Cancer Biology and Signal Transduction

RRAD Promotes EGFR-Mediated STAT3 Activation and Induces Temozolomide Resistance of Malignant Glioblastoma

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Abstract

Glioblastoma multiforme (GBM) is an extremely aggressive brain cancer with a median survival of less than 2 years. GBM is characterized by abnormal activation of receptor tyrosine kinase and constitutively activated STAT3. Although EGFR phosphorylation and STAT3 activation are essential for the maintenance of GBM cancer stem cells, the molecular mechanism underlying endosome-mediated STAT3 activation is not fully understood. In the current study, we showed that GTP-binding protein RRAD (RAS associated with diabetes, RAD) physically associates with EGFR, and EEA1, enhancing the stability and endosome-associated nuclear translocation of EGFR. Functionally, RRAD contributes to the activation of STAT3 and expression of the stem cell factors OCT4, NANOG, and SOX2, thereby enhancing self-renewing ability, tumor sphere formation, EMT, and in vivo tumorigenesis. Most importantly, RRAD contributes to poor survival in patients with GBM. RRAD expression is correlated with temozolomide resistance, and, conversely, depletion of RRAD leads to sensitization of highly temozolomide-resistant GBM cells. Our data collectively support a novel function of RRAD in STAT3 activation and provide evidence that RRAD acts as a positive regulator in the EGFR signaling pathway. These results demonstrate a critical role for RRAD in GBM tumorigenesis and provide a rationale for the development of pharmacologic inhibitors of RRAD in GBM.

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Introduction

Glioblastoma multiforme (GBM) is an extremely aggressive common adult brain tumor. The current standard therapy for GBM includes surgery, radiation, and treatment with temozolomide. Despite improvements in the standard treatment regimens, the survival rate for GBM is only about 9.8%, and most patients eventually experience recurrence (1). Cancer stem cells, a cellular subpopulation with sustained self-renewal and differentiation potential, are responsible for tumor initiation, propagation, recurrence, and treatment resistance (2–4). Expression patterns of surface markers, including CD133, CD15, CD44, L1CAM, CD49f, A3B5, and EGFR collectively support the existence of highly tumorigenic cancer stem cells. However, surface markers display variable expression according to cell-cycle status or environmental conditions (5, 6). Therefore, the stem-like function is more appropriate to distinguish cancer stem cells. The functional properties of cancer stem cells include the ability to form spheres, self-renew and differentiate, and survive drug toxicity. GBM is characterized by abnormal activation of receptor tyrosine kinase (RTK) signaling pathways, and constitutively activated STAT3 is frequently coexpressed with EGFR in high-grade gliomas (7). STAT3 activation is known to be essential for the maintenance of GBM cancer stem cells (8). The cytoplasmic STAT3 protein is recruited to activated receptors and subsequently phosphorylated at Tyr705 by the receptor kinase or an associated kinase. EGFR can serve as a scaffold for trafficking of STAT3 (9). Phosphorylated STAT3 colocalizes with receptor–ligand complexes on the endosome and is transported from the plasma membrane to the perinuclear region (10). Data from the current study support a novel function of RRAD (RAS associated with diabetes, RAD) in STAT3 activation and GBM malignancy, induced through physical interactions of RRAD with EGFR/STAT3/EEA1 and endosome-mediated nuclear translocation of EGFR.

RRAD is a Ras-related GTPase encoded by a gene located at human chromosome 16q22, which is initially identified by subtractive hybridization and selectively overexpressed in type II diabetic muscle as compared with muscle of nondiabetic or type I diabetic individuals (11). RRAD differs from the other Ras-related GTPases in a number of properties, including lack

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of characteristic domains such as prenylation motifs, a GTP-binding domain, and NH2- and COOH-terminal extensions (12). RRAD participates in CaMKII signaling cascade, where it interacts with calmodulin, calmodulin-dependent protein kinase II, and tropomyosin (13, 14).

