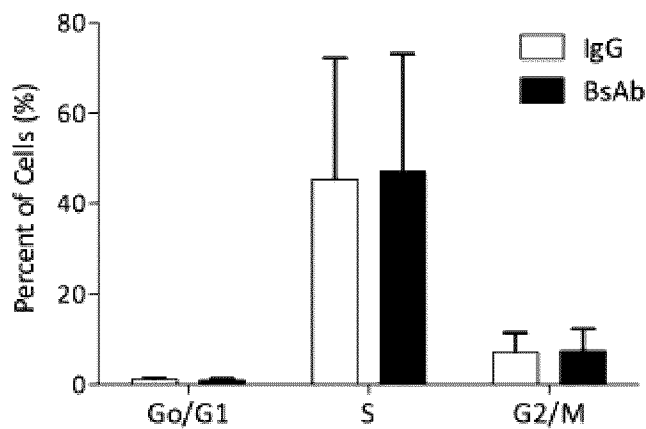


FIGURE 13D

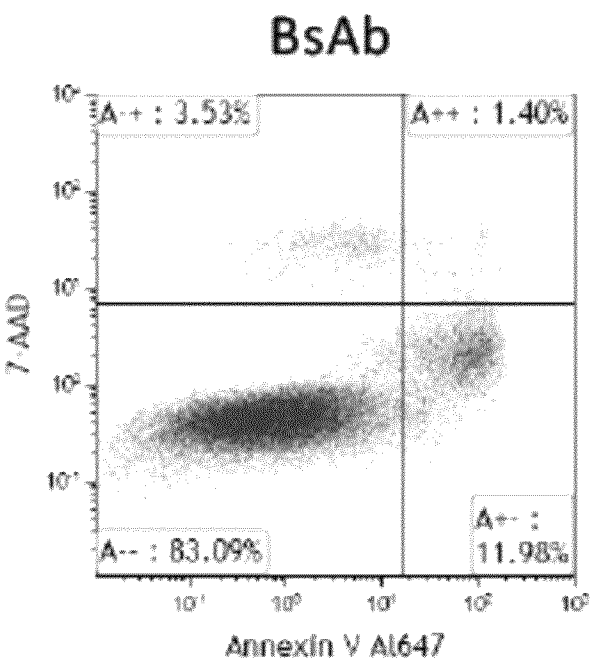
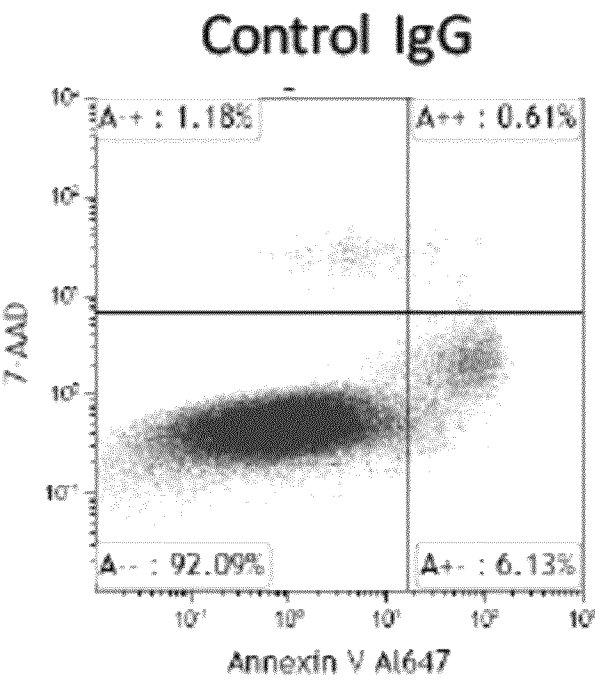


