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(54) Title: NEW METHOD FOR TREATING RESISTANT GLIOBLASTOMA

(57) Abstract: The present invention relates to a compound which is an antagonist of  $\beta 8$  integrin or an inhibitor of the expression of  $\beta 8$  integrin for use in the treatment of resistant glioblastoma.



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**NEW METHOD FOR TREATING RESISTANT GLIOBLASTOMA****FIELD OF THE INVENTION:**

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The present invention relates to a compound which is an antagonist of  $\beta 8$  integrin or an inhibitor of the expression of  $\beta 8$  integrin for use in the treatment of resistant glioblastoma.

**BACKGROUND OF THE INVENTION:**

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Glioblastomas (GBM) are the most common and malignant primary brain tumors in adults, and account for more than 50% of malignant gliomas. Since 2005, the standard therapy for this tumor includes surgery followed by radiotherapy to 60 Gy in combination with temozolomide (TMZ), allowing an improved survival (Stupp R. et al, 2005). Nevertheless, the prognosis of the patients with a GBM remains very bad, with a median survival of 14.6 months, and a 2-year survival rate of 27.2%, because of a local recurrence mainly due to resistance of the GBM cells to radiotherapy.

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Glioblastoma, some malignant, invasive and radio/chemoresistant brain tumors, are characterized by prompt relapse. These aggressive properties at least involve, among these heterogeneous tumors, a subpopulation of highly tumorigenic and radioresistant glioblastoma stem-like cells (GSC). Current research focuses therefore on a specific targeting of this population but there is a need in new therapeutic compounds to treat resistant glioblastoma.

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**SUMMARY OF THE INVENTION:**

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The inventors showed that  $\beta 8$  integrin is overexpressed in glioblastoma stem cells (GSC) and appears to be associated with specific features and functionality to the GSC, including their radiation resistance.

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Thus, the invention relates to a compound which is an antagonist of  $\beta 8$  integrin or an inhibitor of the expression of  $\beta 8$  integrin for use in the treatment of resistant glioblastoma.

**DETAILED DESCRIPTION OF THE INVENTION:***Therapeutic method*

5 A first object of the invention relates to a compound which is an antagonist of  $\beta 8$  integrin or an inhibitor of the expression of  $\beta 8$  integrin for use in the treatment of resistant glioblastoma.

In one embodiment, the resistant glioblastoma is a radioresistant glioblastoma or a chemoresistant glioblastoma.

10 In another embodiment, said compound according to the invention is a  $\beta 8$  integrin antagonist.

In another embodiment, the glioblastoma is resistant to treatment using chemotherapeutic compound like Tipifarnib, Temozolomide, Cilengitide (see Stupp R et al, 2014), Bevacizumab (Thomas AA et al., 2014) and EGFR inhibitors like Erlotinib, Gefitinib or 15 Cetuximab.

In another embodiment, the invention relates to a compound which is an antagonist of  $\beta 8$  integrin or an inhibitor of the expression of  $\beta 8$  integrin for use in the treatment of glioblastoma which are characterized by the enrichment of stem cells (GSC) in glioblastoma.

20 According to the invention, the compound may also be an inhibitor of a molecular complex containing  $\beta 8$  integrin or an inhibitor of  $\beta 8$  integrin signaling pathway.

As used herein, the term "molecular complex containing  $\beta 8$  integrin" denotes a macromolecular structure formed by the functional  $\beta 8$  integrin present at the cellular membrane and any adaptors, effectors proteins and/or ligands at the extracellular and/or intracellular 25 interface and which is required to mediate any effects or functions of  $\beta 8$  integrin.

As used herein the term "Glioblastoma Stem Cells (GSC)" denotes a subpopulation of highly tumorigenic and radioresistant stem cells present in the brain tumor. These cells can express CD133 gene and/or a panel of other genes characteristic of neural stem cells (Nestin, 30 A2B5, Olig2, Sox2, etc..) and possess the self-renewal potential (see Cheng L et al. 2010).

Thus as used herein, the term "enrichment of stem cells (GSC) in glioblastoma" denotes an over-presence of GSC in glioblastoma.

As used herein, the term " $\beta$ 8 integrin" has its general meaning in the art and refers to a member of the integrin's family which are receptors that mediate attachment between a cell and the tissues surrounding it, which may be other cells or the extracellular matrix (ECM). They also play a role in cell signaling and thereby regulate cellular shape, motility, invasion, angiogenesis, survival and the cell cycle.

In one embodiment, the compound according to the invention may bind to  $\beta$ 8 integrin or  $\beta$ 8 integrin complex or inhibits  $\beta$ 8 integrin expression, and blocks the related physiological effects. To identify a compound able to block this molecule, cell adhesion assays on selective  $\beta$ 8 integrin substrates (fibronectin, vitronectin for example) will be performed as well as the measurement of extracellular TGF- $\beta$  activation, a downstream selective event to  $\beta$ 8 integrin involvement (Cambier et al Am J Pathol 2005). Intracellular downstream events to  $\beta$ 8 integrin activation will also be analyzed (interaction with RhoGDI, Reyes et al Mol Biol Cell 2013, or activation of the MAPK pathway) in response to its inhibition. Finally,  $\beta$ 8 integrin mRNA expression level and membrane expression of this protein will be assessed by qPCR and flow cytometry respectively to monitor the effect of inhibitors of  $\beta$ 8 integrin expression. Other validation systems could also be used to assess the inhibition/interaction of the aforementioned  $\beta$ 8 integrin inhibitor with its target, notably through cell free methods. For example, ELISA techniques using purified  $\beta$ 8 integrin could also be used.

In a particular embodiment, the compound according to the invention may be used to improve the sensitivity of glioblastoma to different chemotherapeutic agents or to enhance the sensitivity of glioblastoma to radiotherapy, or to improve glioblastoma sensitivity to associated radio/chemotherapy.

Thus, the invention also relates to a compound which is an antagonist of  $\beta$ 8 integrin or an inhibitor of the expression of  $\beta$ 8 integrin for use to improve the sensitivity of glioblastoma to radiotherapy.

In another particular embodiment, the compound according to the invention is administered in combination with radiotherapy.

Thus, the invention also relates to i) compound according to the invention, and ii) a radiotherapy, as a combined preparation for simultaneous, separate or sequential for use in the treatment of resistant glioblastoma.

In other word, the invention also relates to i) compound according to the invention, and ii) a radiotherapeutic agent, as a combined preparation for simultaneous, separate or sequential for use in the treatment of resistant glioblastoma.

5 In another embodiment, the invention also relates to i) compound according to the invention, and ii) a radiotherapeutic agent, as a combined preparation for simultaneous, separate or sequential to improve the sensitivity of resistant glioblastoma to radiotherapy.

In another particular embodiment, the compound according to the invention is administrated in combination with a chemotherapeutic agent.

10 Thus, the invention also relates to i) compound according to the invention, and ii) a chemotherapeutic agent, as a combined preparation for simultaneous, separate or sequential for use in the treatment of resistant glioblastoma.

In another embodiment, the invention also relates to i) compound according to the invention, and ii) chemotherapeutic agent, as a combined preparation for simultaneous, separate or sequential to improve the sensitivity of resistant glioblastoma to radiotherapy.

In another particular embodiment, the compound according to the invention is administrated in combination with radiotherapy and temozolomide (TMZ).

20 Thus, the invention also relates to i) compound according to the invention, and ii) a radiotherapy, and iii) TMZ as a combined preparation for simultaneous, separate or sequential use in the treatment of resistant glioblastoma.

In other word, the invention also relates to i) compound according to the invention, ii) a radiotherapeutic agent, and iii) TMZ as a combined preparation for simultaneous, separate or sequential for use in the treatment of resistant glioblastoma.

25 In another embodiment, the invention also relates to i) compound according to the invention, ii) a radiotherapeutic agent, and iii) TMZ as a combined preparation for simultaneous, separate or sequential to improve the sensitivity of resistant glioblastoma to radiotherapy.

30 As used herein, "radiotherapy" may consist of gamma-radiation, X-ray radiation, electrons or photons, external radiotherapy or curitherapy.

As used herein, the term "radiotherapeutic agent", is intended to refer to any radiotherapeutic agent known to one of skill in the art to be effective to treat or ameliorate cancer, without limitation. For instance, the radiotherapeutic agent can be an agent such as those administered in brachytherapy or radionuclide therapy. Such methods can optionally further

comprise the administration of one or more additional cancer therapies, such as, but not limited to, chemotherapies, and/or another radiotherapy.

5 In another particular embodiment, the invention also relates to a compound which is an antagonist of  $\beta 8$  integrin or an inhibitor of the expression of  $\beta 8$  integrin for use in the treatment of glioblastoma.

In one embodiment, said  $\beta 8$  integrin antagonist may be a low molecular weight antagonist, e. g. a small organic molecule (natural or not).

10 The term "small organic molecule" refers to a molecule (natural or not) of a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (e. g., proteins, nucleic acids, etc.). Preferred small organic molecules range in size up to about 10000 Da, more preferably up to 5000 Da, more preferably up to 2000 Da and most preferably up to about 1000 Da.

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Antagonists of  $\beta 8$  integrin are well known in the state of the art (see for example Minagawa S et al, 2014 or Sheldrake HM et al, 2014).

20 In another embodiment,  $\beta 8$  integrin, antagonist of the invention may be an anti- $\beta 8$  integrin antibody which neutralizes  $\beta 8$  integrin or an anti- $\beta 8$  integrin fragment thereof which neutralizes  $\beta 8$  integrin, or an anti-  $\beta 8$  integrin nanobody.

25 Antibodies directed against  $\beta 8$  integrin can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production. Although antibodies useful in practicing the invention can be polyclonal, monoclonal antibodies are preferred. Monoclonal antibodies against  $\beta 8$  integrin can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique originally described by Kohler and  
30 Milstein (1975); the human B-cell hybridoma technique (Cote et al, 1983); and the EBV-hybridoma technique (Cole et al. 1985). Alternatively, techniques described for the production of single chain antibodies (see e.g., U.S. Pat. No. 4,946,778) can be adapted to produce anti- $\beta 8$  integrin single chain antibodies.  $\beta 8$  integrin antagonists useful in practicing the present invention also include antibody fragments including but not limited to F(ab')<sub>2</sub> fragments, which

can be generated by pepsin digestion of an intact antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab and/or scFv expression libraries can be constructed to allow rapid identification of fragments having the desired specificity to  $\beta$ 8 integrin.

5 Humanized anti-P8 integrin antibodies and antibody fragments therefrom can also be prepared according to known techniques. "Humanized antibodies" are forms of non-human (e.g., rodent) chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region (CDRs) of the recipient are  
10 replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These  
15 modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an  
20 immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Methods for making humanized antibodies are described, for example, by Winter (U.S. Pat. No. 5,225,539) and Boss (Celltech, U.S. Pat. No. 4,816,397).

Then, for this invention, neutralizing antibodies of  $\beta$ 8 integrin are selected.

25 In a particular embodiment, the anti-P8 integrin antibody according to the invention is an antibody selected in the patent application WO20 13026004.

In a particular embodiment, the anti-P8 integrin antibody according to the invention is an antibody selected in the patent application WO 201 1103490.

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In a particular embodiment, the anti-P8 integrin antibody according to the invention is an antibody selected in the patent applications US5635601 or CA2790488.

In a particular embodiment, the anti-P8 integrin antibody according to the invention is an anti- $\alpha$ V $\beta$ 8 antibody selected in the patent application WO201 1020529.

5 In a particular embodiment, the anti-P8 integrin antibody according to the invention is the antibody MAB4775 obtained by the society R&D or the antibodies ab 172007 and ab80673 obtained by the society Abeam or the antibody sc-25714 obtained by the society Santa Cruz.

In still another embodiment,  $\beta$ 8 integrin antagonists may be selected from aptamers. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by Exponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996).

20 Then, for this invention, neutralizing aptamers of  $\beta$ 8 integrin are selected.

In another embodiment, the compound according to the invention may be selected from peptides, peptides mimetic or RGD peptide mimetics (see Sheldrake HM et al, 2014).

25 In a preferred embodiment, the compound according to the invention is an inhibitor of the  $\beta$ 8 integrin gene expression.

Small inhibitory RNAs (siRNAs) can also function as inhibitors of  $\beta$ 8 integrin gene expression for use in the present invention.  $\beta$ 8 integrin gene expression can be reduced by contacting a subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that  $\beta$ 8 integrin gene expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see for example Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos.

6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

5 Ribozymes can also function as inhibitors of  $\beta 8$  integrin gene expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of  $\beta 8$  integrin mRNA sequences are thereby  
10 useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted  
15 structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

Both antisense oligonucleotides and ribozymes useful as inhibitors of  $\beta 8$  integrin gene  
20 expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramidite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase  
25 promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

30 Antisense oligonucleotides siRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide siRNA or ribozyme nucleic acid to the cells and preferably cells expressing  $\beta 8$  integrin. Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in

the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide siRNA or ribozyme nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, 1990 and in Murry, 1991).

Preferred viruses for certain applications are the adeno-viruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic

integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g. Sambrook et al., 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, eye, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

In a preferred embodiment, the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequence is under the control of a heterologous regulatory region, e.g., a heterologous promoter. The promoter may be specific for Muller glial cells, microglia cells, endothelial cells, pericyte cells and astrocytes. For example, a specific expression in Muller glial cells may be obtained through the promoter of the glutamine synthetase gene is suitable. The promoter can also be, e.g., a viral promoter, such as CMV promoter or any synthetic promoters.

Another object of the invention relates to a method for treating resistant glioblastoma comprising administering to a subject in need thereof a therapeutically effective amount of a compound which is an antagonist of  $\beta 8$  integrin or an inhibitor of the expression of  $\beta 8$  integrin as described above.

Compounds of the invention may be administered in the form of a pharmaceutical composition, as defined below.