Ras signaling activates the downstream effectors RAF, MEK and ERK, and other signaling modules, including PI3K–mTOR–Akt, TIAM1–Rac, Ral, and PLC–PKC. Therefore, there is a strong rationale for the use of the Ras signaling pathway for developing therapeutic interventions. Clinical trials are ongoing to evaluate the efficacy of Ras–RAF inhibitors in multiple cancer types. In malignant gliomas, however, somatic mutations of Ras or RAF are very rare (15–17). Primary GBM tumors are reported to express significantly lower levels of K-Ras and H-Ras transcripts, compared with normal brain tissues, and all tissues do not express detectable levels of Ras proteins (18). K-Ras/H-Ras expression levels are not associated with survival in the GBM cohort (18). Interestingly, inhibition of RAF and STAT3 has a cumulative prognostic impact in human GBM, signifying an additive effect of the two independent signaling pathways (19).

Previously, we and others showed that RRAD expression is positively correlated with malignant progression (20–22). The roles of RRAD are attributed to its ability to inhibit tumor suppressors, nm23 or GCIP, via direct interactions (20, 21). However, these findings do not fully explain how RRAD enhances tumor initiation and treatment resistance. Particularly, the possible role of RRAD in explaining how RRAD enhances tumor initiation and treatment resistance is yet to be established. In this study, we evaluated RRAD expression in GBM, and addressed a positive correlation between RRAD level and tumor malignancy. RRAD promotes malignant progression and enhances resistance to temozolomide via endosome-mediated EGFR/STAT3 signaling.

Materials and Methods

Cell culture and reagents

Human GBM cell lines (U87-MG, U138-MG, U251, LN229, A172, and D85BR-05MG) were purchased from the ATCC and no further authentication was done. Adherent cells were maintained in DMEM with heat-inactivated 10% FBS, penicillin, and streptomycin (Gibco BRL). Tumor spheres were cultured in serum-free DMEM/ F12 (Invitrogen) supplemented with basic fibroblast growth factor (20 ng/mL; Invitrogen), EGF (20 ng/mL; BD Biosciences), and N2 supplement (1×; Invitrogen). Temozolomide, cycloheximide, and Dynasore were obtained from Sigma. Cells were placed in an incubator under conditions of 37°C with 5% CO2.

Spheres formation

Cells were resuspended in DMEM/F12 containing 20 ng/mL EGF, bFGF, and N2 supplement (1×) as the stem cell-permissive medium. Spheres were collected after 5 to 7 days, and protein extracted for additional experiments or dissociated with Accutase (Invitrogen).

Plasmids, transfection, and antibodies

Full-length RRAD was cloned from HeLa mRNA for Flag-tagged cloning into pCMVTag2B (Clontech). To establish stable cell lines overexpressing RRAD (C5 and C9), LN229 cells were transfected with a control vector or that containing Flag-tagged RRAD (21). Successfully transfected cells were selected, and overexpression of RRAD confirmed with immunoblotting using an anti-Flag antibody. Expression of RRAD was in the order: C5>C9>vector. The 21-nucleotide-long siRNAs corresponding to RRAD (siRRAD#1 sense, 5′-GCAAGUGC-CAUUUGAGAGCU-3′; antisense 5′-GAUGUCU-CAGAACUUGCU-3′; siRRAD#2, sense 5′-GGAAGG-GAAAGGCAUUA-3′; antisense 5′-UGAUGC-CUCUCUCUGCU-3′) and control siRNA (siC) were purchased from Dharmacon (Thermo Scientific). STAT3-CA (Flag-tag) plasmid was obtained from Addgene. Cells were transfected with siRNA using jetTene(Qagen). Antibodies against EGFR, STAT3, α-tubulin, PARP, β-actin, and Twist (Santa Cruz Biotechnology), and Vimentin (BD Biosciences) were purchased. Antibodies against RRAD, OCT4, NANOG, and SOX2 (Abcam) and -actin, and Twist (Santa Cruz Biotechnology), and Vimentin (BD Biosciences) were purchased. Antibodies against RRAD, OCT4, NANOG, and SOX2 (Abcam) and were purchased from Dharmacon (Thermo Scientific). STAT3-CA (Flag-tag) plasmid was obtained from Addgene. Cells were transfected with siRNA using jetTene(Qagen). Antibodies against EGFR, STAT3, α-tubulin, PARP, β-actin, and Twist (Santa Cruz Biotechnology), and Vimentin (BD Biosciences) were purchased. Antibodies against RRAD, OCT4, NANOG, and SOX2 (Abcam) and -actin, and Twist (Santa Cruz Biotechnology), and Vimentin (BD Biosciences) were purchased. Antibodies against RRAD, OCT4, NANOG, and SOX2 (Abcam) and were purchased from Dharmacon (Thermo Scientific).

