THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 11, pp. 7138–7148, March 13, 2009

Different Response of Human Glioma Tumor-initiating Cells to Epidermal Growth Factor Receptor Kinase Inhibitors*[S]

Received for publication, September 15, 2008, and in revised form, January 14, 2009 Published, JBC Papers in Press, January 14, 2009, DOI 10.1074/jbc.M807111200

Fabrizio Griffero[‡§], Antonio Daga[‡], Daniela Marubbi[§], Maria Cristina Capra[‡], Alice Melotti[‡§], Alessandra Pattarozzi[§], Monica Gatti[§], Adriana Bajetto[§], Carola Porcile[§], Federica Barbieri[§], Roberto E. Favoni[‡], Michele Lo Casto[‡], Gianluigi Zona[¶], Renato Spaziante[¶], Tullio Florio[§1,2], and Giorgio Corte[‡§1,3]

From the [‡]Department of Translational Oncology, National Institute for Cancer Research, 16132 Genova and [§]Department of Oncology, Biology and Genetics and [¶]Department of Neuroscience, Ophthalmology and Genetics, University of Genova, 16132 Genova, Italy

Because a subpopulation of cancer stem cells (tumor-initiating cells, TICs) is believed to be responsible for the development, progression, and recurrence of many tumors, we evaluated the *in vitro* sensitivity of human glioma TICs to epidermal growth factor receptor (EGFR) kinase inhibitors (erlotinib and gefitinib) and possible molecular determinants for their effects. Cells isolated from seven glioblastomas (GBM 1–7) and grown using neural stem cell permissive conditions were characterized for *in vivo* tumorigenicity, expression of tumor stem cell markers (CD133, nestin), and multilineage differentiation properties, confirming that these cultures are enriched in TICs. TIC cultures were challenged with increasing concentrations of erlotinib and gefitinib, and their survival was evaluated after 1–4 days. In most cases, a time- and concentration-dependent cell death was observed, although GBM 2 was completely insensitive to both drugs, and GBM 7 was responsive only to the highest concentrations tested. Using a radioligand binding assay, we show that all GBM TICs express EGFR. Erlotinib and gefitinib inhibited EGFR and ERK1/2 phosphorylation/activation in all GBMs, irrespective of the antiproliferative response observed. However, under basal conditions GBM 2 showed a high Akt phosphorylation that was completely insensitive to both drugs, whereas GBM 7 was completely insensitive to gefitinib, and Akt inactivation occurred only for the highest erlotinib concentration tested, showing a precise relationship with the antiproliferative effects of the drug. Interestingly, in GBM 2, phosphatase and tensin homolog expression was significantly down-regulated, possibly accounting for the insensitivity to the drugs. In conclusion, glioma TICs are responsive to anti-EGFR drugs, but phosphatase and tensin homolog expression and Akt inhibition seem to be necessary for such effect.

A cancer stem cell population in malignant brain tumors plays an essential role in tumor initiation, growth, and recurrence. It was demonstrated that cancer stem cells, capable of self-renewal and multilineage differentiation, are present in blood and solid tumors (1, 2). This clonogenic tumoral subpopulation is the only one able to originate a tumor mass, and for this reason, these cells were described as tumor-initiating cells (TICs)[4] (3–6). Several groups have demonstrated the presence of TICs in various brain tumors, including gliomas (7–10).

Cerebral tumors represent 2% of all cancers, with an incidence of 14 new cases every year for 100,000 individuals, with gliomas representing the most frequent histotype (86% of total brain neoplasias) (11). These tumors, according to the World Health Organization classification, are divided into four groups, and glioblastoma (GBM) (grade IV) represents the most common and aggressive form, with a median survival time of 14 months (12). GBM is a poorly differentiated astrocytic tumor with preferential localization in the cerebral hemispheres, but it is able to infiltrate the surrounding tissues in a way reminiscent of the ability of neural stem cells to migrate to distance. This ability is the main cause of the poor prognosis and failure of conventional treatments with radio- or chemotherapy observed in this tumor (13), and it is in agreement with the hypothesis of the presence of a cancer stem cell subpopulation. In fact, glioma cells express various molecules associated with the development of neural stem cells (8, 14). In particular, CD133, one of the markers of brain TICs to date identified, is also a normal neural stem cell marker (15, 16).

The proliferation and differentiation of normal neural stem cells are under the modulation of different factors (17). It has been shown that the combination of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) is important for self-renewal and multilineage differentiation (18–20). Other growth factors, such as vascular-endothelial growth factor and platelet-derived growth factor, play a role in the maintenance and development of the central nervous system. Different studies have evaluated the importance of these growth

* This work was supported by grants from Fondazione Carige, MIUR-PRIN, and Ministero della Salute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2 and Table 1.

[1] Both authors are to be considered senior co-authors.

[2] To whom correspondence may be addressed: Laboratory of Pharmacology Dept. of Oncology, Biology and Genetics, University of Genova, Viale Benedetto XV, 2, 16132 Genova, Italy. Tel./Fax: 39-010-3538806; E-mail: tullio.florio@unige.it.

[3] To whom correspondence may be addressed: Dept. of Translational Oncology, National Institute for Cancer Research, Largo Rosanna Benzi 10 16132 Genova, Italy. Tel.: 39-010-5737401; E-mail: giorgio.corte@istge.it.

[4] The abbreviations used are: TIC, tumor-initiating cell; GBM, glioblastoma; EGF, epidermal growth factor; EGFR, EGF receptor; PBS, phosphate-buffered saline; RT, reverse transcription; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; bFGF, basic fibroblast growth factor; GFAP, glial fibrillary acidic protein; PTEN, phosphatase and tensin homolog; TKI, tyrosine kinase inhibitor; PI3K, phosphatidylinositol 3-kinase.

factors and their receptors in glioma growth and maintenance, demonstrating that these factors are also involved in proliferation, tumorigenicity, and malignancy of the tumors (21, 22). A main issue raised by the identification of TICs in brain tumors is the efficacy of chemotherapy on this cell subpopulation. In fact, it was reported that TICs are highly resistant to radiotherapy (23, 24), likely because of increased DNA repair activity, and to many cytotoxic drugs, in virtue of the overexpression of ATP-dependent efflux pumps (25–27). Thus, this study was aimed to evaluate the sensitivity of TIC-enriched cultures to gefitinib and erlotinib, two clinically approved EGFR tyrosine kinase inhibitors (EGFR TKI).

We isolated GBM TICs from seven fresh human GBMs. Cells were expanded *in vitro* in a proliferation-permissive medium, and the expression of different markers, multilineage differentiation capability, and tumorigenicity in immunodeficient mice was evaluated. After this characterization, GBM TICs have been tested for their sensitivity to the antiproliferative activity of erlotinib and gefitinib and for the modulation of the intracellular signaling involved, namely the ERK1/2 and Akt pathways.

EXPERIMENTAL PROCEDURES

Isolation of Human Glioma Tumor-initiating Cells—Glioma specimens were obtained after informed consent from seven patients, 6 males and 1 female, with an average age of 62 years (range 49–74). Tumor samples were obtained during surgery from patients of the Neurosurgery Department, San Martino Hospital (Genova, Italy). All patients underwent surgery for the first time and never received chemotherapy or radiotherapy. Tumor samples, classified as gliomas grade IV (GBM) based on World Health Organization criteria, were immediately processed for isolation of single cells by mechanical dissociation and plated at the concentration of 10^5 cells/ml in Matrigel-coated culture flasks (BD Biosciences) in a proliferation permissive medium containing Dulbecco's modified Eagle's medium/F-12 and Neurobasal (1:1), B27 supplement (Invitrogen), 2 mM L-glutamine (Invitrogen), recombinant human bFGF (10 ng/ml; PeproTech, London, UK), and recombinant human EGF (20 ng/ml; PeproTech). All acutely dissociated primary GBM cells gave rise to neurospheres within 1–2 weeks but also grew as a monolayer on Matrigel for more than 10 passages, without losing their spherogenic properties (28). In fact, the persistence of the stem cell features of the GBM TIC grown on Matrigel was recently demonstrated and deeply characterized by our laboratory (28). We clearly demonstrated that culturing GBM TICs on Matrigel did not affect their tumorigenic potential, their spherogenic properties when transferred to standard culture conditions, nor begin any differentiation program.

Drugs, Growth Factors, Biochemicals, Reagents and Antibodies—The low molecular weight synthetic 4-anilino-quinazoline EGFR TKIs gefitinib (ZD1839, Iressa™, $C_{22}H_{24}ClFN_4O_3$, M_r = 446.9) and erlotinib-HCl (OSI774, Tarceva™, $C_{22}H_{23}N_3O_4$-HCl, M_r = 429.9), provided by Astra-Zeneca SpA (Milano, Italy) and Hoffmann-La Roche, respectively, were diluted as stock solution at the concentration of 1 mM in DMSO and stored in small aliquots at −20 °C (29). When appropriate, the same vehicle dilution was added in the respective control samples. Lyophilized human recombinant EGF and bFGF were purchased by PeproTech EC (London, UK), reconstituted in double distilled H_2O, and stored in working aliquots of 50 ng/100 μl at −20 °C. Human recombinant 3-[^{125}I]iodotyrosyl-EGF, specific activity >750 Ci/mmol, (IM196) aqueous solution, was from GE Healthcare. Hanks' balanced salt solution $CaCl_2$/$MgCl_2$-free was purchased from Invitrogen; bovine serum albumin, fraction V, was from Sigma.