By a "therapeutically effective amount" is meant a sufficient amount of compound to treat resistant glioblastoma disorder.

It will be understood that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment and/or during phase 1 clinical trial. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

Compounds according to the invention may be used for the preparation of a pharmaceutical composition for the treatment of resistant glioblastoma.

Hence, the present invention also provides a pharmaceutical composition comprising an effective dose of an antagonist of  $\beta 8$  integrin or an inhibitor of the  $\beta 8$  integrin expression, particularly a  $\beta 8$  integrin antagonist, according to the invention.

Any therapeutic agent of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable

carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and sex of the patient, etc.

The pharmaceutical compositions of the invention can be formulated for a topical, oral, intranasal, parenteral, intraocular, intravenous, intramuscular, intratumoral or subcutaneous administration and the like.

Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment.

In addition, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; time release capsules; and any other form currently can be used.

Compositions of the present invention may comprise a further therapeutic active agent. The present invention also relates to a kit comprising an antagonist or an inhibitor according to the invention and a further therapeutic active agent.

In one embodiment said therapeutic active agent is an anticancer agent. For example, said anticancer agents include but are not limited to fludarabine, gemcitabine, capecitabine, methotrexate, taxol, taxotere, mercaptopurine, thioguanine, hydroxyurea, cytarabine, cyclophosphamide, ifosfamide, nitrosoureas, platinum complexes such as cisplatin, carboplatin and oxaliplatin, mitomycin, dacarbazine, procarbazine, etoposide, teniposide, campathecins, bleomycin, doxorubicin, idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, L-asparaginase, doxorubicin, epimibicm, 5-fluorouracil, taxanes such as docetaxel and paclitaxel, leucovorin, levamisole, irinotecan, estramustine, etoposide, nitrogen mustards, BCNU, nitrosoureas such as carmustme and lomustine, vinca alkaloids such as vinblastine, vincristine and vinorelbine, imatimb mesylate, hexamethylmelamine, topotecan, kinase inhibitors, phosphatase inhibitors, ATPase inhibitors, typhostins, protease inhibitors, inhibitors

herbimycin A, genistein, erastatin, temozolomide and lavendustin A. In one embodiment, additional anticancer agents may be selected from, but are not limited to, one or a combination of the following class of agents: alkylating agents, plant alkaloids, DNA topoisomerase inhibitors, anti-folates, pyrimidine analogs, purine analogs, DNA antimetabolites, taxanes, podophyllotoxin, hormonal therapies, retinoids, photosensitizers or photodynamic therapies, angiogenesis inhibitors, antimetabolic agents, isoprenylation inhibitors, cell cycle inhibitors, actinomycins, bleomycins, anthracyclines, MDR inhibitors and Ca<sup>2+</sup> ATPase inhibitors.

Additional anticancer agents may be selected from, but are not limited to, cytokines, chemokines, growth factors, growth inhibitory factors, hormones, soluble receptors, decoy receptors, monoclonal or polyclonal antibodies, mono-specific, bi-specific or multi-specific antibodies, monobodies, polybodies.

Additional anticancer agent may be selected from, but are not limited to, growth or hematopoietic factors such as erythropoietin and thrombopoietin, and growth factor mimetics thereof.

In the present methods for treating cancer the further therapeutic active agent can be an antiemetic agent. Suitable antiemetic agents include, but are not limited to, metoclopramide, domperidone, prochlorperazine, promethazine, chlorpromazine, trimethobenzamide, ondansetron, granisetron, hydroxyzine, acetylleucine monoemolamine, alizapride, azasetron, benzquinamide, biantanautine, bromopride, buclizine, clebopride, cyclizine, dunenhydrinate, diphenidol, dolasetron, meclizine, methallatal, metopimazine, nabilone, oxypemdyl, pipamazine, scopolamine, sulphiride, tetrahydrocannabinols, thiephylperazine, thioproperazine and tropisetron. In a preferred embodiment, the antiemetic agent is granisetron or ondansetron.

In another embodiment, the further therapeutic active agent can be an hematopoietic colony stimulating factor. Suitable hematopoietic colony stimulating factors include, but are not limited to, filgrastim, sargramostim, molgramostim and epoietin alpha.

In still another embodiment, the other therapeutic active agent can be an opioid or non-opioid analgesic agent. Suitable opioid analgesic agents include, but are not limited to, morphine, heroin, hydromorphone, hydrocodone, oxymorphone, oxycodone, metopon, apomorphine, nomioipine, etoipbine, buprenorphine, mepedidine, lopermide, anileddine, ethoheptazine, piminidine, betaprodine, diphenoxylate, fentanil, sufentanil, alfentanil, remifentanil, levorphanol, dextromethorphan, phenazodone, pemazocine, cyclazocine, methadone, isomethadone and propoxyphene. Suitable non-opioid analgesic agents include, but are not limited to, aspirin, celecoxib, rofecoxib, diclofenac, diflusal, etodolac, fenoprofen,

flurbiprofen, ibuprofen, ketoprofen, indomethacin, ketorolac, meclofenamate, mefanamic acid, nabumetone, naproxen, piroxicam and sulindac.

5

### *Diagnostic method*

In another aspect, the invention relates to an ex vivo method for diagnosing resistant glioblastoma, comprising the step of determining the level expression of the marker  $\beta 8$  integrin in a tumor sample obtained from a patient.

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Typically, the tumor sample according to the invention may be a cancer biopsy or a cancer biopsy obtained during surgery.

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The term "detecting" as used above includes qualitative and/or quantitative detection (measuring levels) with or without reference to a control. Typically  $\beta 8$  integrin expression may be measured for example by enzyme-labeled and mediated immunoassays (such as ELISA) or by flow cytometry performed on the sample. Moreover, FACS sorting could additionally be performed on  $\beta 8$  integrin positive tumoral cells.

20

Particularly, the invention relates to a method for diagnosis of resistant glioblastoma in a patient comprising a step a) consisting of measuring  $\beta 8$  integrin expression level in a sample obtained from said patient. Preferably, the method of the invention further comprises a step of comparing the  $\beta 8$  integrin expression level obtained in step a) to a threshold value.

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The "control" may be a healthy subject, i.e. a subject who does not suffer from any resistant glioblastoma. The control may also be a subject suffering from resistant glioblastoma. Preferably, said control is a healthy subject.

Detection of  $\beta 8$  integrin expression in the sample may be performed by measuring the level of  $\beta 8$  integrin protein. In the present application, the "level of  $\beta 8$  integrin protein" means the quantity or concentration of said  $\beta 8$  integrin protein.

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Such methods comprise contacting a sample with a binding partner capable of selectively interacting with  $\beta 8$  integrin protein present in the sample. The binding partner is generally an antibody that may be polyclonal or monoclonal, preferably monoclonal.

The presence of the protein can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination

tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; Immuno-electrophoresis; immunoprecipitation, immunohistochemistry, etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of  
5 a complex between the antigen and the antibody or antibodies reacted therewith.

The aforementioned assays generally involve separation of unbound protein in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or  
10 microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies against the proteins to be tested. A sample containing or  
15 suspected of containing the marker protein is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule is added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate is washed and the presence of the secondary binding molecule is detected  
20 using methods well known in the art.

Various immunoenzymatic staining methods are known in the art for detecting a protein of interest. For example, immunoenzymatic interactions can be visualized using different enzymes such as peroxidase, alkaline phosphatase, or different chromogens such as DAB, AEC, or Fast Red; or fluorescent labels such as FITC, Cy3, Cy5, Cy7, Alexafluors, APC, etc.  
25 Counterstains may include H&E, DAPI, Hoechst, so long as such stains are compatible with other detection reagents and the visualization strategy used. As known in the art, amplification reagents may be used to intensify staining signal. For example, tyramide reagents may be used. The staining methods of the present invention may be accomplished using any suitable method or system as would be apparent to one of skill in the art, including automated, semi-automated  
30 or manual systems.

The method of the invention may comprise a further step consisting of comparing  $\beta 8$  integrin expression with a control reference.

The invention thus relates to a method for diagnosis resistant glioblastoma in a patient comprising determining the expression level of  $\beta 8$  integrin in a sample obtained from said

patient and comparing said expression level to a threshold value. As used herein, "expression level of  $\beta 8$  integrin" refers to an amount of a translation product, for instance the protein  $\beta 8$  integrin. Typically, a level of mRNA expression can be expressed in units such as transcripts per cell or nanograms per microgram of tissue. A level of a polypeptide can be expressed as  
5 nanograms per microgram of tissue or nanograms per milliliter of a culture medium, for example. Alternatively, relative units can be employed to describe an expression level.

Typically, a "threshold value", "threshold level" or "cut-off value" can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a  
10 person of ordinary skilled in the art. Preferably, the person skilled in the art may compare the expression levels of  $\beta 8$  integrin obtained according to the method of the invention with a defined threshold value.

Preferably, said threshold value is the mean expression level of  $\beta 8$  integrin of a population of healthy individuals. As used herein, the term "healthy individual" denotes a  
15 human which is known to be healthy, i.e. which does not suffer from resistant glioblastoma, has never been subjected to such resistant glioblastoma, and does not need any medical care.

Typically, the skilled person in the art may determine the expression level of  $\beta 8$  integrin in a biological sample of 100 individuals known to be healthy. The mean value of the obtained expression levels is then determined, according to well known statistical analysis, so as to obtain  
20 the mean expression level of  $\beta 8$  integrin. Said value is then considered as being normal and thus constitute a threshold value. By comparing the expression levels of  $\beta 8$  integrin to this threshold value, the physician is then able to diagnose resistant glioblastoma. Indeed, by comparing the expression level of  $\beta 8$  integrin obtained in a biological sample, of a given subject to a threshold value, one can easily determine whether said subject suffers from resistant  
25 glioblastoma or not.

Accordingly, the physician would be able to adapt and optimize appropriate medical care of a subject in a critical and life-threatening condition suffering from resistant glioblastoma. The determination of said prognosis is highly appropriate for follow-up care and clinical decision making.

Therefore, the invention is drawn to a method for diagnosis resistant glioblastoma in a  
30 patient comprising the following steps:

a) determining the level of expression of  $\beta 8$  integrin in a sample obtained from said patient;

b) determining the mean expression level of  $\beta 8$  integrin in a biological sample of a population of healthy individuals, preferably 100 healthy individuals; and

c) a step of comparing the expression level of  $\beta 8$  integrin obtained of a) to the mean expression level of  $\beta 8$  integrin obtained in b).

5

The present invention also relates to kits for the diagnosis of resistant glioblastoma, comprising means for detecting  $\beta 8$  integrin expression.

According to the invention, the kits of the invention may comprise an anti-  $\beta 8$  integrin protein antibody; and another molecule coupled with a signalling system which binds to said  $\beta 8$  integrin protein antibody.

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Typically, the antibodies or combination of antibodies are in the form of solutions ready for use. In one embodiment, the kit comprises containers with the solutions ready for use. Any other forms are encompassed by the present invention and the man skilled in the art can routinely adapt the form to the use in immunohistochemistry.

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In another embodiment, the expression level of the gene of  $\beta 8$  integrin can be done. Measuring the expression level of a gene can be performed by a variety of techniques well known in the art.

Typically, the expression level of a gene may be determined by determining the quantity of mRNA. Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid contained in the samples (e.g., cell or tissue prepared from the patient) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's instructions. The extracted mRNA is then detected by hybridization (e. g., Northern blot analysis, in situ hybridization) and/or amplification (e.g., RT-PCR).

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Other methods of Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes or amplification primers. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more preferably 85% identical and even more preferably 90-95% identical. In certain embodiments, it will be

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advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization.

Typically, the nucleic acid probes include one or more labels, for example to permit detection of a target nucleic acid molecule using the disclosed probes. In various applications, such as in situ hybridization procedures, a nucleic acid probe includes a label (e.g., a detectable label). A "detectable label" is a molecule or material that can be used to produce a detectable signal that indicates the presence or concentration of the probe (particularly the bound or hybridized probe) in a sample. Thus, a labeled nucleic acid molecule provides an indicator of the presence or concentration of a target nucleic acid sequence (e.g., genomic target nucleic acid sequence) (to which the labeled uniquely specific nucleic acid molecule is bound or hybridized) in a sample. A label associated with one or more nucleic acid molecules (such as a probe generated by the disclosed methods) can be detected either directly or indirectly. A label can be detected by any known or yet to be discovered mechanism including absorption, emission and/ or scattering of a photon (including radio frequency, microwave frequency, infrared frequency, visible frequency and ultra-violet frequency photons). Detectable labels include colored, fluorescent, phosphorescent and luminescent molecules and materials, catalysts (such as enzymes) that convert one substance into another substance to provide a detectable difference (such as by converting a colorless substance into a colored substance or vice versa, or by producing a precipitate or increasing sample turbidity), haptens that can be detected by antibody binding interactions, and paramagnetic and magnetic molecules or materials.

Particular examples of detectable labels include fluorescent molecules (or fluorochromes). Numerous fluorochromes are known to those of skill in the art, and can be selected, for example from Life Technologies (formerly Invitrogen), (e.g., see, the Handbook Guide to Fluorescent Probes and Labeling Technologies). Examples of particular fluorophores that can be attached (for example, chemically conjugated) to a nucleic acid molecule (such as a uniquely specific binding region) are provided in U.S. Pat. No. 5,866, 366 to Nazarenko et al., such as 4-acetamido-4'-isothiocyanatostilbene-2,2' disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS), 4-amino -N- [3 vinylsulfonyl]phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)maleimide, antllranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumarin 151); cyanosine; 4',6-diarninidino-2-phenylindole (DAPI); 5',5"dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7 -

diethylamino -3 - (4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfuric acid; 5-[dimethylamino] naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC Q(RITC); 2',7'-difluorofluorescein (OREGON GREEN®); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthalaldehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron Brilliant Red 3B-A); rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, rhodamine green, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives.