Cell fractionation

For subcellular fractionation, cytosol and nuclear fractions were prepared as described previously (9). Cells were lysed in lysis buffer (20 mmol/L HEPES, pH 7.0, 10 mmol/L KCl, 2 mmol/L MgCl2, 0.5% Nonidet P-40, protease inhibitor mixture). Recovered nuclei were lysed in hypertonic buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L Tris, pH 8.0, 0.5% Nonidet P-40, protease inhibitor mixture), and the nuclear fraction collected after centrifugation at maximal speed.

Immunoprecipitation

For immunoprecipitation, LN229 cells transfected with pCMVTag2B-RRAD were washed with cold PBS and lysed in buffer (20 mmol/L HEPES, pH 7.0, 150 mmol/L NaCl, 1 mmol/L EDTA, 2 mmol/L β-glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail). Anti-EGFR antibody was incubated with cell lysates overnight at 4°C. Immune complexes were pulled down by the addition of protein A/G agarose. After washing with lysis buffer, immune precipitates were analyzed via SDS-PAGE and immunoblotting.

GST pull-down assay

RRAD cDNA was cloned in-frame into pGEX4T-1 (Amersham) to produce the GST fusion protein, GST-
RRAD, for the GST pull-down assay. GST and GST-RRAD fusion proteins were immobilized on glutathione-Sepharose beads (Amersham) and incubated with U251 lysates overnight. Beads were washed four times, and bound proteins eluted with SDS loading buffer containing 5% β-mercaptoethanol, followed by SDS-PAGE and immunoblotting.

**Cell growth assessment and soft-agar colony formation assay**

To assess cell numbers, an equal volume of 0.4% (w/v) Trypan blue was added to each cell suspension, and viability determined on the basis of the ability of live cells to exclude the vital dye. Viable cells were counted using a hemocytometer. To examine anchorage-independent growth, cells (1 × 10^5) were suspended in 0.4% top agar over a bottom layer of 0.8% base agar in 6-well plates. The solidified soft agar was overlaid with DMEM containing 10% FBS, and the medium changed every 4 to 5 days. Colonies were visualized by staining with 0.005% (w/v) crystal violet and colonies (defined as groups containing a minimum of 50 cells) counted under a phase-contrast microscope.

**Phospho-RTK antibody array**

Cells were plated and lysed in NP-40 lysis buffer (1% NP-40, 10% glycerol, 20 mmol/L Tris-HCl, pH 8, 137 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, and protease inhibitors). Cell lysates (250 μg) were incubated overnight on RTK array membranes (ARY-001, R&D Systems). After binding RTKs on the membrane, unbound molecules were washed away. A pan-anti-phospho-tyrosine antibody conjugated to horse-radish peroxidase was used to detect phosphorylated tyrosine on activated receptors with the ECL method.

**Clonogenic survival assay**

Cells were seeded in 6-well plates (500 cells/well) and exposed to temozolomide (10–300 μmol/L) for 48 hours, followed by further observation for 7 to 14 days. Cell densities or colonies were assessed after crystal violet staining. Colonies of more than 50 cells were counted. The dye was subsequently extracted with 10% acetic acid and absorbance determined using spectrophotometry (570 nm).

**Immunocytochemistry**

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. After fixation, cells were incubated with the appropriate primary antibodies in a solution of PBS with 3% BSA at 4°C overnight. Anti-EGFR mouse polyclonal (1:500) and anti-Flag rabbit polyclonal (1:200) antibodies were employed for analysis. Staining was visualized using anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 (Molecular Probes). Nuclei were counterstained using 4′,6-diamidino-2-phenylindole (DAPI). Stained cells were visualized under a fluorescence microscope (Carl Zeiss, LSM 700).

**Transwell migration assay**

Cells (1 × 10^5) were loaded into the top chamber of Transwell plates (8 μm pore size; Corning Costar). FBS (10%) was used as a chemoattractant in the bottom chamber. After incubation for 24 hours, cells in the bottom chamber were fixed and stained with 0.005% (w/v) crystal violet. The number of migrated cells was quantified by counting those in five random fields of each membrane.

