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The EphA2 Receptor Drives Self-Renewal and Tumorigenicity in Stem-like Tumor-Propagating Cells from Human Glioblastomas

Elena Binda,[1,*] Alberto Visioli,[1] Fabrizio Giani,[1] Giuseppe Lamorte,[2] Massimiliano Copetti,[3] Ken L. Pitter,[4] Jason T. Huse,[5] Laura Cajola,[6] Nadia Zanetti,[6] Francesco DiMeco,[7,8] Lidia De Filippis,[1] Annunziato Mangiola,[9] Giulio Maira,[9] Carmelo Anile,[9] Pasquale De Bonis,[9] Brent A. Reynolds,[10] Elena B. Pasquale,[11] and Angelo L. Vescovi[1,3,6,*]
[1]Department of Biotechnology and Biosciences, University of Milan Bicocca, 20126 Milan, Italy
[2]IRCSS Casa Sollievo della Sofferenza, c/o Istituto Mendel, 00198 Rome, Italy
[3]IRCSS Casa Sollievo della Sofferenza, Opera di San Pio da Pietralcina, 71013 S. Giovanni Rotondo (FG), Italy
[4]Weill Cornell Graduate School of Medical Sciences, New York, NY 10065, USA
[5]Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
[6]StemGen SpA, 20126 Milan, Italy
[7]National Neurological Institute "C. Besta", 20133 Milan, Italy
[8]Department of Neurosurgery, John Hopkins University, Baltimore, MD 21287, USA
[9]Institute of Neurosurgery, Catholic University of the Sacred Heart, 00168 Rome, Italy
[10]Evelyn F. and William L. McKnight Brain Institute, Program in Stem Cell Biology and Regenerative Medicine, The University of Florida, Ganesville, FL 32610-0015, USA
[11]Sanford-Burnham Medical Research Institute, La Jolla, CA 92037, USA
*Correspondence: elena.binda@unimib.it (E.B.), vescovi@tin.it (A.L.V.)
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SUMMARY

In human glioblastomas (hGBMs), tumor-propagating cells with stem-like characteristics (TPCs) represent a key therapeutic target. We found that the EphA2 receptor tyrosine kinase is overexpressed in hGBM TPCs. Cytofluorimetric sorting into EphA2^{High} and EphA2^{Low} populations demonstrated that EphA2 expression correlates with the size and tumor-propagating ability of the TPC pool in hGBMs. Both ephrinA1-Fc, which caused EphA2 downregulation in TPCs, and siRNA-mediated knockdown of *EPHA2* expression suppressed TPCs self-renewal ex vivo and intracranial tumorigenicity, pointing to EphA2 downregulation as a causal event in the loss of TPCs tumorigenicity. Infusion of ephrinA1-Fc into intracranial xenografts elicited strong tumor-suppressing effects, suggestive of therapeutic applications.

INTRODUCTION

Human glioblastoma multiforme (hGBM) is the most malignant among gliomas. Because of their very heterogeneous cellular, genetic, epigenetic, and molecular make-up (Maher et al., 2001), hGBMs have a dismal prognosis and almost inevitably recur (Krex et al., 2007; Chen et al., 2012).

Recent findings have demonstrated the existence of a subpopulation of hGBM cells, called cancer stem cells, whose idiosyncratic properties make them resilient to standard therapies. First identified in acute myeloid leukemia (Bonnet and Dick, 1997), cancer stem cells, better defined as tumor-propagating cells (TPCs; Kelly et al., 2007), have been isolated from a variety of solid tumors (Ponti et al., 2005; Ricci-Vitiani et al., 2009; Buzzeo et al., 2007). TPCs with both stem cell characteristics and tumor-initiation and propagation ability have now emerged as key players in hGBM pathogenesis (Hadjipanayis and Van Meir, 2009; Galli et al., 2004).

Significance

Identification and characterization of key regulatory mechanisms in TPCs are crucial for the development of specific therapies for hGBMs. We show that TPCs overexpress EphA2 in hGBMs, which underpins their inherent ability to maintain an undifferentiated state, supporting their self-renewal and tumorigenicity. EphA2 abundance provides a measure of the stem-like potential and tumor-propagating ability of TPCs from hGBMs. Thus, high EphA2 levels can be used to enrich TPCs by cell sorting. EphrinA1-Fc ligand-induced downregulation or siRNA-mediated knockdown of *EPHA2* expression both cause loss of self-renewal as well as induce differentiation and loss of tumor-initiating capacity in hGBM TPCs. Sustained intracranial infusion of ephrinA1-Fc under settings that resemble putative therapeutic conditions elicits effective antitumorigenic activity.

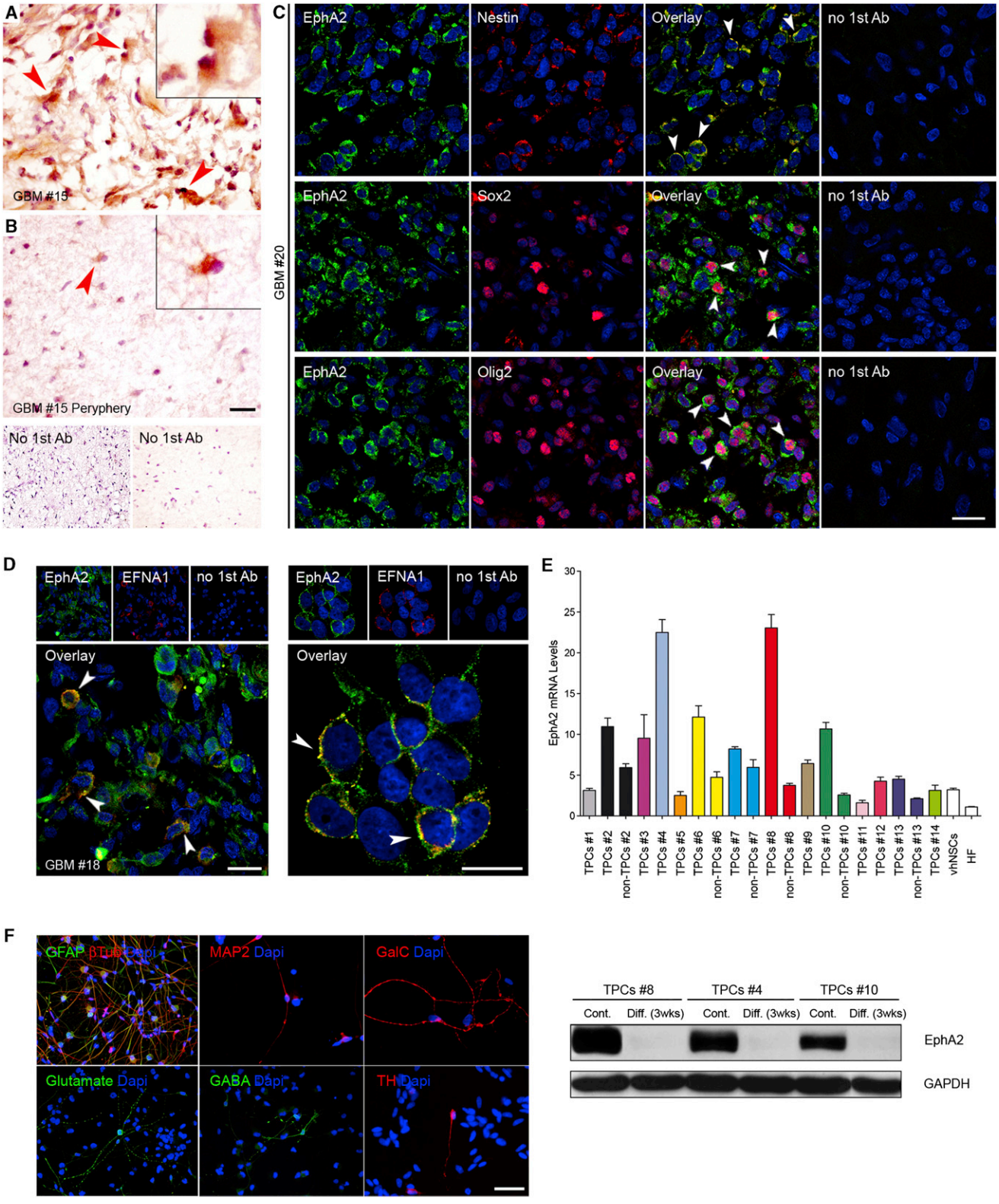


Figure 1. The EphA2 Receptor in Human Glioblastoma Tissues and TPCs

(A and B) Example of strong and frequent EphA2 immunoreactivity (brown) in the tumor core (A, arrows) as compared to infrequent, weaker labeling in the periphery (B) of the same hGBM specimen (six hGBMs yielded similar results). Insets: higher magnification. Bottom: no primary antibody. Blue, hematoxylin counterstain. Scale bar, 20 μm. See also Figures S1A–S1C.

While the origin and nature of hGBM TPCs remain to be unraveled, alterations of G1 arrest regulatory pathways in Nestin- or GFAP-positive cells can cause the onset of high-grade gliomas (Alcantara Llaguno et al., 2011). Thus, hGBM TPCs might derive from the transformation of neural stem cells or their transit amplifying precursor progeny (Alcantara Llaguno et al., 2009; Vescovi et al., 2006). Oligodendroglial precursors might also be the cell of origin in some hGBMs (Sukhdeo et al., 2011). The peculiar characteristics of TPCs encompass a relative quiescent nature, unlimited self-renewal, the clonal capacity to found a tumor (Galli et al., 2004), and resistance to conventional and multimodal treatments (Bao et al., 2006).

Owing to their stem-like nature, TPCs might be regulated by the same cues that control the activity of normal neural precursors and stem cells (NSCs). In fact, pathways that impinge on self-renewal and cell fate in normal NSCs are also active in brain tumors (Alcantara Llaguno et al., 2011; Dell'Albani, 2008). Also, therapeutic agents targeting Wnt, Hedgehog, or Notch deplete the TPC population in hGBMs (Takebe et al., 2011), and tumor suppressor genes can regulate TPC self-renewal (Zheng et al., 2008). The connection of TPCs with NSCs is reinforced by the discovery that critical effectors of NSC activity in the brain stem cell niche, such as bone morphogenetic proteins, suppress the growth of hGBM TPCs, enforcing their differentiation into astroglia (Lee et al., 2008; Piccirillo et al., 2006; Zhang et al., 2006). Another key regulator in the adult NSC niche, nitric oxide, can also drive TPC proliferation in hGBMs (Eyler et al., 2011).