Other suitable fluorophores include thiol-reactive europium chelates which emit at approximately 617 nm (Heyduk and Heyduk, *Analyt. Biochem.* 248:216-27, 1997; *J. Biol. Chem.* 274:3315-22, 1999), as well as GFP, Lissamine™, diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Pat. No. 5,800,996 to Lee et al.) and derivatives thereof. Other fluorophores known to those skilled in the art can also be used, for example those available from Life Technologies (Invitrogen; Molecular Probes (Eugene, Oreg.)) and including the ALEXA FLUOR® series of dyes (for example, as described in U.S. Pat. Nos. 5,696,157, 6,130,101 and 6,716,979), the BODIPY series of dyes (dipyrrrometheneboron difluoride dyes, for example as described in U.S. Pat. Nos. 4,774,339, 5,187,288, 5,248,782, 5,274,113, 5,338,854, 5,451,663 and 5,433,896), Cascade Blue (an amine reactive derivative of the sulfonated pyrene described in U.S. Pat. No. 5,132,432) and Marina Blue (U.S. Pat. No. 5,830,912).

In addition to the fluorochromes described above, a fluorescent label can be a fluorescent nanoparticle, such as a semiconductor nanocrystal, e.g., a QUANTUM DOT™ (obtained, for example, from Life Technologies (QuantumDot Corp, Invitrogen Nanocrystal

Technologies, Eugene, Oreg.); see also, U.S. Pat. Nos. 6,815,064; 6,682,596; and 6,649, 138). Semiconductor nanocrystals are microscopic particles having size-dependent optical and/or electrical properties. When semiconductor nanocrystals are illuminated with a primary energy source, a secondary emission of energy occurs of a frequency that corresponds to the bandgap of the semiconductor material used in the semiconductor nanocrystal. This emission can be detected as colored light of a specific wavelength or fluorescence. Semiconductor nanocrystals with different spectral characteristics are described in e.g., U.S. Pat. No. 6,602,671. Semiconductor nanocrystals that can be coupled to a variety of biological molecules (including dNTPs and/or nucleic acids) or substrates by techniques described in, for example, Bruchez et al, Science 281 :20132016, 1998; Chan et al, Science 281:2016-2018, 1998; and U.S. Pat. No. 6,274,323. Formation of semiconductor nanocrystals of various compositions are disclosed in, e.g., U.S. Pat. Nos. 6,927, 069; 6,914,256; 6,855,202; 6,709,929; 6,689,338; 6,500,622; 6,306,736; 6,225,198; 6,207,392; 6,114,038; 6,048,616; 5,990,479; 5,690,807; 5,571,018; 5,505,928; 5,262,357 and in U.S. Patent Publication No. 2003/0165951 as well as PCT Publication No. 99/26299 (published May 27, 1999). Separate populations of semiconductor nanocrystals can be produced that are identifiable based on their different spectral characteristics. For example, semiconductor nanocrystals can be produced that emit light of different colors based on their composition, size or size and composition. For example, quantum dots that emit light at different wavelengths based on size (565 nm, 655 nm, 705 nm, or 800 nm emission wavelengths), which are suitable as fluorescent labels in the probes disclosed herein are available from Life Technologies (Carlsbad, Calif).

Additional labels include, for example, radioisotopes (such as  $^3\text{H}$ ), metal chelates such as DOTA and DPTA chelates of radioactive or paramagnetic metal ions like  $\text{Gd}^{3+}$ , and liposomes.

Detectable labels that can be used with nucleic acid molecules also include enzymes, for example horseradish peroxidase, alkaline phosphatase, acid phosphatase, glucose oxidase, beta-galactosidase, beta-glucuronidase, or beta-lactamase.

Alternatively, an enzyme can be used in a metallographic detection scheme. For example, silver in situ hybridization (SISH) procedures involve metallographic detection schemes for identification and localization of a hybridized genomic target nucleic acid sequence. Metallographic detection methods include using an enzyme, such as alkaline phosphatase, in combination with a water-soluble metal ion and a redox-inactive substrate of the enzyme. The substrate is converted to a redox-active agent by the enzyme, and the redoxactive agent reduces the metal ion, causing it to form a detectable precipitate. (See, for

example, U.S. Patent Application Publication No. 2005/0100976, PCT Publication No. 2005/003777 and U.S. Patent Application Publication No. 2004/0265922). Metallographic detection methods also include using an oxido-reductase enzyme (such as horseradish peroxidase) along with a water soluble metal ion, an oxidizing agent and a reducing agent, again to form a detectable precipitate. (See, for example, U.S. Pat. No. 6,670,113).

Probes made using the disclosed methods can be used for nucleic acid detection, such as ISH procedures (for example, fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH) and silver in situ hybridization (SISH)) or comparative genomic hybridization (CGH).

In situ hybridization (ISH) involves contacting a sample containing target nucleic acid sequence (e.g., genomic target nucleic acid sequence) in the context of a metaphase or interphase chromosome preparation (such as a cell or tissue sample mounted on a slide) with a labeled probe specifically hybridizable or specific for the target nucleic acid sequence (e.g., genomic target nucleic acid sequence). The slides are optionally pretreated, e.g., to remove paraffin or other materials that can interfere with uniform hybridization. The sample and the probe are both treated, for example by heating to denature the double stranded nucleic acids. The probe (formulated in a suitable hybridization buffer) and the sample are combined, under conditions and for sufficient time to permit hybridization to occur (typically to reach equilibrium). The chromosome preparation is washed to remove excess probe, and detection of specific labeling of the chromosome target is performed using standard techniques.

For example, a biotinylated probe can be detected using fluorescein-labeled avidin or avidin-alkaline phosphatase. For fluorochrome detection, the fluorochrome can be detected directly, or the samples can be incubated, for example, with fluorescein isothiocyanate (FITC)-conjugated avidin. Amplification of the FITC signal can be effected, if necessary, by incubation with biotin-conjugated goat antiavidin antibodies, washing and a second incubation with FITC-conjugated avidin. For detection by enzyme activity, samples can be incubated, for example, with streptavidin, washed, incubated with biotin-conjugated alkaline phosphatase, washed again and pre-equilibrated (e.g., in alkaline phosphatase (AP) buffer). For a general description of in situ hybridization procedures, see, e.g., U.S. Pat. No. 4,888,278.

Numerous procedures for FISH, CISH, and SISH are known in the art. For example, procedures for performing FISH are described in U.S. Pat. Nos. 5,447,841; 5,472,842; and 5,427,932; and for example, in Pirlkel et al, Proc. Natl. Acad. Sci. 83:2934-2938, 1986; Pinkel et al, Proc. Natl. Acad. Sci. 85:9138-9142, 1988; and Lichter et al, Proc. Natl. Acad. Sci. 85:9664-9668, 1988. CISH is described in, e.g., Tanner et al, Am. J. Pathol. 157:1467-1472,

2000 and U.S. Pat. No. 6,942,970. Additional detection methods are provided in U.S. Pat. No. 6,280,929.

Numerous reagents and detection schemes can be employed in conjunction with FISH, CISH, and SISH procedures to improve sensitivity, resolution, or other desirable properties. As discussed above probes labeled with fluorophores (including fluorescent dyes and QUANTUM DOTS®) can be directly optically detected when performing FISH. Alternatively, the probe can be labeled with a nonfluorescent molecule, such as a hapten (such as the following non-limiting examples: biotin, digoxigenin, DNP, and various oxazoles, pyrazoles, thiazoles, nitroaryls, benzofurazans, triterpenes, ureas, thioureas, rotenones, coumarin, coumarin-based compounds, Podophyllotoxin, Podophyllotoxin-based compounds, and combinations thereof), ligand or other indirectly detectable moiety. Probes labeled with such non-fluorescent molecules (and the target nucleic acid sequences to which they bind) can then be detected by contacting the sample (e.g., the cell or tissue sample to which the probe is bound) with a labeled detection reagent, such as an antibody (or receptor, or other specific binding partner) specific for the chosen hapten or ligand. The detection reagent can be labeled with a fluorophore (e.g., QUANTUM DOT®) or with another indirectly detectable moiety, or can be contacted with one or more additional specific binding agents (e.g., secondary or specific antibodies), which can be labeled with a fluorophore.

In other examples, the probe, or specific binding agent (such as an antibody, e.g., a primary antibody, receptor or other binding agent) is labeled with an enzyme that is capable of converting a fluorogenic or chromogenic composition into a detectable fluorescent, colored or otherwise detectable signal (e.g., as in deposition of detectable metal particles in SISH). As indicated above, the enzyme can be attached directly or indirectly via a linker to the relevant probe or detection reagent. Examples of suitable reagents (e.g., binding reagents) and chemistries (e.g., linker and attachment chemistries) are described in U.S. Patent Application Publication Nos. 2006/0246524; 2006/0246523, and 2007/ 01 17153.

It will be appreciated by those of skill in the art that by appropriately selecting labelled probe-specific binding agent pairs, multiplex detection schemes can be produced to facilitate detection of multiple target nucleic acid sequences (e.g., genomic target nucleic acid sequences) in a single assay (e.g., on a single cell or tissue sample or on more than one cell or tissue sample). For example, a first probe that corresponds to a first target sequence can be labelled with a first hapten, such as biotin, while a second probe that corresponds to a second target sequence can be labelled with a second hapten, such as DNP. Following exposure of the sample to the probes, the bound probes can be detected by contacting the sample with a first specific

binding agent (in this case avidin labelled with a first fluorophore, for example, a first spectrally distinct QUANTUM DOT®, e.g., that emits at 585 nm) and a second specific binding agent (in this case an anti-DNP antibody, or antibody fragment, labelled with a second fluorophore (for example, a second spectrally distinct QUANTUM DOT®, e.g., that emits at 705 nm).

5 Additional probes/binding agent pairs can be added to the multiplex detection scheme using other spectrally distinct fluorophores. Numerous variations of direct, and indirect (one step, two step or more) can be envisioned, all of which are suitable in the context of the disclosed probes and assays.

10 Probes typically comprise single-stranded nucleic acids of between 10 to 1000 nucleotides in length, for instance of between 10 and 800, more preferably of between 15 and 700, typically of between 20 and 500. Primers typically are shorter single-stranded nucleic acids, of between 10 to 25 nucleotides in length, designed to perfectly or almost perfectly match a nucleic acid of interest, to be amplified. The probes and primers are "specific" to the nucleic acids they hybridize to, i.e. they preferably hybridize under high stringency hybridization  
15 conditions (corresponding to the highest melting temperature  $T_m$ , e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

The nucleic acid primers or probes used in the above amplification and detection method may be assembled as a kit. Such a kit includes consensus primers and molecular probes. A preferred kit also includes the components necessary to determine if amplification has occurred.  
20 The kit may also include, for example, PCR buffers and enzymes; positive control sequences, reaction control primers; and instructions for amplifying and detecting the specific sequences.

In a particular embodiment, the methods of the invention comprise the steps of providing total RNAs extracted from cumulus cells and subjecting the RNAs to amplification and hybridization to specific probes, more particularly by means of a quantitative or semi-quantitative RT-PCR.  
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In another preferred embodiment, the expression level is determined by DNA chip analysis. Such DNA chip or nucleic acid microarray consists of different nucleic acid probes that are chemically attached to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead. A microchip may be constituted of polymers, plastics, resins,  
30 polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, or nitrocellulose. Probes comprise nucleic acids such as cDNAs or oligonucleotides that may be about 10 to about 60 base pairs. To determine the expression level, a sample from a test subject, optionally first subjected to a reverse transcription, is labelled and contacted with the microarray in hybridization conditions, leading to the formation of complexes between target nucleic acids

that are complementary to probe sequences attached to the microarray surface. The labelled hybridized complexes are then detected and can be quantified or semi-quantified. Labelling may be achieved by various methods, e.g. by using radioactive or fluorescent labelling. Many variants of the microarray hybridization technology are available to the man skilled in the art (see e.g. the review by Hoheisel, Nature Reviews, Genetics, 2006, 7:200-210).

Expression level of a gene may be expressed as absolute expression level or normalized expression level. Typically, expression levels are normalized by correcting the absolute expression level of a gene by comparing its expression to the expression of a gene that is not a relevant for determining the cancer stage of the patient, e.g., a housekeeping gene that is constitutively expressed. Suitable genes for normalization include housekeeping genes such as the actin gene ACTB, ribosomal 18S gene, GUSB, PGK1 and TFRC. This normalization allows the comparison of the expression level in one sample, e.g., a patient sample, to another sample, or between samples from different sources.

In another embodiment, detection of mutations or gene copy-number amplification related to the  $\beta 8$  integrin gene or RNA of patients can be performed. Several techniques can be used like PCR, sequencing methods (Sanger, pyrosequencing, next generation sequencing (NGS) of targeted regions or whole genome). These techniques can be used to find altered  $\beta 8$  integrin in patient glioblastoma. Gene Copy Number can also be used to find amplifications or deletions of one or several exons of the  $\beta 8$  integrin gene or of the total gene leading to protein inactivation or overexpression which may appear in glioblastoma. In this way, techniques like qPCR, MLPA, specific hybridization (FISH, CISH. ...) and NGS can be used.

The present invention also relates to  $\beta 8$  integrin gene or protein as a biomarker for the diagnosis of resistant glioblastoma.