FIGURE 14A

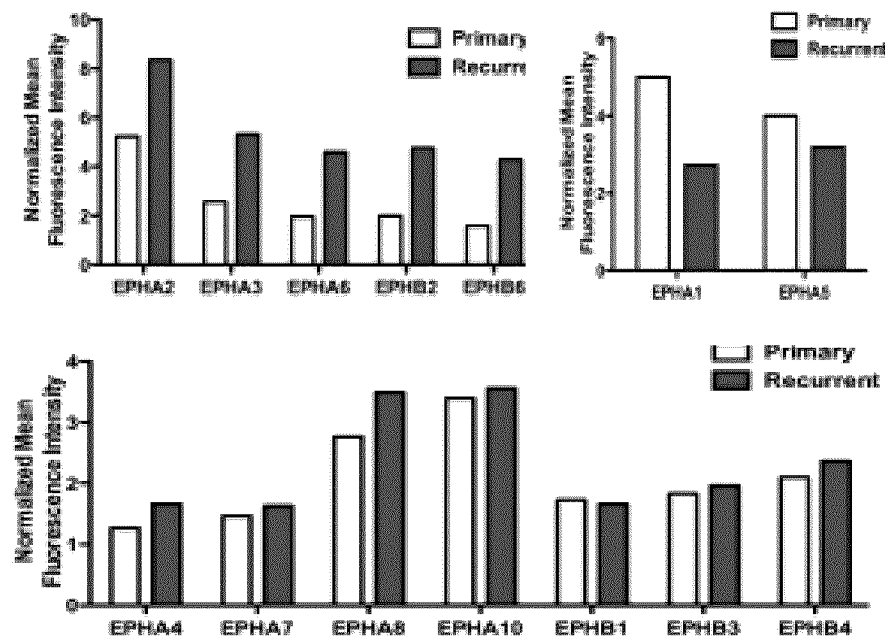


FIGURE 14B

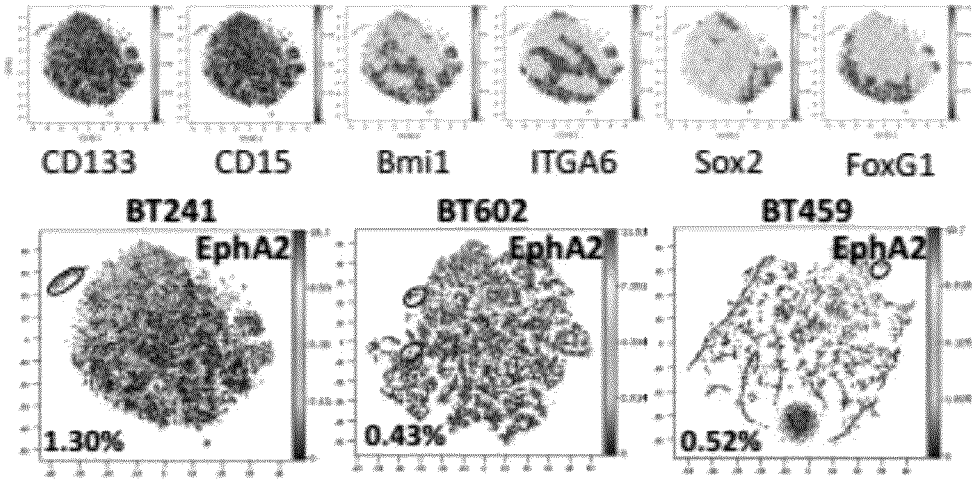


FIGURE 14C

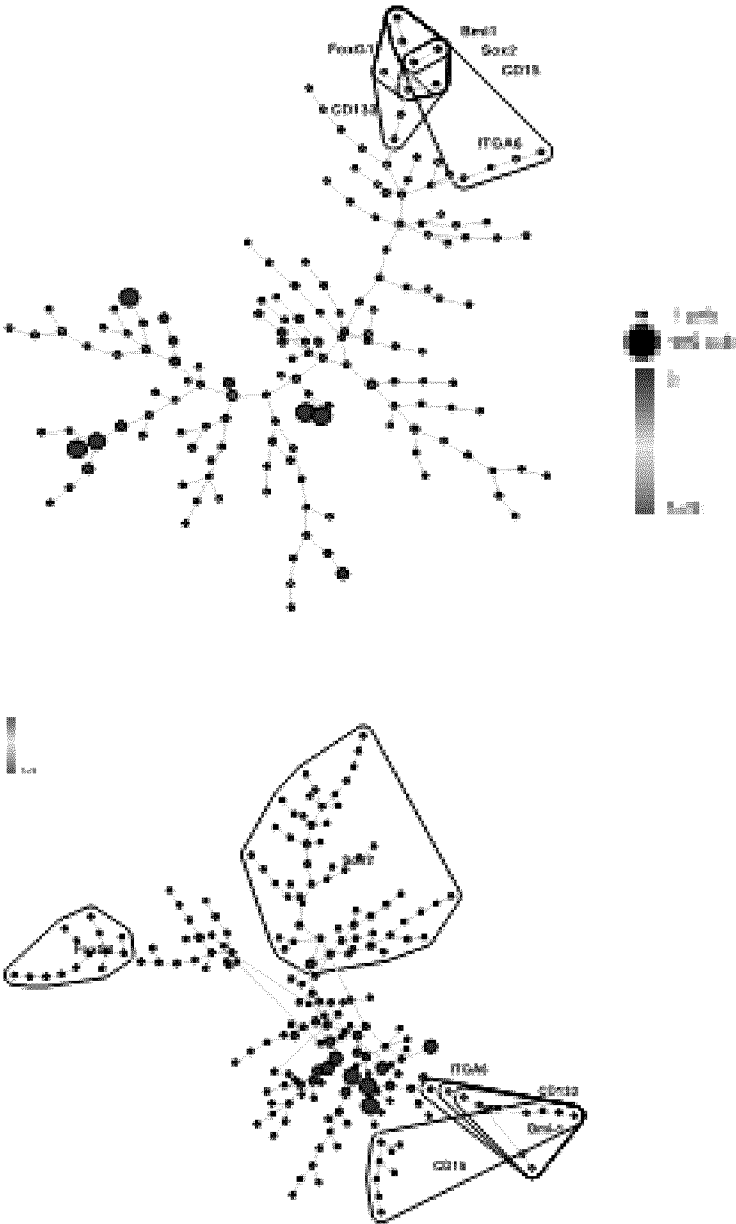