Eph receptor tyrosine kinases and their ephrin ligands influence central nervous system development, stem cell niches, and cancer cells (Goldshmit et al., 2006; Genander and Frisén, 2010; Pasquale, 2010). Deregulation of the Eph receptor/ephrin system is associated with acquisition of tumorigenic properties, tumor growth, angiogenesis, and metastasis in human cancers. In particular, EphA2 receptor is overexpressed in many human epithelial malignancies and hGBMs, where it can promote proliferation and invasiveness through mechanisms that are not well understood and may be independent of ephrin ligand binding (Wykosky and Debinski, 2008; Miao et al., 2009; Pasquale, 2010; Gopal et al., 2011; Nakada et al., 2011; Miao and Wang, 2012). High EphA2 expression also correlates with tumor stage, progression, and patient survival (Wykosky et al., 2005; Liu et al., 2006; Wang et al., 2008; Miao et al., 2009; Li et al., 2010; Wu et al., 2011).

In this study, we investigated the putative regulatory role and function of the EphA2 receptor in TPCs from hGBMs.

RESULTS

High EphA2 Expression in hGBMs and Their TPCs

Analysis of hGBM surgery specimens showed high EphA2 mRNA expression as compared to other EphA and EphB receptors (Figure S1A available online). Real-time PCR (qPCR) revealed how EphA2 mRNA levels were up to 100-fold higher in hGBMs than in normal human brain tissue, as compared to a 10-fold upregulation in low-grade gliomas, epitheliomas, and primitive neuroectodermal tumors (Figure S1B).

Strong EphA2 immunoreactivity was found in many cells of the non-necrotic hGBM core (Figure 1A) versus a few positive cells in the tumor periphery (Figure 1B) and normal brain (Figure S1C). Accordingly, immunolabeling of hGBM tissues showed coexpression of EphA2 and antigens of normal and transformed neural precursors, namely Nestin, Sox2, and Olig2 (Fatoo et al., 2011; Ligon et al., 2007) (Figure 1C). In contrast, the signal for ephrinA1, an EphA2 preferred ligand (Wykosky et al., 2008; Miao et al., 2009), was variable in intensity and distribution in the hGBM core and undetectable in the periphery (Figure S1C).

We also found high EphA2 mRNA and protein levels in cells acutely dissociated from hGBMs or cultured as neurospheres and enriched for the putative TPC markers SSEA-1 or CD44 (Figures S1D–S1F). Analysis of hGBM TPCs confirmed this overexpression (Figure 1E), which was from 2- to 300-fold that of their original hGBM tissue (Figure S1G). EphA2 and ephrinA1 were detected in both hGBMs and their TPCs (Figure 1D). Notably, EphA2 was heavily downregulated when TPCs were differentiated, losing stemness and tumorigenicity (Piccirillo et al., 2006; Zhang et al., 2006) (Figure 1F), reinforcing a correlation between high EphA2 levels and the TPC state.

EphA2 mRNA levels in individual TPC neurosphere cultures also correlated with their specific growth kinetics (Figures S1H and S1I), i.e., with their self-renewal activity (Rietze and Reynolds, 2006), and with *EPHA2* gene copy number at the 1p36.12 locus ($p < 0.0001$) (Figures S1H and S1J).

Enriching for Cells with Enhanced Tumor-Propagating Ability Based on EphA2 Expression

To reinforce the correlation between EphA2 enhanced levels and the TPC state we FACS-sorted acutely isolated hGBM cells into two distinct pools, expressing either high ($EphA2^{High}$) or low ($EphA2^{Low}$) EphA2 levels, and then assessed their in vitro clonogenicity and intracranial tumorigenic capacity. As expected, the $EphA2^{High}$ fraction contained more clonogenic cells than the $EphA2^{Low}$ fraction (Figure 2A) and mice implanted with $EphA2^{High}$ cells showed a higher mortality (median survival 4 months) than those receiving $EphA2^{Low}$ cells (median survival 7 months) (Figure 2B). Limiting dilution intracranial transplantation using acutely dissociated $EphA2^{High}$ and $EphA2^{Low}$ cells (Figures S2A–S2C) confirmed that high EphA2 levels are a hallmark of TPCs and can be used for their enrichment. In addition, when primary hGBM cells were sorted based on their combined expression of EphA2 and the putative TPC antigen SSEA-1 (Figures 2C and 2D), intracranial tumorigenicity was significantly

(C) Immunolabeling of hGBM tissue shows that cells positive for the putative neural stem cell markers Nestin, Sox2, or Olig2 (red) coexpress (arrows) the EphA2 receptor (green; five hGBMs yielded similar results). Scale bar, 20 μm.
(D) Coexpression of EphA2 (green) and ephrinA1 (red) on the surface of cells in hGBM tissues (left) and TPC cultures (right). Arrows mark examples of coexpression (yellow). Scale bars, 10 μm.
(E) Higher EphA2 expression as detected by qPCR in cultured TPCs from the core (TPCs; n = 14 independent cultures) versus cells from the periphery of the same hGBM (non-TPCs; n = 6 independent cultures; Student's t test, p = 0.017). Controls: vhNSCs and human fibroblasts (HF). Error bars: SEM. See also Figures S1D–S1J.
(F) TPCs differentiated by mitogen starvation show upregulation of astroglial (GFAP), oligodendroglial (GalC), and neuronal (β-Tub, MAP2, tyrosine hydroxylase [TH], glutamate, and GABA) markers (left), while losing EphA2 expression (right). Scale bar, 20 μm

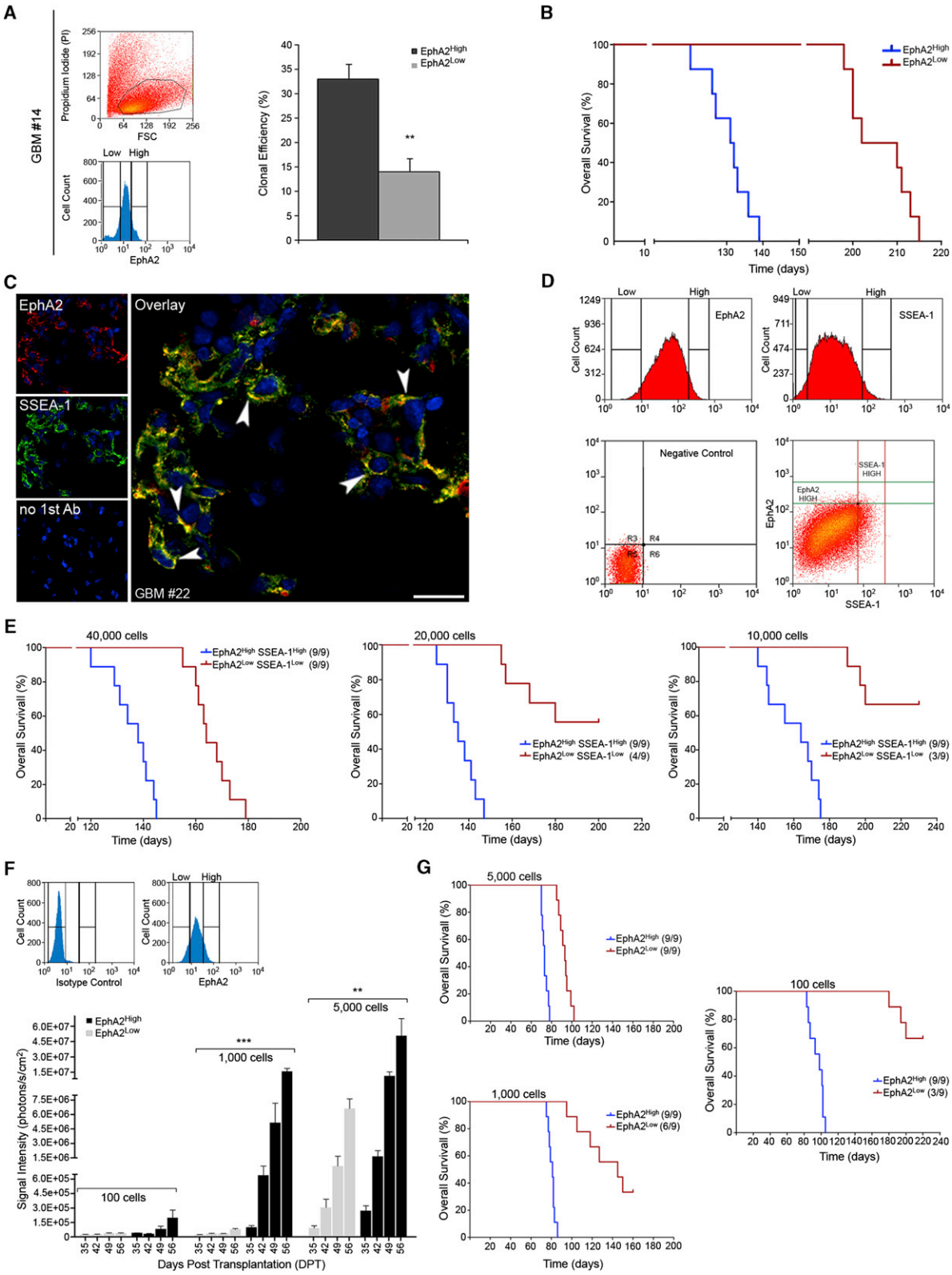


Figure 2. Enrichment of the Stem-like Tumorigenic Pool Based on EphA2 Levels

(A) Viable (propidium iodide negative) tumor cells acutely isolated from hGBM specimens (top left) were sorted into EphA2^{High} and EphA2^{Low} fractions (bottom left). The EphA2^{High} fraction displayed higher clonogenic index than the EphA2^{Low} fraction (right) (n = 4 tumors). Error bars: SEM; **p = 0.0004 for EphA2^{High} versus EphA2^{Low} by Student's t test.

higher in EphA2^{High} SSEA-1^{High} than in EphA2^{Low} SSEA-1^{Low} cells (Figure 2E). Similar results were obtained with EphA2^{High} CD44^{High} and EphA2^{Low} CD44^{Low} cells (Figures S2D–S2F). By applying the extreme limiting dilution analysis approach, we were able to show that the frequency of tumor-initiating cells (TICs) was always significantly higher in cell fractions with an increased EphA2 expression, alone or in combination with SSEA-1 or CD44 (Figure S2G). Finally, when EphA2^{High} and EphA2^{Low} cultured TPCs were assayed for intracranial tumorigenicity in a limiting dilution assay, as few as 100 EphA2^{High} cells established large gliomas in 60 days, as compared to a minimum requirement of 5,000 cells when using EphA2^{Low}, below which level tumorigenicity was negligible, even after 7 months (Figures 2F and 2G).

