MET Signaling Regulates Glioblastoma Stem Cells.

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ABSTRACT

Glioblastomas (GBMs) contain highly tumorigenic, self-renewing populations of stem/initiating cells (GSCs) that contribute to tumor propagation and treatment resistance. However, our knowledge of the specific signaling pathways that regulate GSCs is limited. The MET tyrosine kinase is known to stimulate the survival, proliferation, and invasion of various cancers including GBM. Here, we identified a distinct fraction of cells expressing a high level of MET in human primary GBM specimens that were preferentially localized in perivascular regions of human GBM biopsy tissues and were found to be highly clonogenic, tumorigenic, and resistant to radiation. Inhibition of MET signaling in GSCs disrupted tumor growth and invasiveness both in vitro and in vivo, suggesting that MET activation is required for GSCs. Together, our findings indicate that MET activation in GBM is a functional requisite for the cancer stem cell phenotype and a promising therapeutic target.
INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and lethal primary brain tumor with a median survival of 14.6 months despite maximal therapy (1). As included in classification criteria for GBMs, excessive and unstable blood vessel formation and tumor necrosis associated with hypoxia are key pathological characteristics of GBMs (2). GBMs heavily infiltrate into the neighboring brain parenchyma and are almost uniformly resistant to standard therapeutic regimes such as irradiation and chemotherapy (3). These biological characteristics of GBMs are major reasons of lethality and need to be targeted for therapy.

Cancer stem cell (CSC) hypothesis posits that a subpopulation of cancer cells is highly enriched with tumorigenic potential (3-7). Compared to bulk tumor cells, GSCs survive better against irradiation and chemotherapies, thereby contributing to therapeutic resistance and tumor recurrence (8, 9). In addition, GSCs frequently reside in perivascular and hypoxic regions, actively promoting angiogenesis and facilitating the survival in harsh environment (10-12). However, the underlying molecular pathways that govern these processes in GSCs are poorly understood.

The MET receptor tyrosine kinase (RTK) regulates cell growth and motility. Hepatocyte growth factor (HGF)/Scatter factor (SF) is a cognate ligand for MET signaling (13, 14). MET pathway activation induces glioma cell proliferation, survival, and migration (15, 16). We and others reported that MET overexpression is associated with poor prognosis and tumor invasiveness in GBM patients (17, 18). Large-scale genomic studies
confirmed frequent MET pathway activation and genomic amplification of \textit{MET} in GBMs, indicating that aberrant activation of MET is an important genetic event in GBMs (19-21).

As hypoxia directly induces MET expression and HGF induces expression of vascular endothelial growth factor (VEGF) in glioma cells, MET signaling may be particularly important for tumor cell survival in hypoxia and co-option with angiogenesis (15, 22, 23). In addition, it was reported that MET signaling is a key mechanism to maintain stem cell niche in brain (24). A recent study reported that MET signaling enhances GSC populations, suggesting a link between MET signaling and GSCs (25). The precise roles of MET signaling operated in GSCs, however, remain unclear (25).

Based on this background, we hypothesized that MET signaling promotes self-renewal and therapeutic resistance of GSCs. By extensive \textit{in vivo} and \textit{in vitro} studies utilizing a large number of freshly isolated patient-derived GBM cells, we provide evidence suggesting that MET signaling plays critical roles in GSC maintenance, migration, and resistance to radiation.
MATERIALS AND METHODS

Isolation of primary GBM cells and establishment of short-term cultured GSCs.

Following informed consent, glioblastoma specimens were obtained from patients undergoing surgery at the Samsung Medical Center in accordance with the Institutional Review Boards. Tumor specimens were classified as GBM based on World Health Organization (WHO) criteria by examination of pathologists (2). Tumor specimens were enzymatically dissociated into single cells and cultured, following the procedures previously reported (26, 27).

FACS analysis and sorting

Patient GBM-derived tumor cells were dissociated into single cells and labeled with the following antibodies; anti-MET (R&D systems), anti-CD133-PE (Miltenyi Biotec), anti-CD15-FITC (MMA clone, BD)(28). For MET staining and sorting, one million cells were labeled with 2 μg of antibodies at 4°C for 10 min. Antibodies against mouse immunoglobulin conjugated to PE or FITC were used as antibody isotype controls (BD). The stained cells were analyzed on the FACS Aria (BD). Cell Quest Acquisition and Analysis software (BD) was used to acquire and quantify the fluorescence signal distributions and intensities from individual cells.

