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Autocrine Endothelin-3/Endothelin Receptor B Signaling Maintains Cellular and Molecular Properties of Glioblastoma Stem Cells

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Abstract

Glioblastoma stem cells (GSC) express both radial glial cell and neural crest cell (NCC)-associated genes. We report that endothelin 3 (EDN3), an essential mitogen for NCC development and migration, is highly produced by GSCs. Serum-induced proliferative differentiation rapidly decreased EDN3 production and downregulated the expression of stemness-associated genes, and reciprocally, two glioblastoma markers, EDN1 and YKL-40 transcripts, were induced. Correspondingly, patient glioblastoma tissues express low levels of EDN3 mRNA and high levels of EDN1 and YKL-40 mRNA. Blocking EDN3/EDN receptor B (EDNRB) signaling by an EDNRB antagonist (BQ788), or EDN3 RNA interference (siRNA), leads to cell apoptosis and functional impairment of tumor sphere formation and cell spreading/migration in culture and loss of tumorigenic capacity in animals. Using exogenous EDN3 as the sole mitogen in culture does not support GSC propagation, but it can rescue GSCs from undergoing cell apoptosis. Molecular analysis by gene expression profiling revealed that most genes downregulated by EDN3/EDNRB blockade were those involved in cytoskeleton organization, pause of growth and differentiation, and DNA damage response, implicating the involvement of EDN3/EDNRB signaling in maintaining GSC migration, undifferentiation, and survival. These data suggest that autocrine EDN3/EDNRB signaling is essential for maintaining GSCs. Incorporating END3/EDNRB-targeted therapies into conventional cancer treatments may have clinical implication for the prevention of tumor recurrence. Mol Cancer Res; 9(12); 1668–85. ©2011 AACR.

Introduction

Glioblastoma (WHO grade IV) is the most common and most aggressive type of primary brain tumor in humans. It remains virtually incurable despite extensive surgical excision and postoperative adjuvant radiotherapy and chemotherapy (1). Glioblastoma stem cells (GSC) have been recently isolated from glioblastoma tumors of patients and were characterized as a small subset of stem-like tumor cells capable of initiating and sustaining tumor growth when grafted into mice (2–6). Although CD133/prominin, a normal neural stem cell (NSC) marker, is not an obligatory marker for GSCs (6, 7), CD133 was first applied as a surface marker for isolation and enrichment of GSCs (6). Studies showed that CD133+/CD117+ tumor-initiating cells possess marked resistance to radio- and chemotherapy (10, 11) and thus are now suggested to be responsible for posttreatment failure and tumor recurrence. The molecular profiles of GSCs revealed characteristics of neuroectodermal-like cells, expressing both neural and mesenchymal developmental genes, and portraying an undifferentiated, migratory, astrocytic, and chondrogenic phenotype (8). This suggests that a subset of GSCs may inherit neural crest cell (NCC)-like developmental pathways to initiate a tumor. In particular, endothelin 3 (EDN3), a potent mitogen for NCC and its derived lineage precursor cells (12, 13), was identified as one of the top genes highly expressed in tumorigenic GSCs (8). EDN3 is a member of
the endothelin (EDN) family, which consists of a group of vasoactive peptides referred to as EDN1, EDN2, and EDN3 (14). EDNs are synthesized initially as inactive larger precursor molecules and then posttranslationally cleaved by EDN-converting enzyme (ECE1) to yield the biologically active 21-amino acid form (15). The effects of EDNs are mediated by 2 distinct but highly homologous G-protein–coupled receptors, EDN receptor A (EDNRA) and EDN receptor B (EDNRB), in an autocrine and paracrine manner (16, 17). The EDNRA predominantly binds to EDN1 and EDN2 with similar affinities, and EDN3 with 1,000- to 2,000-fold lower affinity, whereas EDNRB has similar affinities for all 3 isopeptides (18). Mutations in EDN3 or EDNRB can lead to abnormal development of the enteric nervous system (ENS) and melanocytes and are known to account for the majority of patients with Waardenburg syndrome (WS) type IV, who exhibit both pigmentation and megacolon phenotypes (19, 20). EDN3 and EDNRB mRNA expression has been reported in fetal human enteric mesenchyme and NCCs (21), and EDN3/EDNRB signaling is known to influence NCC proliferation, differentiation, and migration during ENS development (22). The expression of the EDN system genes has been shown in the brain and in human glioblastoma (23, 24), as well as a broad range of other types of human cancers (25). The role of the EDN-axis, especially in EDN1-axis, has been implicated in promoting tumor progression. Blocking EDN receptors has been suggested as a novel strategy in cancer therapy (25).

In this study, we provide the first comprehensive analysis of the expression and function of the EDN system in patient-derived GSCs. We found an essential role of autocrine EDN3/EDNRB system in GSCs. Blocking either EDNRB function or EDN3 production leads to GSC apoptosis and loss of migration, self-renewal, and tumorigenesis. Furthermore, genome-wide expression array analyses have elucidated molecular pathways and gene network connections to this essential signaling for maintaining GSCs. This finding implicates that the EDN3/EDNRB signaling pathway may serve a novel therapeutic target for the development of potentially more effective treatment protocols for preventing GSC-mediated tumor recurrence.

Materials and Methods

Glioblastoma sphere culture

Glioblastoma tumor specimens were obtained from patients who underwent surgery at Ronald Reagan UCLA Medical Center. All samples were collected under protocols approved by the UCLA Institutional Review Board. Dissociated tumor cells from fresh tumor tissue were cultured in a serum-free media containing Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 (Mediatech) supplemented with 20 ng/mL human recombinant epidermal growth factor (EGF; Sigma), 20 ng/mL basic fibroblast growth factor (FGF; Millipore), 10 ng/mL leukemia inhibitory factor (LIF; Millipore), and 1 × B27 without vitamin A (Invitrogen) and is designated as stem cell growth factor (SGF) media. The tumorigenicity of GSCs was verified in xenograft animals (8). In parallel, autologous glioblastoma cell lines were established by propagating cells in DMEM/Ham’s F-12 media supplemented with 10% FBS.

Fluorescence-activated cell sorting analyses and purification of CD133+ GSCs

A total of 2 × 10^6 to 5 × 10^6 dissociated cells from glioblastoma tumor sphere cultures were stained with anti–CD133–APC (Miltenyi Biotech) for 20 minutes at 4°C. The analyses for CD133 positive cells were done on a FACSCalibur flow cytometer (Becton Dickinson) and 10,000 or more events were collected in each analysis. To purify CD133+ cells, dissociated glioblastoma sphere cultures or enzyme digested tumor xenografts were immunostained with anti-CD133 APC (allophycocyanin) under sterile conditions. The CD133+ and CD133– cells were sorted and collected on a BD FACSAria II cell sorter at 70 psi using a 70-μm nozzle.