Antibody directed against phospho-Akt (Ser-473), Akt, phospho-ERK1/2, and ERK1/2 were from Cell Signaling Technology (Beverly, MA); anti-phospho-EGFR (Tyr-1173) and anti-EGFR antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Differentiation Assay—To induce differentiation into different brain cell histotypes, GBM cells were seeded on Matrigel-coated coverslips at the concentration of 1×10^4 cells per slide in a medium without growth factors and containing 10% fetal calf serum (EuroClone, Pavia, Italy) for 2 weeks (30). To keep a better reproducibility, all the following experiments were performed using cells grown *in vitro* for 15–25 passages.

Immunofluorescence—For xenograft tumor analysis, mice were sacrificed, and cryopreserved brain sections were cut using a 10-μm cryostat (CM 1100; Leica, Germany). Staining with hematoxylin-eosin identified sections bearing tumors. Briefly, brain sections were mounted on slides and stained with Harris hematoxylin for 40 s and then counterstained with alcoholic eosin for 30 s. Cryosections containing tumors were permeabilized in PBS containing 0.5% Triton X-100 and blocked in 10% normal goat serum/PBS. After incubation with anti-human nestin antibody (mouse monoclonal, diluted 1:1,000; Abcam (Cambridge, UK)) in NGS-PBS overnight at 4 °C, sections were washed in Tris-buffered saline. Immunopositivity was revealed using Alexa488-conjugated goat anti-mouse IgG (1:700; Invitrogen). Cells were counterstained with Hoechst 33342 (Sigma) to identify the nuclei.

To examine the expression of different brain cell lineage markers in undifferentiated and differentiated human glioma TICs, cells were plated onto Matrigel-coated glass coverslips and then fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilized and stained with different primary antibodies as follows: anti-human nestin, anti-Map2 (mouse monoclonal, 1:1,000; Chemicon/Millipore, Milano, Italy), and anti-GFAP (rabbit polyclonal, 1:10,000; Dako, Glostrup, Denmark). Following washing with PBS, cells were exposed to the appropriate secondary fluorescent antibody (Alexa488-conjugated goat anti-mouse IgG, 1:700 or rhodamine-conjugated goat anti-rabbit IgG, 1:250; Jackson ImmunoResearch Europe, Cambridgeshire, UK) and counterstained with Hoechst 33342 dye to identify all nuclei. Data are reported as percentage of immunolabeled cells from five randomly selected microscopy fields. Images were obtained with a Nikon Eclipse 80i microscope (Nikon Europe, Lijnden, The Netherlands).

Tumorigenicity—Human glioma TICs *in vivo* tumorigenicity was tested by cell intracranial inoculation in 6–8-week-old NOD/SCID mice (Charles River Laboratories, Calco, Italy). Preliminary experiments showed that as few as 5,000 cells (not selected for CD133 expression) were able to induce tumors with a latency of up to 180 days. To obtain a more rapid evaluation of the results, we decided to inject 10^5 cells from each TIC

culture. Three mice for each GBM cell culture were used. Mice were anesthetized intramuscularly with 20 μl of ketamine (2%) and xylazine (100 mg/ml) and positioned into a stereotactic frame (David Kopf Instruments, Tujunga, CA). A hole was made in the skull using a 21-gauge needle, 2.5 mm lateral and 1 mm anterior from the intersection of the coronal and sagittal sutures (bregma), and cells were injected in the left corpus striatum using a Hamilton syringe (series 7000; Sigma) at a depth of 3.5 mm (total volume 2 μl). Mice were monitored for about 6 months for disease symptoms and were sacrificed by CO_2 asphyxiation when they showed weight loss or any severe sign of disease. All experiments, including animals, were performed in compliance with guidelines approved by the Ethical Committee for animal use in cancer research at the National Institute for Cancer Research (Genova, Italy).

$CD133^+$ Cell Isolation and Quantification—Human GBM TICs were sorted by CD133 expression with Miltenyi microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, cells were dissociated and resuspended in PBS, 0.5% bovine serum albumin, and 2 mM EDTA. CD133/1 microbeads were used for positive magnetic cell separation using two magnetic cell separation columns in series. Aliquots of $CD133^+$ and $CD133^-$ cells were evaluated for purity by flow cytometry using CD133/2-PE (Miltenyi Biotech) or isotype control antibody (IgG2b-PE, Miltenyi Biotech) and analyzed on a FACSCalibur (BD Biosciences). For one-color cytofluorimetric analysis, human glioma TICs were stained with the appropriate monoclonal antibodies followed by phycoerythrin-conjugated isotype-specific goat anti-mouse second reagent (Southern Biotechnology Associated, Birmingham, AL).

MTT Assay—For MTT assay, human GBM TICs were plated in 96-well microplates previously coated with Matrigel. Because the cells employed in this study had different proliferation rates, the number of cultured cells was adjusted to a density that allowed the cells to grow exponentially for all the duration of the assay (up to 4 days) ranging from 2,500 to 10,000 cells/well. We tested the effects of erlotinib and gefitinib using three concentrations as follows: 0.5, 1, and 5 μM. After exposure to the drugs for different times (0, 24, 48, 72, and 96 h) at 37 °C, cells were incubated for 4 h in MTT solution (2 mg/ml in PBS). After removing MTT, 150 μl of DMSO were added to each well, and the absorbance was determined at 540 nm wavelength, as reported (31), using an enzyme-linked immunosorbent assay plate reader.

Radioreceptor Assay with ^{125}I-Labeled EGF/Unlabeled EGF Competitive Binding to EGFR—Human GBM TICs were plated at the concentration of 1.5×10^5 in duplicate wells using the proliferation permissive medium in Matrigel pre-coated 24-well plates (28) and incubated overnight at 37 °C, before the medium was changed with a growth factor-deprived medium for an additional 48 or 96 h according to the experimental protocol. At the end of this period, cell monolayers were washed once with 1 ml/well of Binding Buffer (BB: Dulbecco's modified Eagle's medium, 40 mM Hepes, 0.1% bovine serum albumin) and incubated at 37 °C, 5% CO_2 for an additional 60 min. Cells were then treated with ^{125}I-labeled EGF (average 2×10^4 cpm in 200 μl/well of BB) and serially increasing amounts of unlabeled peptide (0.195–50 ng/ml for standard curve + 400 ng/ml excess for nonspecific binding evaluation) in 200 μl/well BB (total volume/well = 400 μl). Plates were incubated for 2 h at 4 °C on a shaker with gentle agitation. After binding, medium was carefully removed from each well and monolayers were washed three times with 500 μl/well ice-cold Washing Buffer (Hanks' balanced salt solution, 0.1% bovine serum albumin). Cells were lysed by adding 750 μl of Solubilizing Solution (20 mM Hepes, 1% Triton X-100, 10% glycerol, 87% H_2O) to each well and then incubated for 20–30 min at room temperature. An aliquot (80%) of solubilized and carefully homogenized material was than measured in a γ-counter (Cobra II Autogamma, Packard Instrument Co.) to quantify the released radioactivity of the bound ligand. Specific binding was calculated after subtraction of nonspecific binding and by means of Scatchard analysis, B_{max}, and K_d values, and sites/cell were determined.

Western Blotting—To avoid the activities of growth factors and other supplements present in the proliferation medium, cells were cultured for 48 h in a growth factor-free medium before being treated (32). Cells were lysed in buffer containing 1% Nonidet P-40, 20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM NaF (all from Sigma), and the "Complete" protease inhibitor mixture (Roche Applied Science) for 10 min at 4 °C. Nuclei were removed by centrifugation (5,000 rpm at 4 °C, for 10 min), and total protein content was measured using the Bradford assay (Bio-Rad). Proteins (10 μg) were resuspended in 2× reducing sample buffer (2% SDS, 62.5 mM Tris, pH 6.8, 0.01% bromphenol blue, and 1.43 mM β-mercaptoethanol, 0.1% glycerol), electrophoresed on 7.5–10% SDS-polyacrylamide gels, transferred on polyvinylidene difluoride membrane (Bio-Rad) (32), and probed with specific antibodies. The detection of immunocomplexes was performed using ECL system (GE Healthcare).

RNA Isolation and Reverse Transcription (RT)-PCR—Total RNA was isolated using the TRIzol reagent (Invitrogen) and the "Total RNA Purification System" (Invitrogen). cDNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad) and amplified with *Taq* DNA polymerase (Qiagen, Milano, Italy) by PCR (iCYCLER Bio-Rad) using the following primers: 5′-aaagctggaaagggacgaac-3′ and 5′-caggtaacggctgagggaac-3′ (GenBank™ accession number NM_000314). Amplification profile was as follows: 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by 7 min at 72 °C.

β-Actin amplification, used as positive control for the PCR, was performed using the following primers: sense 5′-tccggagacggggtca-3′ and antisense 5′-cctgcttgctgatcca-3′. The selected sequences encompass an intronic sequence in the β-actin gene that allow the identification of genomic DNA contamination (33). Negative controls were obtained performing PCR amplification in the absence of RT reaction.

Statistical Analysis—Data are reported as means ± S.E. of at least two independent experiments performed in triplicate. Statistical analysis (analysis of variance) was performed using the Software GraphPad QuickCalcs. A p value ≤ 0.05 was considered statistically significant.