Antibodies

The following antibodies were used as primary antibodies: MET (Zymed and Santa Cruz for detection of total MET protein; Cell Signaling for detection of phosphorylated MET proteins), Nestin, Sox2 (R&D systems), GFAP (Dako), TuJ1 (Covance), O4 (Chemicon), α-tubulin and β-actin (Sigma), and AKT and Erk (Cell Signaling).

Tumorsphere forming limiting dilution assay
Tumor cells were dissociated into single-cell suspensions, sorted for MET expression, and then plated into 96-well plates with various seeding densities (1 to 500 cells per well depending on the experiment, >30 wells for each condition).

**Intracranial tumor cell injection into NOD/SCID II2rg⁻/⁻ mice**

Unsorted GBM cells or the sorted MET<sup>high</sup> and MET<sup>low/-</sup> tumor cells were resuspended in 5 μl of HBSS and injected stereotactically into the striatum of adult NOD/SCID II2rg⁻/⁻ mice by using a stereotactic device (Kopf instruments) (coordinates: 2 mm anterior, 2 mm lateral, 2.5 mm depth from the dura). Mice with neurological signs were killed for the analysis of tumor histology and immunohistochemistry. All mice experiments were performed according to the guidelines of the Animal Use and Care Committees.

**In vivo delivery of MET siRNA-polyelectrolyte-micelle complexes**

For the conjugation of MET siRNA or non-targeting siRNA with polyethylene glycol 5000 (PEG5K), we followed the procedures as previously described (29). PEG-MET siRNA or PEG-control siRNA (0.5 mg/kg body weight) complexes were delivered through tail vein injections twice a week for four weeks.

**Determination of intracranial tumor volumes**

To estimate the size of intracranial tumor, we performed immunohistochemical analysis using an anti-human nuclei specific antibody (Chemicon) on the serial brain sections. Staining images were analyzed by ImageJ software (NIH) and the number of immunopositive cells and the total number of cells in a given field were calculated. The volume of the intracranial tumor was estimated by the largest width² x length x 0.5.

**Statistical Analysis**

All values are shown as mean ± standard deviation (SD). For group comparison, paired
t-test (two group comparison) or ANOVA (more than three group comparison) were used. Kaplan-Meier survival analysis was performed using Prism 4.0 software.

**Supplemental Data**

Supplemental Data include three tables and nine figures.
RESULTS

MET-positive tumor cells are preferentially located in perivascular regions of human GBM specimens and co-express GSC markers.

We reasoned that components of a GSC regulator pathway would be preferentially expressed in perivascular regions and hypoxic edges, the proposed *in vivo* niches for GSCs (10). By immunohistochemical analysis using human GBM patient specimens (n=93), we determined the expression patterns and intensities of various growth factor receptors, oncogenes, and stem cell associated proteins.

High frequency of cells positive for MET (more than 10% of tumor cells) in GBM specimens was positively correlated with the shorter progression-free-survival (PFS) and overall survival (OS) of the patients, suggesting a potential role of MET overexpression in malignancy (Figure 1 A and B). We also determined whether high expression of MET mRNA in GBM specimens portends poor patient survival by the analysis of The Cancer Genome Atlas (TCGA) database (Figure S1) (19). Patients with high MET mRNA expression have shorter PFS and OS, however, the prognostic value was marginal although significant (Figure S1).

We found that MET protein expression displayed inter- and intratumoral heterogeneity. Interestingly, cells strongly stained for MET were predominantly located near blood vessels and hypoxic edges (Figure 1 C and D). To quantify the proximity between MET-positive cells and endothelial cells in primary GBM specimens, we performed dual immunofluorescence for CD31 (endothelial marker) and MET using the frozen sections
derived from four GBM patient specimens (Figure 1 E to G). MET-positive cells were located closely to the nearest blood vessels, compared to MET negative cells (Figure 1G). In addition, a subset of tumor cells express both GSC enrichment markers CD133/CD15 and MET (Figure S2) (7, 28).