**Evaluation of tumorigenicity**

To examine the effect of RRAD on tumor formation, adherent cells (3 × 10^6) or tumor spheres (3 × 10^5) were implanted subcutaneously into 6-week-old male BALB/c nude mice and tumor growth monitored using calipers. Tumor volume was calculated using the formula: \( V = \frac{4}{3} \pi \frac{a^3}{b} \), in which \( a \) is the long axis and \( b \) the short axis in millimeters.

**Patient datasets and data analysis**

All glioma patient data were publicly available in the deidentified form and obtained from the NCI Repository for the Molecular Brain Neoplasia Data (REMBRANDT) database. Differences between the groups were analyzed with the log-rank \( P \) value. Graphs were created using Rembrandt data for Affymetrix probes 204803 with the highest geometric mean intensity and associated survival data. Up (high)- and downregulation (low) among glioma specimens refers to a 2-fold (or greater) change in RRAD expression, compared with specimens from patients with nonglioma.

**Statistical analysis**

Data in the graphs represent mean ± SD of values from at least three independent measurements. To determine the differences in mean values, Student \( t \) test was applied. Intergroup comparisons were made with the paired two samples \( t \) test. Differences were considered significant at \( P < 0.05 \).

**Results**

**RRAD expression is upregulated in glioblastoma and associated with poor survival**

Previously, we showed that RRAD enhances malignant progression in prostate cancer (21). Accordingly, we examined whether the RRAD protein is upregulated in the GBM cell lines, including U87-MG, U138-MG, U251, LN229, A-172, and DBTRG-05MG. Notably, cellular RRAD protein levels were upregulated in malignant cells, compared with normal control (Fig. 1A, \( P < 0.05 \)). We further validated RRAD overexpression in human glioma specimens. As shown in Fig. 1B, RRAD expression levels were enhanced in 6 of 6 tumor samples, compared with normal tissue controls (tumor vs. N1; \( P < 0.05 \)). All glioma patient data were publicly available in the deidentified form and obtained from the NCI Repository for the Molecular Brain Neoplasia Data (REMBRANDT) database. Using the database, we evaluated the survival rates of patients according...
to their RRAD mRNA levels (Fig. 1C). Specifically, data were analyzed to determine the survival of 343 patients with intermediate, low or high RRAD expression. Overall, 37 patients displayed more than 2-fold RRAD upregulation, 233 patients displayed intermediate RRAD expression, and 73 had more than 2-fold downregulation. Median survival of patients with upregulated, intermediate, and downregulated expression in glioma specimens was 297, 540, and 606 days, respectively. We observed significant differences in terms of survival in high RRAD versus intermediate RRAD ($P < 0.0001$) and high RRAD versus low RRAD populations ($P < 0.0001$). No significant differences were evident in terms of survival between low RRAD and intermediate RRAD populations ($P = 0.2458$). To understand whether RRAD expression was associated with any particular glioma subtype, we compared the expression of RRAD among oligodendroglioma ($n = 49$), astrocytoma ($n = 104$), mixed ($n = 6$), and GBM ($n = 185$). Expression of RRAD was significantly higher in GBM, compared with other lower grade glioma (Fig. 1C, b and Supplementary Fig. S1).

To address whether RRAD expression is correlated with genetic alteration, we analyzed mRNA expression in high RRAD (36 cases) and low RRAD (73 cases) glioma samples. Our results disclosed several genes preferentially upregulated in high RRAD population (Supplementary Fig. S1). These genes included genes that increase cell proliferation (CHI3L1, PTX3, ANXA1), invasion (PDPN, LOX), metastasis (NNMT), inflammation (CCL2), and mesenchymal signature (TIMP1, IL13RA2). To identify a putative group in which RRAD has a strong impact on survival, we divided patients into four percentiles and analyzed survival for the four independent groups. As shown in Supplementary Fig. S2, RRAD expression $>75$th percentile was correlated with lower survival ($P = 0.0027$). Collectively, the data show that high expression of RRAD is significantly associated with GBM and predicts a poor prognosis of glioma.