EphrinA1-Fc Downregulates EphA2 and Inhibits TPC Growth and Expression of Neural Stem Markers

Due to the emerging correlation between high EphA2 expression and TPC state, we used ephrinA1-Fc (a soluble ephrinA1 dimer fused to Fc) to downregulate EphA2 (Wykosky et al., 2005; Liu et al., 2007) and examined its effects on hGBM TPCs. A dose-dependent EphA2 downregulation ensued, which peaked at ephrinA1-Fc concentrations of 1–5 μg/ml, producing up to 90% receptor depletion (Figure S3A). EphrinA1-Fc induced a negligible EphA2 downregulation both in cells derived from the hGBM periphery (non-TPCs)—bearing stem-like features but negligible tumorigenicity (Piccirillo et al., 2009)—and v-myc-immortalized, non-transformed hNSCs (vhNSCs; De Filippis et al., 2007) (Figure 3A). Immunofluorescence assays confirmed EphA2 loss in both acutely isolated and cultured TPCs upon ephrinA1-Fc treatment (Figure 3B).

TPCs acutely isolated from hGBM specimens adhered to the dish when treated with ephrinA1-Fc, losing their capacity to grow and to generate stable TPC lines in the neurosphere assay (Figures 3C, 3D, and 3G), an effect also seen in 14 established TPC cultures (Figures 3E–3G, S3B, and S3C). In contrast, ephrinA1-Fc had negligible effects on non-TPCs (Figure 3H) and vhNSCs (Figure 3I). Hence, ephrinA1-Fc dowregulates EphA2 and hinders the expansive growth of cells that possess both stem cell characteristics and tumor-propagating ability.

EphrinA1-Fc Depletes the TPC Pool, Inhibits Self-Renewal, and Induces Astroglial Differentiation

The effects of ephrinA1-Fc suggested that it might inhibit the expansion of the TPC pool. This was emphasized by the ability of ephrinA1-Fc to downregulate both EphA2 and the neural precursor markers Nestin, Sox2, or Olig2 (Figure 3J) that are coexpressed in TPC spheroids. Also, FACS analysis showed loss of the putative TPC markers SSEA-1, CD44 and CD133 (Dell'Albani, 2008; Fatoo et al., 2011) (Figure S3D), but not of BMI-1 (Fatoo et al., 2011). Finally, ephrinA1-Fc depleted the TPC side population (SP), thought to comprise stem-like cells (Fukaya et al., 2010) (Figure S3E).

To confirm depletion of the TPC stem-like pool by ephrinA1-Fc, we measured self-renewal ability in a clonogenic assay, whereby single cells from acutely dissociated or from established TPC lines were plated in single wells by automated FACS and grown as neurospheres. EphrinA1-Fc drastically decreased clonogenicity in TPCs but not in non-TPCs, slightly increasing vhNSC clonogenicity (Figure 4A). Loss of self-renewal was not caused by changes in cell cycling (Figure 4B) or viability (Figure 4C). Notably, ephrinA1-Fc induced a marked, time-dependent upregulation of the astroglial antigen GFAP, without affecting the neuronal βIII tubulin and oligodendroglial GalC markers (Figure 4D). Thus, ephrinA1-Fc hinders self-renewal in TPCs in a non-cytotoxic way and increases astroglial differentiation.

Molecular Regulation of TPCs Stemness by EphA2

We studied the molecular events underlying the changes induced in hGBM TPCs by ephrinA1-Fc. EphrinA1-Fc transiently increased EphA2 tyrosine phosphorylation, in a dose-dependent manner, causing a strong and persistent downregulation of EphA2 expression (Figure 4E). We also observed marked ERK phosphorylation, which returned to basal levels after 24 hr, a moderate increases in Akt and FAK phosphorylation (Figure 4E, bottom panels), and reorganization of the actin cytoskeleton (Figures 4F–4J).

Altogether, this suggested that EphA2 expression sustains TPC self-renewal and that receptor loss played a causal role in the depletion of the hGBM stem-like pool. To prove this, we used a mixture of siRNAs to assess the effects of direct EphA2 downregulation (Figure 5A). *EPHA2* silencing caused changes consistent with loss of stemness and increased differentiation, ie, (1) loss of clonogenicity (Figure 5B) and amplification rate (Figure 5C), (2) downregulation of putative stem cell markers (Figure 5E), and (3) increased GFAP expression (Figure 5F). These changes were quite similar to those induced by ephrinA1-Fc (Figures 3G, 3J, 4A, and 4D). Per se, EphA2 downregulation activated ERK in TPCs, as shown by a prominent increase in its phosphorylation (Figures 5F and 5G). Feeble Akt and FAK activation were detected. Furthermore, when EphA2 levels began to

(B) Intracranial transplantation of 6 x 10^4 EphA2^{High} or EphA2^{Low} cells confirmed the much higher tumor-propagating capacity of the former (MC test, log-rank $p < 0.0001$ for EphA2^{High} versus EphA2^{Low}; n = 8).

(C) Confocal images show widespread colocalization (arrowheads; yellow) of EphA2 (red) and SSEA-1 (green) in hGBM tissue. Scale bar, 20 μm.

(D) Cells from the same hGBM were sorted and gated according to EphA2 and SSEA-1 levels.

(E) Kaplan-Meier survival curves show that mice receiving intracranially 2 × 10^4 and 1 × 10^4 EphA2^{High} SSEA-1^{High} purified TPCs die earlier (median survival: 135 and 164 days, respectively) than mice receiving EphA2^{Low} SSEA-1^{Low} cells (56% and 67% survival at 200 days, respectively). MC and GBW tests, log-rank $p < 0.0001$ EphA2^{High} SSEA-1^{High} versus EphA2^{Low} SSEA-1^{Low}; n = 9). Survival was also shorter when implanting 4 × 10^4 EphA2^{High} SSEA-1^{High} as compared to EphA2^{Low} SSEA-1^{Low} TPCs. See also Figure S2.

(F) Limiting dilution intracranial transplant of cultured, luciferase-tagged TPCs sorted into EphA2^{High} and EphA2^{Low} pools (top). Light emission imaging analysis (bottom; 5,000, 1,000, and 100 cells per mouse) shows a higher tumor-initiating ability of EphA2^{High} versus EphA2^{Low} TPCs. Error bars, SEM; ***$p < 0.0001$, **$p = 0.002$, EphA2^{High} versus EphA2^{Low}.

(G) Kaplan-Meier analysis shows that mice receiving EphA2^{High} TPCs die earlier than mice receiving EphA2^{Low} cells (MC and GBW tests, log-rank $p < 0.0001$ EphA2^{High} versus EphA2^{Low}; n = 9).

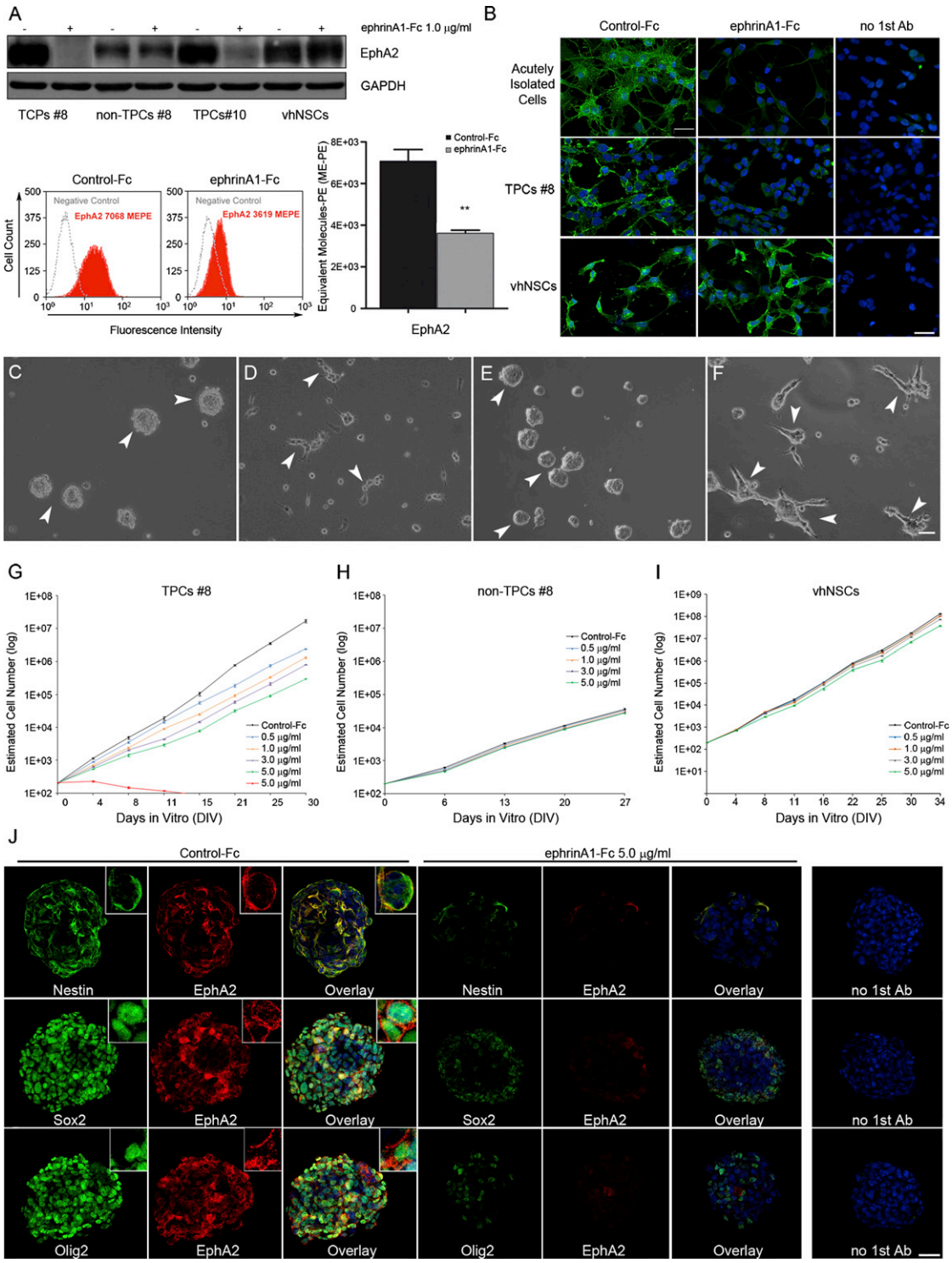


Figure 3. EphA2 Downregulation by EphrinA1-Fc Inhibits In Vitro Proliferation and Depletes the Stem Cell-like Pool in hGBM TPCs