To obtain more quantitative results, we determined the expression level of MET in the freshly dissociated cells obtained from 23 different GBM patients by fluorescence-activated cell sorting (FACS) analysis (Figure 2A). The percentages of MET-positive cells vary between the samples (3.7 to 76.4% of bulk cells; standard deviation 16.1%). In 15 of 23 samples, the percentages of MET-positive cells are between 10 to 30% of the bulk cells. The cells dissociated from non-tumor epileptic brain specimens did not have any distinct MET-positive population (data not shown). To determine whether there are distinct subpopulations expressing both MET and CD133 and/or CD15, we performed dual FACS staining on the freshly isolated GBM cells derived from 14 different patients (Figure 2A and Table S1). CD133 and/or CD15 positive cells were enriched (2- to 7- fold) in MET-positive subpopulation compared to the bulk tumor cells. Conversely, MET-positive cells were enriched in CD133+ or CD15+ populations (Table S1).

To corroborate these findings, we extended our analysis to the previously characterized GSCs derived from human GBM specimens (26, 28). GSCs and their derivatives used in this study are described in supplementary information (Table S2). We fractionated these short-term cultured GSCs by FACS and performed immunoblot analysis (Figure 2B). MET protein was highly expressed in CD133+ (or in CD15+) subpopulations.
compared to the corresponding negative populations in all three GSCs (Figure 2B). Total MET and activated MET protein (Y1234) were preferentially expressed in GSC-enriched population compared to GSC-depleted cells (Figure 2C). Collectively, these data indicate that MET-positive GBM cells have activated MET signaling and that they significantly overlap with CD133+ and/or CD15+ GBM populations.

**MET\textsuperscript{high} cells sustain *in vitro* growth and are highly clonogenic compared to MET\textsuperscript{low/-} cells**

To determine whether MET\textsuperscript{high} GBM cells have cellular characteristics associated with GSC phenotypes, we employed FACS using an antibody recognizing the extracellular domain of MET protein and prospectively isolated MET\textsuperscript{high} and MET\textsuperscript{low/-} subpopulations from the patient GBM specimen 448. We operationally defined MET\textsuperscript{high} cells as the cells with top 10 to 20 % highest intensities out of total MET-positive cells. After confirming the purity and viability of these sorted populations by FACS re-examination, we first determined the growth kinetics of each subpopulation by culturing these cells in the standard stem cell culture condition. MET\textsuperscript{high} cells readily formed neurosphere-like aggregates and continued to proliferate, whereas MET\textsuperscript{low/-} cells failed to sustain active cell growth *in vitro* (Figure 3 A to C).

Clonogenic growth as neurospheres is an *in vitro* indicator of self-renewal in normal neural stem/progenitor cells (NSCs) and GSCs. To determine the clonogenic potentials of MET\textsuperscript{high} and MET\textsuperscript{low/-} populations, we performed limiting dilution assays (LDA) using both 448 freshly isolated GBM cells and 206 short-term cultured GSCs (Figure 3D).
FACS-sorted \( \text{MET}^{\text{high}} \) and \( \text{MET}^{\text{low/-}} \) cells were plated into 96-well plates with various seeding densities and allowed to grow. Neurospheres were readily formed by \( \text{MET}^{\text{high}} \) cells but not by \( \text{MET}^{\text{low/-}} \) cells (Figure 3D). In addition, we performed a competitive growth assays in which \( \text{MET}^{\text{high}} \) and \( \text{MET}^{\text{low/-}} \) cells were differentially labeled with green fluorescence protein (GFP) and red fluorescence protein (RFP), respectively, mixed at various ratios, and then cultured for additional two weeks. Even when the initial fraction of \( \text{MET}^{\text{high}} \) GFP cells was 10%, more than 85% of the resultant cells after the culture were GFP positive (Figure 3E). Taken together, these data indicate that \( \text{MET}^{\text{high}} \) GBM cells are highly clonogenic and sustain in vitro growth of total population.

To determine whether the HGF/MET signaling pathway is functional in GSCs, we examined the activation status of MET downstream effectors (Figure S3A). GSCs displayed basal activation of MET, suggesting the presence of an autocrine loop with further response to the addition of exogenous HGF. Treatment with exogenous HGF further increased activated MET (Y1234 and Y1349), AKT (S473) and ERK (16). Consistent with this, we detected high levels of the secreted HGF protein in GSC-conditioned media (seven out of 11 GSCs) by ELISA assays (Figure S3B). Finally, we determined whether HGF could induce the proliferation of GBM cells by performing LDA. Similar to EGF, HGF increased the clonogenic growth of GBM cells (Figure S3C). Collectively, these data indicate the presence of active HGF/MET signaling in GSCs.