**RRAD enhances EGFR protein stability and EGFR-induced STAT3 activation**

Glioblastoma is characterized by the abnormal activation of RTK signaling pathways (7). To identify RRAD-
associated RTKs, we screened a human phospho-RTK array using whole-cell lysates from vector-(LN229-Vector) and RRAD-(LN229-RRAD)-transfected cells. Among multiple RTKs, the spot intensity of phosphorylated EGFR was the most significantly enhanced by RRAD (Fig. 2A). Previous studies have reported that STAT3 activation in GBM is commonly induced by growth factor receptors on the cell surface (23). EGFR signaling induces STAT3 activation in GBM, and phosphorylation of EGFR at tyrosine 845 is essential for activating STAT3. Consistent with these findings, we showed that RRAD promotes phosphorylation of EGFR and STAT3 (Fig. 2B). We did not observe significant differences in total EGFR expression during phosphorylation by EGF. Moreover, RRAD physically associates with EGFR, as observed from coimmunoprecipitation experiments (Fig. 2C). As RRAD does not exert kinase activity to phosphorylate EGFR, we assessed for changes in the stability of EGFR protein by treating LN229-Vector and LN229-RRAD cells with cycloheximide (Fig. 2D). To assess the turnover rate of EGFR, the protein half-life was measured at various time points after cycloheximide treatment. The EGFR level declined more slowly in LN229-RRAD, compared with LN229-Vector (ratio of EGFR to actin expression at 9 hours for LN229-RRAD vs. LN229-Vector: 0.85 vs. 0.18; \( P = 0.0002 \)).

To rule out the possibility of clonal selection, experiments were repeated using U87-MG after transient transfection as well as in the context of RRAD targeting. As shown in Supplementary Fig. S3, transient overexpression of RRAD promoted phosphorylation of EGFR, and conversely, depletion of RRAD effectively reduced EGFR phosphorylation.

**RRAD enhances endosome-mediated EGFR translocation to the nucleus**

RTKs, including IGF, HGF(c-Met), FGFR, VEGFR, and the EGFR family, have been shown to localize to the nucleus (23, 24). Cell surface EGFR translocates to the inner nuclear membrane through nuclear pore complexes, which is mediated by importin-\( \beta \) and endosome (25, 26). Phosphorylated STAT3 colocalizes with receptor–ligand complexes on the endosome and is transported from the plasma membrane to the perinuclear region (10). RRAD associates with importin-\( \beta \) through three conserved NLS regions (27). Surprisingly, in our experiments, the membrane-bound early endosomal marker, EEA1, also coprecipitated with RRAD (Fig. 3A).
To examine whether RRAD affects subcellular location of EGFR, cell lysates were fractionated and subjected to immunoblot analysis. The results indicate that EGFR translocates to the nucleus in the presence of RRAD (Fig. 3B). Immunofluorescence staining was performed to further investigate whether EGFR associates with RRAD in vivo. EGFR, which is normally localized on the plasma membrane in the absence of EGF, was frequently detected in the nuclear envelope after EGF stimulation (25). Consistent with cell fractionation data, we observed prominent perinuclear and nuclear colocalization of RRAD with endogenous EGFR in vesicle structures (Fig. 3C).

Next, we examined the involvement of endosomal sorting in RRAD-induced EGFR nuclear transport by investigating whether inhibition of endocytosis can block this process. Dynasore is a small molecule that prevents receptor internalization (28). RRAD promoted the nuclear expression of EGFR, as shown with immunoblotting (Fig. 3B), which was abrogated with Dynasore treatment. After EGF stimulation, EGFR and EEA1 colocalized as nuclear and perinuclear structures in the presence of RRAD (Fig. 3D). RRAD-induced upregulation of nuclear EEA1 was confirmed by subcellular fractionation and immunoblotting (Fig. 3E). The data collectively suggest that RRAD...
enhances the endosome-mediated nuclear translocation of EGFR.