(A) (Top) Cells were treated with ephrinA1-Fc for 24 hr. EphA2 was heavily downregulated in TPCs but not vhNSCs and non-TPCs. (Bottom) FACS analysis and flow cytometry quantitative data showing EphA2 downregulation in TPCs based on equivalent molecules of phycoerythrin (ME-PE) (n = 10 independent cultures); error bars, SEM; **p < 0.005 by Student's t test. See also Figure S3A.

(B) EphrinA1-Fc (5 μg/ml for 24 hr) downregulates EphA2 expression in TPCs acutely isolated from patients or from neurospheres (TPCs no. 8 shown as an example), but not in vhNSCs. Scale bar, 20 μm.

(C–F) EphrinA1-Fc (5 μg/ml for 48 hr) triggers obvious morphologic changes (arrowheads) in acutely isolated TPCs (C, control; D, treated) or serially subcultured TPC neurospheres (E, control; F, treated), promoting cell adhesion. Scale bar, 50 μm.

normalize, due to the elapsing effect of siRNAs transfection (Figure 5A, arrow), TPCs growth and ERK phosphorylation also began to normalize (Figures 5C, arrow, and 5F). siRNA-mediated knockdown of *EPHA2* expression in vhNSCs also inhibited growth (Figure 5D), in contrast to ephrinA1-Fc treatment, which neither inhibited growth nor downregulated EphA2 in these cells (Figures 3A, 3B, and 3I). Transduction of TPCs with individual *EPHA2* siRNA, and rescue with a siRNA-resistant *EPHA2* construct, confirmed these effects as the result of the EphA2 mRNA knockdown (Figure 5G). Importantly, inhibition of ERK activation partially rescued loss in clonogenicity as induced by *EPHA2* siRNA, implicating ERK activation in the loss of TPC stemness, as caused by EphA2 downregulation (Figure 5H).

Notably, EphA2 phosphorylation on Serine897 (Ser897) promotes oncogenic activities of the receptor that do not depend on its interaction with ephrin ligands (Miao et al., 2009). We detected a strong signal for Ser897-phosphorylated EphA2 in the hGBM core and in TPCs but not in the hGBM periphery, non-TPCs or vhNSCs (Figure 6A). Furthermore, treatment with high doses of soluble monomeric EphA2 extracellular domain that block endogenous EphA2-ephrin interaction did not affect GFAP levels or ERK activity in TPCs (Figure 6B). Thus, the effects of EphA2 in TPCs appear to be independent from activation by endogenous ephrinA ligands.

EphrinA1-Fc and EPHA2 Silencing by siRNAs Suppress TPC Tumorigenicity In Vivo

The main effect of ephrinA1-Fc treatment or siRNA-mediated *EPHA2* silencing is the depletion of the tumorigenic hGBM TPC pool, which ought to reduce their tumorigenic capacity in vivo. To examine the in vivo effects of ephrinA1-Fc, we used three experimental paradigms. hGBM TPCs were (1) treated with ephrinA1-Fc in culture prior to transplantation (pre-treatment), (2) treated starting immediately after transplantation (co-treatment), or (3) allowed to establish sizeable tumors before beginning treatment with ephrinA1-Fc (post-treatment). All three protocols were evaluated in a subcutaneous xenograft model. Furthermore, pre- and post-treatment protocols were also evaluated in an intracranial (orthotopic) xenograft model (Galli et al., 2004; Piccirillo et al., 2006).

When TPCs were injected subcutaneously, ephrinA1-Fc inhibited growth in all three protocols (Figure 7A). Similar results were obtained with the more complex, yet clinically more relevant, orthotopic model. In the intracranial hGBM model, tumor growth was greatly reduced in the pre-treatment settings, as shown by quantitative imaging of luciferase-tagged hGBM TPCs (luc-TPCs; Figures 7B left panels, 7C left, 7E, and 7F) and by the increase in overall survival (Figure 7C, right). Importantly, ephrinA1-Fc infusion into the brain for 14 days by means of osmotic mini-pumps also effectively suppressed the growth of well pre-established hGBMs (Figures 7B middle and right panels, 7D left, 7G, and 7H). Kaplan-Meier analysis revealed a median survival of 130 days for mice receiving ephrinA1-Fc versus 72 days for controls, infused with Fc (Figure 7D, right)—confirming the therapeutic efficacy of ephrinA1-Fc administration.

Immunohistochemistry confirmed that EphA2, which was high in TPC-derived, control intracranial hGBMs (Figure S4A), strongly downregulated upon ephrinA1-Fc infusion (Figure S4B). Control tumors contained numerous malignant cells (Figure S4C), whereas ephrinA1-Fc-treated ones contained few neoplastic cells and many more differentiated elements (Figure S4D). Accordingly, ephrinA1-Fc-treated tumors embodied a lower percentage of mitotic cells (Figures S4E and S4F) and were significantly less vascularized than Fc-treated controls (2-fold reduction in total vascular area, with less and smaller blood vessels; Figures S4G and S4H and data not shown).

To determine if decreasing EphA2 receptor levels was sufficient to inhibit hGBM tumorigenicity, we analyzed the effects of siRNAs-mediated *EPHA2* silencing on the in vivo tumor-propagating ability of luc-TPCs, in comparison with luc-TPCs treated with control-siRNAs or untreated ones. Knockdown of endogenous *EPHA2* suppressed the growth of TPC-derived intracranial tumors (Figure 7I, top) and substantially increased overall survival (Figure 7I, bottom).

Given the prominent involvement of EphA2 in the pathogenic mechanisms of hGBMs documented above, we analyzed the TCGA data set (Network, 2008) for relative EphA2 mRNA expression in hGBM subcategories (Verhaak et al., 2010). We found that the classical and mesenchymal subtypes had the highest EphA2 expression (Figure 8). In addition, when the mesenchymal or proneural subtypes were divided into two groups based on median EphA2 expression, high EphA2 expression trended with poor patient survival (mesenchymal: 10.9 versus 15.03 months, $p = 0.0415$ Mantel-Cox [MC], $p = 0.0215$ Gehan-Breselow-Wilcoxon [GBW]; proneural: 9.9 versus 16.73 months, $p = 0.2026$ MC test, $p = 0.0411$ GBW test). There was no significant correlation between EphA2 and patient survival in the neural and classical subtypes.

DISCUSSION

We describe a specific role for the EphA2 receptor in the pathogenesis of hGBMs. We report how enhanced EphA2 expression is a property of the TPCs in these cancers and show a causal relationship between high EphA2 expression and the capacity of these cells to expand their pool size and form hGBMs. Accordingly, high levels of EphA2 expression can be used to enrich for TPCs by FACS. Furthermore, treatment with a soluble form of the EphA2 ligand, ephrinA1-Fc, hinders the self-renewal ability of hGBM TPCs causing a drastic loss in their capacity to establish and propagate hGBM phenocopies subcutaneously or intracranially. We demonstrate that EphA2 receptor downregulation is a causal event in the suppression of the tumor-propagating

(G–I) Acutely isolated TPCs cannot establish stable TPC primary lines if exposed to ephrinA1-Fc (G, red line), which also inhibits steady growth in pre-established TPCs (G); $p < 0.0001$ versus Control-Fc. Negligible inhibition of ephrinA1-Fc was observed in non-TPCs (H) and vhNSCs (I) ($p < 0.005$ versus Control-Fc; TPCs no. 8 and non-TPCs no. 8 are shown as representative examples); error bars, SEM. See also Figures S3B and S3C.

(J) Coexpression of EphA2 (red) with the putative stem antigens Nestin, Sox2, or Olig2 (green) in TPC spheroids treated for 24 hr with Control-Fc or ephrinA1-Fc. EphrinA1-Fc nearly abolishes expression of both EphA2 and the stem antigens. Insets: higher magnification. Scale bar, 40 μm. See also Figures S3D and S3E.

Unless otherwise indicated, the data are representative of three independent experiments giving similar results.