**\( \text{MET}^{\text{high}} \) GBM cells are highly tumorigenic in orthotopic transplantation models**

Tumorigenicity in vivo is the gold standard for determining cancer stemness. We
injected MET\textsuperscript{high} and MET\textsuperscript{low/-} cells into the brains of highly immunodeficient NOD/SCID Il2rg\textsuperscript{-/-} mice (non-obese diabetic/severe combined immunodeficiency mice lacking Interleukin-2 gamma receptor) and monitored tumor formation (30). MET\textsuperscript{high} cells from four GBMs (448 and 464, freshly isolated patient GBM cells; 822 and 206, short-term cultured GSCs) generated tumors more efficiently than their corresponding MET\textsuperscript{low/-} cells (Figure 4 A and B). Of note, all of the above GBM cells express HGF (data not shown). To estimate the relative enrichment of tumorigenicity in MET\textsuperscript{high} cells compared to MET\textsuperscript{low/-} cells, we performed \textit{in vivo} tumorigenicity titration assays (Figure 4C). Varying numbers of MET\textsuperscript{high} and MET\textsuperscript{low/-} cells isolated from primary GBM specimen (559 and 905) were injected into brains of NOD/SCID Il2rg\textsuperscript{-/-} mice. Two of five mice injected with 100 MET\textsuperscript{high} cells developed tumors and so did all the mice (n=5) that had 1000 MET\textsuperscript{high} cells (median survival of 88 days). In contrast, all the mice that received 100 MET\textsuperscript{low/-} cells and four of five mice injected with 1000 MET\textsuperscript{low/-} cells showed no sign of tumor development six months after the injection (Figure 4C). Kaplan-Meier survival analysis further demonstrated significant survival differences between animals receiving these subpopulations (Figure 4 D and E).

Similar to the parental GBM tumor, 448 MET\textsuperscript{high} cell-derived xenograft tumors harbored a mixture of MET\textsuperscript{high} cells and MET\textsuperscript{low/-} cells, indicating the reconstitution of tumor heterogeneity (31) (Figure S4). In addition, immunohistochemical analysis on the xenograft tumors revealed that MET\textsuperscript{high} cells were preferentially located near the blood vessels and infiltrating edges of tumors (Figure 4 F to H). Robust expression of tumor-derived HGF was also detected in xenograft sections by immunohistochemical analysis,
suggesting an active HGF/MET signaling in these tumors (Figure S5).

**MET\textsuperscript{high} cells are efficient in tumor formation regardless of CD133 expression**

Our data show that some of MET\textsuperscript{high} cells co-express CD133 (Figure 2 and Table S1). To determine whether MET expression alone can enrich GSCs, we fractionated GBM cells into four subpopulations (CD133\textsuperscript{+}/MET\textsuperscript{high}, CD133\textsuperscript{-}/MET\textsuperscript{high}, CD133\textsuperscript{+}/MET\textsuperscript{low/-}, and CD133\textsuperscript{-}/MET\textsuperscript{low/-} cells) and determined their tumor formation efficiencies. Both CD133\textsuperscript{+}/MET\textsuperscript{high} and CD133\textsuperscript{-}/MET\textsuperscript{high} cells, derived from the freshly isolated GBM cells (060) and GBM xenografts (559 and 464), were highly efficient in tumor formation, whereas CD133\textsuperscript{+}/MET\textsuperscript{low/-} and CD133\textsuperscript{-}/MET\textsuperscript{low/-} cells were not (Figure 5A). In vitro clonogenic potential of each subpopulation correlated with in vivo tumorigenicity of the corresponding cells (Figure 5B). These data suggest that MET but not CD133 is a major determinant for GSC enrichment at least in these GBMs.