**RRAD expression is correlated with STAT3 activation and malignancy-associated properties**

In GBM, aberrant STAT3 activation is correlated with tumor grade and clinical outcomes (29). Activated STAT3 enhances the expression of a number of downstream genes and regulates multiple behaviors of tumor cells, such as survival, growth, angiogenesis, and invasion. Malignant progression of cancer involves loss of cell–cell interactions together with acquisition of migratory properties, and is often associated with epithelial–mesenchymal transition (EMT; ref. 30). Upon overexpression of RRAD, the p-STAT3 (Y705) level was increased, and EMT-associated proteins, including N-cadherin, Twist, and Vimentin, were upregulated (Fig. 4A). In parallel, RRAD depletion reduced STAT3 phosphorylation and expression of TWIST (Supplementary Fig. S4). To address the requirement for RRAD in maintaining the tumorigenic capability of GBM cells, we examined the anchorage-independent colony-forming ability according to RRAD overexpression. As shown in Fig. 4B, overexpression of RRAD led to a marked increase in colony-forming ability in soft agar, whereas RRAD depletion in highly aggressive U251 cells diminished colony formation (Fig. 4C). To clarify whether changes in colony formation are associated with differences in proliferation and/or survival, Trypan blue exclusion staining was performed. As shown in Supplementary Fig. S5, depletion of RRAD led to decreased proliferation and survival of GBM cells. In addition, Transwell migration was upregulated in cells with higher RRAD expression (Fig. 4D), but diminished upon RRAD depletion (Fig. 4E). To ascertain whether the effect of RRAD is EGFR-dependent, the Transwell migration assay was performed in the presence of the EGFR inhibitor, gefitinib. As shown in Fig. 4F, the EGFR inhibitor effectively blocked RRAD-induced colony formation and migration, clearly indicating an EGFR-dependent effect of RRAD. Consistent with these results, the scratch assay revealed that RRAD increases tumor cell motility (Supplementary Fig. S6). Taken together, our findings

![](image_url)
indicate that RRAD enhances the malignant properties of human GBM cells through STAT3 activation.

**RRAD promotes self-renewal and sphere formation of glioblastoma cells**

In addition to invasion and metastasis, EMT is often associated with self-renewal and tumor-initiating capability (31). EGFR and STAT3 activation are frequently implicated in the stem cell properties of GBM (7). Stem cell transcription factors, such as SOX2, OCT4 and NANO2, are critical for maintaining self-renewal, proliferation, survival, and multilineage differentiation potential of GBM stem cells (32). Expression analysis of EMT-regulating (Rex1 and Snail1) and stemness-regulating transcription factor (OCT4, NANO2, and SOX2) levels disclosed upregulation of these factors with RRAD overexpression (Fig. 5A). As tumor sphere formation is based on the unique properties of stem/progenitor cells to survive and grow in serum-free suspension, we performed the tumor sphere formation assay to examine whether RRAD enhances self-renewal of GBM. Our data showed that RRAD enhances sphere formation in a concentration-dependent manner (Fig. 5B). The limiting dilution sphere-forming assay demonstrated that RRAD-expressing LN229 (C5) cells generate

![Figure 5](image-url)
one tumor sphere in 12.4 cells, whereas vector-expressing cells generate one sphere in 64 cells (Fig. 5B). Moreover, depletion of RRAD attenuated the expression of stem cell transcription factors, OCT4 and SOX2, the lineage marker, GFAP, and EMT proteins, TWIST and Vimentin, as shown in Fig. 5C. Previous studies have shown that GBM stem cells are enriched with the CD44$^+$ cell population (33, 34). FACS analysis revealed that the percentage of CD44$^+$ cells increases with RRAD expression, but is markedly reduced upon depletion of RRAD (Supplementary Fig. S7).

Notably, RRAD knockdown diminished the ability of GBM to form tumor spheres (Fig. 5D). As shown in Fig. 5D, the limiting dilution sphere-forming assay demonstrated that RRAD-depleted U87-MG cells generate one tumor sphere in 55.2 (siRRAD#1) or 63.3 (siRRAD#2) cells, whereas control cells generate one sphere in 18.1 cells (Fig. 5D). To confirm mechanistic link between RRAD and STAT3, we examined whether ectopic expression of a constitutively active STAT3 (STAT3-CA) could rescue the phenotypes caused by RRAD knockdown. STAT3 activation efficiently rescued the impaired sphere formation and downregulation of SOX2 and OCT4 (Fig. 5E). The results collectively indicate that RRAD-induced STAT3 activation is critical for the self-renewal of GBM cells.