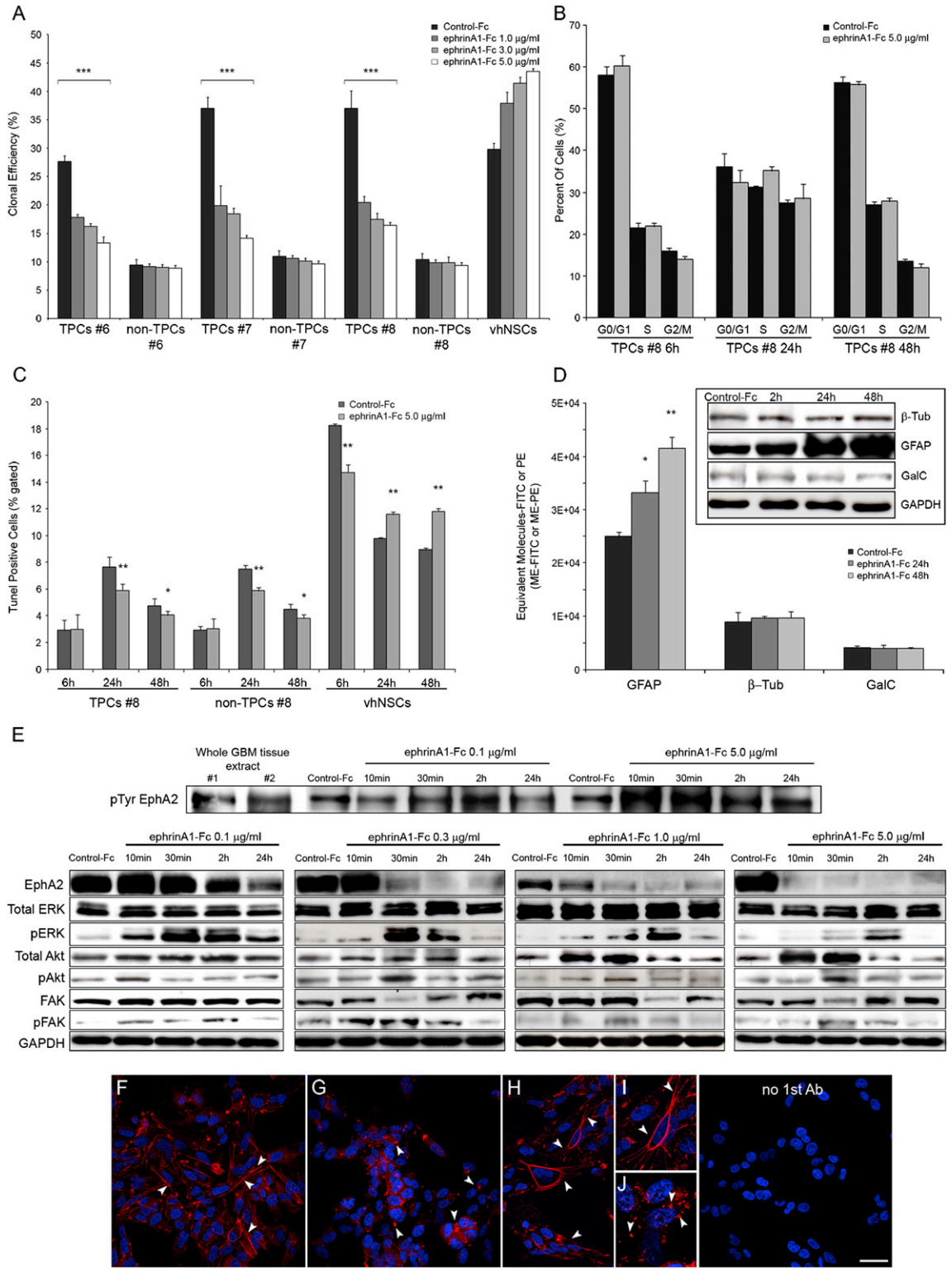


Figure 4. EphrinA1-Fc Inhibits TPC Self-Renewal by Inducing a Differentiated Phenotype

(A) Clonogenic assays show a dose-dependent inhibition of self-renewal by ephrinA1-Fc in TPCs but not in non-TPCs and vhNSCs; error bars, SEM; ***$p < 0.0001$ versus Control-Fc cells.

(B) Up to 5 μg/ml ephrinA1-Fc does not alter TPC cell cycling, as determined by FACS analysis of BrdU incorporation; error bars: SEM.

(C) Cytofluorimetric Tunel analysis shows no induction of apoptosis in TPCs or non-TPCs treated with ephrinA1-Fc; error bars: SEM; **$p < 0.005$, *$p < 0.05$ versus Control-Fc.

capacity of TPCs. Intracranial administration of ephrinA1-Fc causes EphA2 downregulation in hGBM orthotopic xenografts, significantly hindering their growth and expansion in the brain, even if these tumors are established ahead of ephrinA1-Fc administration.

Aberrant expression of Eph receptors has been reported in various cancers, including gliomas, and correlates with malignancy (Wykosky and Debinski, 2008; Pasquale, 2010; Nakada et al., 2011; Miao and Wang, 2012). In our survey of 12 hGBM specimens, we found EphA2 to be the most upregulated of the Eph receptors. This is consistent with the EphA2 overexpression previously reported in most high-grade human gliomas, which correlates with poor prognosis (Wykosky et al., 2005; Liu et al., 2006; Wang et al., 2008; Miao et al., 2009; Li et al., 2010; Wu et al., 2011), suggesting a role for EphA2 in the pathogenesis of hGBMs.

While studies with glioma cell lines have implicated EphA2 in cell growth and invasiveness (Wykosky et al., 2005; Liu et al., 2006, 2007; Miao et al., 2009; Li et al., 2010; Gopal et al., 2011), the identity and nature of the actual target cells in the patients' own GBM remain unclear. In addition, the cellular functions affected by EphA2, the regulatory mechanisms underpinning EphA2′s actions in hGBMs pathophysiology, and the possibility of manipulating this system to suppress glioma growth are not well characterized. hGBMs contain subpopulations of cells that act as stem-like TPCs, which have now been proven to be crucial therapeutic targets (Chen et al., 2012). We found that the EphA2 receptor is coexpressed with neural precursor markers in hGBMs, being concentrated in regions rich in TPCs. Furthermore, hGBM cell preparations enriched in TPCs display enhanced EphA2 levels which, conversely, are low in low-grade gliomas (Figure S1B) that contain few TPCs (Galli et al., 2004). Altogether, these findings suggest that high EphA2 levels are a distinguishing feature of hGBM TPCs.

Conclusive evidence to this idea came from fractionating TPCs into $EphA2^{Low}$ and $EphA2^{High}$ populations, also in combination with high levels of the putative TPC markers. The highest tumorigenic potential was associated with high EphA2 or combined, high EphA2 SSEA-1 or EphA2 CD44 expression. Thus, in vitro and in vivo limiting dilution experiments show that high EphA2 expression can be exploited to obtain preparations highly enriched in TPCs, which are capable of establishing intracranial hGBMs by injection of as few as 100 cells.

Overexpression of EphA2 in TPCs suggests a role for this receptor in hGBM pathogenesis. A first indication that EphA2 might drive the expansion of the TPC pool came from the significant correlation between the rate of self-renewal of multiple TPC preparations and their EphA2 mRNA levels and *EPHA2* gene copy number. This was reinforced by the fact that EphA2 expression decreases dramatically when hGBM TPCs lost their stemness upon differentiation.

To verify the role of EphA2 in TPC self-renewal and amplification, we first perturbed EphA2 by using the ephrinA1-Fc ligand (Noblitt et al., 2004; Lee et al., 2011; Khodayari et al., 2011). This treatment decreased the proliferation of both acutely isolated and of serially subcultured hGBM TPCs, was coupled to the failure of primary hGBM TPCs to generate stably expanding lines and caused both loss of expansion capacity and clonogenic ability in well-established TPC cultures.

The ephrinA1-Fc action was associated to decreased expression of putative hGBM TPC antigens (such as Olig2, Nestin, Sox2, CD133, CD44, and SSEA-1), occurred in the absence of detectable changes in cell cycle or apoptosis and produced increased astroglial differentiation and the depletion of the chemoresistant SP. Notably, these effects were specific to TPCs, since stem-like cells from the same tumors but lacking tumor-propagating ability (non-TPCs) and nontransformed neural stem cells (vhNSCs) were resilient to ephrinA1-Fc administration. Thus, in vitro exposure to ephrinA1-Fc leads to the depletion of those stem-like cancer cells that are responsible for hGBM initiation, growth, and recurrence after therapy.

EphrinA1-Fc caused prolonged EphA2 downregulation in TPCs, suggesting a critical role for this phenomenon in this ligand's tumor suppressor activities. Although ephrin Ligands trigger Eph receptor tyrosine phosphorylation and activation, thereby eliciting complex downstream signaling, they also cause Eph receptor downregulation (Wykosky et al., 2005; Liu et al., 2007; Pasquale, 2010). We observed only negligible inhibition of TPC clonogenicity (less than 10%; data not shown) at the lower ephrinA1-Fc concentrations tested, which induced strong intracellular signaling but limited EphA2 downregulation. Conversely, at higher ephrinA1-Fc concentrations, signaling was weaker, but EphA2 downregulation, inhibition of TPC clonogenicity and growth were much more evident. Indeed, while signaling was transient and returned to basal levels within 24 hr, the inhibition of TPC tumorigenicity and EphA2 downregulation persisted well beyond 24 hr. The importance of EphA2 downregulation was further supported by the observation that in vhNSCs ephrinA1-Fc failed to both induce EphA2 downregulation and inhibit cell growth and self-renewal. Furthermore, siRNA-mediated knockdown of *EPHA2* expression closely mimicked the effects of ephrinA1-Fc on TPC self-renewal, expansion, differentiation in vitro, and tumorigenic ability in vivo. Interestingly, *EPHA2* expression knockdown also inhibited the growth and self-renewal of the "ephrinA1-Fc-resilient" vhNSCs. Therefore, EphA2 downregulation alone can inhibit TPC tumor-propagating activity although it remains possible that ephrinA1-Fc-induced EphA2 signaling may also contribute

(D) Quantitative FACS analysis shows a time-dependent increase in equivalent molecules of fluorescein (ME-FITC) for astroglial GFAP but not neuronal βIII tubulin (β-Tub) or oligodendroglial GalC markers in ephrinA1-Fc-treated TPCs; error bars, SEM; *p = 0.0095, **p = 0.0005 versus Control-Fc. (Inset) Western blotting confirms a marked, time-dependent increase in GFAP under the same settings.

(E) (Top) Tyrosine phosphorylation of EphA2 immunoprecipitated from whole hGBM lysates and from TPCs treated with ephrinA1-Fc. (Bottom) Western blots for EphA2, ERK, Akt and FAK (Tyr397) expression and phosphorylation in TPCs treated with increasing ephrinA1-Fc concentrations over a 24 hr time course. Lower ephrinA1-Fc concentrations preferentially stimulate signaling, higher ones more rapidly downregulate the receptor.