Another object of the invention relates to a method for treating resistant glioblastoma comprising the steps of i) determining the level expression of the marker  $\beta 8$  integrin in a tumor sample obtained from a patient, ii) comparing the expression levels determined at step i) with predetermined reference values and iii) administering the subject a therapeutically effective amount of a compound which is an antagonist of  $\beta 8$  integrin or an inhibitor of the expression of  $\beta 8$  integrin as described above when the levels determined at step i) is higher than its predetermined reference value.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

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## FIGURES:

**Figure 1:  $\beta 8$  integrin (ITGP8) is overexpressed in GSC compared to GBM differentiated cells.** GSC-enriched neurosphere (NS) cell lines isolated from 10 patient tumors were maintained in stem cell medium or allowed to differentiate as adherent GBM cells for 15 days in FCS-enriched medium. (A) Real-time Quantitative PCR (qPCR) analysis of the ITG $\beta 8$  expression in NS cells and in FCS-differentiated GBM cells. Shown are the fold inductions expressed as means  $\pm$  SEM of at least four independent experiments. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$  compared with the related FCS condition. (B) Western-blot analysis of the ITG $\beta 8$  expression in NS cells and in FCS-differentiated cells in comparison with the classic stem (Olig2, Sox2) and differentiation (TUJ1) markers. Equal gel loading and transfer efficiency were checked with anti-actin antibody. Blots were representative of at least three independent experiments in 3 patient cell lines (A, B and C). (C) Immunofluorescence analysis performed by FACS for membrane ITG $\beta 8$  expression. The SFI (Specific Fluorescence Index) allowed to evaluate the marker expression level (see Material and Methods). Results are expressed as the means  $\pm$  SEM of at least five independent experiments. \* $P < 0.05$  compared with the related FCS condition. For each patient cell line (A, B and C), representative FACS plot overlays were depicted.

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**Figure 2: ITGP8 is efficiently downregulated in GSC by specific shRNA targeting.** GSC-enriched NS cells isolated from two patient tumors (B and C) were transfected or not (NT) for 4 days with two different ITGp8-targeting shRNA (shB8-1 and -4) or with a negative control shRNA (shCTR). ITG $\beta 8$  expression levels were then analyzed in NS cells at the mRNA or protein level either by (A) qPCR or (B) FACS immunofluorescence, respectively. Results are expressed as the means  $\pm$  SEM of at least four independent experiments. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$  compared with the related shCTR condition.

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**Figure 3: shRNA-mediated ITGP8 knock-down impairs GSC sternness and viability.** GSC (B and C cell lines) were transfected or not (NT) with two different ITG $\beta$ 8-targeting shRNA (shB8-1 and -4) or with a negative control shRNA (shCTR). (A) 4 days after transfection, GSC were subjected to a FACS sorting of either GFP<sup>+</sup>/ITG $\beta$ 8<sup>+</sup> or GFP<sup>+</sup>/ITG $\beta$ 8<sup>-</sup> GSC for the shCTR or the shB8 conditions, respectively. These sorted GSC were immediately plated in 96-well plates at different low cell densities to study their ability to generate primary NS through limiting dilution assays. (B) A similar limiting dilution assay was performed in B cell line to assess the secondary NS forming ability of transfected GSC after selection of the shRNA-positive cells by G418 during at least 20 days. Results (A-B) are expressed as the means  $\pm$  SEM of at least three independent experiments. \*P < 0.05. \*\*P < 0.01, \*\*\*P < 0.001 compared with the related shCTR condition. (C-D) 7 days after transfection, GSC were subjected to subG1 analysis by FACS (C) or to Caspase 3-7 activity assay (D) in order to determine the percentage of non-viable cells or apoptosis, respectively. (E) mRNA of transfected GSC were extracted 4 days post-transfection to perform qPCR analysis of the expression of two stem (Olig2) and differentiation (TUJ1) markers. (F) These transfected cells were processed by FACS 4 days after transfection to analyze the percentage of the GSC population (see Material and Methods). Results (C-F) are expressed as the means  $\pm$  SEM of at least three independent experiments. \*P < 0.05. \*\*P < 0.01, \*\*\*P < 0.001.

**Figure 4: siRNA-mediated ITGP8 knock-down impairs GSC sternness and induced apoptosis.** GSC (B and C cell lines) were transfected or not (NT) with an ITG $\beta$ 8-targeting siRNA (siB8-6) or with a negative control siRNA (siCTR). (A) 4 days after transfection, ITG $\beta$ 8 and Nestin expression levels were then analysed in NS cells at the mRNA level by qPCR. (B) 4 days after transfection, GSC were subjected to Caspase-8 activity assay in order to analyse the pro-apoptotic process. Results (A-B) are expressed as the means  $\pm$  SEM of at least three independent experiments. \*P < 0.05, \*\*\*P < 0.001.

**Figure 5: shRNA-mediated ITGP8 knock-down radiosensitizes the GSC population.** GSC (B and C cell lines) were transfected with two different ITG $\beta$ 8-targeting shRNA (shB8-1 and -4) or with a negative control shRNA (shCTR) and then selected by G418 during at least 20 days. These established stable NS cell lines were then dissociated and irradiated by the indicated dose of ionizing radiations. (A-B) 4 days after irradiation (5 Gy), cells were subjected to either subG1 analysis (A) or AnnexinV (AV) - Propidium Iodide (PI) double staining by FACS in order to determine the percentage of non-viable cells or apoptotic

cells, respectively. (B) Prior to irradiation, parental (NT) or stably transfected NS were dissociated and plated in 96-well-plates (500 cells/well) in order to determine the surviving fraction by clonogenic assay. Cells were kept in culture during 10 days and the number of surviving NS clones were determined by microscopy. Results (A-B) are expressed as the means  $\pm$  SEM of at least three independent experiments. \*P < 0.05. \*\*P < 0.01 compared with the related shCTR condition.

## EXAMPLE:

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### Material & Methods

#### Human tumor collection

The study was conducted on newly diagnosed GBM tumor samples isolated from 8 different patients to establish 10 primary neurosphere (NS) cell lines. These samples were all obtained after written informed consent from patients admitted to the Neurosurgery Department at Toulouse University Hospital and processed in accordance with the Institution's Human research Ethics Committee. Tumors used in this study were histologically diagnosed as grade IV astrocytoma according to the WHO criteria.

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#### Cell culture

The GBM samples were processed as described by Dahan et al in order to obtain the corresponding primary neurosphere (NS) cell lines shown to be enriched in GSC. Neurosphere GSC lines were maintained in DMEM-F12 (Lonza) supplemented with B27 and N2 (Invitrogen), 25 ng/ml of FGF-2 and EGF (Peprotech) at 37°C in 5% CO<sub>2</sub> humidified incubators. All GSC lines were used for the experiments in this Stem Cell Medium between the 2nd and 12th passages in order to avoid any stem cell characteristic loss. Forced differentiation was performed according to previous published protocol adapted as follows. Briefly, the dissociated NS cells were cultured and plated as adherent monolayer ( $7.5 \times 10^3$  cells/cm<sup>2</sup>) in DMEM-F12 only supplemented with 10% FCS (FCS medium) for at least 15 days to insure an optimum differentiation.

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#### Quantitative real-time RT-PCR

Total RNA was isolated either from primary NS, FCS-differentiated cells or from NS cells after transfection using RNeasy kit (Qiagen) and then reverse-transcribed using iScript

cDNA synthesis kit (Bio-Rad). Real-time qPCR reactions were carried out using Evagreen dye and ABI-Stepone+ Detection System (Applied Biosystems). GAPDH was used as endogenous control in the ACT analysis. Amplification folds were measured by the  $2^{-\Delta\Delta C_t}$  method. Different primers (Eurogentec) were used in this study.

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#### Western blotting

Cells were lysed in RIPA buffer complemented with cocktails of protease and phosphatase inhibitors (Sigma). 25 $\mu$ g of proteins were then separated on a 10% or 12.5% SDS-PAGE, electroblotted onto PVDF membranes (Amersham), which were blocked with 10% milk. The primary antibodies used for this study are listed in Table 1.

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<b>PROTEIN</b>	<b>Primary Antibody</b>
Actin ( <i>Human</i> )	Mouse anti-Actin (MAB1501, 1/10000, Millipore)
ITG $\beta$ 8 ( <i>Human</i> )	Rabbit anti-ITG $\beta$ 8 (ab80673, 1/1000, Abcam)
Olig2 ( <i>Human</i> )	Rabbit anti-Olig2 (AB9610, 1/2500, Millipore)
Sox2 ( <i>Human</i> )	Rabbit anti-Sox2 (ab92494, 1/1000, Abcam)
TUJ1 ( <i>Human</i> )	Mouse anti-TUJ1 (MAB1637, 1/1000, Millipore)

Table 1: Immunoblot antibodies.

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#### Flow cytometry immunofluorescence assay

Direct immunofluorescence assay was performed by FACS as previously described. For all samples,  $2 \times 10^5$  cells were incubated for 30 min in PBS with 10% BSA at 4°C to avoid nonspecific binding, and then incubated with appropriate APC-conjugated anti-ITG $\beta$ 8 antibody (R&D, 1/20) for 40 min at 4°C. Fluorescence related to immunolabeling was measured using a FACSCalibur flow cytometer (BD Biosciences). Each measurement was conducted on at least 7,000 events, acquired on CellQuest software (BD Biosciences) and analyzed with VenturiOne software (AppliedCytometry). To evaluate the marker expression, we determined the specific fluorescence index (SFI) using the mean fluorescence intensity (MFI). The SFI was calculated as previously described with the following formula  $SFI = (MFI \text{ antibody} - MFI \text{ isotype control}) / MFI \text{ isotype control}$ .

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#### SubG1 and AnnexinV-PI double-staining analysis

Dead cells were quantified using FACS by determining the percentage of cells with subG1-DNA content. This subG1 population was analyzed after cell permeabilization and

subsequent Propidium Iodide staining, as previously described. SubG1 measurements were conducted on at least 10,000 events, acquired on CellQuest software (BD Biosciences) and analyzed with VenturiOne software (Applied Cytometry). For AV/PI staining experiments, apoptosis and necrosis were quantified at the same time on non-permeabilised cells by flow cytometry with an Alexa Fluor 647-conjugated AV and PI kit, according to the manufacturer's protocol (Biovision).

#### shRNA and siRNA transfection

Neurospheres from 2 different patient cell lines (B and C) were dissociated and  $7 \times 10^5$  cells per well were plated in 6 well-plates before being transfected using either Fugene HD (3/1 ratio, Promega) or RNAiMax (Invitrogen) for shRNA or siRNA, respectively. Two different shRNA sequences and one siRNA sequence targeting ITG $\beta$ 8 (sh $\beta$ 8-1 and -4 and si $\beta$ 8-6, Qiagen) were used to specifically knock-down ITG $\beta$ 8 expression in GSC (Table 2). Off-target shRNA (shCTR, Qiagen) or siRNA (siCTR, Qiagen) were used as negative controls. ShRNA were either coupled to a GFP-reporter gene or to a Neomycin-resistance gene for short-term transfection or stable selection in G418 (1 mg/mL, 20 days, Merck), respectively. Medium replacement was done 24h after transfection in order to avoid any unspecific toxicity.

Name	Target	Reporter or Resistance	Sequence	SEQ ID
shCTR	Off-target	GFP or Neomycin	GGAATCTCATTTCGATGCATAC	SEQ ID N°: 1
sh $\beta$ 8-1	ITG $\beta$ 8	GFP or Neomycin	CCAAGCTACTTGAGAATATTT	SEQ ID N°: 2
sh $\beta$ 8-4	ITG $\beta$ 8	GFP or Neomycin	TCTCGCTCTTGATAGCAAATT	SEQ ID N°: 3
si $\beta$ 8-5	ITG $\beta$ 8	GFP or Neomycin	AACGTCTATGTCAAATCGACA	SEQ ID N°: 4
si $\beta$ 8-6	ITG $\beta$ 8	GFP or Neomycin	CAGCCTGTTTGCAGTGGTCGA	SEQ ID N°: 5

20 Table 2: shRNA and siRNA sequences.

#### GSC gating and sorting by FACS

In order to discriminate by FACS the GSC population from the more differentiated population, both present among the NS cells, we adapted a previously published protocol.

Briefly, NS cells were dissociated and  $2 \times 10^5$  cells were analyzed by flow cytometry. The percentages of each population were subsequently quantified according to their specific FSC/SSC patterns. Prior to this, a pre-gating was performed to avoid cell debris in the analysis.

For the measurement of the NS formation capacity by limiting dilution assay, cells were transfected for 4 days with GFP-coupled shRNA (shB8-1/-4 or shCTR) before being sorted by FACS (Beckman MoFlo Astrios). This sorting was performed on the GSC population present within the NS cells and was designed to only sort the GFP-positive cells with normal (for shCTR condition) or negative (for shB8-1/-4 conditions) expression for ITG $\beta$ 8. Cells were then collected and plated for limiting dilution assay.

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#### Neurosphere formation assays

Cells obtained after GFP-coupled shRNA transfection were sorted by FACS as described above and immediately plated in 96-well plates at different low cellular densities (1 to 500 cells/well) in order to assess their ability to generate primary NS through limiting dilution assays. After 15 days, the generated NS were counted by microscopy in each well.

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When cells were transfected by shRNA vectors carrying the Neomycin-resistance gene, cells were treated 4 days after transfection by G418 during at least 20 days in order to select the positive clones, capable of forming NS during the selection process. These NS cells were then dissociated and plated in 96-well plates to perform similar limiting dilution assays and measure their ability to generate secondary NS.

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#### Caspase assays

Neurosphere cells obtained after transfection and indicated treatment were processed according to the manufacturer instructions to be analyzed through Caspase-Glo 8 or Caspase-Glo 3/7 assays (Promega) in order to measure respectively the activity of the extrinsic apoptotic pathway (initiative caspase-8) and the activity of effective caspases-3/7. The assays were read using a luminometer (FluoStar, Optima).

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#### Irradiation

The stable NS clones selected in G418 (shB8-1/-4 or shCTR) were subjected or not to different doses of irradiation (Gamma-cell Exactor 40, Nordion) in order to evaluate the radiosensitizing effect of ITG $\beta$ 8 knock-down in GSC through subG1 analysis and clonogenic assays.