FIGURE 15A

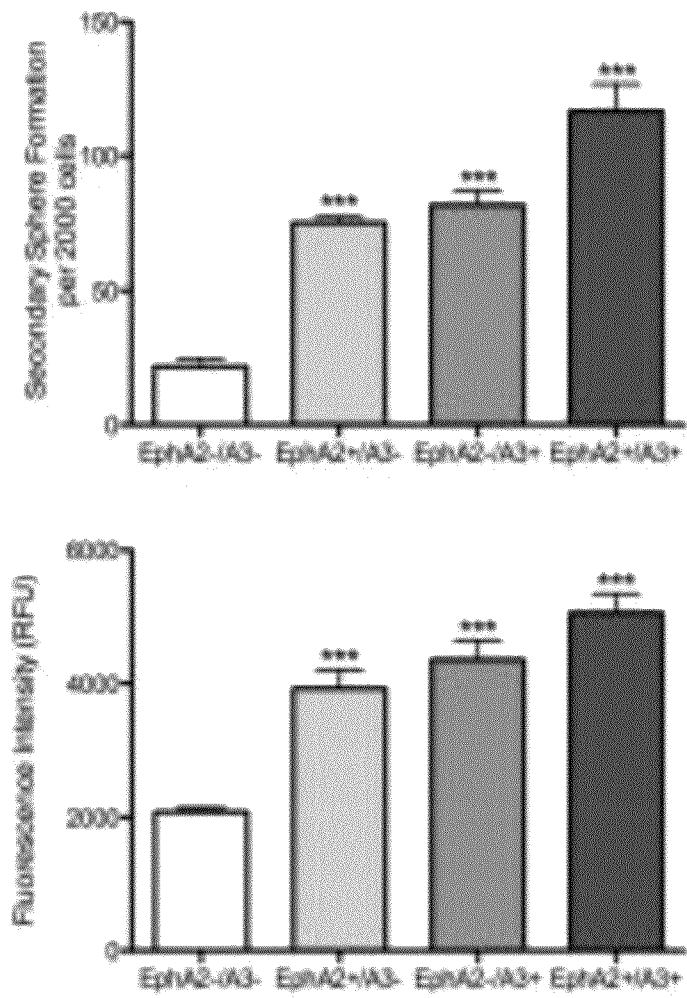


FIGURE 15B

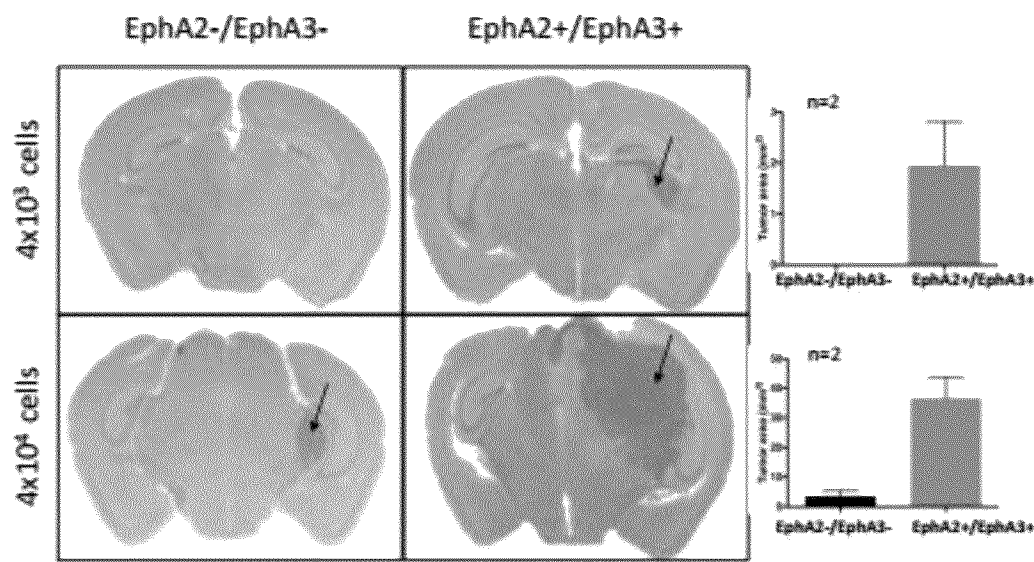


FIGURE 15C

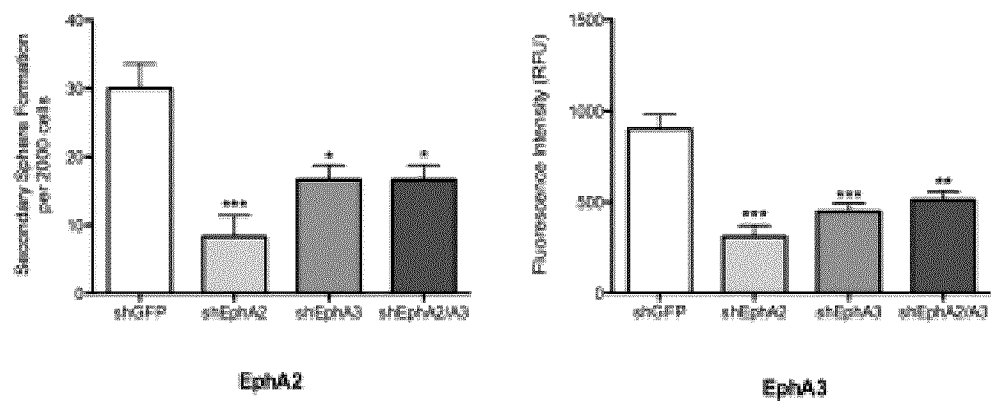