TABLE 1

Characterization of human glioma TICs

Human glioma cells that we derived from seven patients express stem cells markers (nestin and CD133) and are positive for Map2 (neuronal marker) and GFAP (astroglial marker). These cells can also generate tumors *in vivo,* being serially transplantable, so we called them TICs. Data represent range of time required to induce large brain tumors that required the sacrifice of the animals after injection of 10^5 cells of each tumor as primary xenograft or after reimplant in immunodeficient mice of cells derived from explants of the primary tumors (secondary tumors). Moreover, it is reported, for each TIC culture, the percentage (±S.E.) of the cells that express the indicated biomarkers, analyzed by immunocytochemistry (nestin, GFAP and Map2) or flow cytometry (CD133) experiments. For the stem cell markers nestin and CD133, data derived from short (3–6 passages *in vitro*) and long term cultures (>35 passages *in vitro*) are indicated. Representative fluorescence-activated cell sorter diagrams and immunofluorescence microphotographs are reported in the supplemental Fig. 1.

Tumor code	*In vivo* tumorigenicity (survival, days)		Percentage of positive cells *in vitro*					
	Primary tumor	Secondary tumor	Nestin short term	Nestin long term	CD133 short term	CD133 long term	GFAP	Map2
GBM1	150–180	90–110	93 (±2)	95 (±2.5)	3 (±1.5)	2 (±1)	27 (±3)	75.5 (±5.5)
GBM2	80–90	55–65	78 (±2.5)	75 (±5)	1.8 (±0.8)	1.6 (±1)	4.5 (±0.5)	8.5 (±3.5)
GBM3	40–120	40–60	95 (±3.5)	97 (±2.5)	4.5 (±1.5)	5.2 (±2)	15 (±5)	7.5 (±2.5)
GBM4	100–120	80–90	76 (±2.5)	78 (±4)	83 (±3)	82 (±4)	9.5 (±1)	23 (±2.5)
GBM5	70–80	60–70	40 (±6)	35 (±5)	85 (±4.5)	80 (±3)	3 (±1)	40 (±5)
GBM6	120–170	90–110	69 (±3)	65 (±5)	2.5 (±1)	5.1 (±0.5)	7.5 (±2.5)	75 (±5)
GBM7	64–110	55–70	93 (±2)	90 (±5)	1.8 (±0.5)	2.1 (±0.5)	80 (±6)	12.5 (±2.5)

RESULTS

Tumorigenicity of Human Glioma-derived Cells—We analyzed post-surgical explants from seven consecutive human gliomas, histopathologically classified as grade IV according to the World Health Organization grading. Tumor samples were immediately dissociated into single cells, and plated at clonal density in a proliferation medium, in Matrigel-coated flasks (see "Experimental Procedures"). Under these conditions these cells can be cultured *in vitro* retaining the ability to form neurospheres that, in limiting dilution experiments, are detected after 1 or 2 weeks, according to the tumor analyzed. To identify the presence of TICs in these cultures, we tested their *in vivo* tumorigenicity, to date the only test able to discriminate TICs from differentiated nontumorigenic cancer cells.

A hundred thousand cells derived from all GBM cultures analyzed (called GBM 1–7) were orthotopically injected in immunodeficient NOD/SCID mice as described under "Experimental Procedures." After 2–6 months, according to the GBM from which the cells were isolated (see Table 1), all cultures produced tumors with highly invasive properties that resembled the primitive neoplasia, thus confirming that our cultures are enriched in TICs. In particular, intracranial tumors generated by TICs injections demonstrated various grades of infiltration into the surrounding cerebral cortex, and GBM cells showed a propensity to migrate along the corpus callosum or other brain structures. GBM generated from the injected TICs were identified by histological analysis that confirmed the exclusive presence of human tumor cells, without significant contamination from murine cells, as assessed by the positive staining detected using anti-human nestin antibody (Fig. 1) and the lack of labeling using anti-mouse antibodies (data not shown). Importantly, in agreement with our previous studies (28), no differences in tumorigenicity were observed culturing the cells as nonadherent neurosphere or in monolayer on Matrigel, in both cases injecting 10^5 cells the take rate was 100% even after more than 40 passages *in vitro*.

Cells, isolated from dissociated intracranial xenograft tumors and re-cultured under stem cells conditions, were able to form neurospheres. When cells were injected back into the brain of new recipient mice, they not only retained the ability to generate new tumors but also showed an increased tumorigenic potential, as shown by the shorter time required for tumor development (Table 1).

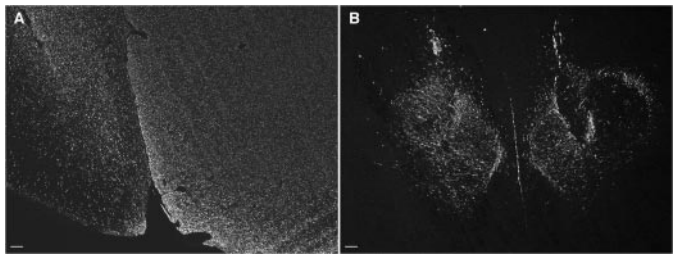

FIGURE 1. **Tumorigenicity of human glioma-derived cells.** Representative immunohistochemical analysis of brain tumors generated after orthotropic injection of isolated human glioblastoma cells. Immunofluorescence of mouse brains cryosections labeled with anti-human nestin antibody. *A,* diffuse infiltration of the injected and contralateral hemisphere (GBM 1). *B,* cells infiltrating the striatum ipsilateral and contralateral to the side of injection (GBM 2). *Scale bar,* 100 μm. GBM xenografts recapitulate the morphology of corresponding tumors in human cancer patients.

Characterization and Differentiation of Human Glioma Tumor-initiating Cells—All the human glioma TICs that we isolated from GBM 1–7 displayed the potential for multilineage differentiation and clonogenicity *in vitro*, which are typical features of neural stem cells. In particular, immunofluorescence and cytofluorimetric analysis showed that the expression of nestin and CD133, respectively, was clearly detectable in subpopulations of these cultures (Table 1). In agreement with the stemness of these cultures, nestin was expressed in an extremely high percentage of cells (up to 97% in GBM 3, see Table 1). The identification of a CD133-positive subpopulation within these cultures was also particularly relevant, because this protein is recognized as a marker of glioma TICs (34, 35). In agreement with previously reported data (36), nestin was detected in a higher number of cells than CD133, although in two cases (GBM 4 and -5) the proportion of CD133-expressing cells was as high as 80% (see Table 1 and supplemental Fig. 1). Using microbeads for immunoselection, it was possible to obtain cultures enriched in CD133-expressing cells. However, after a few passages the level of $CD133^+$ cells returned to baseline values because of the stem-like nature of these cells whose cell division results in the generation of a percentage of differentiated cells.

Immunofluorescence experiments showed that a high percentage of all TIC cultures also expressed percentage of Sox2 (data not shown). All these features were reported to be specific for both neural stem cells and brain tumor TICs.

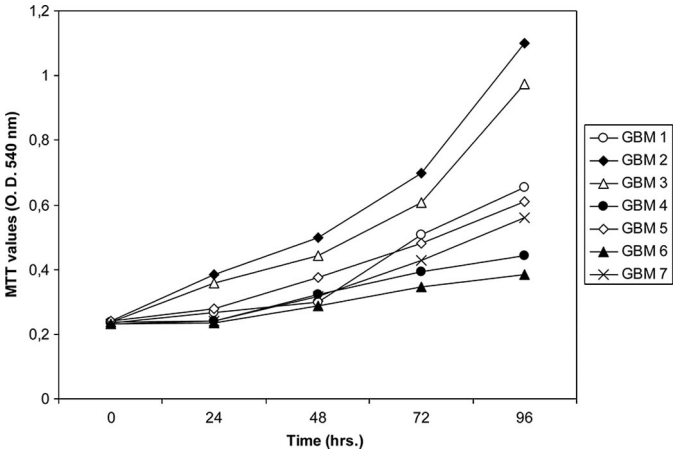

FIGURE 2. ***In vitro* proliferation of human glioma tumor-initiating cells.** Time-dependent proliferation of human glioma TICs derived from 7 patients (GBM 1–7). Cells were cultured at different cell concentrations/well to obtain a linear cell proliferation for all the experimental observations (96 h). In particular 2,500 cells/well were plated for GBM 2 and GBM 3; 5,000 cells/well were plated for GBM 7; 10,000 cells/well were plated for GBM 1, GBM 4, GBM 5, and GBM 6. The *graph* reports the average of two independent experiments performed in triplicate. Data represent mean values of three independent experiments. In all samples S.E. was lower than 7% of the value.

Analysis of the basal expression of differentiation markers showed that these cells were also positive for microtubule-associated protein 2 (Map2, neuronal marker) and GFAP (astroglial marker), although at very different levels among the various GBMs (see Table 1). Interestingly, a small (but in some cases significant) percentage of the tumor cells we analyzed co-expressed both neural and glial markers (GBM 1, 13.6 ± 0.4%; GBM 2, 0.1 ± 0.1; GBM 3, 5.3 ± 1.5; GBM 4, 2.7 ± 0.8; GBM 5, 1.1 ± 0.3; GBM 6, 3.4 ± 2.8; and GBM 7, 5.6 ± 0.6).

However, culturing the cells under differentiating conditions resulted in the acquisition of typical astroglial morphology and a high expression of GFAP (data not shown). These data suggest that when the differentiation program is activated, TIC cultures are mainly oriented to astroglial commitment.

Importantly, we periodically evaluated the expression levels of nestin, CD133, GFAP, and Map2, as well as their tumorigenicity, and we found that it was quite stable over time in all the selected TIC cultures (see Table 1), confirming that it represents a specific feature of each GBM TIC culture.