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### Clonogenic assays

Prior to irradiation, parental or stably transfected NS (shB8 or shCTR clones) were dissociated and plated in 96-well-plates (500 cells/well) in order to determine the surviving fraction by clonogenic assay. Cells were then irradiated or not with increasing doses of ionizing radiations (0 to 10 Gy) and kept in culture during 10 days. Finally, the number of surviving NS clones was determined by microscopy.

### Statistical analysis

The results are presented as means  $\pm$  SEM of at least three independent experiments. Significant differences (\*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001) were evaluated with the Student t-test. Log. Western-blot and FACS plots are representative of at least three different experiments.

## Results

### 1. ITGB8 overexpression in GSC compared to GBM differentiated cells

We screened by RT-qPCR the expression level of ITG $\beta$ 8 mRNA in 10 different patient GSC cell lines, cultured as neurospheres (NS) in stem medium, compared to their differentiated counterparts, kept in FCS-enriched differentiation medium (Figure 1A). We observed that this integrin was significantly overexpressed in all GSC cell lines (1.5 to 3.5 fold according to the patient). Moreover, we confirmed on 3 patient cell lines that the protein level of ITG $\beta$ 8 was also overexpressed in GSC-enriched NS in comparison with their differentiated counterparts. This overexpression was demonstrated in whole cell lysates, as shown by western-blot (Figure IB), and at the membrane cell surface, as observed by flow cytometry (Figure 1C). In order to fully establish the preferential expression of this particular integrin in GSC, we additionally performed western-blot analysis of several stem (Olig2, Sox2) and differentiation (TUJ1) markers and confirmed that NS cells specifically expressed these stem markers and failed to express the neuronal marker TUJ1 (Figure IB). We can conclude that ITG $\beta$ 8 is selectively express at the membrane of glioblastoma stem-like cells and barely present in differentiated GBM cells.

## 2. ITGB8 inhibition by shRNA or siRNA greatly alter GSC viability and sternness

We subsequently designed a shRNA-based strategy to selectively downregulate ITG $\beta$ 8 in GSC in order to assess its functions in this subpopulation of undifferentiated cells. We used two different ITGp8-targeting shRNA sequences (shB8-1 and -4) or a negative control shRNA (shCTR) and transfected NS-dissociated cells for 4 days. We observed in two different patient NS cell lines (B and C) that shB8-1 and -4 sequences potently inhibited the expression of ITG $\beta$ 8 mRNA level (Figure 2A) and the presence of this integrin at the cell membrane surface (Figure 2B) in comparison with both the non-transfected (NT) or shCTR conditions. One of the main characteristic of cancer stem cells is their self-renewal ability in limiting dilution condition. We assessed this sternness-associated parameter by measuring the NS forming capacity of GSC at low cell density after ITGp8-targeting. We demonstrated that GFP-coupled shB8-1 and -4 dramatically abrogated the NS forming capacity of ITGp8-/GFP+ GSC compared to the ITG $\beta$ 8+/GFP+ shCTR GSC in FACS-sorted cells from the B and C patient NS cell lines (Figure 3A). Moreover, transfection of the same shB8-1 and -4 sequences coupled to the neomycin-resistance gene and subsequent selection by G418 induced a similar drop in the NS generation ability at low density compared to the shCTR condition (Figure 3B).

This major alteration of the GSC *in vitro* self-renewal ability in response to ITG $\beta$ 8 inhibition can be mediated by several causes such as viability loss. We notably observed that ITG $\beta$ 8-targeting by shB8-1 and -4 induced 7 days after transfection a significant increase of the subG1 cell population in NS compared to NT and shCTR conditions (Figure 3C), which may be associated with the induction of a pro-apoptotic mechanism in transfected ITGp8-negative GSC. This apoptotic process in response to ITGB8 downregulation was confirmed by the increase in caspase 3/7 activities in 3 different patient GSC cell lines (Figure 3D). In addition, a decrease of the GSC self-renewal ability could also be linked to a weakening of their stem phenotype due to ITG $\beta$ 8 knock-down. In that way, we demonstrated that shRNAs against ITG $\beta$ 8 induce 4 days after transfection a significant decrease of the stem factor Olig2 and an increase of the TUJ1 differentiation marker at the mRNA level (Figure 3E). These variations in stem and differentiation marker expression were associated to a noticeable decrease of the GSC subpopulation within the NS structures in response to ITG $\beta$ 8, as defined by flow cytometry analysis in B and C patient cell lines (Figure 3F). We repeated some of these experiments through ITGB8 downregulation by siRNA (siB8-6) and we also showed in B and C cell lines that the mRNA level of this integrin was potently decreased 4 days after transfection (compared to siCTR). This siB8 was also able to downregulate the expression of Nestin mRNA (Figure 4A),

a well-known GSC stem marker and to induce an increase in caspase-8 activity (Figure 4B). Altogether, these observations suggest that ITG $\beta$ 8 knock-down may induce a differentiation process in GSC associated to an alteration of their stemness characteristics, such as self-renewal ability and stem markers, and to the induction of apoptotic cell death.

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### 3. ITGB8 inhibition by shRNA radiosensitizes the GSC population

GSC are, among the GBM cellular heterogeneity, one of the most radioresistant tumoral subpopulation. They were shown to resist to the deleterious effects of ionizing radiations (IR) by several mechanisms such as a better efficiency of DNA damage repair systems or a higher level of anti-apoptotic or pro-survival factors. However, the selective expression of particular integrins in GSC and their roles on GSC radioresistance, which are largely unknown in the literature, appeared to us of potential interest as part of a combined therapeutic approach. As a consequence, we then studied the combined effect of ITG $\beta$ 8 inhibition by shRNA on GSC radioresistance. For that purpose, we submitted G418-selected shB8-1 and -4 clones (B and C cell lines) to a toxic IR dose of 5 Gy for 4 days and measured that ITG $\beta$ 8-targeting significantly increased the level of subG1-positive cells (Figure 5A) and apoptotic AnnexinV-positive cells (Figure 5B), compared to the shCTR condition in response to IR. Moreover, to fully demonstrate the radiosensitizing effect of ITG $\beta$ 8 downregulation in GSC, we performed clonogenic assays at low density in 96 well plates and measured the NS generation potential of shCTR or shB8 stably-transfected GSC (B and C patients) in response to increasing doses of IR (up to 10 Gy). As depicted in Figure 5C, we observed that the shRNA-mediated invalidation of ITG $\beta$ 8 induced a significant decrease in the NS surviving fraction in response to IR (6 to 10 Gy) in comparison with shCTR cells or non-transfected NT cells.

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Altogether, these results demonstrate that (i) ITG $\beta$ 8 is specifically expressed by GSC compared to more differentiated GBM cells, (ii) this integrin could mediate in GSC a pro-survival signal associated to the maintenance of a stem phenotype and (iii) the specific inhibition of this GSC-specific target could radiosensitize GBM-stem cells, known as a really radioresistant subpopulation in GBM able to favor the recurrence of these aggressive and invasive brain tumors.

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**REFERENCES:**

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A compound which is an antagonist of  $\beta 8$  integrin or an inhibitor of the expression of  $\beta 8$  integrin for use in the treatment of resistant glioblastoma.
- 5 2. A i) compound according to claim 1 and ii) a radiotherapy, and iii) TMZ as a combined preparation for simultaneous, separate or sequential use in the treatment of resistant glioblastoma.
3. A i) compound according to claim 1 and ii) a chemotherapeutic agent, as a combined preparation for simultaneous, separate or sequential for use in the treatment of resistant  
10 glioblastoma.
4. A i) compound according to claim 1 and ii) a radiotherapy, and iii) a chemotherapeutic agent, as a combined preparation for simultaneous, separate or sequential for use in the treatment of resistant glioblastoma.
5. A i) compound according to claim 1 and ii) a radiotherapy, as a combined preparation  
15 for simultaneous, separate or sequential for use in the treatment of resistant glioblastoma.
6. A compound according to claim 1 or a combined preparation according to claims 2, 3, 4 or 5 wherein the compound is an antagonist of  $\beta 8$  integrin.
7. A compound according to claim 6 or a combined preparation according to claim 6  
20 wherein the antagonist is an antibody anti-  $\beta 8$  integrin.
8. A pharmaceutical composition comprising an effective dose of an antagonist of  $\beta 8$  integrin or an inhibitor of the  $\beta 8$  integrin expression, for use in the treatment of resistant glioblastoma.
9. An ex vivo method for diagnosing resistant glioblastoma, comprising the step of  
25 determining the level expression of the marker  $\beta 8$  integrin in a tumor sample obtained from a patient.

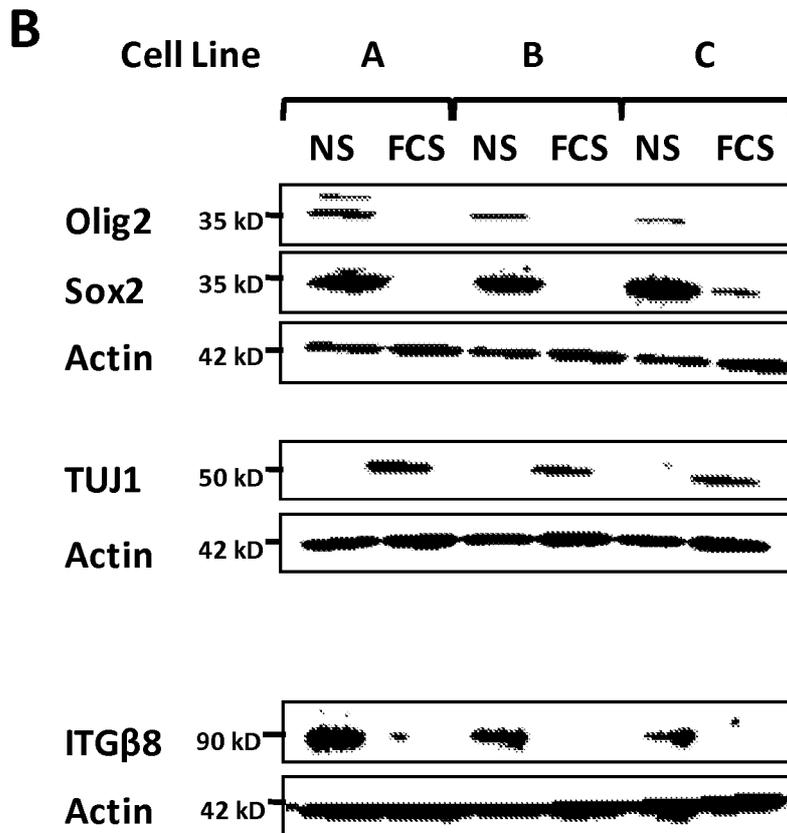
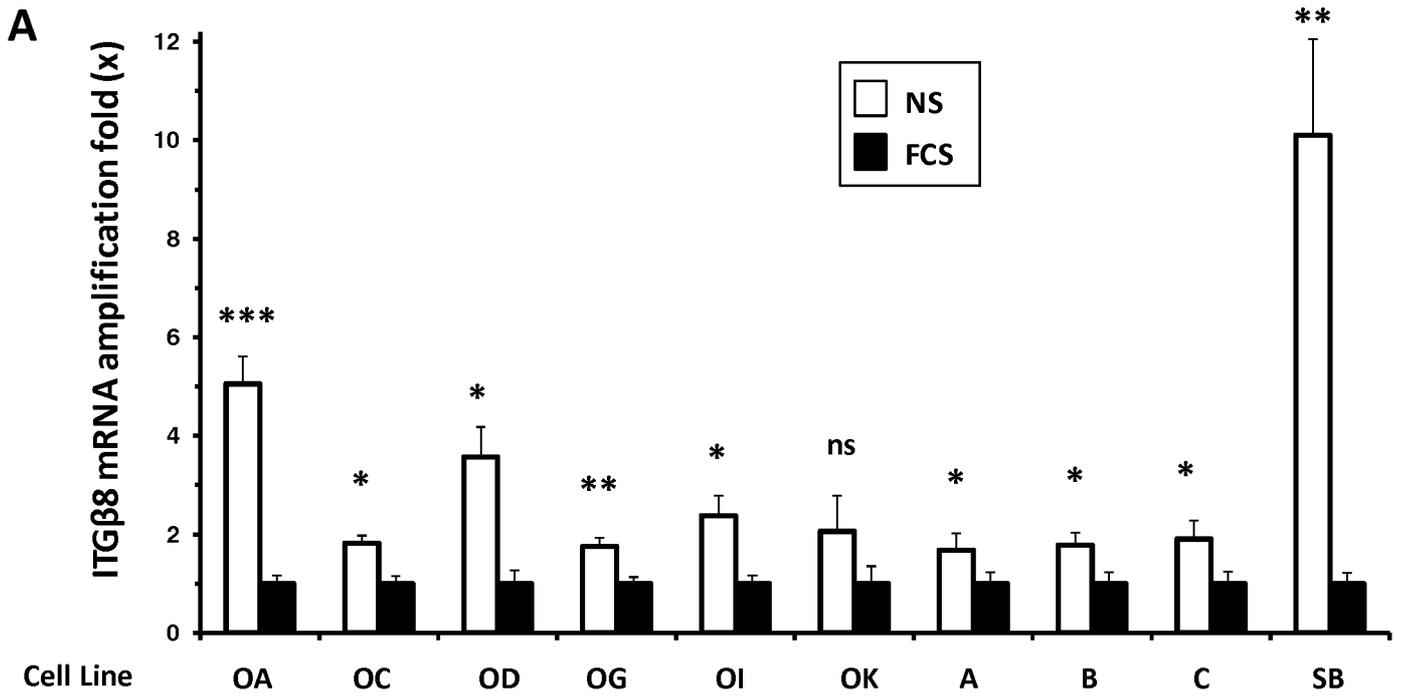