FIGURE 15D

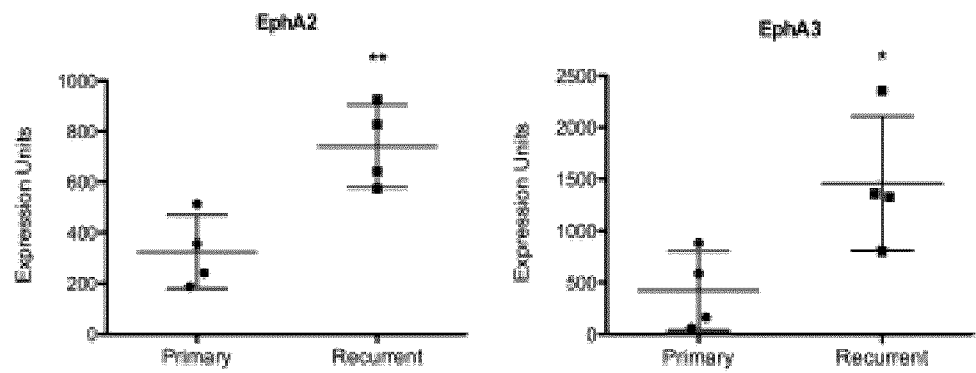


FIGURE 16A

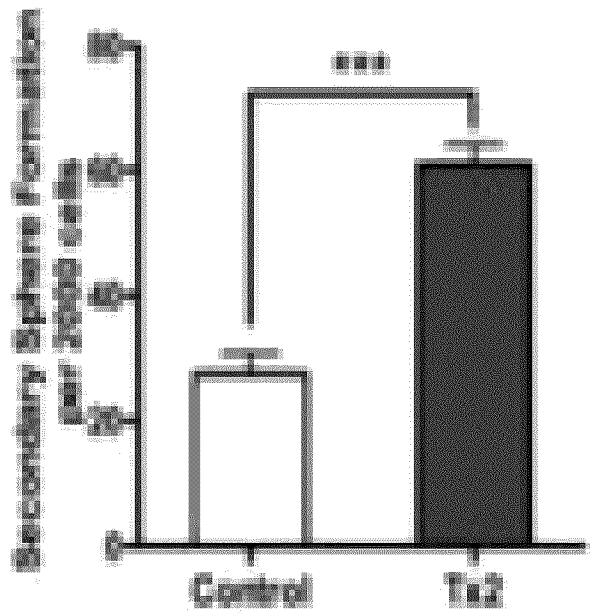


FIGURE 16B

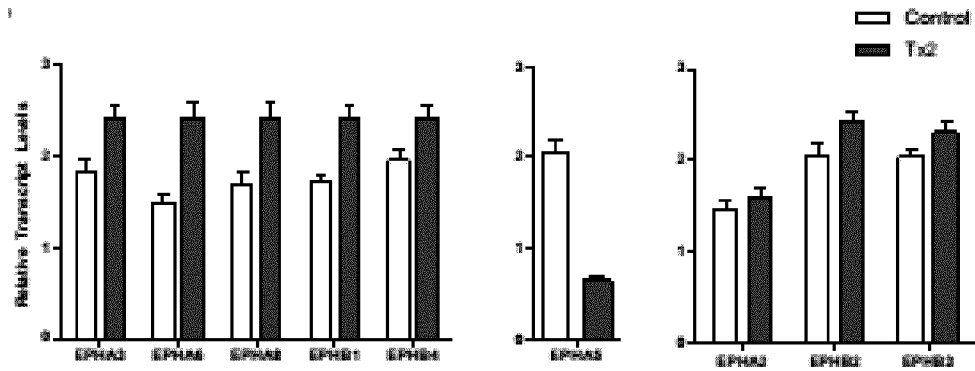