(F–J) (F) TPCs spread on Cultrex show an organized actin cytoskeleton and F-actin assembled in stress fibers (arrows). Typical ring-like actin bundles are seen at higher magnification (H and I; arrowheads). (G and J) EphrinA1-Fc-treatment (5 μg/ml for 5 min) causes TPC elongation and actin concentration at cell-cell junctions (arrowhead). Scale bar, 20 μm.

Unless otherwise indicated, the data are representative of three independent experiments giving similar results.

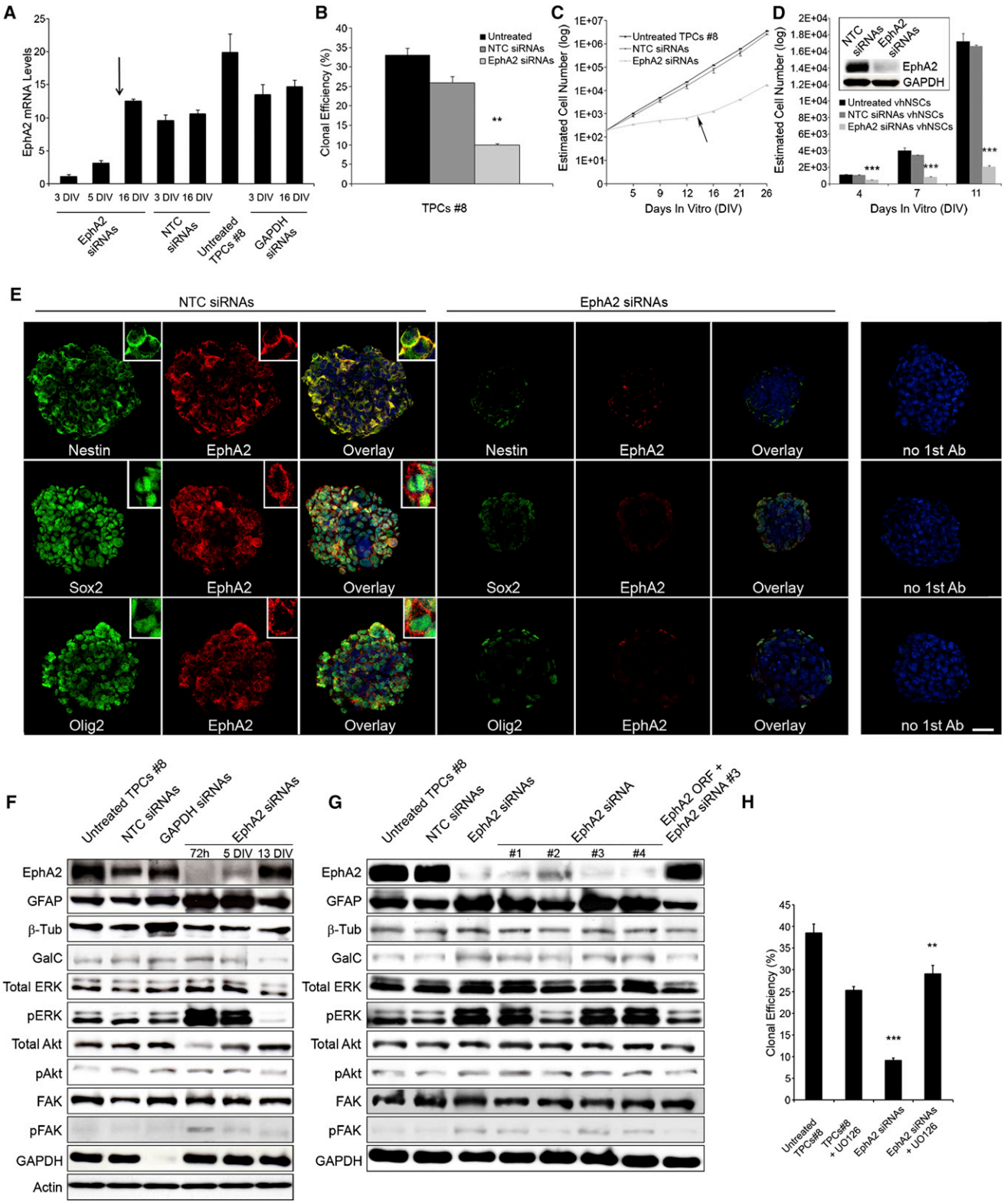


Figure 5. *EPHA2* siRNA Knockdown in hGBM TPCs Inhibits Self-Renewal and Increases Differentiation, Concomitant with ERK and Akt Activation

(A) TPCs were treated with an *EPHA2* siRNA pool, non-targeting control (NTC) or *GAPDH* control pool siRNAs. Three days post-transfection, only the *EPHA2* siRNAs caused a substantial decrease in EphA2 mRNA, as detected by qPCR. Between 12 and 16 days post-transfection (DIV), EphA2 levels began to normalize (arrow); error bars, SEM.

to the suppression of TPC tumorigenicity (Wykosky and Debinski, 2008; Nakada et al., 2011; Miao and Wang, 2012). Thus, EphA2 is prominently expressed in TPCs and is required for sustained self-renewal and expansion of this tumor-propagating cell pool in hGBMs.

The depletion of the TPC stem-like pool induced by ephrinA1-Fc treatment and EphA2 downregulation seems to arise from increased cell differentiation. First, loss of the undifferentiated state is demonstrated by the depletion of Sox2, Olig2, Nestin, CD133, CD44, and SSEA-1, as well as the increased expression of the astroglial marker GFAP, as induced by both ephrinA1-Fc and knockdown of *EPHA2* expression. Second, both manipulations also inhibit the typical undifferentiated stem-like functions of TPCs, such as self-renewal and extensive amplification capacity. Given the unchanged cell cycling and death rate, these data imply that ephrinA1-Fc and EphA2 downregulation cause the loss of undifferentiated functional and antigenic properties as well as the acquisition of a more mature cell phenotype by TPCs.

The activities of EphA2 in hGBM TPCs appear to involve unconventional ephrin-independent activities of EphA2, which are not yet well understood but may also involve crosstalk with other signaling systems (Miao et al., 2009; Pasquale, 2010; Gopal et al., 2011; Miao and Wang, 2012). This is supported by the prominent phosphorylation of EphA2 at the Ser897 residue (Miao et al., 2009) that we detected in the TPC-enriched core but not the periphery of hGBMs, and in cultured TPCs but not non-TPCs or vhNSCs. Phosphorylation of EphA2 Ser897 by Akt is known to promote EphA2′s oncogenic activities independently of ephrin binding, and in fact interaction of EphA2 with ephrinA1 inhibits phosphorylation at this site (Miao et al., 2009). Consistent with a role of EphA2 in TPCs that does not depend on activation by endogenous ephrins, using the soluble EphA2 extracellular domain to block EphA2-ephrin interaction did not affect GFAP levels or ERK activation in TPCs. In contrast, *EPHA2* siRNA-mediated knockdown depleted the stem cell pool concomitantly with ERK, suggesting that this pathway may mediate the effects of EphA2. This was confirmed by using the ERK inhibitor UO126, which re-established self-renewal ability in TPCs with downregulated EphA2 expression. This suggests that ERK can promote differentiation in TPCs that have lost EphA2 expression.

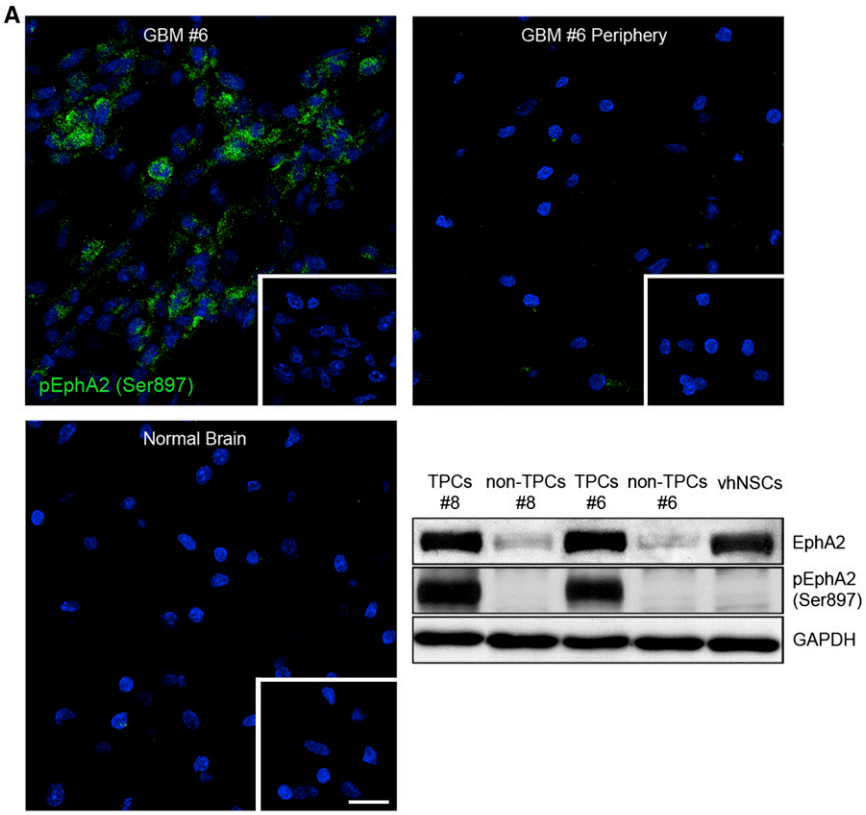
The hindrance of self-renewal capacity induced by ephrinA1-Fc predicts that this ligand will lessen the ability of TPCs to establish tumors in vivo. In fact, a 48 hr exposure to soluble ephrinA1-Fc reduced the capacity of TPCs to establish hGBM phenocopies, both subcutaneously and intracranially. A similar effect was observed when we implanted TPCs subcutaneously and began ephrinA1-Fc infusion either at the same time or after sizeable tumors had been established. Treatment of intracranial tumors with ephrinA1-Fc significantly reduced the tumor mass, vascularization, and proliferation and dramatically decreased EphA2 levels. When *EPHA2* expression in TPCs was knocked down with siRNAs, intracranial tumorigenicity was nearly abolished. Thus, EphA2 is critical to maintain TPC properties and plays a pivotal role in the pathogenesis of hGBMs in the brain, as driven by TPCs.