Figure 1 A and B

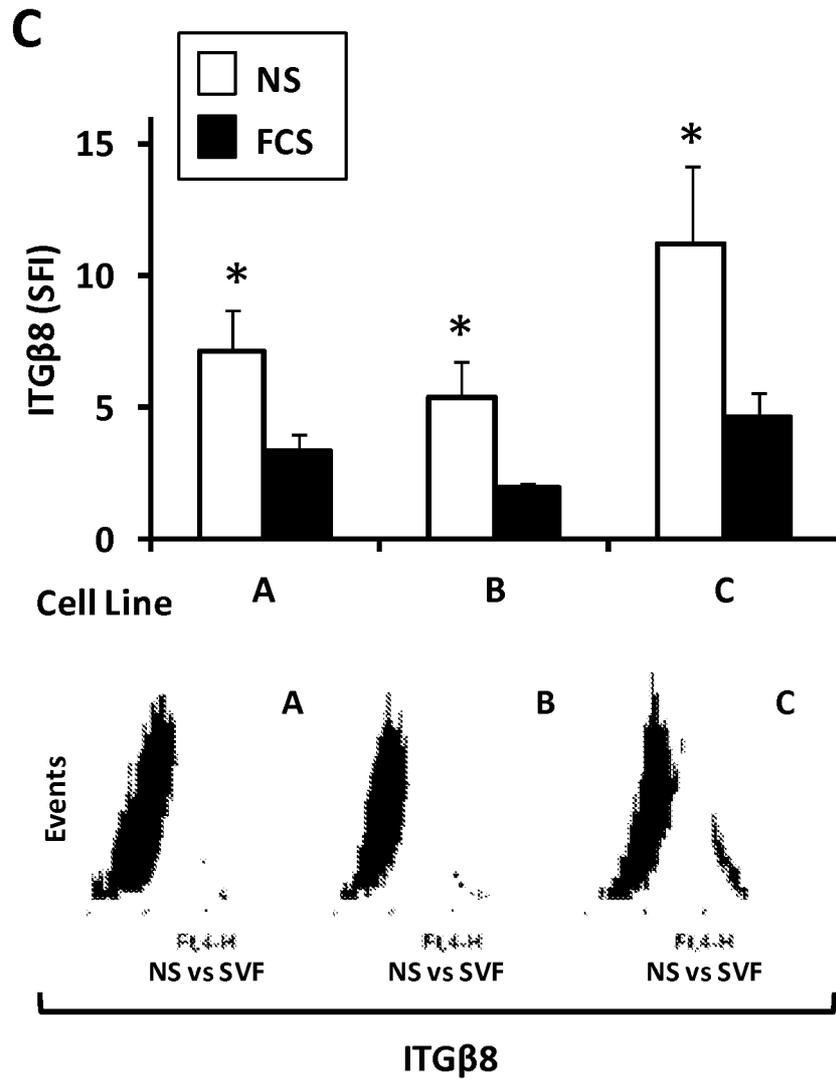


Figure 1 C

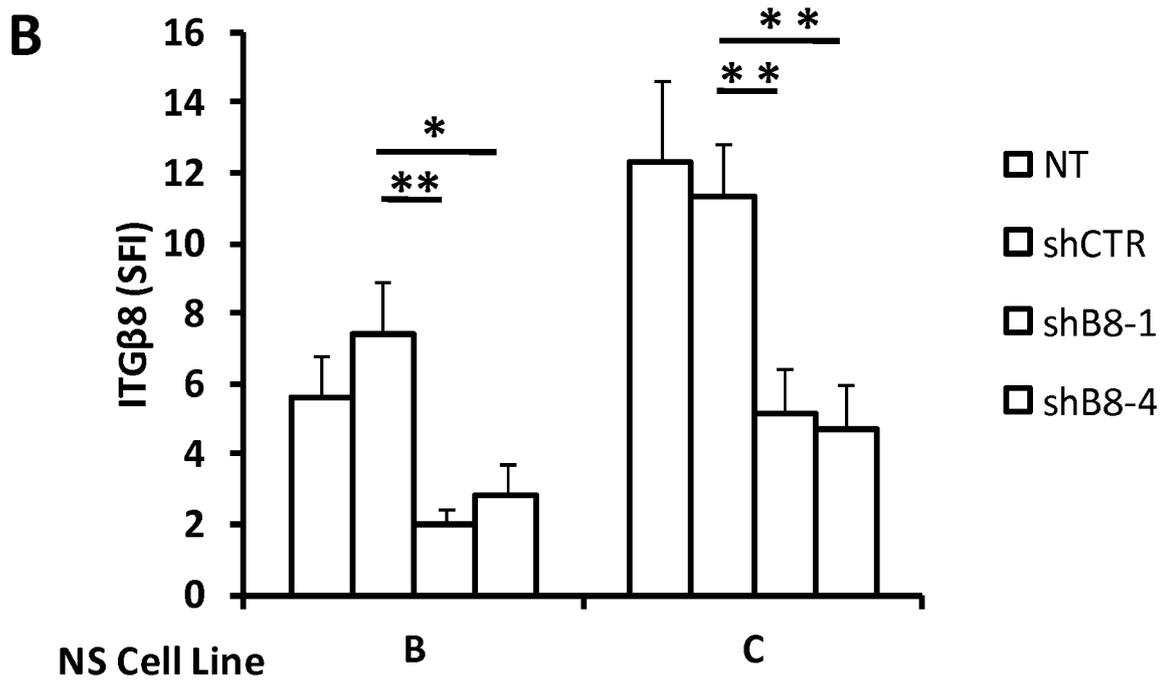
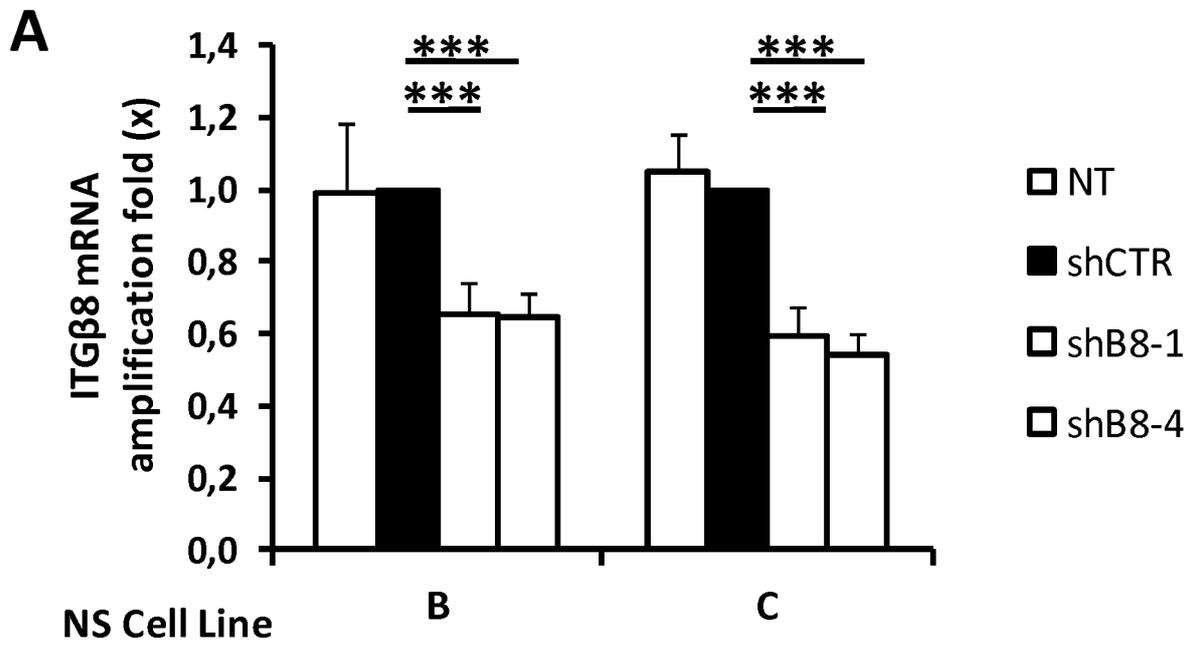