FIGURE 16C

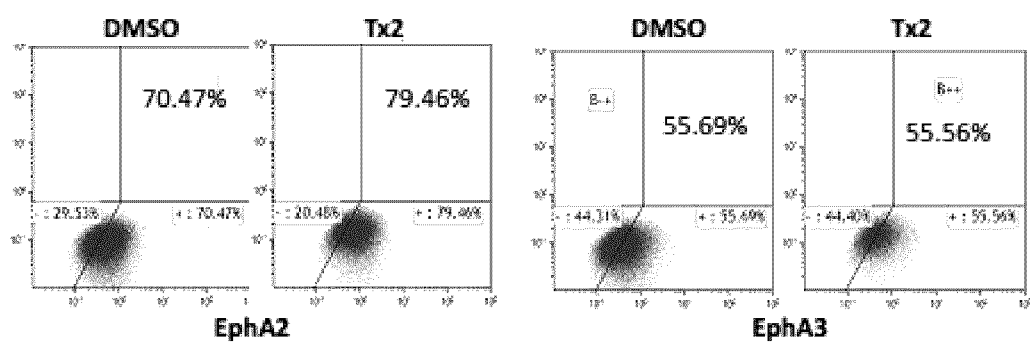


FIGURE 16D

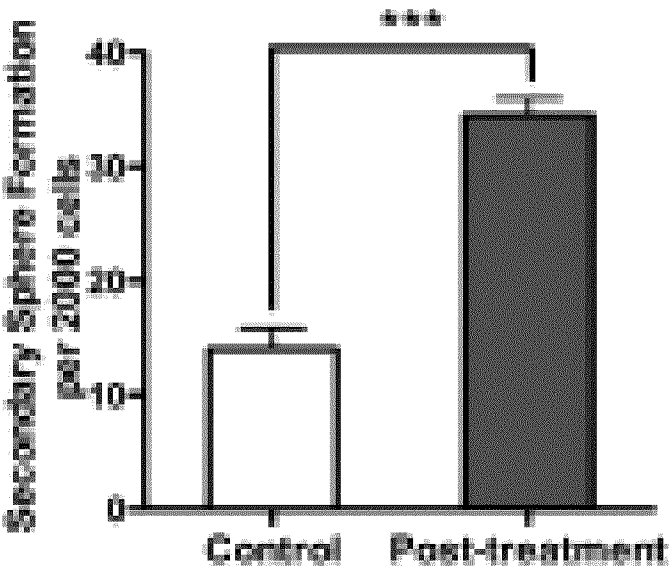


FIGURE 16E