Quantitative reverse transcriptase PCR analysis

Total RNA was extracted using a RNeasy Kit (QIAGEN). Two micrograms of RNA from each sample were transcribed to cDNA using a TaqMan RT Reagent Kit (Applied Biosystems). PCR was done using 5 μL cDNA equivalents to 100 ng total RNA and was carried out by using SYBR Green PCR Core Reagents (Applied Biosystems). After amplification, PCR products (5 μL) were electrophoresed on 2% agarose gel. The primer sequences and expected size of amplified PCR products are described in Supplementary Information.

Immunocytochemical, histopathologic, and immunohistochemical analysis

GSCs were fixed in 4% paraformaldehyde and then singly or doubly stained with anti–CD133 (1:100; Abcam), anti-nestin (1:200; Chemicon), or anti–EDN3 (1:200; Santa Cruz Biotech) antibodies. After washing, cells were incubated with rhodamine red–conjugated anti-mouse or Alexa Fluor 488–conjugated anti-rabbit antibodies (Invitrogen). Nuclei were counterstained with Hoechst 33342 (Invitrogen). Histopathologic analyses were done on frozen section or paraffin slides stained with hematoxylin and cosin (H&E) staining as per standard technique. Immunohistochemical staining was done on paraffin slides. Slides were subject to a 1-hour blocking step followed by the application of primary antibody or control antibody for 1 hour at room temperature. The following primary antibodies were used: CD133 (1:100; Abcam), EDN3 (1:100; Epitomics), EDN1 (1:250; Abcam), EDNRB (1:100; Epitomics), and EDNRA (1:200; ENZO life Science). The immunodetection was done using Vectastain ABC Standard Kit and Vector NovaRED (Vector Laboratories).

ELISA

To determine the amount of EDN3 released from GSCs, dissociated GSCs were plated in 24-well plates in triplicate at the density of 5 × 10^5 cells per mL per well. Four hours later, stem cell culture media was replaced with fresh SGF media.

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with or without ECE1 inhibitor, plain media or serum-containing media. The respective conditioned media were collected at various time points and were stored at −80°C. The amount of EDN3 peptides in condition media was determined by an ELISA Kit specific for EDN3 (Immunobiological Laboratories). The absorbance was read at 450 nm by Epoch Microplate Reader (BioTek Instruments).

**Flow cytometric analysis for cell-cycle distribution and cell apoptosis**

To determine the effects of BQ788, BQ123, or combination treatment on cell-cycle distribution and cell apoptosis, 2 × 10^5 to 5 × 10^5 treated and untreated GSCs were stained with propidium iodide (PI)-based hypotonic DNA staining buffer. Cell-cycle distribution for approximately 10,000 cells was analyzed using a FACScan (Becton Dickinson). Apoptotic cells with degraded DNA were detected as a hypodiploid or "sub-G1" peak in a DNA histogram.

**Proliferation assay for GSCs**

The effects of BQ788 and/or BQ123 treatment on the proliferative activity of GSCs were determined by a MTS/ PMS (phenazine methosulfate) colorimetric assay according to the manufacturer’s instructions (Promega). Cells were seeded into 96-well tissue culture plates at a density of 2,000 cells per well in the presence or absence of EDNRA or EDNRB antagonists and incubated for 72 hours. The absorbance was measured at 490 nm after a 4-hour incubation with MTS/PMS reagents.

**Quantitative measurement of cell apoptosis and cell viability**

To determine whether EDN3 alone can support GSC survival, cell apoptosis assays were done by using a Cell Death Detection ELISAPLUS kits (Roche), which quantitatively detects the amount of cleaved DNA/histone complexes (nucleosomes) in a given sample. The GSCs were seeded at the concentration of 2,000 cells/well/100 µL in triplicates. The culture media was changed and switched to plain media that contain various concentrations of EDN3. Cell apoptosis was measured after 72 hours incubation by reading the absorbance at 405 nm. The same method was applied to the measurement of BQ788 and EDN3siRNA treatment-induced cell apoptosis. The viability of GSCs was determined by Trypan Blue staining (Invitrogen).

**Knockdown of EDN3 by siRNA transfection**

A reverse transfection protocol was done to deliver EDN3 siRNA (Ambion), nonsilencing control siRNA (Ambion) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA (Dharmacon) into GSCs. Briefly, a transfection complex was prepared by diluting siRNA in 10 µL OPTI-MEMI (Invitrogen), then adding 10 µL OPTI-MEMI containing 0.3 µL Lipofectamine RNAiMAX transfection reagent (Invitrogen). This complex was then added into each well in 96-well plate followed by seeding 6,000 GSCs in 100 µL SGF media to give a final siRNA concentration of 30 nmol/L in each well. EDN3 gene silencing was determined 72 hours after transfection by quantitative reverse transcriptase PCR (qRT-PCR), using Power SYBR Green Cells-to-CT Kit (Ambion).

**Intracranial tumor formation assay and histopathologic analysis**

The role of EDN3/EDNRB signaling in maintaining the tumorigenic capacity of GSCs was examined in Beige/severe combined immunodeficient mice (SCID) mice. A total of 10^5 live GSCs treated with and without 50 µmol/L BQ788 for 24 hours were injected in a total volume of 3 µL into brains of mice under a UCLA Institutional Animal Research Committee—approved protocol. Mice were maintained for 25 weeks or until development of neurologic signs. Brains of euthanized mice were collected, fixed in 10% formalin, paraffin-embedded, and sectioned. Alternatively, brain tissue was placed in O.C.T. embedding medium (Tissue Tekm), dipped in liquid nitrogen, and sectioned in a −20°C cryostat. Histopathologic analyses were done on frozen section or paraffin slides stained with H&E staining as per standard technique.