Figure 2 A and B

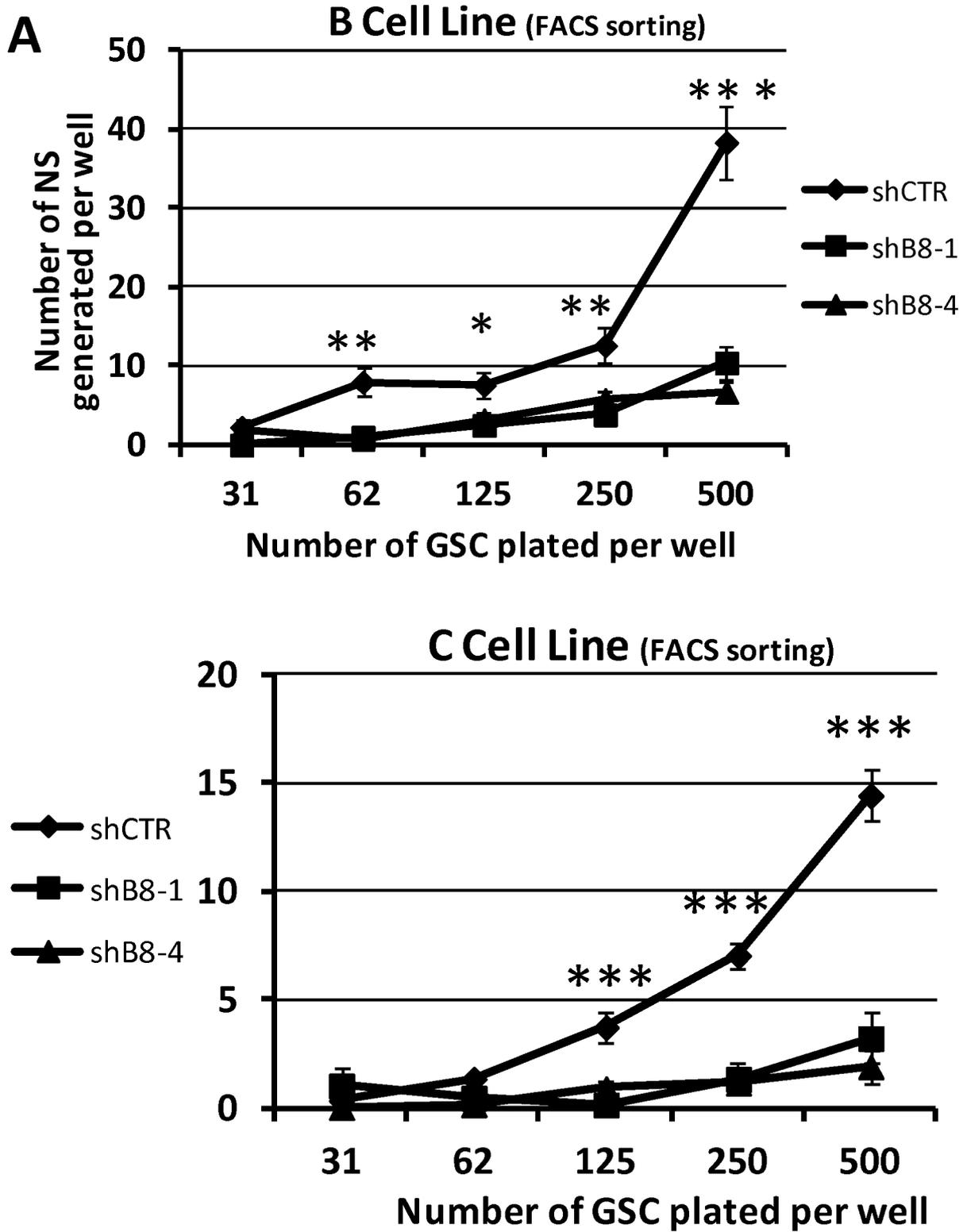


Figure 3 A

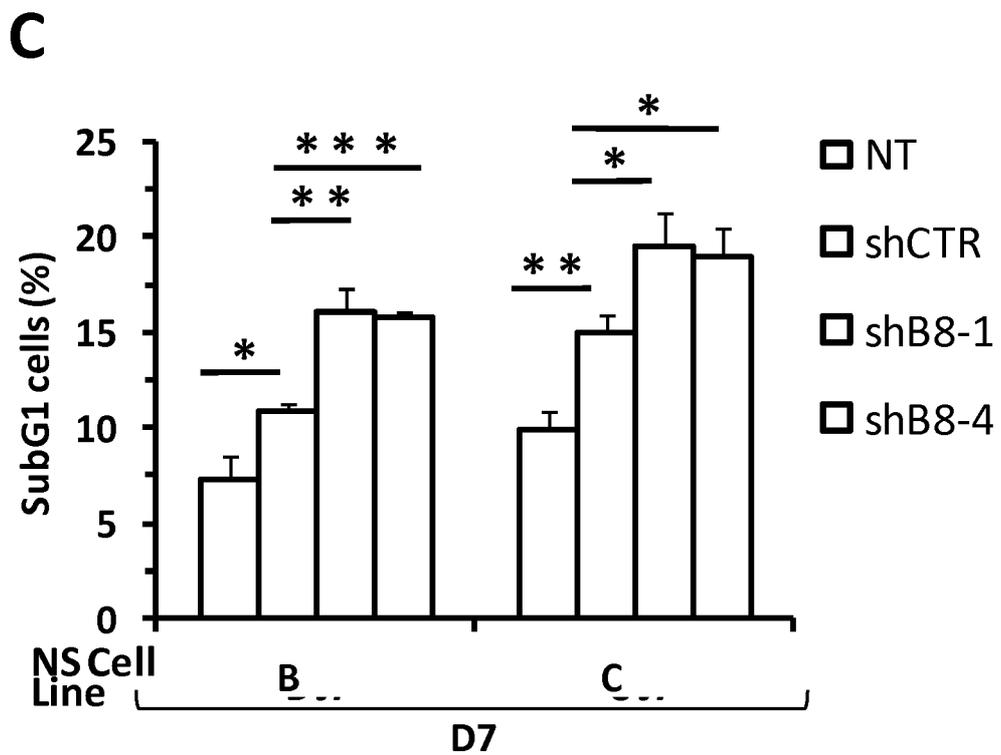
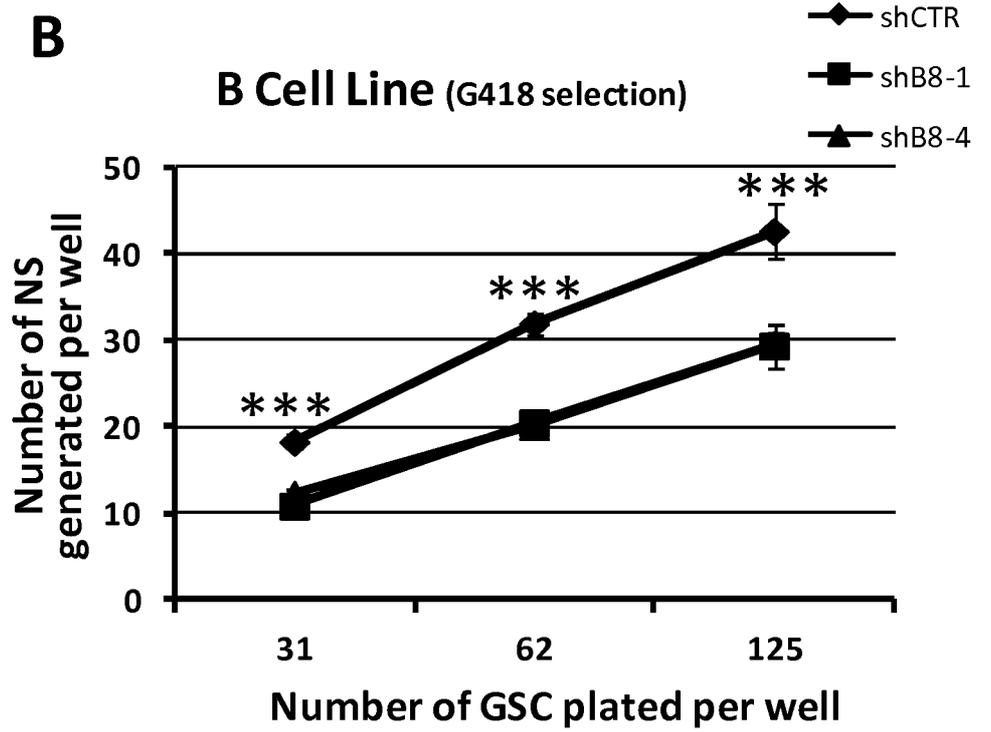


Figure 3 B and C

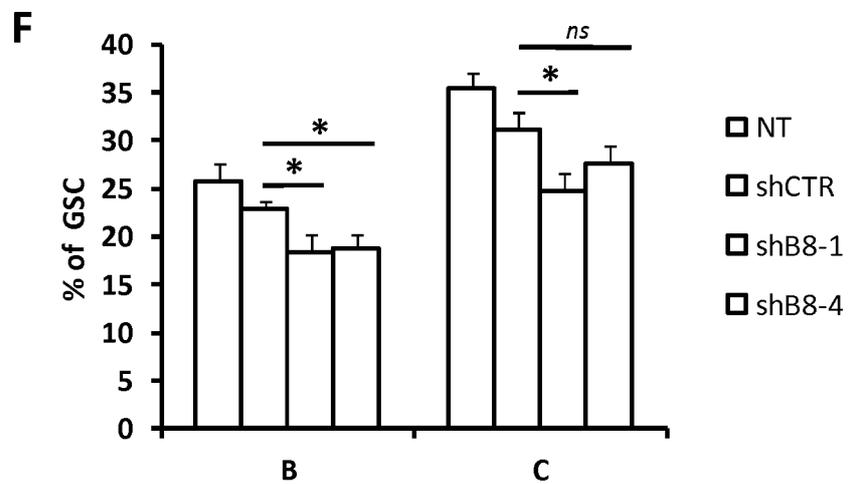
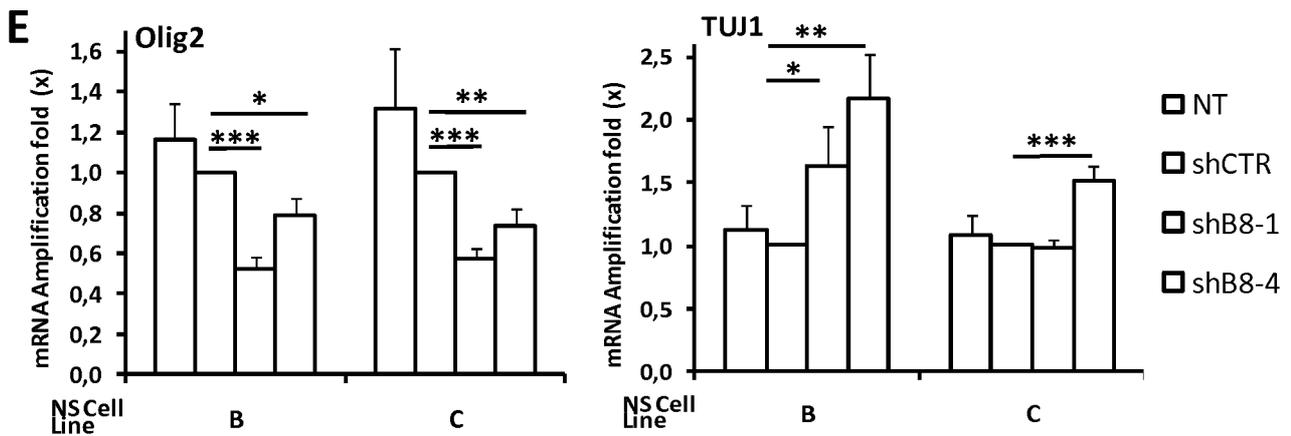
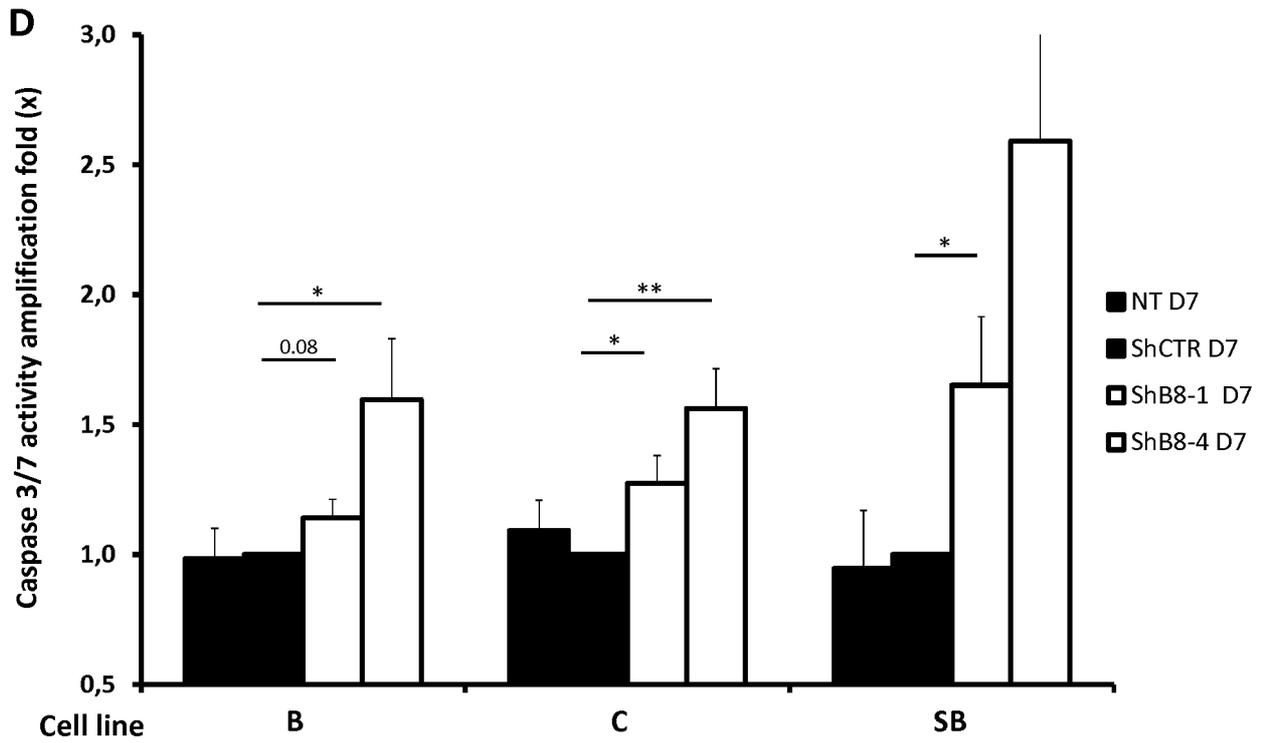