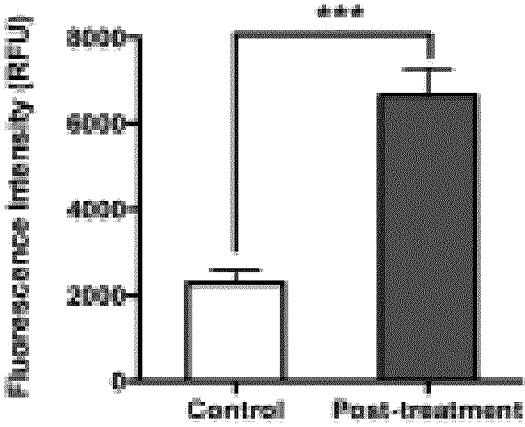


FIGURE 16F

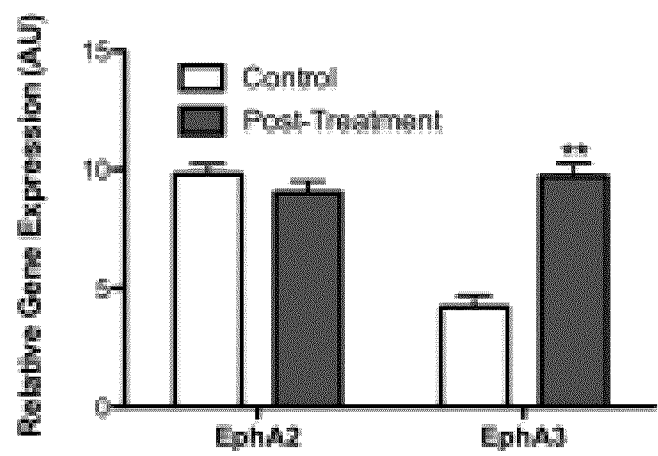


FIGURE 17A

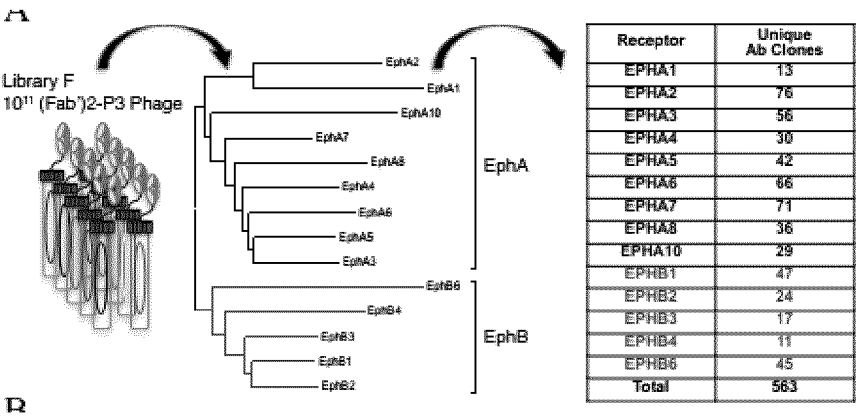


FIGURE 17B