Effects of the EGFR Inhibitors Erlotinib and Gefitinib on Human Glioma TIC Proliferation—Before studying the sensitivity of TICs in response to EGFR TKI, we set up conditions of cell culture that allowed exponential growth of all the GBM TICs for the entire length of the experiments (4 days). Thus, in preliminary experiments we determined the appropriate cell number to be used for each individual TIC culture, plated in 96-multiwell plates in proliferation medium. From these experiments we defined a cell number range (from 2,500 cells/well for GBM 2 and -3 to 10,000 cells/well for GBM 1, -4, and -5) that allows the cells to keep a linear proliferation rate during the entire experimental period (up to 4 days, Fig. 2).

To analyze the sensitivity of these cells to target-specific antiproliferative drugs, we studied the responsiveness of TICs to two different EGFR kinase inhibitors, erlotinib and gefitinib. The proliferation rate of TICs derived from all the seven GBM treated with the drugs at increasing concentrations (0.5, 1, and 5 μM) was compared with vehicle-treated cells at different time points (time 0, and after 24, 48, 72, and 96 h of treatment).

Erlotinib caused a time- and dose-dependent inhibition of cell proliferation in all cultures with the only exception of GBM 2 that was completely insensitive to all the concentrations used (Fig. 3*A*). At the concentration of 5 μM, erlotinib caused a highly significant growth arrest in GBM 3, which reached the maximal inhibition already after 24 h of treatment (−62%, $p < 0.01$) and lasted for the length of the experiment (−68%, after 4 days). Similar inhibitory effects were observed after 48 h in GBM 1 and after 72 h in GBM 4, GBM 5, GBM 6, and GBM 7 (ranging from −40 to −55%, $p < 0.01$), although a further increase in the inhibitory effects after 96 h of treatment was observed only in GBM 1 (−62%). A higher responsiveness of GBM 1 and -3 was further confirmed using a drug concentration of 1 μM that caused cell growth inhibition not statistically different ($p < 0.01$) from that observed using 5 μM (from −55% after 1 day of treatment to −66% after 4 days for GBM 3). Lower effects, but still statistically significant, were observed with 1 μM erlotinib in the other GBM TIC cultures, although no statistically significant effects were observed in GBM 7 cells. 0.5 μM erlotinib caused a smaller inhibitory effect in all the GBM TICs, and only GBM 3 showed a significant reduction of cell viability for all the time points analyzed, reaching a maximum after 3 days of treatment (−41%, $p < 0.01$). GBM 1, GBM 5, and GBM 6 cells displayed a lower inhibitory effect that reached a statistical significance ($p < 0.05$) after 48 h (GBM 1) or 72 h (GBM 5 and GBM 6). Using low erlotinib concentrations, no significant inhibition was observed in both GBM 4 and GBM 7.

Interestingly, GBM 2 TICs were completely insensitive to all the drug concentrations tested. All the data concerning the erlotinib effects on the seven GBM TIC cultures are summarized in Fig. 3*A*, and the results of the statistical analysis (*p* values) are summarized in supplemental Table 1.

Similar, but not identical, results were obtained after treatment with gefitinib. In fact, although GBM 3 displayed a high sensitivity to gefitinib that was comparable with what was observed with erlotinib (maximum inhibition was already reached at the concentration of 0.5 μM after 4 days of treatment, about −50%; but all time and concentration points analyzed showed a statistical significance, see Fig. 3*B* and supplemental Table 1), GBM 1 and -4 showed an increased responsiveness as compared with erlotinib, −73 and −62% already after 3 days of treatment with 5 μM, and a statistically significant inhibition ($p < 0.05$) already after 24 h of treatment with 1 and 0.5 μM gefitinib, respectively (Fig. 3*B* and supplemental Table 1). GBM 5 and -6 showed maximal inhibition similar to that observed after erlotinib treatment, and the antiproliferative effect was statistically significant already at the concentration of 0.5 μM after 72 h of treatment ($p < 0.05$, Fig. 3*B*). However, for the highest gefitinib concentration tested, a more significant inhibition of cell proliferation was observed in GBM 5 and -6, as compared with erlotinib treatment, being statistically significant already after 1 day of treatment (see supplemental Table 1 and Fig. 3*B*). Interestingly, GBM 7 showed a moderate reduction in the proliferation rate only at highest concentration (5 μM) and for prolonged times of treatment (3 and 4 days), whereas at lower concentrations, gefitinib was completely inef-

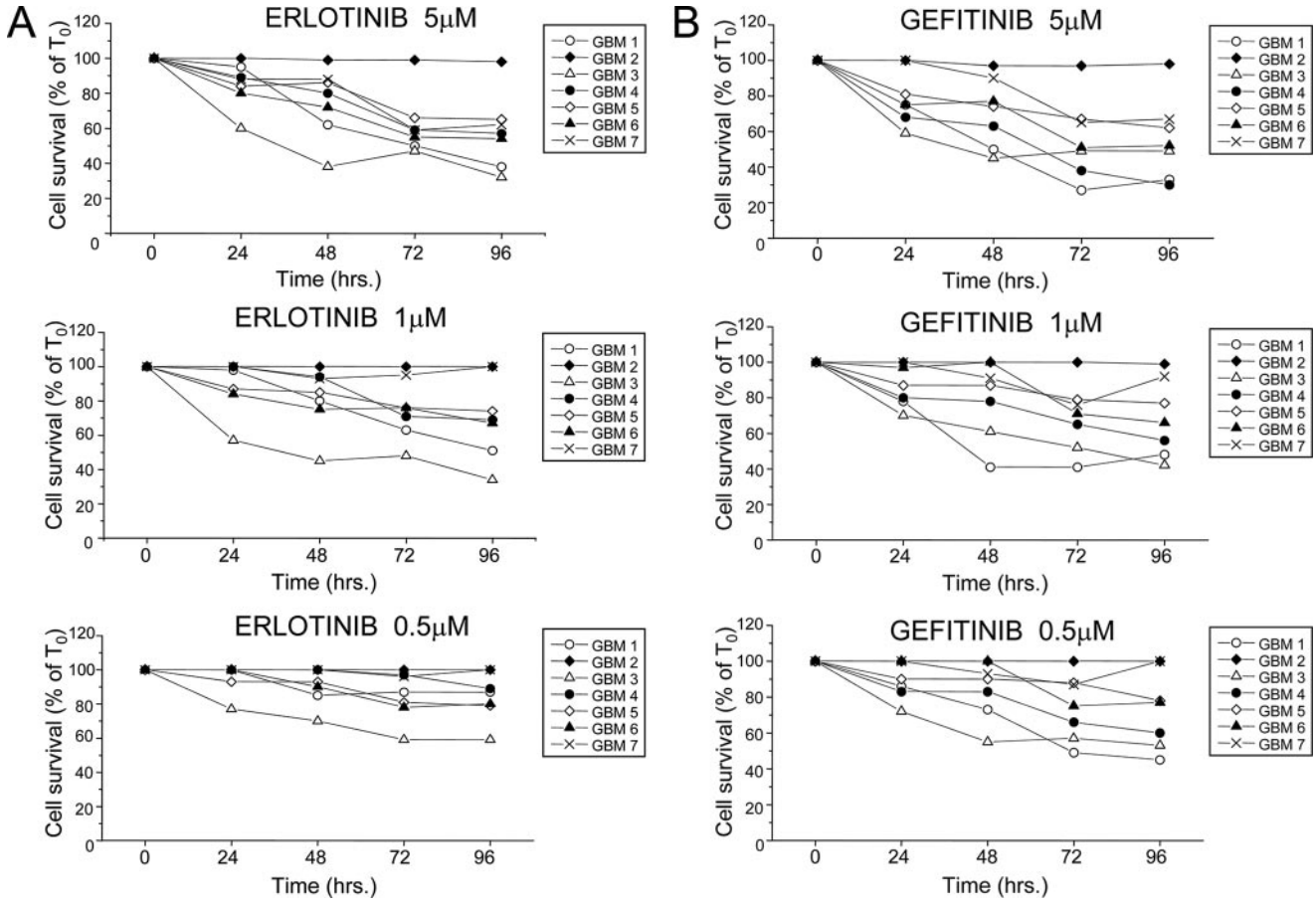

FIGURE 3. **Inhibitory effects of EGFR tyrosine kinase inhibitors on the proliferation of human glioma TICs.** TICs were plated in 96-well multiplates for 24 h and then treated with different concentrations of tyrosine kinase inhibitors. The results are reported as means from three different experiments. S.E. were constantly lower than 5% of each mean value. Individual *p* values obtained by analysis of variance are reported in the supplemental Table 1. *A,* time-dependent growth inhibition induced by erlotinib used at the concentration of 5 μM (*upper panel*), 1 μM (*middle panel*), and 0.5 μM (*lower panel*). *B,* time-dependent growth inhibition induced by gefitinib used at the concentration of 5 μM (*upper panel*), 1 μM (*middle panel*), and 0.5 μM (*lower panel*).

fective. Finally, GBM 2 confirmed the absolute insensibility to EGFR inhibition, with its proliferation rate unaffected by all the gefitinib concentrations used (Fig. 3*B*).