RRAD depletion sensitizes temozolomide-resistant glioblastoma cells

Next, we examined whether the decrease in RRAD is related to drug-induced cell growth inhibition. An assay of cell viability using Trypan blue staining revealed that siRRAD, but not control siRNA, specifically inhibits growth in aggressive GBM cell lines, including U87-MG, U138-MG, and U251 (Supplementary Fig. S5). Stem-like properties of cancer cells have been implicated in resistance to chemotherapy and radiotherapy (35). The alkylating agent, temozolomide, is frequently used to treat
patients with GBM. However, more than 90% of recurrent GBMs do not respond to repeated challenges with temozolomide. To determine the precise role of RRAD in temozolomide resistance of GBM, we performed a clonogenic survival assay. Temozolomide resistance of GBM, increased in a RRAD expression-dependent manner (Figs. 6A). To select temozolomide-resistant GBM cells, LN229 cells were treated with high doses of TMZ (2 mmol/L) for 24 hours and surviving live cells collected after flow cytometer-assisted cell sorting, termed “temozolomide-resistant” cells. TMZR cells showed marked resistance to temozolomide, compared with parental cells (Fig. 6B). Specifically, the median inhibitory concentration (IC50) of temozolomide was >300 μmol/L in LN229-TMZR cells, whereas that for control LN229 cells was 10 μmol/L (P = 0.0096). RRAD expression was higher in LN229-TMZR, compared with parental LN229 cells in immunoblot analysis (Fig. 6C). Previously, it was shown that stem-like properties of GBM cells contribute to chemoresistance to temozolomide (36). In vitro tumor sphere formation assay (Supplementary Fig. S8) showed that temozolomide-resistant LN229-TMZR can generate one tumor sphere in 22.1 cells, whereas LN229 can generate one sphere in 79.9 cells. We additionally examined whether depletion of RRAD controls the chemoresistance and stem-like properties of temozolomide-resistant GBM cells. Notably, expression of stemness-regulating molecules (Fig. 6D) and survival of temozolomide-resistant cells (LN229-TMZR, U87-MG, and A172) was dramatically reduced upon depletion of RRAD (Fig. 6E and Supplementary Fig. S9), indicating that RRAD expression is critical for maintaining stem cell properties and temozolomide resistance in GBM cells.

**RRAD promotes in vivo tumorigenesis of glioblastoma and is required for maintenance of pluripotency**

We compared tumorigenesis of stable RRAD transfectant LN229 and control cells after subcutaneous implantation of adherent monolayer cultured cells or tumor spheres into athymic nude mice. Our results revealed significantly higher tumor growth in RRAD-overexpressing monolayer cells (3 × 10^6) than those expressing vector controls over 4 weeks (Fig. 7A; P = 0.0004). LN229-RRAD sphere cells exhibited a high capacity to form tumors with as low as 3 × 10^3 tumor spheres. Control cells formed tumors with an average size of 1 ± 1 mm^3 over 4 weeks. In contrast, RRAD-transfected spheres developed tumors that averaged 564 ± 57 mm^3 in size during the same period (Fig. 7B; P = 0.0002).

Human GBM cells from U87-MG display a predominant mesenchymal stem cell phenotype (37), express typical markers, and are capable of adipogenic differentiation in vitro. Accordingly, we investigated whether RRAD knockdown suppresses these properties of GBM. Upon cultivation of U87-MG cells in adipogenic media for three weeks, parental or siControl-transfected U87-MG cells were stained with Oil-red O, indicating intracellular lipid accumulation. However, no lipid droplets were

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**Figure 7.** RRAD promotes tumorigenesis of GBM. Effect of RRAD expression on GBM growth in vivo. For each injection, stable LN229 cell clones were cultivated as monolayer cells (3 × 10^5 cells; A) or as tumor spheres (3 × 10^3 cells; B) implanted subcutaneously into the flanks of athymic nu/nu mice. Five mice were used for each group (bars, SEM). C, a model based on our studies illustrates that RRAD activates STAT3 via endosome-mediated EGFR translocation, thereby increasing the levels of EMT-regulating (TWIST, SNAIL, and SLUG) and stemness-regulating (OCT4, NANOg, and SOX2) genes.
observed in RRAD-depleted U87-MG cells (Supplementary Fig. S10).

Discussion
The hypothesis that within the heterogeneous tumor mass, cells with characteristics of cancer stem cells (CSC) are responsible for tumor initiation, recurrence and resistance to therapy is redirecting the therapeutic efforts against cancer (38, 39). Hence, elucidation of the molecular pathways involved in CSC control may be essential to eradicate tumors. In the current study, we aimed to identify the targets responsible for resistance of GBM cells to temozolomide and maintenance of GBM stem-like cells. Previously, we showed that RRAD overexpression is associated with chemoresistance of cancer cells and RRAD knockdown sensitizes resistant cancer cells to cytotoxic drugs (21, 22). RRAD has additionally been associated with poor prognosis in breast cancer (14). However, the exact function of RRAD in temozolomide resistance of GBM is currently unknown. STAT3 activation is significantly correlated with stem-like GBM cells (8). To our knowledge, this is the first study to show that RRAD is capable of modulating endosome-mediated EGFR–STAT3 signaling, thereby promoting stem-like function and temozolomide resistance of GBM cells. Moreover, RRAD is overexpressed in GBM cell lines and tumor tissues and its expression is inversely correlated with survival of patients with GBM in publicly available clinical data.