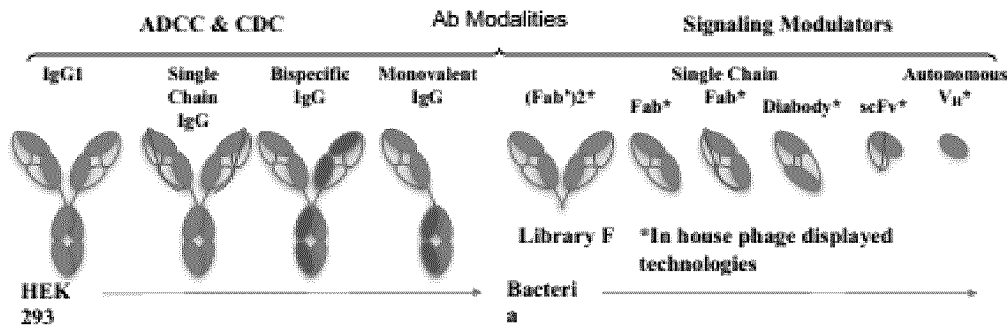


FIGURE 17C

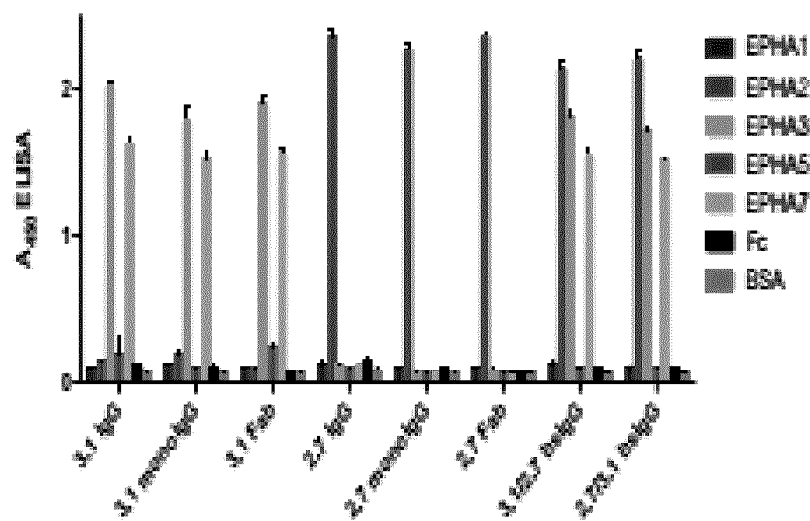


FIGURE 17D

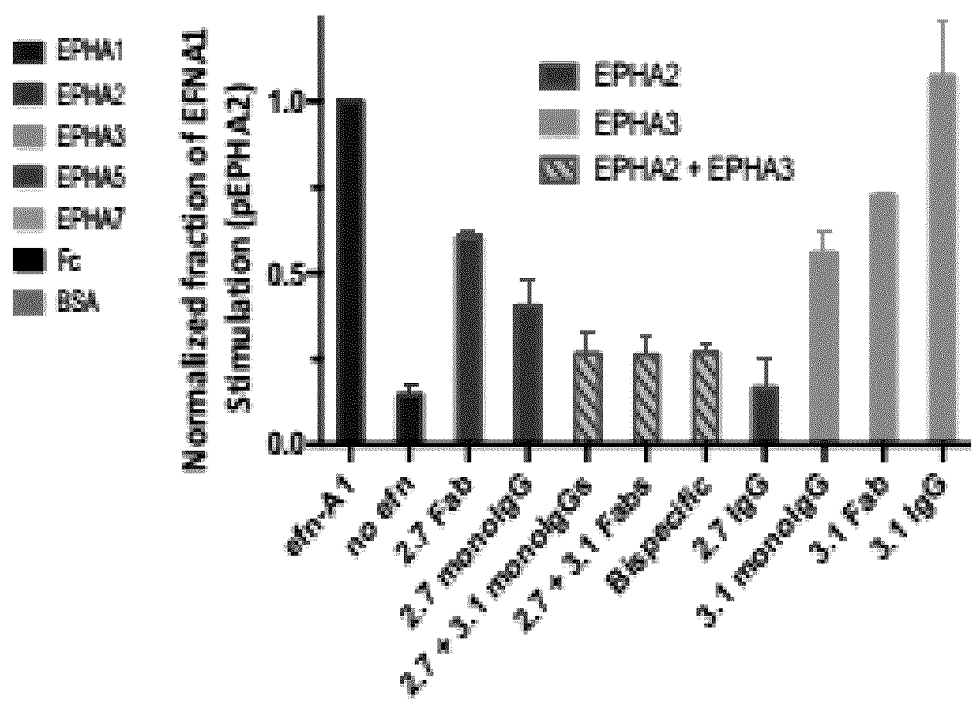


FIGURE 17E