**Microarray procedures, data analysis, and gene annotation**

Molecular profiling of 6 untreated (n = 3, duplicate) and 3 BQ788-treated GSCs (n = 3) were carried out using standard Affymetrix protocols and hybridized to Affymetrix GeneChip U133 Plus 2.0 Array as described previously (8). The DCP files were globally normalized, and gene expression values were generated using the dChip implementation of perfect-match minus mismatch model-based expression index. The group comparisons were done in dChip and samples were permuted to assess the false discovery rate (FDR). To avoid inclusion of low level and unreliable signals, the difference of the means needed to exceed 100 and be called present by MAS 5.0 in greater than 20% of the samples. In addition, the Gene Set Enrichment Analysis (GSEA) tool was done to identify significant gene ontology groups that were enriched (26). Functional annotation of individual gene was obtained from NCBI/Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez), UniProt (http://www.uniprot.org/), information hyperlinked over protein (http://www.ihop-net.org/), and the published literature in PubMed Central (http://www.ncbi.nlm.nih.gov/pubmed).

**Statistical analysis**

Each experiment was set up in triplicate and repeated at least twice. Data were expressed as means ± SD and analyzed using 1-way ANOVA tests, depending on homogeneity of variances. All P values were 2-sided, and values less than 0.05 were considered significant. SPSS v13.0 for Windows software was used for all statistical analysis.

**Results**

**Expression of the EDN system components in GSCs**

Through genome-wide expression microarray analysis, we previously identified EDN3 as one of the most overexpressed genes.
GSC cultures form semiadherent tumor spheres with cells spontaneously migrating out of spheres. Scale bar – 25 μm. B, Total RNA from the indicated GSCs cultured in SGF media, autologous glioblastoma cell lines cultured in serum-containing media were extracted. The mRNA expression levels of EDN1, EDN3, EDNRA, EDNRB, and ECE1 were analyzed by qRT-PCR with specific primers. β-Actin was used as an internal control gene.

EDN3 is a Survival Factor for Glioblastoma Stem Cells

EDN3, a member of the endothelin family, is a survival factor for GSCs and may contribute to maintaining GSC properties. To test this hypothesis, we first investigated the expression of the EDN system genes in GSCs. Three patient-derived tumorigenic GSC lines (D431, S496, and E445), which contain 39.6%, 9.6%, and 1.5% CD133+ cells, respectively, were employed for this study (Fig. 1A). GSCs, which form semiadherent tumor spheres with highly motile cells, spontaneously migrate outward from sphere bodies (Fig. 1A). We tested both unsorted and sorted CD133+ and CD133− cells from D431 and S496 GSC cultures, as well as the autologous CD133− glioblastoma cell lines passaged in serum-containing media. Because E445 GSC cultures contain only 1.5% CD133− cells, we only tested the unsorted cell population. By using qRT-PCR analysis, mRNA expression of EDN1, EDNRA, EDNRB, and ECE1 was detected in all glioblastoma cell samples tested. Notably, the expressions level of EDN1 and EDNRA mRNAs were particularly upregulated in all glioblastoma cell lines cultured in 10% serum, whereas high levels of EDN3 mRNA was only detected in GSC lines established in SGF media, unsorted GSCs, or sorted CD133− cells (Fig. 1B). EDN3 mRNA seems not to be a SGF responsive gene or direct target of SGF because switching serum-cultured glioblastoma cells to SGF for 6, 24, and 48 hours (Fig. 1B, not shown) did not stimulate comparable EDN3 mRNA expression in glioblastoma cells (Fig. 1B). These data indicate that EDN3, but not EDN1, is a molecular/cellular signature for GSCs. Although both CD133+ and CD133− cells sorted from glioblastoma sphere cultures express EDN3, differentiated CD133− glioblastoma cell lines cultured in serum do not express EDN3, indicating that EDN3 is only expressed in GSCs and their immediately differentiated CD133− daughter cells maintained in stem cell culture condition. The data also suggest that GSC lines represent different cell populations from autologous glioblastoma cell lines, which cannot be subsequently reversed following transition to SGF conditions (27).

Upregulation of EDN3 mRNA coupled with downregulation of EDN1 mRNA expression characterizes stemness state of GSCs

EDN1 has been implicated in the growth and progression of a wide range of human tumors (25), whereas recently, EDN3 has been suggested as a tumor suppressor gene in female malignances (28, 29). Because we detected a reciprocal relationship in the expression of EDN1 and EDN3 mRNA in glioblastoma cells cultured under different conditions, we examined whether there is a reciprocal regulation of EDN1 and EDN3 in GSCs. FBS, which contains a variety of cytokines and growth factors, was used as a surrogate for inflammatory mediators to force GSC cultures to undergo proliferative differentiation as transit-amplifying-like cells capable of proliferation and differentiation, which eventually can be propagated as glioblastoma cell lines (Fig. 2A). Progressively diminished

Figure 1. Presence of EDN system components in GSCs. A, tumorigenic GSC cultures, D431, S496, and E445, were established from patient-derived glioblastoma tumors. Percentages of CD133+ cells in each culture were determined by flow cytometry analysis using anti-CD133 directly conjugated to APC. GSC cultures form semiadherent tumor spheres with cells spontaneously migrating out of spheres. Scale bar – 25 μm. B, Total RNA from the indicated GSCs cultured in SGF media, autologous glioblastoma cell lines cultured in serum-containing media and switched to SGF media for 6 hours and fibroblasts cultured in serum-containing media were extracted. The mRNA expression levels of EDN1, EDN3, EDNRA, EDNRB, and ECE1 were analyzed by qRT-PCR with specific primers. β-Actin was used as an internal control gene.
EDN3 mRNA expression coupled with marked induction of EDN1 mRNA was seen in serum-stimulated GSC cultures (Fig. 2B). Serum-induced GSC differentiation was further characterized by the codownregulation of several radial glial cells (RGC)- and NSC-associated genes expressed by GSCs (ref. 8; Fig. 2B), including fatty acid-binding protein 7 (FABP7; ref. 30), CD133 (31), sex-determining region Y-box 2 (SOX2; ref. 32), inhibitor of DNA binding 4 (ID4; ref. 33), and GAP43 (34), suggesting that EDN3 is a marker for undifferentiated, stem-like glioblastoma cells. In contrast, the expressions of EDN1 (glioblastoma tumor marker; ref. 23) and YKL-40 mRNA (glioblastoma prognostic marker; ref. 35, only expressed in D431 and S496), were induced as a result of proliferative differentiation (Fig. 2B). The mRNA level of topoisomerase (DNA) II alpha (TOP2A), a maker that is associated with cell growth, was unchanged. This data supports the notion that the reciprocal regulation of EDN3 and EDN1 expression in GSCs may modulate and determine tumor development (36, 37). Indeed, the transcripts of EDN system genes were determined in patient-derived glioblastoma tumors, and elevated expression of EDN1 mRNA was determined in all 11 tumors analyzed, whereas EDN3 mRNA was expressed at a lower level in the majority of tested primary tumors when compared with EDN1 mRNA (Fig. 2C). Likewise, YKL-40 mRNA was expressed in most glioblastoma tumors (Fig. 2C).