Characterization of Human Glioma TIC EGFR Site Number by EGF Competitive Binding—To better understand the differential response of the individual GBM TIC cultures to erlotinib and gefitinib, we measured EGFR expression by competitive binding of EGF to its specific transmembrane cell receptor in all the cultures. Cell monolayers were simultaneously treated (2 h at 4 °C) with unlabeled and ^{125}I-labeled EGF. Evaluation of non-specific binding was performed by adding excess amount of EGF (400 ng/ml) that completely blocked (>95%) the binding of the iodinated isotope. Scatchard analysis of ^{125}I-EGF binding to TIC cells resulted in a best fit according to the one-affinity model with one single class of binding sites totally in the high affinity state (see supplemental Fig. 2), ranging from 0.16 to 0.67 nM as far as the K_d and from 3.5 to 39.8 pM regarding the B_{max} after 96 h of growth factor starvation (Table 2). The number of binding sites ranged from about 2 to 12 × 10^4 per cell (Table 2). A shorter starvation period lowered the binding ability of GBM 3, whereas values remained substantially unchanged for GBM 2, -5, and -6 cells (data not shown). Thus, the increasing of sites/cell after a longer period of serum starvation (96 h *versus* 24–48) does not seem to be a rule, as it could have been expected considering the potential release of free available receptor sites, which is likely to happen in total absence of exogenous EGF. On the contrary, assays carried out in two TIC cultures (GBM 2 and -3) without previous growth factor starvation did not show significant values in B_{max}, K_d, and sites (data not shown), possibly because of the unavailability of free receptor sites due to the exogenous EGF present in culture medium. Interestingly, we found only a partial correlation

TABLE 2

Radioligand receptor assay on human glioma TICs

Cells plated in 24-well plates in duplicate were growth factor-starved for 96 h, washed, and incubated for 60 min at 37 °C in 1 ml of Binding Buffer (BB). Monolayers were then treated for 2 h at 4 °C with shaking and with serially increasing amounts of EGF (200 μl/well) and about 2 × 10^4 cpm of ^{125}I-EGF (200 μl/well) in 400 μl/well BB total volume. After incubation, cells were washed three times, lysed, and counted in a γ-counter. Binding affinities and receptor numbers were derived from Scatchard analysis. B_{max} indicates maximum binding capacity; K_d indicates dissociation constant.

Tumors	B_{max}	K_d	Sites/cell
	pM	*nM*	
GBM1	30.5	0.17	124,000
GBM2	15.4	0.16	45,000
GBM3	39.8	0.36	112,000
GBM4	27.5	0.21	115,000
GBM5	17.2	0.67	68,000
GBM6	3.5	0.37	23,000
GBM7	12.1	0.20	37,000

TABLE 3

Inhibition of EGFR, ERK1/2, and Akt phosphorylation/activation induced after treatment with the EGFR kinase inhibitors, erlotinib and gefitinib

Data are derived by densitometric analysis of at least two independent sets of experiments and expressed as mean of the percentage values (±% S.E.) of the maximal inhibition observed of the EGF-induced protein phosphorylation (set as 100%). NS indicates not statistically significant *versus* EGF-treated cells

Tumors	Erlotinib			Gefitinib		
	p-EGFR	p-ERK1/2	p-Akt	p-EGFR	p-ERK1/2	p-Akt
GBM1	8 ± 1^a	18 ± 5^b	9 ± 3^a	16 ± 2^a	45 ± 6	34 ± 7^a
GBM2	10 ± 3^a	15 ± 4^a	101 ± 12 NS	3 ± 1^a	3 ± 1^a	105 ± 9 NS
GBM3	41 ± 7^b	32 ± 3^b	42 ± 5^b	21 ± 4^b	28 ± 4^a	28 ± 6
GBM4	3 ± 2^a	35 ± 8^b	12 ± 3^a	8 ± 2^a	18 ± 3^b	37 ± 11^b
GBM5	46 ± 9^b	43 ± 11^b	$39 \pm 9^*$	12 ± 2^a	42 ± 11^b	43 ± 9
GBM6	15 ± 4^b	16 ± 3^a	34 ± 12^b	9 ± 2^a	39 ± 9^b	40 ± 7^b
GBM7	48 ± 6^b	9 ± 2^a	38 ± 8^b	98 ± 13 NS	90 ± 12 NS	102 ± 10 NS

[a] Values are $p < 0.01$.
[b] Values are $p < 0.05$.

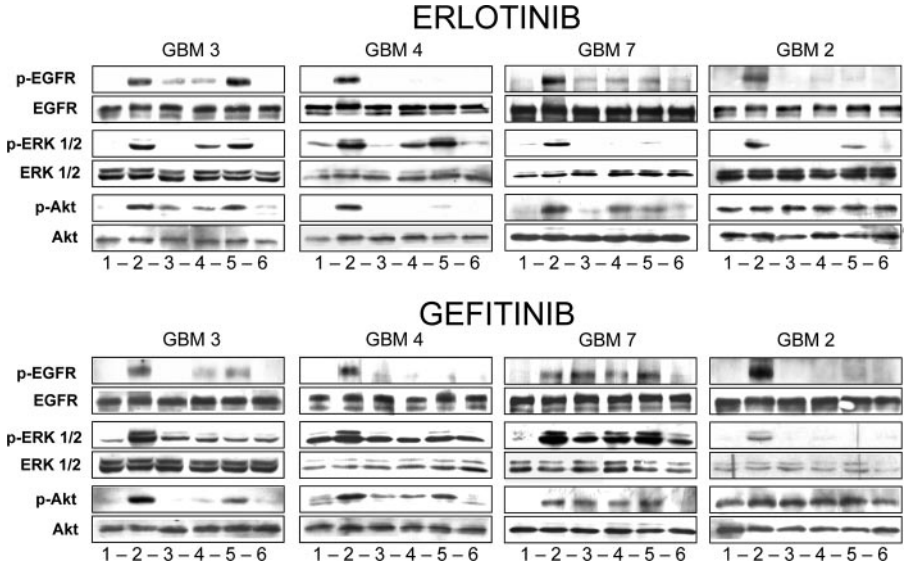

FIGURE 4. **Effects of tyrosine kinase inhibitors on EGFR, ERK 1/2, and Akt phosphorylation.** Representative Western blots of GBM 3 and −4 (responsive to the antiproliferative effects of erlotinib and gefitinib) and GBM 7 and −2 (slightly responsive and unresponsive, respectively) are shown. *Upper panel,* effect of erlotinib on EGF-induced EGFR, ERK1/2, and Akt phosphorylation. Lanes represent the following: *lane 1,* control; *lane 2,* EGF (40 ng/ml); *lane 3,* EGF + erlotinib (5 μM); *lane 4,* EGF + erlotinib (1 μM); *lane 5,* EGF + erlotinib (0.5 μM); and *lane 6,* erlotinib (5 μM). EGF-induced EGFR and ERK1/2 phosphorylation was reverted by erlotinib treatment in all the GBM TICs analyzed. Erlotinib was also highly effective in inducing a significant inhibition of Akt activation observed after EGF treatment in all the GBM TICs except for GBM 2 cells. *Lower panel,* effect of gefitinib on EGF-induced EGFR, ERK1/2, and Akt phosphorylation. Lanes represent the following: *lane 1,* control; *lane 2,* EGF (40 ng/ml); *lane 3,* EGF + gefitinib (5 μM); *lane 4,* EGF + gefitinib (1 μM); *lane 5,* EGF + gefitinib (0.5 μM); *lane 6,* gefitinib (5 μM). Gefitinib treatment also completely abolished EGF-induced EGFR and ERK1/2 phosphorylation in GBM 2, GBM 3, and GBM 4, whereas in GBM 7 cells it was completely ineffective. A significant inhibition of Akt activation was observed after gefitinb treatment only in GBM 3 and GBM 4 but failed to inhibit Akt activation in GBM 2 and GBM 7. The analysis of the results of the Western blots in all the GBM TICs is reported in Table 3.

between the receptor number and the responsiveness to EGFR TKI antiproliferative effects. In fact, although the more responsive GBM TIC cultures (GBM 1, -3, and -4) displayed the highest number of EGFR site/cell, and the less responsive GBM 2 and -7 showed low levels of expression of the receptor, GBM 5 and -6 combined a significant inhibition of cell proliferation with a small number of binding sites.

Effects of the EGFR Inhibitors Erlotinib and Gefitinib on Human Glioma TIC EGFR Activation—The lack of a precise correlation between EGFR expression level and antiproliferative effects of the TKIs in the TIC cultures analyzed suggested that the different responses to erlotinib and gefitinib may reside in the different efficacy of the signal transduction activated by this receptor.

First, to confirm the specificity of the effects of erlotinib and gefitinib on the proliferation rate of human glioma TICs, we investigated their effects on EGFR phosphorylation/activation induced by EGF. Cells were cultured for 48 h in a growth factor-free medium, pretreated for 15 min with increasing concentrations of erlotinib or gefitinib (0.5, 1, and 5 μM), and then treated for 5 min with EGF (40 ng/ml). EGF induced activation/phosphorylation of EGFR in all TIC cultures (data not shown). Importantly, Western blot analysis of the apparent EGFR molecular weight showed that in all GBM TIC cultures the EGFRvIII mutant isoform, often expressed in human GBM (37), was never expressed (data not shown). Similarly, other EGFR family members, such as HER-2, were detected in the TIC cultures (data not shown).

In Table 3 are reported the effects of erlotinib (left columns) and gefitinib (right columns) on EGF-induced phosphorylation of EGFR, and representative blots from GBM 3 and -4 (highly responsive to the antiproliferative effects of the tyrosine kinase inhibitors) and GBM 7 and -2 (slightly responsive or completely unresponsive, respectively) are reported in Fig. 4.