We demonstrated that RRAD-expressing GBM cells harbor properties, including the ability to self-renew, generate tumor spheres, and grow tumors in vivo. Expression of the stem cell transcription factors, OCT4, SOX2, and NANOG decreased with the decrease in RRAD levels, suggesting a tight correlation between RRAD levels and self-renewal processes. OCT4, upregulated by RRAD, is responsible for the maintenance of stem cells (40, 41). Thus, RRAD-induced STAT3 activation and OCT4 expression may facilitate the maintenance of GBM cells in a stem-like state and contribute to EMT transition of GBM.

We found that transient depletion of RRAD led to decreased STAT3 activation, implying that RRAD expression is required for maintenance of the stem-like state. Consequently, the capacity of GBM cells to form tumor spheres was prevented, suggesting that RRAD-mediated STAT3 activation is responsible for in vivo tumorigenesis. These cellular changes were accompanied by downregulation of OCT4, SOX2, Vimentin, and TWIST. Therefore, we further investigated the mechanism by which RRAD maintains and/or promotes the stem-like properties of GBM. We propose that a RTK signaling pathway in GBM cells is activated by RRAD, leading to phosphorylation and activation of STAT3. Previously, it was reported that constitutively activated STAT3 is frequently coexpressed with EGFR in high-grade gliomas (7), and EGFR cooperates with STAT3 to facilitate EMT in human epithelial cancers (42). Our data showing that depletion of RRAD promptly inhibits TWIST expression and EMT are consistent with these findings. EMT is a unique process in which epithelial cells undergo morphologic changes leading to increased motility and invasion (31, 43).

The lack of effective GBM stem cell markers and heterogeneity of GBM stem cells are limiting factors for the effective targeting of GBM (44, 45). Targeting of the molecules responsible for temozolomide resistance is crucial to block GBM evasion of therapeutic responses. Given that RRAD promotes STAT3 signaling of GBM cells, it is possible that long-term exposure to RRAD induces a temozolomide-resistant phenotype in GBM cells. To address this issue, we exposed GBM cells to temozolomide. Our data showed that temozolomide causes an increase in RRAD and ultimately leads to the formation of highly resistant GBM tumors. Upregulation of RRAD expression mimicked the effects of long-term exposure to temozolomide, whereas targeting RRAD reversed temozolomide resistance. Importantly, the increase in RRAD expression was apparent not only in vivo but also in human tumors in cases of poor survival, indicating clinical relevance.

Overexpression of EGFR is associated with poor prognosis of GBM (46). EGFR signaling has also been found to mediate resistance to chemotherapy and radiotherapy (47). Our results collectively showed that RRAD expression induces temozolomide resistance in GBM via enhancement of EGFR and STAT3 activation, as summarized in Fig. 7C. We further demonstrated that RRAD inhibition attenuates acquired temozolomide resistance and stem-like properties in GBM, suggesting that RRAD inhibition constitutes an important strategy in the prevention of recurrence after GBM treatment. Previous studies by our group have shown that RRAD promotes the cell cycle through interactions with GCIP and consequent inhibition of the GCIP-mediated decrease in cyclin D activity and Rb phosphorylation (21, 48). As RRAD depletion also renders GBM cells less proliferative, it appears that the RRAD pathway performs a dual function in maintaining stem-like properties and accelerating proliferation of the cycling subset in GBM.

Our data demonstrate that RRAD promotes endosome-mediated STAT3 signaling, which is critical for the maintenance of stem-like cancer cells and temozolomide resistance in GBM. Elucidation of the novel STAT3 activation pathway and identification of RRAD as a molecular target are thus crucial findings that may facilitate sensitization of temozolomide-resistant GBM.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: D.-H. Nam, C. Park
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.-Y. Yeom, C. Park
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.-Y. Yeom, C. Park
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Park
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