GSCs are actively secreting EDN3 peptides when maintained in SGF conditions

Because EDN3 mRNA is uniquely expressed in GSCs, but not in more differentiated autologous glioblastoma cells cultured in the serum, we then tested whether EDN3 was expressed at the protein level and secreted by GSCs maintained in SGF media. Immunostaining analysis revealed that EDN3 was only expressed in GSCs, but not in more differentiated autologous glioblastoma cell lines only expressed nestin, nor EDN3 or CD133. Either CD133<sup>+</sup> or CD133<sup>−</sup> cells in all 3 tested serum cultures express EDN3 as determined by 2-color flow cytometric analysis (Supplementary Fig. S1). ELISA assays further confirmed that the conditioned media collected from GSC cultures contained high concentration of EDN3, although no EDN3 peptides were measured in conditioned media collected from glioblastoma cell line cultures (Fig. 3B). The production of EDN3 peptides by GSC cultures was markedly decreased when cultures were switched to plain media or serum-containing media (1 week), or treated with the ECE1 inhibitor SM-19721, by which GSC were forced to undergo starvation, proliferative differentiation, and acute EDN3 deficiency (Fig. 3B). These data confirmed that bioactive EDN3 peptides were only actively secreted by GSCs maintained in SGF media. Moreover, under light microscope,

Figure 2. Reciprocal regulation and expression of EDN1 and EDN3 mRNA in GSCs and glioblastoma tumors. A, light-microscopic morphology of indicated GSC cultures before (a, c, e) and after 2-week serum-induced proliferative differentiation (b, d, f). Scale bar = 25 μm. B, the indicated GSC cultures were switched to serum-containing media for the time shown. The mRNA expression levels of EDN1 and EDN3 and the indicated NSC (CD133, SOX2, ID4), RGC (FABP7), and glioblastoma-associated genes (YKL-40, TOP2A) were analyzed by qRT-PCR with specific primers. β-Actin and GAPDH were used as internal control genes. C, total RNA from patient-derived glioblastoma tumors (n = 11) were extracted. The mRNA expression levels of the indicated EDN system components were analyzed by qRT-PCR with specific primers. YKL-40 represents a glioblastoma progression-associated gene, and β-actin was used as an internal control gene.
acute cell death was observed when GSCs were treated with the ECE1 inhibitor (Fig. 3C), suggesting that autocrine EDN3 may be an essential survival factor only when GSCs are maintained in SGF conditions, not when GSCs undergo cell differentiation.

**Blockade of EDNRB, not EDNRA, signaling induces GSC apoptosis without prominent cell-cycle arrest**

Because proteolytic processing of EDN precursors to bioactive peptides by ECE1 is not specific to EDN3, we then analyzed and compared the effects of EDNRB and EDNRA antagonists on GSCs. GSCs were treated for 72 hours with various doses of the EDNRB antagonist BQ788 or the EDNRA antagonist BQ123. Evidently, structural/morphologic alterations and cell apoptosis were only detected in GSCs treated with BQ788, not BQ123 or vehicle control (ethanol), as examined microscopically, and BQ788-induced morphologic changes and cell apoptosis were significantly enhanced in a dose-dependent manner (Fig. 4A). Correspondingly, the increase in apoptotic cells (2.5- to 4-fold) appeared in a sub-G1 peak in DNA histograms analyzed by flow cytometric analysis and was only found in GSCs treated with BQ788 (50 μmol/L), not BQ123 (50 μmol/L), in all 3 GSC cultures tested (Fig. 4B). Notably, no major changes were found in the cell-cycle distribution in BQ788-treated GSCs when compared with untreated control cells. Only slightly increased numbers of cells in G1 phase (11%), S phase (8%), and G2/M phases...
Figure 4. Blockade of EDNRB not EDNRA induces GSC apoptosis independent from cell-cycle arrest. A, light-microscopic morphology of GSC cultures treated with the indicated concentrations of BQ788, BQ123, or vehicle control (ethanol alcohol) for 3 days. Scale bar = 25 or 50 μm. B, the indicated GSC lines were treated with 50 μmol/L BQ788 or BQ123 for 3 days, and cell-cycle distributions including sub-G1 fraction were determined by flow cytometric analysis. C, the sorted CD133+ and CD133− cells and unsorted GSC lines, autologous glioblastoma cell lines, and fibroblasts were treated with BQ788, BQ123 (a–d), or combinations (e) at the indicated concentrations for 3 days. The cell growth was determined by MTS/PMS cell proliferation assays. Data represent mean values ± SD of triplicate measurements in triplicate experiments.
EDNRB on GSC growth, GSCs were treated for 72 hours with various doses of BQ788 or BQ123 (Fig. 4C) and assayed for cell proliferation. Inhibition of proliferation was not seen when GSCs (sorted or unsorted GSC) were treated with 25 to 100 μmol/L BQ123, although treatment with 75 or 100 μmol/L BQ788 reduced cell proliferation by 30% to 80% in D431, 30% to 60% in S496 and 40% to 60% in E445 (Fig. 4C, a–c). The combination of BQ788 and BQ123 had no additive effect on suppressing cell growth, although a notable growth stimulation was observed in unsorted D431 and S496 by treatment with 50 μmol/L BQ123 (Fig. 4, c). Of note, both fibroblasts and autologous glioblastoma cell lines passed in serum-containing media that do not produce EDN3 were resistant to BQ788 treatment (Fig. 4C, a–d). In contrast, both CD133+ and CD133− daughter cells sorted from sphere cultures (D431 and S496) are sensitive to BQ788 treatment. BQ788 treatment also decreased clonogenic efficiency of both CD133+ and CD133− daughter cells sorted from GSC cultures that were initiated from purified CD133+ GSCs (Fig. 4D). Notably, untreated CD133− daughter cells are fast-growing cells, which exhibited higher clonogenic efficiency compared with those of untreated CD133+ daughter cells on day 7 after cell seeding.