Erlotinib inhibited EGF-induced EGFR phosphorylation/activation in all TIC cultures (Table 3, left columns). In particular, EGFR phosphorylation induced by EGF was markedly inhibited by erlotinib pretreatment in GBM 1, -2, -4, -6, and -7 cells at all the concentrations tested (0.5, 1, and 5 μM), although in GBM 3 and -5 cells the inhibitory effects were more dose-dependent with an almost complete inhibition obtained at the highest concentration tested (5 μM).

On the other hand, gefitinib pretreatment did not reduce EGF-induced EGFR phosphorylation in all the cell lines, since

in GBM 7 cells it was completely ineffective (see Table 3, right panel, and Fig. 4). Gefitinib completely abolished EGF-induced EGFR phosphorylation in GBM 2 and -4 cells at all the concentrations used (Fig. 4). In GBM 1, -3, -5, and -6 cells, the drug caused a highly significant reduction of EGFR phosphorylation in a dose-dependent manner, being ineffective only at the lowest concentration used (0.5 μM). Conversely, as shown in the Western blot reported in Fig. 4 and in Table 3, gefitinib did not affect EGFR phosphorylation induced by EGF in GBM 7 cells.

Effects of the EGFR Inhibitors Erlotinib and Gefitinib on Human Glioma TIC ERK1/2 Activation Induced by EGF—To assess a possible role for the activation of the mitogen-activated protein kinase cascade, via EGFR, in the proliferative effects of EGF, we performed Western blot evaluating ERK1/2 activation as a consequence of EGF treatment, in the presence or absence of the two EGFR TKIs. For this purpose, we investigated the changes in ERK1/2 phosphorylation using the same cell extracts used in the above described experiments. Interestingly, erlotinib treatment caused a significant reduction of ERK1/2 phosphorylation induced by EGF in all TICs that almost completely paralleled the effects of EGFR phosphorylation (Table 3, left panel). In particular, the drug was highly effective in all GBM TICs showing in most cases a complete abolishment of ERK1/2 phosphorylation also at low concentrations (Fig. 4).

A significant inhibition of ERK1/2 phosphorylation was also observed after gefitinib treatment in all TICs (Table 3, right panel), although the drug was less effective in GBM 7 cells with a barely detectable reduction of ERK1/2 phosphorylation only at the highest concentration tested (Table 3 and Fig. 4). Again, these effects were comparable with those observed for EGFR phosphorylation. Interestingly, in GBM 2 the significant effects of erlotinib on EGFR and ERK1/2 activation were at variance with the lack of antiproliferative effects induced by the drug.

Effects of the EGFR Inhibitors Erlotinib and Gefitinib on Human Glioma TIC Akt Phosphorylation Induced by EGF—It was reported that the effects of EGFR, as a key mediator of oncogenesis in human tumors, are mediated not only through ERK1/2 signaling but also by the serine/threonine kinase Akt, activated by phosphatidylinositol 3′-kinase (PI3K) signaling. Constitutively activated Akt pathway is often associated with lack of tumor response to EGFR inhibitors (38, 39). To study the role of this downstream pathway in EGFR activation, we also performed Western blot experiments evaluating the phosphorylation/activation of Akt in basal or EGF- treated conditions in the presence or absence of erlotinib and gefitinib. In all TICs, a significant increase in Akt activation was observed after EGF treatment, except in GBM 2 cells. As reported in Fig. 4, in this TIC culture an elevated level of Akt phosphorylation was already detected in untreated cells, and EGF treatment did not modify Akt basal activation.

Table 3 details the effects of gefitinib and erlotinib on EGF-induced Akt phosphorylation. Erlotinib pretreatment caused a significant reduction of the kinase activation in all TICs but had no effect either on basal or EGF-induced Akt phosphorylation of GBM 2 cells. Gefitinib also powerfully inhibited the activation of Akt by EGF while lacking efficacy in GBM 2 and GBM 7 (Fig. 4 and Table 3).

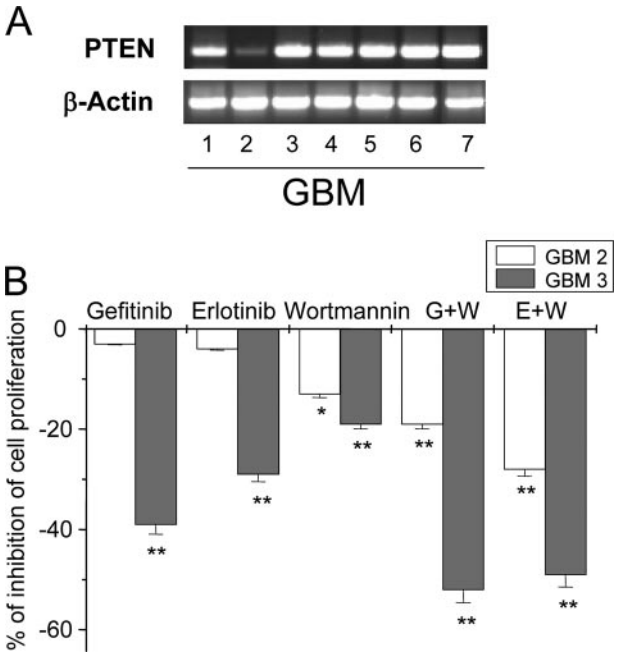

FIGURE 5. *A,* PTEN mRNA expression in cultured human glioma TICs. RT-PCR analysis of PTEN mRNA expression in human gliomas TICs. All cells analyzed expressed PTEN mRNA at a similar level with only GBM 2 cells that showed a significantly lower mRNA amount. *B,* effect of PI3K/Akt inhibition on gefitinib or erlotinib modulation of GBM TIC survival. GBM 2 (*white bars*) and GBM 3 (*gray bars*) TIC cultures were plated in 96-well multiplates for 24 h and then treated with gefitinib and erlotinib (5 μM) in the presence or absence of wortmannin (1 μM) for 96 h. The results are reported as mean of two independent experiments ± S.E. Data represents the percentage of inhibition of the respective control values (set as 100%). *G,* gefitinib; *E,* erlotinib; *W,* wortmannin. *, $p < 0.05$ and **, $p < 0.01$ *versus* control values.

Phosphatase and Tensin Homolog (PTEN) mRNA Expression in Cultured Human Glioma TICs—From the previous experiments it appears that the main determinant of the responsiveness to the antiproliferative activity of erlotinib and gefitinib may be represented by the ability to revert Akt activation. Thus, we examined, by RT-PCR, the levels of mRNA expression of PTEN, a phospholipid phosphatase representing the main intracellular inhibitor of the PI3K/Akt pathway.

TICs derived from all the GBM analyzed expressed significant amounts of PTEN mRNA, with only GBM 2 cells that showed a significantly lower mRNA amount (Fig. 5*A*). To better correlate the role of Akt activation to the EGFR-TKI response, we treated two TIC cultures with gefitinib or erlotinib in the presence or absence of the PI3K inhibitor wortmannin. In these experiments we evaluated a highly responsive TIC culture (GBM 3) and GBM 2 that displayed low PTEN expression associated with a drug nonresponsive phenotype. The inhibition of PI3K/Akt pathway resulted, *per se*, in slight antiproliferative effect in both TIC cultures (Fig. 5*B*). In the TKI-responsive GBM 3 cells the significant inhibition induced by gefitinib and erlotinib was further increased in the presence of wortmannin (Fig. 5*B*). More importantly, the inhibition of PI3K/Akt activity evidenced a moderate, although statistically significant, inhibitory effect of gefitinib and erlotinib also in the nonresponsive TIC culture from GBM 2 (Fig. 5*B*). These results strongly support the hypothesis that in the presence of constitutively active PI3K/Akt signaling, the EGFR kinase inhibitors lose their efficacy.

DISCUSSION

The discovery in many solid tumors (including GBM) of a cancer stem cell subpopulation endowed with tumorigenic properties opened a completely new scenario in the therapeutic approach in oncology. In fact, although not yet completely characterized, these cells showed a very different pattern of resistance to the traditional pharmacological or radiation treatments, as compared with the bulk of neoplastic cells into the tumor mass (23, 25, 40). However, because of the difficulties in obtaining and maintaining *in vitro* cultures enriched in this TIC subpopulation, a characterization of their specific sensitivity to target-directed compounds has been performed only in limited samples.

Here we report the characterization of GBM-derived TIC cultures as far as *in vivo* tumorigenicity, cancer stem cell marker expression, multilineage differentiation, and sensitivity to the EGFR kinase inhibitors erlotinib and gefitinib. Interestingly, we show that whereas most of the GBM TICs analyzed retain the *in vitro* responsiveness to both drugs, this effect seems to be related to PTEN activity and the ability to reduce Akt phosphorylation.

Seven human GBM TIC cultures from post-surgical specimens were isolated from patients that never received chemo- or radiotherapy. In agreement with previous studies, we cultured these cells in a proliferation medium containing the growth factors EGF and bFGF, according to the conditions described for the propagation of brain cancer stem cells (9). Moreover, we confirmed the crucial role of EGF in the mitogenic regulation of GBM TICs, as recently reported (41). Although all the GBM-derived cultures gave rise to tumorigenic neurospheres when cultured in nonadherent conditions according to standard protocols (9), we developed a novel *in vitro* growth protocol using Matrigel-coated dishes. In these experimental conditions TICs grow in monolayers, retain a good proliferation rate, and do not enter the differentiation program, as shown by the continuous and stable expression of stem cells markers (CD133, nestin, see Table 1) in a rather constant percentage of cells. More importantly, the persistence of TICs under these culture conditions was further confirmed by the retained ability to generate tumors when orthotopically injected in NOD-SCID mice. Interestingly, these tumors displayed a highly invasive pattern of growth, very much resembling the growth features of human GBM *in vivo*. This observation strongly support the relevance of this cell model to evaluate the biological characteristics and pharmacological responsiveness of human GBM cells, as already demonstrated at the genetic level (9).