Figure 3 D, E and F

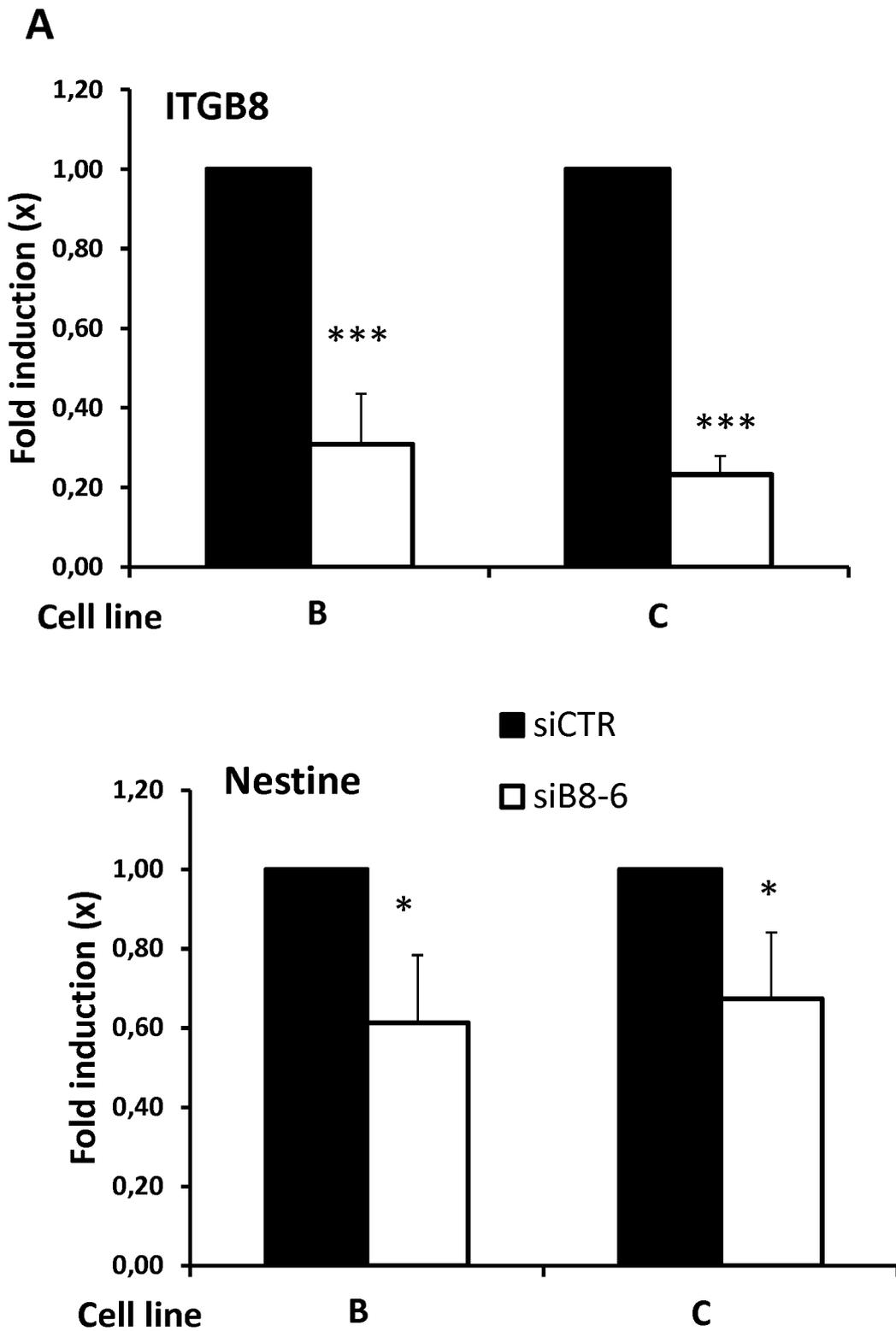


Figure 4A

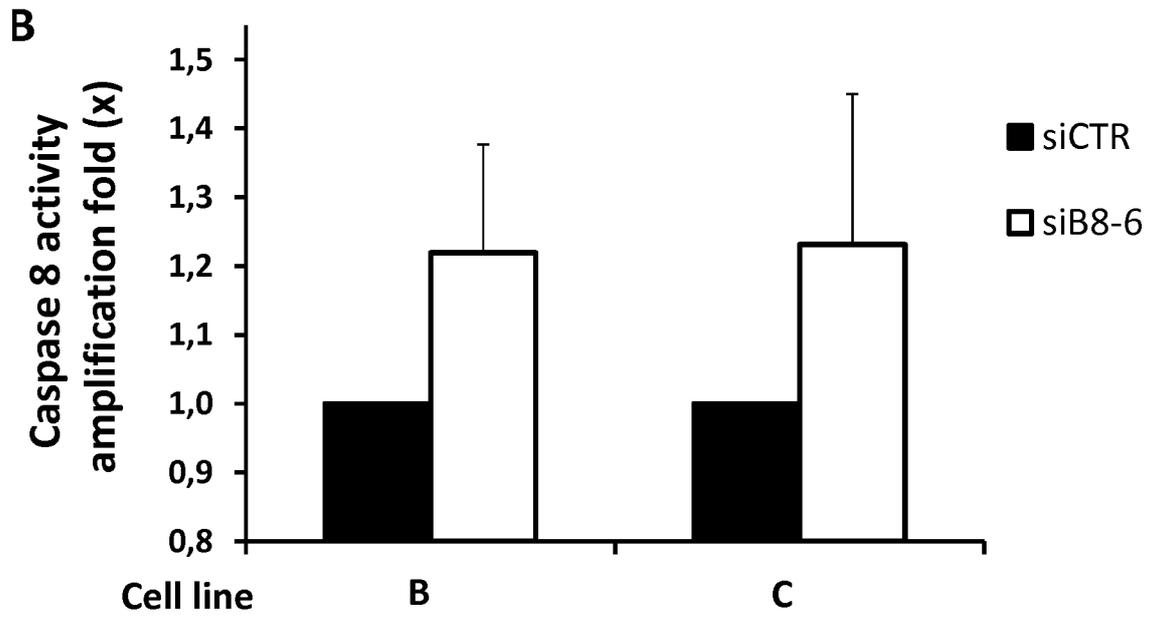


Figure 4B

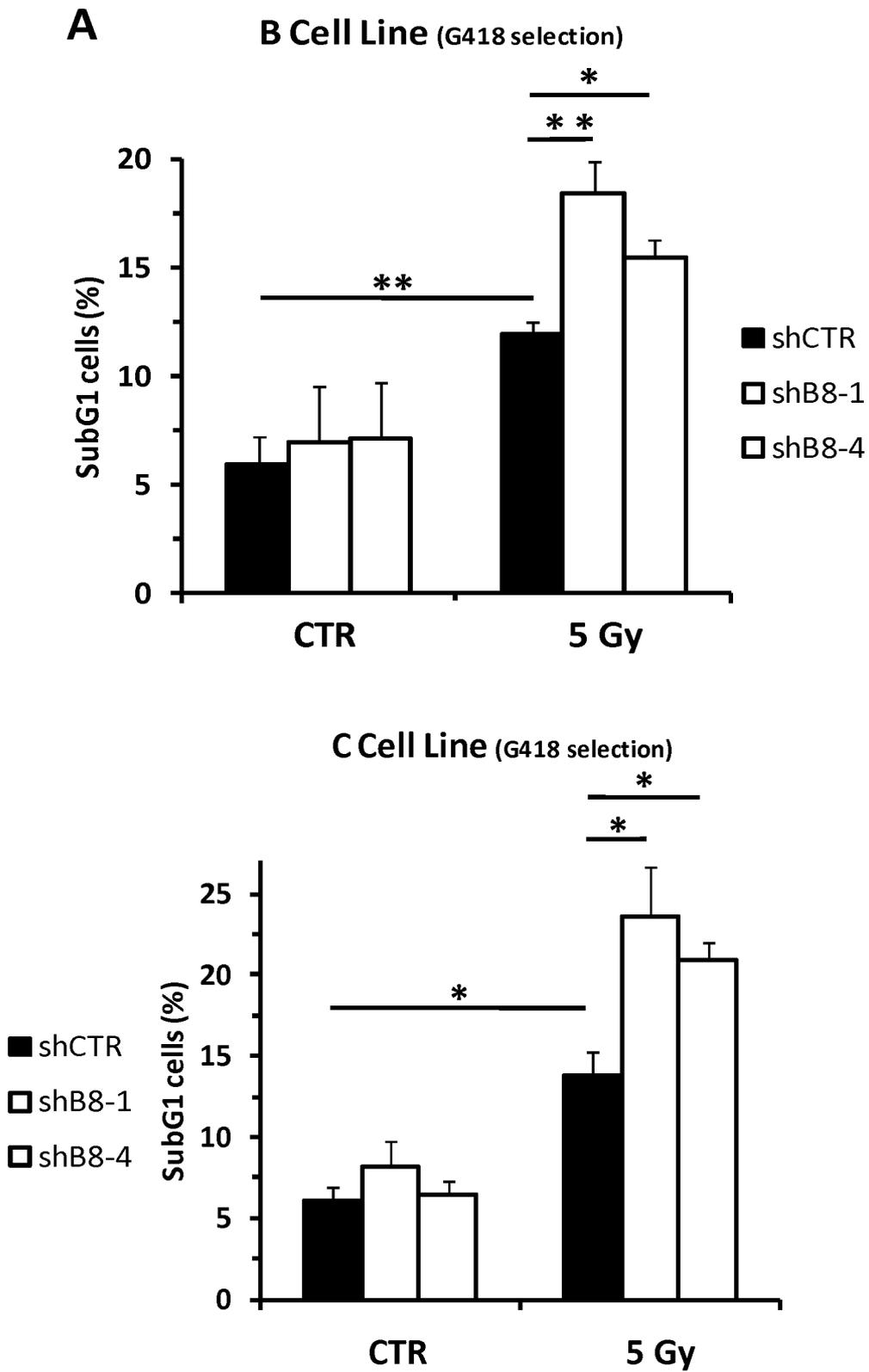


Figure 5 A

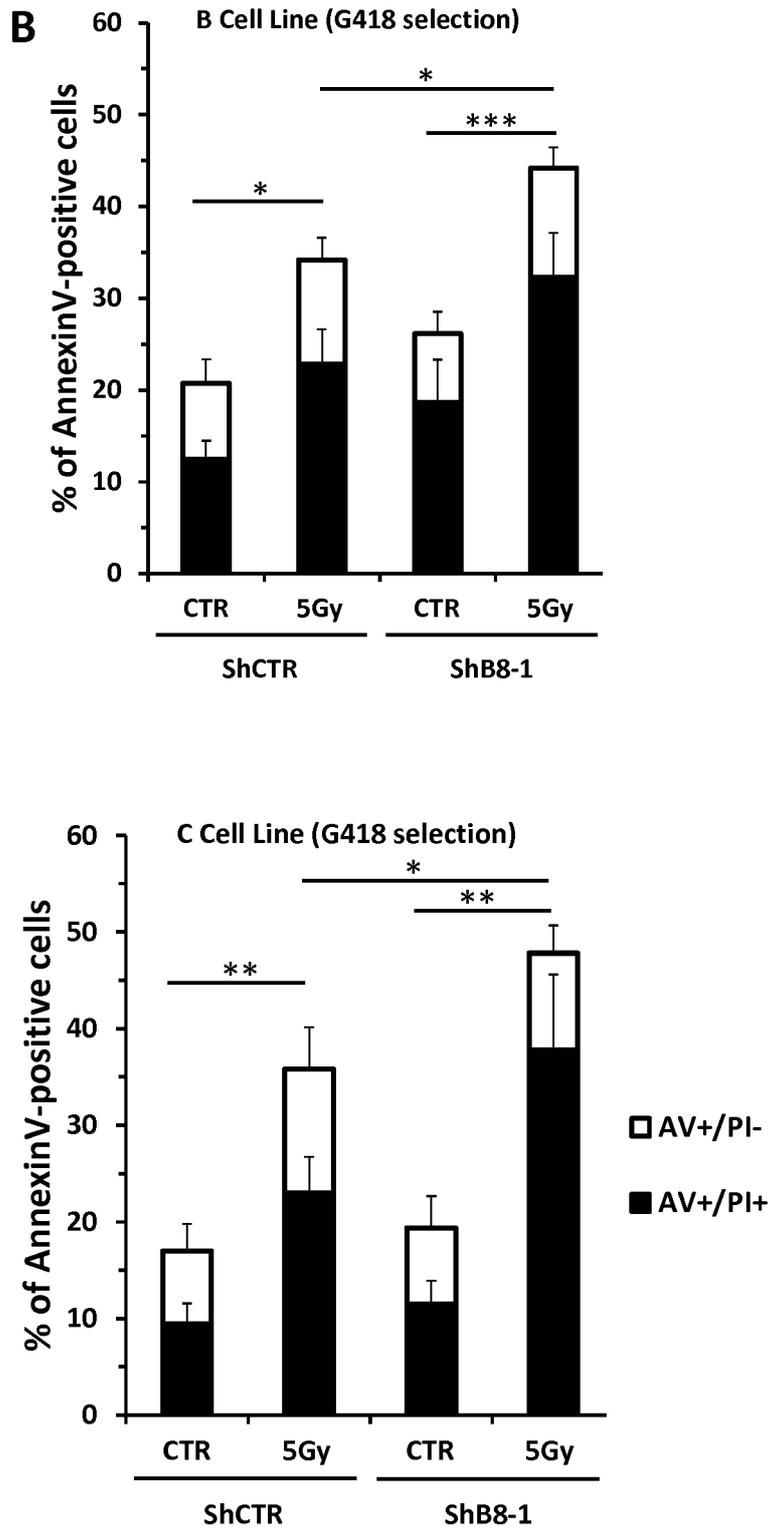


Figure 5 B

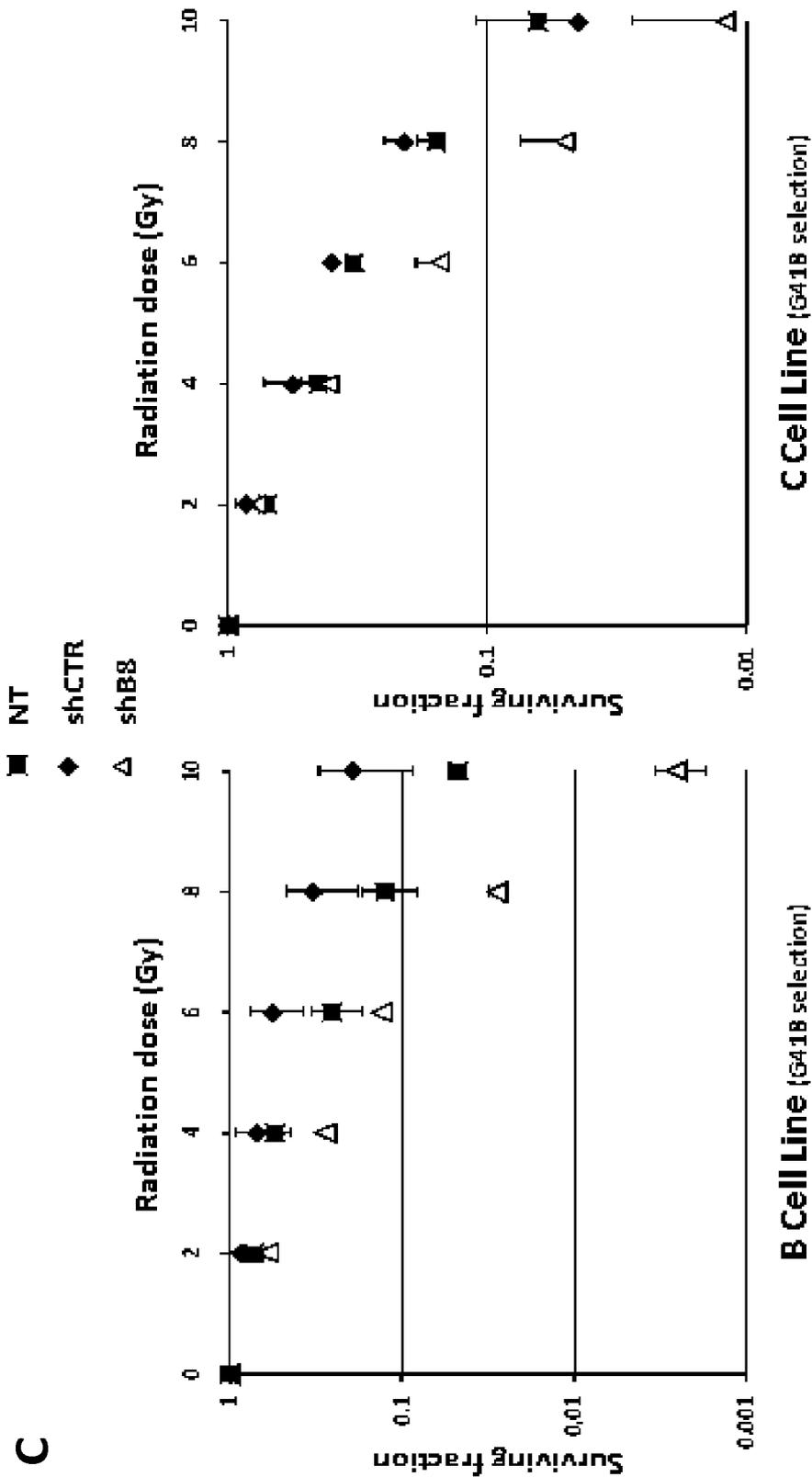


Figure 5C

# INTERNATIONAL SEARCH REPORT

International application No PCT/IB2016/00Q626
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<b>A. CLASSIFICATION OF SUBJECT MATTER</b> INV. C12N15/113      A61P35/0Q      G01N33/574 ADD. A61K31/713				
According to International Patent Classification (IPC) or to both national classification and IPC				
<b>B. FIELDS SEARCHED</b>				
Minimum documentation searched (classification system followed by classification symbols) C12N A61K G01N				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal , BIOSIS, WPI Data				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	wo 2007/084670 A2 (MERCK PATENT GMBH [DE] ; GOODMAN SIMON [DE] ; PICARD MARTIN ANDREAS [DE] ) 26 July 2007 (2007-07-26) the whole document <div style="text-align: center; margin-top: 10px;">                     -----                      - / - -                 </div>	1-8		
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;"><input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.</td> <td style="width: 50%; border: none;"><input checked="" type="checkbox"/> See patent family annex.</td> </tr> </table>			<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.	<input checked="" type="checkbox"/> See patent family annex.
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* Special categories of cited documents :				
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family			
Date of the actual completion of the international search	Date of mailing of the international search report			
8 December 2016	16/12/2016			
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer  Spi ndler, Mark-Peter			

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International application No  
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