To verify that the observed decreased proliferative activity and clonogenic efficiency caused by BQ788 is mainly due to cell apoptosis, not cell-cycle arrest, the induction of GSC apoptosis by the EDNRB antagonist was further determined using an ELISA-based cell death assay, which quantitatively detects the amount of cleaved DNA/histone complexes. Significant cell apoptosis were detected in all 3 GSC lines treated with 50 to 100 μmol/L BQ788 when compared with untreated cells (Fig. 4E). Thus, cell morphology observations, clonogenic assays, cell-cycle analyses, and cell apoptosis assays suggest that EDN3/EDNRB signaling is a survival pathway for GSCs.

**Blockade of EDN3/EDNRB signaling leads to the loss of cell spreading/migration, self-renewal, and tumor initiation**

Next, we determined whether the observed decreased proliferative activity and clonogenic efficiency caused by BQ788 treatment was due to a loss in malignant behavior. To verify that the observed decreased proliferative activity and clonogenic efficiency caused by BQ788 treatment was due to a loss in malignant behavior, GSCs were treated with 50 μmol/L BQ788 when compared with untreated cells (Fig. 4F). More importantly, these BQ788-treated GSCs failed to regenerate spheres when live cells were reseeded at clonal density (Fig. 5A, h, l), indicating that the self-renewal capacity of GSCs was lost. The impairment of cell migration and self-renewal by BQ788 treatment was also shown in purified CD133+ GSCs (Supplementary Fig. S2). To study whether blocking the production of endogenous EDN3 in GSCs will have impact on these GSC functions, we carried out an on-target knockdown of EDN3. Treatment with EDN3 siRNA, not negative control siRNA, not only efficiently knocked down EDN3 mRNA expression (Fig. 5B), but also abrogated the EDN3 peptide release (Fig. 5C). More importantly, prominent cell apoptosis was only determined in GSCs treated with EDN3 siRNA (Fig. 5D). Microscopic changes in cellular morphology were observed, and in contrast to negative control siRNA-treated GSCs (Fig. 5E, c, g, k), loss of endogenous EDN3 by EDN3 siRNA treatment resulted in loss of self-renewal potential and cell migration capacity (Fig. 5E, d, h, l). By contrast, GSCs in negative control siRNA-treated cultures spontaneously migrated/spread out of tumor spheres and formed the surrounding monolayer (Fig. 5E, g–j), implicating a role of EDN3/EDNRB signaling in GSC self-renewal and migration.

On the basis of the requirement of EDN3/EDNRB signaling for self-renewal, migration, and survival of GSCs in culture, we test whether the EDN3/EDNRB signaling is required for maintaining the tumorigenic potential of GSCs. Unsorted GSCs or sorted CD133+ GSCs were...
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treated with or without 50 μmol/L BQ788 for 24 hours. The viability of the inhibitor-treated cells, which ranged from 80% to 87%, was determined by trypan blue staining. Following washing, 10^4 live cells were stereotactically injected into the brains of SCID mice. Mice (15/16) which received untreated GSCs developed neurologic signs at weeks 14 to 22 postinjection. The H&E staining of tumor xenografts identified histologic hallmarks of human glioblastomas (Fig. 5F, a–l). In particular, the hypercellular zones surrounding necrotic foci similar to histopathologic features of pseudopalisading necrosis was observed (Fig. 5F, a–d). The infiltrative tumor exhibits hypercellularity, hyperchromatism, pleomorphism, mitosis, vascular endothelial hyperplasia, and oligodendrogial components were also identified (Fig. 5F, e–l). On the other hand, injection of GSCs pretreated with 50 μmol/L BQ788 failed to develop a tumor (0/14) at week 25. The area of tumor injection site shows no evidence of neoplastic cells (Fig. 5F, m–v) and show no significant changes when compared with normal brain tissue (Fig. 5F, s–v), except a scar-like feature is occasionally observed near the injection site (Fig. 5F, r). These data thus suggest the potential involvement of autocrine EDN3/EDNRB signaling in maintaining the tumorigenic capacity of GSCs in culture.

To investigate whether in vitro cultured GSCs change the expression of the EDN ligands and receptors, we analyzed and compared the expression of EDN system genes in tumor xenografts (initiated from purified CD133^+ GSCs), and CD133^+ and CD133^- cells directly sorted from them, by RT-PCR (Fig. 5G, a). CD133, EDN1, EDN3, and EDNRB mRNA were determined in tumor xenografts (Fig. 5G, a), whereas a lower level of EDN3 mRNA was detected compared with that of EDN1 mRNA. However, although EDNRB mRNA was detected in CD133^- GSCs used for injection, it could not be detected in tumor xenografts after the 30 cycles of PCR. Similar expression patterns of the EDN system genes were also shown in tumors by immunohistochemical staining (Fig. 5G, b). Likewise, EDN1, EDN2, EDN3, and EDNRB mRNA were also determined in both CD133^+ and CD133^- cells sorted from tumor xenografts, whereas CD133^- cells express a lower level of EDN1 and a higher level of EDN3 mRNA when compared with those of CD133^- cells (Fig. 5G, c). YKL-40 mRNA could only be detected in tumor xenografts and sorted CD133^- cells (Fig. 5G, a, c). When CD133^- cells were recultured in SGF media for 7 days, the expression of EDNRB mRNA could be redetected (Fig. 5G, d). On the other hand, tumor xenografts which exhibit a higher level of EDN3 mRNA and lower levels of EDN1 and EDN2 mRNA were also identified (Supplementary Fig. S3). Nevertheless, a higher level of EDNRB mRNA is always expressed in tumor xenografts and sorted CD133^+ and CD133^- cells, whereas a low level of EDNRB mRNA could only be detected in tumor xenografts. These data may suggest that the initiation of tumor growth in vivo from GSCs is accompanied with the activation/upregulation of EDNRA signaling and downregulation of EDNRB signaling.

Exogenous EDN3 alone can maintain GSC survival but not promote GSC expansion

To explore the possibility that EDN3 alone might be a growth factor or a survival factor for GSCs, we removed stem cell growth factors (FGF, EGF, LIF) from the media and replaced them with various doses of recombinant EDN3. Apoptotic cells were observed in all tested GSCs maintained in plain media (40%–50% viable cells) and the addition of EDN3 (1–100 nmol/L) improved the viability of GSCs as determined by the trypan blue exclusion test (50%–80% viability; P < 0.05). However, reduced viability was observed when high doses of EDN3 (300 and 1,000 nmol/L) were added (Fig. 6A). Rescue of GSCs from apoptosis by adding exogenous EDN3 was further evidenced by a quantitative apoptosis assay coupled with morphologic examination (Fig. 6B and C). In general, 30 nmol/L EDN3 alone lowered the degree of cell apoptosis to a level close to that of SGF compared with plain media. Furthermore, low proliferative stimulation was seen with the addition of exogenous EDN3 (~1.5-fold; Fig. 6A), yet exogenous EDN3 alone was not able to propagate GSCs in culture as that of SGF media, suggesting that the significance of "growth stimulation" (P < 0.05) is likely due to significantly fewer apoptotic cells maintained when compared with cells cultured in plain media. This notion was further tested by investigating the effects of exogenous EDN3 on sorted CD133^+ and CD133^- daughter cells. We found that EDN3 alone could enhance the viability of both types of cells in a dose–response fashion as determined by trypan blue staining (Fig. 6D). However, in contrast to cells cultured in SGF, sphere formation and cell expansion were not achievable by EDN3 alone, even when EDN3 were repetitively added daily (Supplementary Fig. S4). These data therefore suggest that EDN3 alone is not a potent mitogen for GSCs but may provide cell survival benefits.