Our findings are consistent with previous studies suggesting that EphA2 expression correlates with malignancy in hGBMs (Liu et al., 2006; Wang et al., 2008; Li et al., 2010; Wu et al., 2011). Hence, we examined the relationship between EphA2 expression and patient survival in relation to the recent stratification of hGBMs into different subtypes (Verhaak et al., 2010). We found that EphA2 is most highly expressed in the classical and mesenchymal groups and that, at least in the mesenchymal subtype, patients with EphA2 levels above the median trended toward poorer survival. This trend was also present and statistically significant in the proneural subtype, although this subtype has lower mean levels of EphA2 than the mesenchymal and classical ones. The correlation with poor survival in these two groups further underscores the importance of EphA2 expression in aggressive tumor behavior, which may be more relevant in the mesenchymal and proneural hGBM subtypes.

We were able to drastically inhibit the growth of pre-existing orthotopic hGBM xenografts under experimental conditions that mimic those needed for experimental human therapy, such as protracted delivery through intra-parenchymal infusion. This supports the value of devising anti-GBM therapies that harness the Eph/ephrin system to target the TPC compartment. Hence, the concept emerges of impinging in non-toxic fashion on specific molecular targets, such as EphA2, to tackle the subset of tumor cells that plays the most critical role in hGBM pathogenesis.

We show that a signaling system that is operational in the neural stem cell niche also regulates self-renewal and tumorigenicity in hGBM TPCs, in agreement with previous observations (Ying et al., 2011). Eph receptors and ephrins regulate the migration, survival, proliferation, cell fate and differentiation of neural precursors during development (Goldshmit et al., 2006; Genander and Frisén, 2010). In the adult, such activities persist in stem cell niches, including those of the adult central nervous

(B) siRNA-mediated knockdown of *EPHA2* expression causes loss of TPC clonogenicity; error bars, SEM; **$p < 0.005$ EphA2 versus NTC siRNAs.
(C) TPC growth decrease is concomitant with siRNA-mediated EphA2 downregulation and normalizes when EphA2 levels begin recover (arrow); error bars, SEM; $p < 0.0001$ *EPHA2* siRNAs versus NTC.
(D) siRNA-mediated EphA2 downregulation decreased vhNSCs growth; error bars, SEM; ***$p < 0.0001$ *EPHA2* siRNAs versus NTC. Inset: western blot for EphA2 upon *EPHA2* siRNA treatment.
(E) Confocal analysis shows that siRNA-mediated EphA2 downregulation in TPC spheroids is associated with depletion of putative stem markers, as compared to NTC-treated spheroids (NTC siRNAs). Insets: magnification. Scale bar, 40 μm.
(F and G) Western blot analysis show increased GFAP but not β-Tub or GalC levels in TPCs treated with *EPHA2* siRNAs versus NTC or *GAPDH* siRNAs (F). ERK phosphorylation is also strongly increased concomitant with EphA2 downregulation, with more prominent effects at 72 hr than at 5 days, when EphA2 levels begin to recover. (G) Representative western blot showing that only the siRNA sequences that effectively reduce EphA2 expression increase GFAP expression and activate ERK. An *EPHA2* construct lacking the 3′UTR sequence targeted by *EPHA2* siRNA no. 3 was used to transfect TPCs in a control rescue experiments.
(H) The reduced TPC clonogenicity caused by knockdown of *EPHA2* expression was partially restored by 10 μM UO126, which inhibits ERK; error bars, SEM; ***$p < 0.0001$, **$p < 0.005$ versus untreated TPCs.
Unless otherwise stated, data are representative of three independent experiments giving similar results.



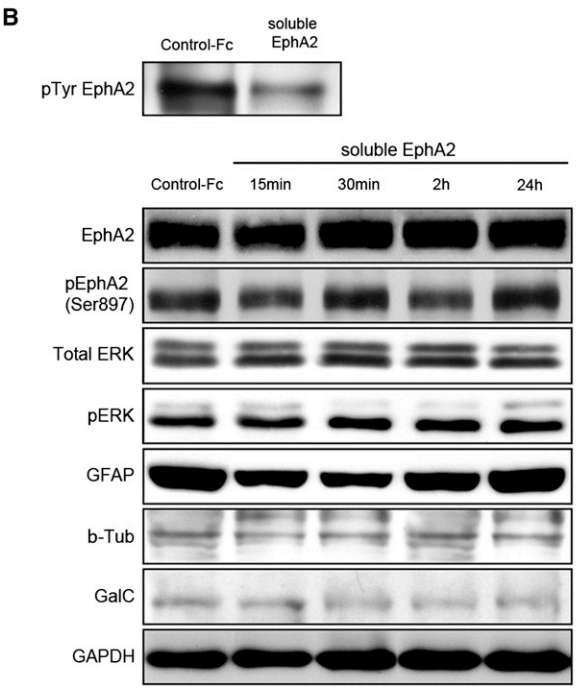


Figure 6. EphA2 Does Not Appear to be Activated by Ephrins in hGBM

TPC is phosphorylated on Ser897 in hGBM and treatment with the soluble EphA2 extracellular domain to inhibit ephrin binding to endogenous EphA2 does not affect TPC signaling pathways.

(A) Top: EphA2 is constitutively phosphorylated on Ser897 in the core of hGBMs (left) but not in the periphery of hGBMs (center) or normal brain (right). Bottom: EphA2 is highly phosphorylated on Ser897 in hGBM TPCs but not non-TPCs or vhNSCs. EphA2 Ser897 phosphorylation suggests ephrin-independent oncogenic activities.

(B) Representative western blots showing that treatment of TPCs with 10 μg/ml EphA2 extracellular domain to inhibit possible interactions with endogenous ephrin ligands does not affect GFAP levels or ERK phosphorylation as compared to Fc protein used as a control.

Data are representative of three independent experiments giving similar results.

system, although our understanding of Eph/ephrin regulatory functions in adult neurogenesis remains limited. Since other Eph receptors besides EphA2 are expressed in adult neural stem cell niches (Goldshmit et al., 2006; Genander and Frisén, 2010), by using agents that selectively target EphA2 we may be able to target stem-like cells in hGBMs without affecting the normal brain stem cell population.

These findings emphasize the importance of approaches that exploit fundamental similarities in the physiology of normal neural stem cells and their stem-like, tumor-propagating counterpart in brain tumors (Vescovi et al., 2006). Such approaches can make use of the wealth of information derived from studies on regulatory systems in normal neural stem cells to identify candidate effectors capable to affect TPCs, thus helping to design more effective and specific anti-GBM therapies.

EXPERIMENTAL PROCEDURES

Primary Culture, Culture Propagation, Population Analysis, and Cloning

Adult hGBM, low-grade gliomas, ependimomas, PNET tissues, and normal human brain samples were obtained and classified according to the World Health Organization guidelines. All of the tumors were banked in accordance with research ethics board approval from the Institute of Neurosurgery, Catholic University of the Sacred Heart (Prot. RBAP10KJC5) and patients gave informed consent prior to surgery. hGBM non-necrotic core and periphery tissues were dissected and digested in a papain solution (Worthington Biochemical, Lakewood, NJ, USA). Primary stem-like tumor-propagating cells (acutely isolated cells) were plated in NeuroCult NS-A medium (StemCell Technologies, Vancouver, BC, Canada) containing 20 ng/ml of epidermal- and 10 ng/ml of fibroblast-growth factor (Peprotech, Rocky Hill, NJ, USA) (culture medium; Galli et al., 2004). EphA2 was stimulated with ephrinA1-Fc (R&D Systems, Minneapolis, MN, USA) for the indicated concentrations and times. Mouse monoclonal IgG1 isotype (R&D Systems) was used as control. For population analysis, clonogenic assays and differentiation