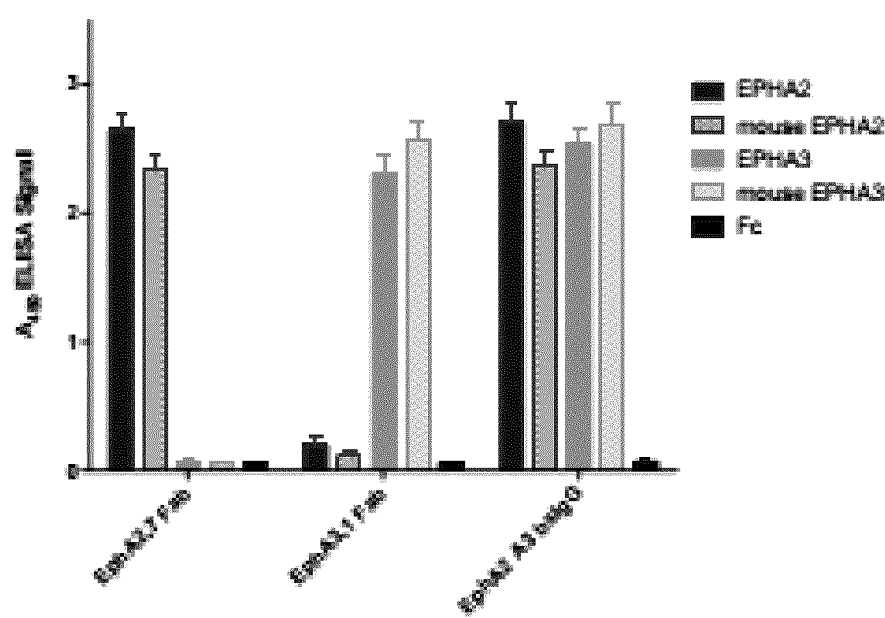


FIGURE 18A

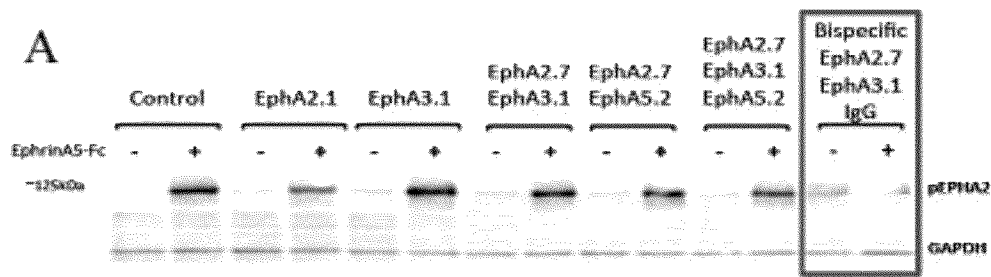


FIGURE 18B

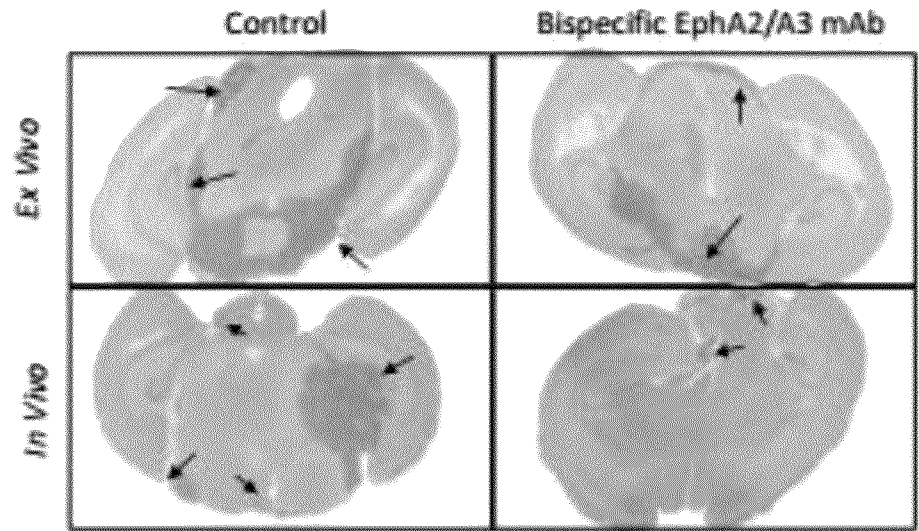


FIGURE 18C