Genes regulated by EDN3/EDNRB signaling

To gain further insight into the molecular mechanisms underlying the requirement of EDN3/EDNRB signaling in maintaining GSCs, we carried out a large-scale gene expression comparison of GSCs treated with and without EDNRB inhibitor (BQ788). Probe set signals on the expression array that were equal to less than 1.5-fold lower in 3 treated (n = 3 patients) versus 6 untreated GSC samples (50 μmol/L, 24 hours; n = 3 patients, sample duplicate) with a pairwise t test (P < 0.05) were selected. Samples were permuted 100 times by dChip for a FDR of 11% (median), and 61 significant genes were obtained (Table 1). The major downregulated genes identified among 61 genes are those involved in organizing cytoskeleton structure and function (e.g., EFEMP1, IQGAPI, CADLI, WASF2, S100A6, MYO10, MYO6, CDC248, RHOQ, SRGA2, PALLD, CT3B, WASL, FRMD4B, HIP1, RAB31, and VPS35), mitotic spindle assembly, mitotic checkpoint (e.g., ASPM, NUSAP1, ASPM, and USAP1), and DNA repair or anti-apoptosis (e.g., UHMK1, NIPBL, XRAS, G2E3, DHX9, GTSE1, RPI1, and NAA15), reflecting disruptions in cell structure, cell polarity, cell movement, intracellular...
Figure 5. Blockade of EDN3/EDNRB signaling impairs GSC self-renewal, cell migration, and tumorigenic capacity. A, light-microscopic morphology of GSC cultures. The indicated GSC cultures were treated with and without BQ788 at the concentration of 50 μmol/L for 3 days and the pictures were taken (a–f). Cells were harvested and were reseeded at clonal density, which prevent cells from forming aggregates. The cultures were incubated for 3 days and pictures were taken (g–l). Scale bar = 25 μm. B, the indicated GSC cultures were transfected with negative control siRNA, EDN3 siRNA (clone #1 and clone#2), or treated with or without transfection reagents. Cells were incubated for 3 days and the levels of EDN3 mRNA were assayed by qRT-PCR. The GAPDH was used as an internal control gene. Fibroblasts serve as a negative control cell line, which does not express EDN3 (data not shown). C, EDN3 protein peptides concentrations secreted into the conditioned media by transfected cells were assayed by anti-EDN3 ELISA kit. Data represent mean values ± SD of triplicate.
transports, cell division, and cell survival pathway. Concomitantly, a series of genes that provides negative regulation of development, cell growth, and differentiation were identified (e.g., IFI27, SOCS3, MTUS1, SCML1, IFI16, LUZPI, IL6ST, JMJDC1, TEAD1, LINGO1, C9orf86, PSD3, and USP10), suggesting the stemness properties and cellular quiescence in GSCs were disturbed. The distinctive gene expression profiles of randomly selected genes were verified by qRT-PCR (Supplementary Fig. S5). Correspondingly, GSEA identified 97 significant gene ontology (GO) clusters (Supplementary Table S1), mostly associated with microtubule cytoskeleton organization, cell division, motor activity, spindle, DNA packaging, leading edge, transcription repressor activity, negative regulation of DNA binding, detection of chemical stimulus, cell-cycle arrest, and neuron development, offering a potential molecular explanation for how loss of EDN3/EDNRB signaling may impact GSC migration, self-renewal, cell survival, and