We show that isolated GBM TICs, cultured as detailed before, were positive for typical stem cells markers such as nestin, CD133, and Sox2. All these markers were reported to be overexpressed in gliomas as compared with normal brain (42) as well as in cancer stem cells (28). In particular, nestin was detected in most of the cultured cells from each GBM (up to 97%, in GBM 3), whereas CD133 was identified in a subset of the nestin-positive tumor cells. In agreement with previous studies on human glioma specimens (42), a certain variability in the expression of these markers was observed, with two TIC cultures that expressed an extremely high level of CD133 (GBM 4 and -5) as compared with all the others in which its content was much lower (1.6–5.2%), although not dissimilar to recent published studies (41). Previous reports suggested that increased expression of CD133 was directly related to a highly aggressive behavior of the tumor (36). Interestingly, in xenotransplant experiments we did not observe a faster tumor development injecting GBM cells in which a significant higher percentage CD133 expression was detected *in vitro* (*i.e.* GBM 4 and −5). It is likely that the number of cells injected in the CD133 less expressing cultures were already over a threshold of maximal capability to develop tumors in immunodeficient mice.

The characteristics of these cultures, showing tumor-specific phenotype differences, well match the accepted theory that tumors are composed of mixed populations of cells in which only a fraction retaining stem cells features can maintain and perpetuate the tumor (34, 43). Importantly, the *in vivo* tumorigenicity and the *in vitro* immunolabeling experiments, reported here, provide evidence that the culture conditions used allow the persistence of a proportion of nestin and CD133-expressing cells, sustaining the *in vitro* "self-renewal" of a subpopulation of cells possessing stem cell characteristics.

It has been shown that EGF plays an important role in the maintenance of normal and malignant cells of many tumor histotypes (including GBMs) promoting not only cell proliferation but also cell survival (44). Downstream to EGFR activation, three major intracellular signaling pathways are activated to elicit these effects as follows: the Ras-Raf-MEK-ERK cascade, the PI3K/Akt pathway, and the STAT3-dependent signaling events (44–50). It was recently reported that the spherogenic and self-renewal properties of cells isolated from three human GBMs were mainly dependent on EGFR activation and were sensitive to gefitinib treatment (41). In this study we used two selective inhibitors of EGFR tyrosine kinase, erlotinib and gefitinib to evaluate the role of EGFR in the maintenance of human brain TICs derived from a larger sample (seven tumors), with the aim to identify possible tumor-related specific responses and the molecular determinant of such effects.

We obtained a highly significant inhibition of TIC proliferation after treatment with both erlotinib and gefitinib in five of seven cultures that showed a concentration- and time-dependent growth arrest, with a slightly higher responsiveness observed after gefitinb treatment and the expected tumor-related individual different sensitivity. In addition to a direct EGFR signaling inhibition, it was proposed that in primary cultures of human GBM (51) or other tumor cell lines (52), it was possible to induce growth arrest through a G protein-coupled receptor-mediated activation of phosphotyrosine phosphatase (53). Thus, it will be important to demonstrate that a synergism between inhibition of tyrosine kinase and an activation of phosphotyrosine phosphatases may induce a more complete growth arrest also in TIC-enriched cell cultures.

As discussed before, TICs represent only a small percentage of tumor cells, and this feature is maintained *in vitro* even after long term cultures using stem cell permissive conditions (see above and Ref. 41). Thus, it is difficult to assess whether drug treatments really affect this tumorigenic subpopulation or exert their effects only on the bulk of differentiated tumor cells. However, it is important to point out the following: 1) it was shown that EGF signaling is the main mediator of the self-renewal

activity of these cells (41), thus the observed cell growth arrest very likely also involves the duplication of TICs; and 2) a significant inhibition of proliferation was also observed in two TIC cultures (derived from GBM 4 and −5) in which a persistent high level of $CD133^+$ cells (>80%) was observed, clearly indicating the sensitivity also of this subpopulation to the EGFR inhibitors.

Another important achievement of this study was the assessing of possible different responsiveness to drugs of individual GBM TICs cultures. In fact, previous studies analyzed only a very limited number of isolated tumor cells and were unable to identify GBM-specific responses.

In our study, using a larger sample, we report that only TICs derived from two patients (GBM 2 and −7) display a much less responsive profile to EGFR TKI. In particular, GBM 2 was totally unresponsive, and GBM 7 showed a slight inhibition of proliferation only at the highest concentrations of erlotinib and gefitinib and after prolonged treatment (at least 3 days). Thus, it was very relevant to identify possible molecular determinants for such unsatisfactory effects in this subset of tumors. In all responsive GBM TIC cultures, erlotinib and gefitinib caused the reversal of EGFR phosphorylation with the consequent inhibition of both ERK1/2 and Akt activation. However, in GBM 2, whose proliferation was completely unaffected by the tyrosine kinase inhibitors, unexpectedly EGFR and ERK1/2 phosphorylations induced by EGF were abolished in a way not dissimilar from that observed in the responsive cells. Conversely, Akt activation not only was not inhibited in the presence of either erlotinib or gefitinib, but the kinase was already maximally phosphorylated under basal conditions (after growth factor deprivation) without any further increase caused by the treatment with EGF. This constitutive activation pattern of Akt was likely related to the down-regulation of PTEN mRNA observed, in RT-PCR experiments, only in GBM 2 cells. In fact, the pharmacological inhibition of the PI3K/Akt pathway induced by wortmannin allowed the identification of an inhibitory effect of both gefitinib and erlotinib also in GBM 2 TIC cultures. Thus, the inhibition of Akt activation seems to be necessary for the antiproliferative effects of EGFR kinase inhibitors on GBM TIC-enriched cultures, as already shown for human GBM *in vivo* (54).

A more complex picture was observed in GBM 7 in which a different response to erlotinib and gefitinib was observed, and Akt was not constitutively phosphorylated. In fact, considering the response to erlotinib, the proliferation rate of these cells was affected only at the highest concentration used (5 μM), which was the only one able to abolish Akt phosphorylation, despite a significant inhibition of ERK1/2 at all the concentrations tested. No apparent alterations in PTEN mRNA expression levels were detected in these cells, although we cannot exclude the occurrence of inactivation mutations in the PTEN gene that will require further analysis. Thus, as far as the response to erlotinib is concerned, again the inhibition of Akt activity seems to be one of the main requirements for the antiproliferative effects of the drug.

On the other hand, in GBM 7 TIC cultures, the minimal response to gefitinib in terms of growth arrest, with a statistically significant inhibition only at the highest concentration of drug for at least 3 days, was not paralleled by a clear biochemical effect. Differently from what was observed with erlotinib, no reduction in the EGF-dependent phosphorylation/activation of EGFR, ERK1/2, and Akt was detected, even for high gefitinib concentrations and prolonged treatment. Thus, we cannot exclude that in this case a "nonspecific" toxic activity of the drug may occur after 3 days of exposure to high concentrations of gefitinib, as already reported in previous *in vitro* studies (29).

Although the different responsiveness to these drugs may be due to different causes yet undetermined, our results underline the possible unpredictable differential responses occurring in human tumors treated with drugs apparently acting with the same molecular mechanism (*i.e.* EGFR TKI). In conclusion, we report that human TIC-enriched cultures isolated from seven GBMs undergo growth arrest in the presence of inhibitors of EGFR kinase activity and that this effect seems to be related to their ability to inhibit the PI3K/Akt pathway.

Acknowledgments—We are grateful to Astra-Zeneca (Milano, Italy) and Hoffmann-La Roche for kindly providing us with gefitinib and erlotinib, respectively. We thank Dr. Rosaria Maria Rita Gangemi for helpful discussion and comments on the manuscript.