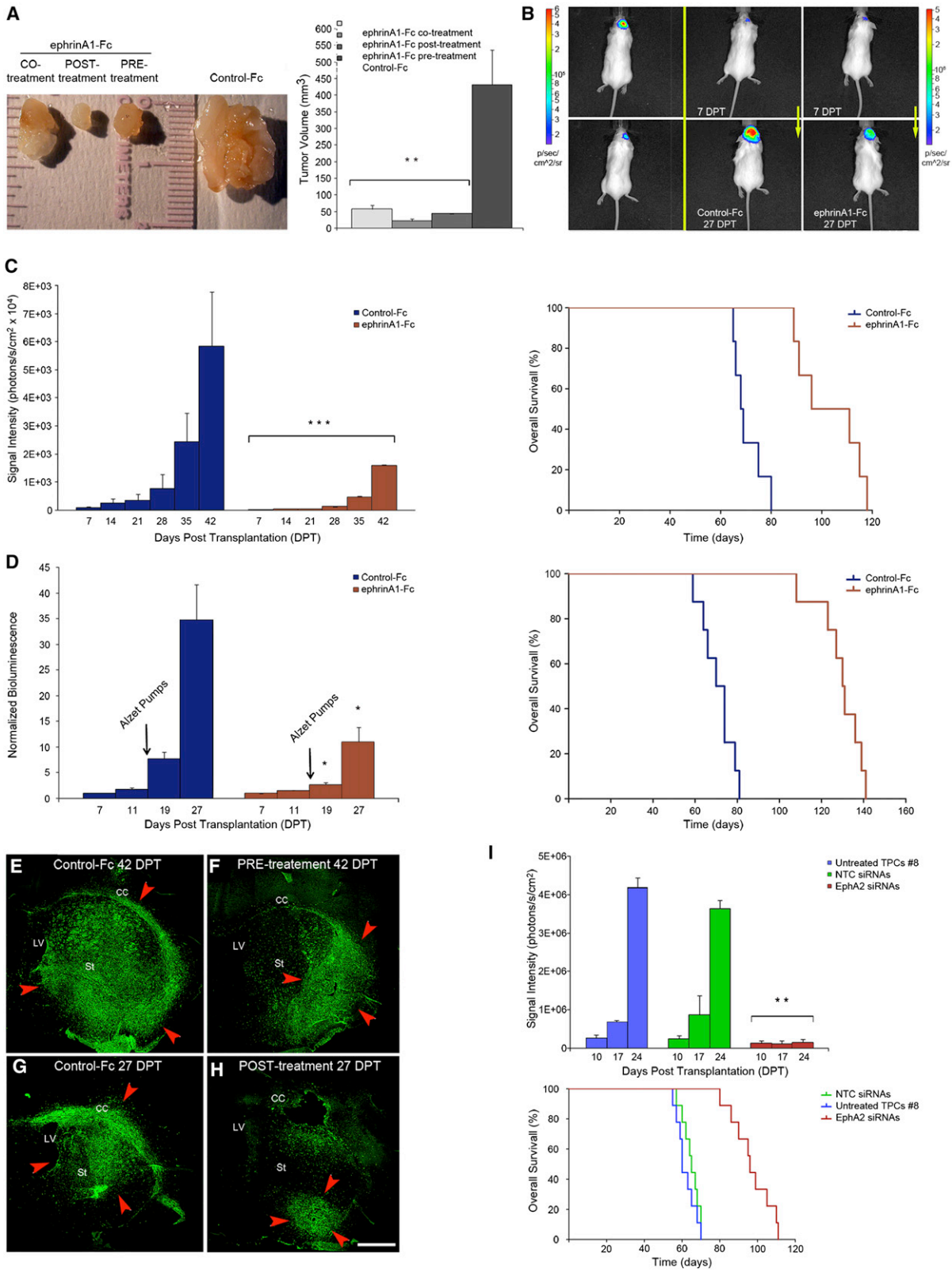


Figure 7. EphA2 Downregulation by EphrinA1-Fc or by siRNA-Mediated Knockdown Inhibits TPC Tumorigenicity in Immunodeficient Mice

(A) Treating TPCs with ephrinA1-Fc (5 μg/ml for 48 hr) prior to subcutaneous implantation (PRE-treatment) lessens their tumor-initiating capacity (left). Similar results were obtained by coinjecting TPCs and ephrinA1-Fc (CO-treatment) or injecting ephrinA1-Fc (10 μg/day) around the tumor starting 11 days after cell

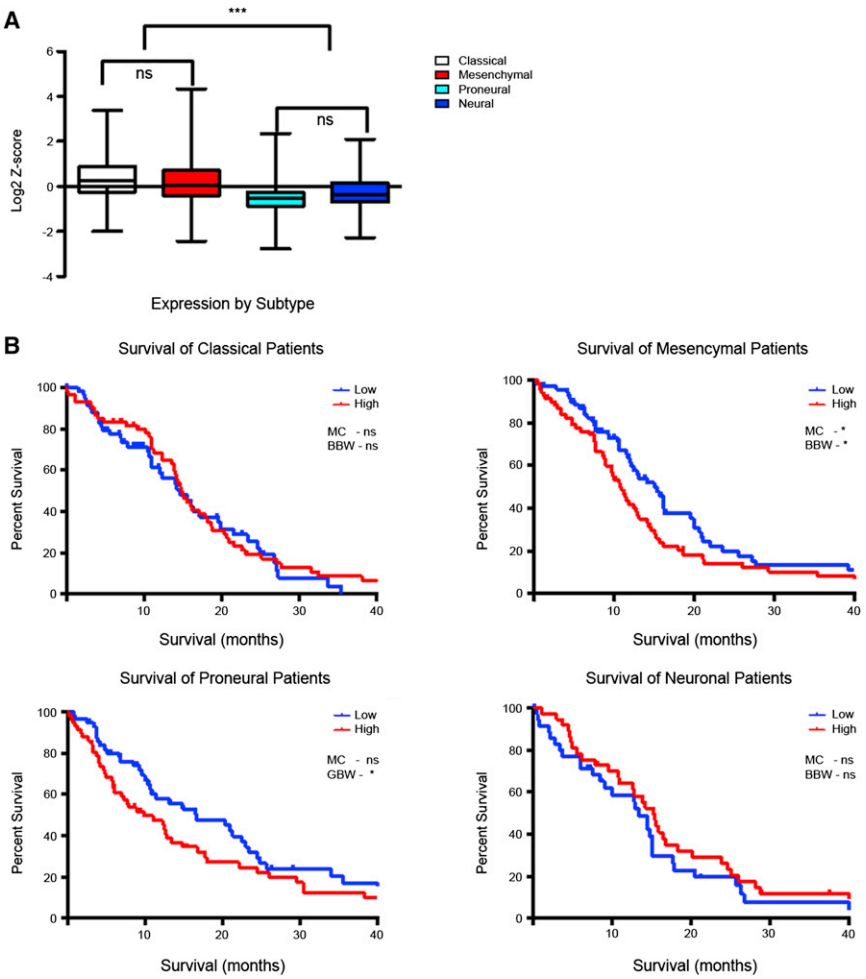


Figure 8. EphA2 Is Differentially Expressed in GBM Subtypes, and Its Abundance Correlates with Patient Survival

(A) Relative EphA2 mRNA expression in the four GBM sutbypes of the TCGA data set. (***p < 0.0001 by ANOVA; n = 495).

(B) Kaplan-Meier survival curves for the classical, mesenchymal, proneural, and neural subtypes. High and low expression are defined as above and below the median expression value for each subtype, log-rank p values were determined by MC and GBW tests.

experiments see the matching section in the Supplemental Experimental Procedures.

Statistical Analysis for the Correlation

Growth curves were analyzed with a hierarchical linear model for repeated-measurements to assess trend over time (Diggle et al., 1994; Singer and Willett, 2003). Log-transformed cell number was considered as outcome. Association with EphA2 mRNA expression levels and *EPHA2* copy number were assessed including them into the statistical models (Figures S1H–S1J). A spatial power correlation type was used to account for unequally spaced time occasions during the experiment (Singer and Willett, 2003). Unless indicated otherwise, comparisons between ephrinA1-Fc (also with different doses) treated cells and Control-Fc-treated cells were carried out with a hierarchical linear model for repeated-measurements. The p values less than 0.05 were considered statistically significant. All analyses were performed using SAS Statistical Package Release 9.1 (SAS Institute, Cary, NC, USA) or GraphPad Prism.

Evaluation of Tumorigenicity by Subcutaneous or Orthotopic Implantation

Tumorigenicity was studied by TPCs subcutaneous or orthotopic injections (Galli et al., 2004). All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees (IACUCs) at the University of Milan Bicocca. See the matching section in the Supplemental Experimental Procedures for details.

Molecular Analysis

TCGA data analysis was performed as described by Squatrito et al., 2010; survival and expression data were based on clinical TCGA data and relative

transplant (POST-treatment). (Right) Volumes of subcutaneous tumors 35 days after TPCs injection. Histograms, mean volume ± SEM; **p = 0.0002 versus Control-Fc mice, n = 6.

(B) Imaging of luciferase-tagged TPCs (luc-TPCs) injected into the brain of *Scid/bg* mice. After 42 days, untreated TPCs established larger tumors (vehicle, top left) than ephrinA1-Fc PRE-treated TPCs (bottom left). Luc-TPC tumors established for 7 days (7 DPT, top center) grew quickly when a mini-pump delivered Control-Fc for 14 days starting at 11 days post-transplant (27 DPT, bottom center). In contrast, tumor growth was markedly inhibited by ephrinA1-Fc (top and bottom right panels).

(C) (Left) Quantitative analysis of luc-TPC signals for the PRE-treatment intracranial transplants. Histograms, mean ± SEM; ***p < 0.0001 versus Control-Fc mice; n = 6. (Right) Kaplan-Meier survival curves showing that mice receiving ephrinA1-Fc-treated TPCs have a significant longer life span than mice injected with Control-Fc cells (MC and GBW tests, log-rank p = 0.0005 and 0.0013 respectively; n = 9).

(D) (Left) Quantitative time course analysis of the luc-TPC signal for POST-treatment paradigm (arrows mark the time of mini-pumps implantation). Histograms, mean ± SEM; *p < 0.05 versus Fc-treated mice; n = 8. (Right) Kaplan-Meier survival curves are shown (MC and GBW tests, log-rank p < 0.0001 and p = 0.0002 versus Control-Fc mice; n = 9).

(E–H) Mouse brain sections immunolabeled for luciferase show that tumors established from luc-TPCs PRE-treated with Control-Fc (E) spread through the brain parenchyma more than those established from cells PRE-treated with ephrinA1-Fc (F) at 42 DPT. Similarly, ephrinA1-Fc infused into the brain for 2 weeks starting 11 days after tumor establishment (H) inhibits the growth of luc-TPC tumors more than Control-Fc (G). Arrowheads mark the edges of the tumors. CC, corpus callosum; LV, lateral ventricle; St, striatum. See also Figure S4. Scale bar, 1 mm.

(I) Loss of intracranial tumorigenicity in luc-TPCs treated with *EPHA2* siRNAs for 72 hr prior to transplantation as compared to NTC siRNA-treated or untreated TPCs. Tumor growth was monitored by quantitative imaging analysis (top). Histograms, mean ± SEM; ***p < 0.0001 *EPHA2* siRNAs versus NTC; n = 6. (Bottom) Kaplan-Meier survival analysis. Mice receiving *EPHA2* siRNA-transfected TPCs die significantly later than those receiving untreated TPCs or TPCs transfected with non-targeting control siRNAs (MC and GBW tests, log-rank p < 0.0001 versus NTC siRNAs treatment; n = 9).

mRNA expression obtained from the TCGA data portal (http://cancergenome.nih.gov/dataportal/data/about) and the MSKCC cBio Genome Data Analysis Portal (http://cbio.mskcc.org/gdac-portal/index.do), respectively. Tumor subtype classification was previously described (Verhaak et al., 2010). Relative mRNA results are based on relative distribution of the expression values for diploid tumor samples and were subjected to statistical analysis by one-way ANOVA (GraphPad Prism v5.0 software). Survival curves were analyzed using the Kaplan-Meier method, with groups compared by respective median survival of number of days taken to reach 50% morbidity; log-rank p value was measured using both MC and GBW tests.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2012.11.005.

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