Figure 5. (Continued) measurements in triplicate experiments. *P < 0.05, **P < 0.001 versus control siRNA transfected cells. D, cell apoptosis in negative control siRNA or EDN3 siRNA transfected cells was determined by a cell death detection ELISA kit. Data represent mean values ± SD of triplicate measurements in triplicate experiments. *P < 0.05 versus control siRNA-treated cells. E, light-microscopic morphology of indicated cells treated with assay buffer (a, e, i, m), transfection reagents (b, f, j, n), negative control siRNA (c, g, k, o), and EDN3 siRNA-1 (d, h, l, p) for 72 hours, as well as negative control siRNA for 7 days (q-s). Scale bar = 25 or 50 μm. F, representative photographs of hematoxylin and eosin (H&E) staining of mouse brain tissue. Brain tissues from mice injected with untreated GSCs display invasive growth of gliomas with diffuse infiltration into the surrounding tissue and vessels (a–f). Infiltrating tumor exhibits hypercellular zones surrounding necrotic foci and forms a “pseudo-palisading” necrosis pattern (S496, frozen section; a–d). Other important histopathologic features of glioblastoma are also seen in tumor lesions, including hypercellularity (e), hyperchromatism (f, g), pleomorphism and mitosis (h–j), vascular endothelial hyperplasia (k), and oligodendroglial component (l); D431, S496, E445. Mouse brains injected with GSCs pretreated with 50 μmol/L BQ788 showed no evidence of tumor development (m–r). Mouse brain injected with stem cell media serves as control for normal brain tissue (s–u). Magnification, 4× (o, s), 8× (a, m), 100× (e, f, p, t, u), 200× (b, c, g, n, r, v), 400× (d, h–l). G, presence of EDN system components in glioma xenografts. The expression of the indicated EDN system components in both subcutaneous and intracranial glioma xenografts were analyzed by qRT-PCR with specific primers. β-Actin was used as an internal control gene (a). Representative immunohistochemical stainings of EDN components in intracranial tumor xenografts (b). The mRNA levels of the EDN system components in uncultured CD133+ and CD133− cells directly sorted from the glioma xenografts were analyzed by qRT-PCR (c). The expressions of EDN system transcripts in CD133+ cells recultured in SGF media for 7 days (d).
EDN3 is a survival factor for GSCs. A, the indicated GSC lines were cultured in the plain media supplemented with various doses of EDN3 as indicated. Cells were incubated for 3 days and viability was determined by manually counting the trypan blue-stained samples in a hemacytometer chamber and present as percentage of viable cells in cultures. The cell growth was determined by MTS/PMS cell proliferation assays and present as fold increase compared with nontreated cells. Data represent mean values ± SD of triplicate measurements in triplicate experiments. **P < 0.05 versus cells cultured in plain media. B, cell apoptosis in (A) was assayed by a cell death detection ELISA kit. Data represent mean values ± SD of triplicate measurements in triplicate experiments. *, P < 0.05 versus D431 and S496 GSCs treated with 1, 30, 100, 1000 nmol/L EDN3 and SGF media; **, P < 0.05 versus E445 cells treated with 1, 30, 100 nmol/L EDN3 and SGF media. +, P < 0.05 versus D431, S496, and E445 cultured in SGF media. C, light-microscopic morphology of cell cultures in (A). Scale bar = 25 μm. D, cell survival was assayed in sorted CD133+ and CD133− GSC cells cultured in the SGF, plain media, or plain media supplemented with various doses of EDN3 as indicated. Cells were incubated for 7 days and viability was determined by trypan blue staining. Data represent mean values ± SD of triplicate measurements in triplicate experiments. *, P < 0.05, **, P < 0.01 (CD133+ cells), Δ, P < 0.05, ΔΔ, P < 0.01 (CD133− cells) versus cells cultured in plain media.
tumorigenic capacity. We used the default parameters of GSEA and Gene Ontology gene sets were permuted 1,000 times. We called a gene set significant if the FDR Q value was under 0.15. BQ788 works by potently and competitively inhibiting EDN3 binding to EDNRB; therefore, it may explain that downregulation of EDNRB transcripts by BQ788 treatment was not determined either by expression microarray or RT-PCR (data not shown). However, several GSC genes (e.g., EFEMP, IQGAP1, SOCS3, WAVE2, MYO10, and K-RAS) downregulated by BQ788 treatment have been previously reported to be linked to activation of mitogen-activated protein kinase (MAPK) pathways (Supplementary Table S2), which are the downstream effector pathways of EDNRB signaling pathway (38–43), suggesting that the identified genes characterizing stemness, migration, and survival of GSCs are regulated by EDN3/EDNRB signaling.

Discussion

The finding of EDN3 overexpression in GSCs through the expression microarray analysis led to our novel investigation of the EDN system in cultured GSCs. In normal NCC cultures, EDN3/EDNRB signaling prevents the premature differentiation of crest-derived precursors (22, 44). In vivo, EDN3/EDNRB signaling is required for NCC migration during ENS development (22). Mutations in EDN3 or EDNRB can lead to the abnormal development of ENS and melanocytes, implying that the loss of function in these 2 genes may interfere with tumor development by EDN3/EDNRB signaling-dependent GSCs. Indeed, blockade of EDN3/EDNRB signaling of GSCs leads to the loss of tumorigenic potential in our GSC model. We found that cultured GSCs express both neural and mesenchymal signatures characterizing NCC-like cells and, therefore, hypothesized that autocrine EDN3/EDNRB signaling may be a survival/maintenance factor for GSCs. In fact, when GSCs undergo serum-induced proliferative differentiation, a significant loss of EDN3 production accompanied by codownregulation of a series of transcripts related to NCCs, RGCs, and NSCs was observed. For instance, SOX2 is a marker of NSCs and is essential for maintaining the pluripotent, self-renewal, and undifferentiated phenotypes of embryonic stem cells. Moreover, silencing of SOX2 in GSCs can cause the loss of tumorigenicity (45). FABP7, a glial-specific marker, is a direct target of Notch signaling in RGCs, and the involvement of Notch signaling in the maintenance of the tumorigenic potential of GSCs has been reported (46). Thus, autocrine EDN3/EDNRB signaling may prevent GSCs from differentiating prematurely.

Direct evidence of the differentiation-inhibiting effect of EDN3 was shown in clonogenic cultures of ENS progenitors supplemented with EDN3, by which neuronal and glial differentiation pathways were blocked and the multipotent state of progenitors was maintained (44, 47). These observations thus support the view that autocrine EDN3 maintains the undifferentiated state of GSCs. Moreover, EDN3 and EDN1 mRNAs are altered reciprocally in GSCs in response to differentiation, indicating that EDN3 and EDN1 genes can be epigenetically regulated, and that increased EDN1 may be associated with the onset of tumorigenesis. Indeed, we detected an increased expression of EDN1, but not EDN3, mRNA in patient glioblastoma tumors (compared with GSCs), suggesting that EDN3 may play a tumor suppressor-like role to maintain the quiescence and tumorigenic potential of GSCs. In agreement with this, a frequent loss of EDN3 expression in breast tumors due to epigenetic inactivation has previously been suggested (29). Likewise, there were decreased levels of EDN3 and increased levels of EDN1 and EDN2 mRNA in cancerous cervical epithelial cells compared with normal cervical epithelial cells (28). Thus, the reduction of EDN3 levels (e.g., induced by exogenous cues) may implicate the initiation of proliferative differentiation from quiescent GSCs toward more differentiated progeny, by which the proliferative progeny gradually overpopulate a tumor. It seems that sole EDN3/EDNRB signaling alone cannot grant tumorigenic potential to GSCs, yet the removal of EDN3/EDNRB signaling contributed undifferentiated, antiapoptotic, and migratory properties to GSCs, which may weaken or eliminate their tumorigenic capacity. Autocrine EDN3 in EDNRB−/− GSCs, may therefore, play a role in antidevelopmental, antidifferentiating, antiapoptotic, and antitumorigenic functions, thereby help in maintaining a continuous GSCs pool.