REFERENCES

1. Dalerba, P., Cho, R. W., and Clarke, M. F. (2007) *Annu. Rev. Med.* **58,** 267–284
2. Cho, R. W., and Clarke, M. F. (2008) *Curr. Opin. Genet. Dev.* **18,** 48–53
3. Bonnet, D., and Dick, J. E. (1997) *Nat. Med.* **3,** 730–737
4. Clarke, M. F., Dick, J. E., Dirks, P. B., Eaves, C. J., Jamieson, C. H., Jones, D. L., Visvader, J., Weissman, I. L., and Wahl, G. M. (2006) *Cancer Res.* **66,** 9339–9344
5. Jordan, C. T., Guzman, M. L., and Noble, M. (2006) *N. Engl. J. Med.* **355,** 1253–1261
6. Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001) *Nature* **414,** 105–111
7. Reynolds, B. A., Tetzlaff, W., and Weiss, S. (1992) *J. Neurosci.* **12,** 4565–4574
8. Hemmati, H. D., Nakano, I., Lazareff, J. A., Masterman-Smith, M., Geschwind, D. H., Bronner-Fraser, M., and Kornblum, H. I. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 15178–15183
9. Lee, J., Kotliarova, S., Kotliarov, Y., Li, A., Su, Q., Donin, N. M., Pastorino, S., Purow, B. W., Christopher, N., Zhang, W., Park, J. K., and Fine, H. A. (2006) *Cancer Cell* **9,** 391–403
10. Singh, S. K., Clarke, I. D., Hide, T., and Dirks, P. B. (2004) *Oncogene* **23,** 7267–7273
11. Wen, P. Y., and Kesari, S. (2008) *N. Engl. J. Med.* **359,** 492–507
12. Louis, D. N., Ohgaki, H., Wiestler, O. D., Cavenee, W. K., Burger, P. C., Jouvet, A., Scheithauer, B. W., and Kleihues, P. (2007) *Acta Neuropathol.* **114,** 97–109
13. Preusser, M., Haberler, C., and Hainfellner, J. A. (2006) *Wien. Med. Wochenschr.* **156,** 332–337
14. Galli, R., Binda, E., Orfanelli, U., Cipelletti, B., Gritti, A., De Vitis, S., Fiocco, R., Foroni, C., Dimeco, F., and Vescovi, A. (2004) *Cancer Res.* **64,** 7011–7021
15. Beier, D., Hau, P., Proescholdt, M., Lohmeier, A., Wischhusen, J., Oefner, P. J., Aigner, L., Brawanski, A., Bogdahn, U., and Beier, C. P. (2007) *Cancer Res.* **67,** 4010–4015
16. Shmelkov, S. V., St. Clair, R., Lyden, D., and Rafii, S. (2005) *Int. J. Biochem. Cell Biol.* **37,** 715–719
17. Doetsch, F., Petreanu, L., Caille, I., Garcia-Verdugo, J. M., and Alvarez-Buylla, A. (2002) *Neuron* **36,** 1021–1034
18. Gritti, A., Frolichsthal-Schoeller, P., Galli, R., Parati, E. A., Cova, L., Pagano, S. F., Bjornson, C. R., and Vescovi, A. L. (1999) *J. Neurosci.* **19,**

3287–3297
19. Palmer, T. D., Markakis, E. A., Willhoite, A. R., Safar, F., and Gage, F. H. (1999) *J. Neurosci.* **19,** 8487–8497
20. Dai, C., Celestino, J. C., Okada, Y., Louis, D. N., Fuller, G. N., and Holland, E. C. (2001) *Genes Dev.* **15,** 1913–1925
21. van der Valk, P., Lindeman, J., and Kamphorst, W. (1997) *Ann. Oncol.* **8,** 1023–1029
22. Zhou, Y. H., Tan, F., Hess, K. R., and Yung, W. K. (2003) *Clin. Cancer Res.* **9,** 3369–3375
23. Bao, S., Wu, Q., McLendon, R. E., Hao, Y., Shi, Q., Hjelmeland, A. B., Dewhirst, M. W., Bigner, D. D., and Rich, J. N. (2006) *Nature* **444,** 756–760
24. Diehn, M., and Clarke, M. F. (2006) *J. Natl. Cancer Inst.* **98,** 1755–1757
25. Hirschmann-Jax, C., Foster, A. E., Wulf, G. G., Nuchtern, J. G., Jax, T. W., Gobel, U., Goodell, M. A., and Brenner, M. K. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 14228–14233
26. Liu, G., Yuan, X., Zeng, Z., Tunici, P., Ng, H., Abdulkadir, I. R., Lu, L., Irvin, D., Black, K. L., and Yu, J. S. (2006) *Mol. Cancer* **5,** 67
27. Zhou, S., Schuetz, J. D., Bunting, K. D., Colapietro, A. M., Sampath, J., Morris, J. J., Lagutina, I., Grosveld, G. C., Osawa, M., Nakauchi, H., and Sorrentino, B. P. (2001) *Nat. Med.* **7,** 1028–1034
28. Gangemi, R. M., Griffero, F., Marubbi, D., Perera, M., Capra, M. C., Malatesta, P., Ravetti, G. L., Zona, G. L., Daga, A., and Corte, G. (2008) *Stem Cells* **23,** 23
29. Pattarozzi, A., Gatti, M., Barbieri, F., Wurth, R., Porcile, C., Lunardi, G., Ratto, A., Favoni, R., Bajetto, A., Ferrari, A., and Florio, T. (2008) *Mol. Pharmacol.* **73,** 191–202
30. Gangemi, R. M., Daga, A., Muzio, L., Marubbi, D., Cocozza, S., Perera, M., Verardo, S., Bordo, D., Griffero, F., Capra, M. C., Mallamaci, A., and Corte, G. (2006) *Eur. J. Neurosci.* **23,** 325–334
31. Arena, S., Barbieri, F., Thellung, S., Pirani, P., Corsaro, A., Villa, V., Dadati, P., Dorcaratto, A., Lapertosa, G., Ravetti, J. L., Spaziante, R., Schettini, G., and Florio, T. (2004) *J. Neurooncol.* **66,** 155–166
32. Florio, T., Casagrande, S., Diana, F., Bajetto, A., Porcile, C., Zona, G., Thellung, S., Arena, S., Pattarozzi, A., Corsaro, A., Spaziante, R., Robello, M., and Schettini, G. (2006) *Mol. Pharmacol.* **69,** 539–546
33. Bajetto, A., Barbieri, F., Pattarozzi, A., Dorcaratto, A., Porcile, C., Ravetti, J. L., Zona, G., Spaziante, R., Schettini, G., and Florio, T. (2007) *Neuro. Oncol.* **9,** 3–11
34. Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., and Dirks, P. B. (2003) *Cancer Res.* **63,** 5821–5828
35. Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., Henkelman, R. M., Cusimano, M. D., and Dirks, P. B. (2004) *Nature* **432,** 396–401
36. Zeppernick, F., Ahmadi, R., Campos, B., Dictus, C., Helmke, B. M., Becker, N., Lichter, P., Unterberg, A., Radlwimmer, B., and Herold-Mende, C. C. (2008) *Clin. Cancer Res.* **14,** 123–129
37. Pedersen, M. W., Meltorn, M., Damstrup, L., and Poulsen, H. S. (2001) *Ann. Oncol.* **12,** 745–760
38. Knobbe, C. B., Merlo, A., and Reifenberger, G. (2002) *Neuro. Oncol.* **4,** 196–211
39. Castellino, R. C., and Durden, D. L. (2007) *Nat. Clin. Pract. Neurol.* **3,** 682–693
40. Dean, M., Fojo, T., and Bates, S. (2005) *Nat. Rev. Cancer* **5,** 275–284
41. Soeda, A., Inagaki, A., Oka, N., Ikegame, Y., Aoki, H., Yoshimura, S., Nakashima, S., Kunisada, T., and Iwama, T. (2008) *J. Biol. Chem.* **283,** 10958–10966
42. Ma, Y. H., Mentlein, R., Knerlich, F., Kruse, M. L., Mehdorn, H. M., and Held-Feindt, J. (2008) *J. Neurooncol.* **86,** 31–45
43. Vermeulen, L., Sprick, M. R., Kemper, K., Stassi, G., and Medema, J. P. (2008) *Cell Death Differ.* **15,** 947–958
44. Kari, C., Chan, T. O., Rocha de Quadros, M., and Rodeck, U. (2003) *Cancer Res.* **63,** 1–5
45. Dowlati, A., Nethery, D., and Kern, J. A. (2004) *Mol. Cancer Ther.* **3,** 459–463
46. Fukazawa, H., Noguchi, K., Murakami, Y., and Uehara, Y. (2002) *Mol. Cancer Ther.* **1,** 303–309
47. Gibson, S., Tu, S., Oyer, R., Anderson, S. M., and Johnson, G. L. (1999) *J. Biol. Chem.* **274,** 17612–17618
48. Florio, T., Barbieri, F., Spaziante, R., Zona, G., Hofland, L. J., van Koetsveld, P. M., Feelders, R. A., Stalla, G. K., Theodoropoulou, M., Culler, M. D., Dong, J., Taylor, J. E., Moreau, J. P., Saveanu, A., Gunz, G., Dufour, H., and Jaquet, P. (2008) *Endocr.-Relat. Cancer* **15,** 583–596
49. Ono, M., Hirata, A., Kometani, T., Miyagawa, M., Ueda, S., Kinoshita, H., Fujii, T., and Kuwano, M. (2004) *Mol. Cancer Ther.* **3,** 465–472
50. Woodburn, J. R. (1999) *Pharmacol. Ther.* **82,** 241–250
51. Massa, A., Barbieri, F., Aiello, C., Arena, S., Pattarozzi, A., Pirani, P., Corsaro, A., Iuliano, R., Fusco, A., Zona, G., Spaziante, R., Florio, T., and Schettini, G. (2004) *J. Biol. Chem.* **279,** 29004–29012
52. Pan, M. G., Florio, T., and Stork, P. J. (1992) *Science* **256,** 1215–1217
53. Florio, T. (2008) *Mol. Cell. Endocrinol.* **286,** 40–48
54. Mellinghoff, I. K., Wang, M. Y., Vivanco, I., Haas-Kogan, D. A., Zhu, S., Dia, E. Q., Lu, K. V., Yoshimoto, K., Huang, J. H., Chute, D. J., Riggs, B. L., Horvath, S., Liau, L. M., Cavenee, W. K., Rao, P. N., Beroukhim, R., Peck, T. C., Lee, J. C., Sellers, W. R., Stokoe, D., Prados, M., Cloughesy, T. F., Sawyers, C. L., and Mischel, P. S. (2005) *N. Engl. J. Med.* **353,** 2012–2024