We found that loss of EDN3 production in GSCs through serum-induced differentiation did not cause cell apoptosis, whereas directly blocking EDN3/EDNRB signaling causes severe GSC apoptosis. These observations seem to support the notion that autocrine EDN3/EDNRB signaling only provides survival benefits when GSCs are maintained in SGF condition. A previous study has shown that stimulation of the EDNRB in astrocyte induces cAMP response element-binding protein (CREB) and c-fos expression via multiple MAPK signaling pathways, including the extracellular regulated kinase (ERK) 2, c-Jun N-terminal kinase (JNK1), and p38 kinase (38). EDN3 stimulation also rapidly increased phosphorylation of ERK2 in neural progenitor cells (39) and activated IkappaB and MAPK in the human colonic epithelial cells (40). Likewise, EDN3 treatment resulted in the activation of MAPK-p90 ribosomal S6 kinase-CREB and cAMP-protein kinase A-CREB pathways in melanocyte culture (41, 42) and induction of ERK1/2 and focal adhesion kinase phosphorylation in melanoma cells (43). One important note is that the activation of EDNRB by EDN3 also leads to loss of expression of the cell adhesion molecule E-cadherin and associated catenin proteins, while increasing Snail and N-cadherin expression (43). Thus, the expression profiles of BQ788-treated GSCs supports the notion that these pathways are the downstream effector pathways of EDN3/EDNRB signaling pathway that contribute GSC survival and migration (Supplementary Table S2). The usage of an inducible knockdown of EDNRB would strengthen the role of EDNRB signaling in GSCs. Moreover, because ex vivo treatment with BQ788 may already
Table 1. Genes expressed at lower levels in BQ788-treated GSCs compared with untreated GSC

<table>
<thead>
<tr>
<th>Gene ID</th>
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<th>Fold change</th>
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<td>CCDC75</td>
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<td>0.000894</td>
<td>Unknown</td>
</tr>
<tr>
<td>11031</td>
<td>RAB31</td>
<td>1.62</td>
<td>0.001205</td>
<td>Vesicle and granule targeting</td>
</tr>
<tr>
<td>51773</td>
<td>RSF1</td>
<td>1.57</td>
<td>0.002448</td>
<td>Chromatin remodeling</td>
</tr>
<tr>
<td>55537</td>
<td>VPS35</td>
<td>1.54</td>
<td>0.000354</td>
<td>Subunit of the retromer, vesicle transport</td>
</tr>
</tbody>
</table>

NOTE: Probe set signals on the expression array that were <1.5-fold lower in BQ788 treated (n = 3 patients) versus untreated GSC samples (n = 3 patients, sample duplicate) with a pairwise t test (P < 0.05) were selected. Samples were permuted 100 times by dChip and 61 genes with median FDR = 11% were obtained.
damage the capabilities of self-renewal and proliferative differentiation in GSCs, inducible knockdown of EDNRB or EDN3 using viral-delivered hairpin RNA would allow more in depth functional and in vivo studies.

It has been shown that EDN3 first stimulates expression of EDNRB, then when under prolonged exposure to EDN3, EDNRB expression decreases (12). This may explain why EDNRB are expressed at lower levels in the sphere cultures, which consistently produce EDN3. We found that EDN3 alone is not a potent mitogen for GSCs and similar finding was also reported (44). Interestingly, it was shown that EDNRB antagonists reduced the viability and proliferation of glioma cells, which do not express EDN3 (48), implying the possibility of blocking EDN1/EDNRB signaling. The decreased glioma cell viability by EDNRB antagonists independent of their cognate receptor was also reported (49). We speculate that low levels of EDNRB mRNA being expressed in cultured CD133⁺ GSCs used for injection, but not in some xenografts, could be due to tumor initiation from CD133⁺ GSCs being accompanied by activation/upregulation of EDNRA signaling and downregulation of EDNRB signaling in our model system. Alternatively, it is possible that EDNRB may be only expressed by a small subset of quiescent GSCs that express the tumor-suppressive phenotype (e.g., CD133⁻/EDNRB⁺ cells) for maintaining GSCs pool in vivo, which can be retrieved and enriched by SGE culturing. On the other hand, CD133⁺ cells sorted from xenografts may mostly contain activated GSCs (e.g., CD133⁻/EDNRB⁻/EDNRA⁺ cells) that have entered the pathway to tumorigenesis. Interestingly, some tumor xenografts express high levels of EDN3 and EDNRA mRNA, but low levels of EDN1 and EDN2 mRNA, with a negligible level of EDNRB mRNA, suggesting that the growth of EDN3⁺ tumor may not be EDNRB, but EDNRA signaling dependent. Promoter hypermethylation of the EDNRB gene in various human malignancies has been reported and suggested that EDNRB may be a candidate tumor suppressor gene (50).

Finally, our genome-wide expression analysis has provided some molecular explanation for the requirement of EDN3/EDNRB signaling in maintaining GSC sphere cultures. Apparently, EDN3/EDNRB blockade mostly impacts cell structure, cell movement, self-renewal/cell division, and cell survival, rendering GSCs nontumorigenic. We previously showed that CD133⁻ cells, not CD133⁺ cells, sorted from same sphere cultures contain enriched tumorigenic cells, yet they express tumor suppressor phenotype (8). Thus, true GSCs may be quiescent before undergoing asymmetric cell division to simultaneously self-renew and generate more differentiated progeny. It seems that differentiated progeny are active-growing cells that make up the most population in tumor spheres and are likely the effector progeny which can undergo proliferative differentiation to produce more differentiated progeny that expresses hyperproliferative and hyperangiogenic phenotype, leading to tumor formation in animals. Therefore, identifying essential genes (e.g., EDN3) which are shared by both GSCs and their immediate differentiated progeny/daughter cells would enhance its clinical value because both populations can be targeted. Thus, treatment with BQ788 not only abolishes the self-renewal capacity of quiescent GSCs but also prevents differentiated progeny from populating tumor spheres and forming tumor in animals.

Depletion of EDN3⁺ cells will likely prevent either CD133⁻ or CD133⁺ GSCs from regenerating a tumor. Futures studies should determine whether addition of EDNRB antagonist can sensitize cells to radiochemotherapy in treatment of established glioblastoma tumors in animals. Our data support the view that the prevention of GSC-mediated tumor recurrence may need to focus on targeting active stem cell pathways in GSCs (such as EDN3/EDNRB pathway), not proliferative pathways. Obviously, the cure for brain cancer requires eliminating both GSC and non-GSC populations; therefore, it is important to evaluate the synergistic benefits of incorporating GSC-targeted therapies into conventional cancer treatments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by grants from the American Cancer Society (RSG-07-109-01-CCE), National Cancer Institute (1 R21 CA149912-01), NIH (1DP2OD006444-01), and The Bradley Zankel Foundation to C-L Tso. 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.

Received December 12, 2010; revised September 21, 2011; accepted October 2, 2011; published OnlineFirst October 19, 2011.

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www.aacrjournals.org Mol Cancer Res; 9(12) December 2011 1683

Published OnlineFirst October 19, 2011; DOI:10.1158/1541-7786.MCR-10-0563

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