**MET inhibition decreases the survival, migration, and clonogenicity of GSCs**

To interrogate the role of MET in GSC biology, we inhibited MET by short hairpin RNA (shRNA)-mediated knockdown and evaluated its effects on GSCs. To ensure the specific knockdown of MET, we used two independent sequences of shRNA directed against MET mRNA. Both MET shRNA constructs led to a significant reduction in MET protein compared to the non-targeting shRNA control (Figure S6). First, we determined the cell growth/survival of MET knockdown GBM cells. Compared to the control, MET knockdown cells showed a significant decrease in the proliferation index (Figure S6 B and C). MET knockdown cells were largely in G\textsubscript{0}/G\textsubscript{1} phase without notable increase in
sub G₁ populations, suggesting that cell cycle arrest is a main contributor of the decreased cell proliferation. MET knockdown significantly decreased the clonogenicity of various GSCs, determined by LDA (Figure S6 D and E). We also tested whether pharmacological inhibition of MET signaling can decrease GSC clonogenicity. GSCs treated with SU11274, a widely used MET kinase inhibitor (25, 32), were less efficient in tumor-sphere formation compared to the control (Figure 6A).

HGF is an effective chemokine for glioma cell migration (13, 33). To test whether MET signaling mediates invasive growth of GSCs, we performed transwell-based in vitro migration assays (Figure S7 A and B). We plated control shRNA- or MET shRNA-transduced GBM cells in serum-free media and added serum (or HGF)-containing media on the other side of the transwell membranes to stimulate migration. MET knockdown decreased the number of migrating/invading cells up to 70% compared to the control (Figure S7 A and B). In addition, we performed ex vivo brain slice assays that simulate in vivo migration behavior much better than transwell assays (Figure S7C). RFP-expressing GSCs were treated with a chemical MET inhibitor PHA665752 for one day, mixed with GFP-expressing GSCs treated with the vehicle control, and then implanted into the cortex region of 300-micron thick brain slices. Three days after, more than 90% of the migrated cells were GFP-positive, suggesting that MET inhibition decreases glioma cell invasion (Figure S7 C and D). Together, these data demonstrate that MET signaling is required for the self-renewal, proliferation, and migration of GSCs.

**In vivo MET targeting via liposome-conjugated siRNA increases the survival of**
tumor bearing mice.

By using GSC-derived tumors as a clinically relevant model system, we wanted to test whether MET targeting in vivo could provide therapeutic benefit. Polyelectrolyte complex micelles are stable in vivo and efficient for intracranial delivery of small interference RNA (siRNA) due to the highly lipophilic nature of brain tissues (29). We conjugated MET siRNA or non-targeting siRNA with polyethylene glycol 5000 (PEG5K) and mixed with small liposomes (29). Intracranial tumors were generated from three freshly isolated GBM cells (559 and 578, five for each group; 464, four for each group). These cells express both MET and HGF at high levels, determined by Western blot analysis and HGF ELISA (data not shown). Once tumors were established in the mouse brains, we initiated the administration of either PEG-MET siRNA or PEG-control siRNA (0.5 mg/kg body weight) intravenously twice a week for four weeks. Compared to the tumors from the control siRNA-treated mice, tumor cells in MET siRNA-treated mice revealed a significant decrease in MET staining positivity (Figure 6B). After four weeks of treatment, we monitored the size of tumors by magnetic resonance imaging (MRI) (Figure 6C) and brain histology section (Figure 6D and Figure S8). All of MET siRNA treated groups showed a significant decrease in tumor volumes, indicating the efficacy of in vivo MET targeting in intracranial GSC-derived tumors (Figure 6E).

Irradiation induces MET upregulation and MET targeting decreases the clonogenicity of surviving GBM cells.

Irradiation treatment decreases tumor burden and prolong the survival of GBM patients. However, patients eventually succumb due to tumor recurrence. Given the observations
that GSCs preferentially survive radiation and that MET targeting disrupted GSC self-renewal and clonogenicity, we hypothesized that combination of radiation and MET inhibition in GSCs might be an effective therapeutic approach (9, 34, 35). Cells of a short-term cultured GSC (822) were irradiated in vitro with doses of 5 or 10 Gy and harvested two days later. We found about a 3-fold increase in the number of MET positive cells and upregulation of MET protein in irradiated cells compared to non-irradiated control (Figure 7 A and B). To determine the clonogenic potentials of MET<sup>high</sup> and MET<sup>low/-</sup> GBM cells after radiation treatment, we sorted MET<sup>high</sup> and MET<sup>low/-</sup> cells from the freshly isolated 448 GBM cells, exposed them to radiation, and immediately seeded at the clonal density for LDA assays. MET<sup>high</sup> cells remained highly clonogenic after radiation treatment, whereas irradiated MET<sup>low/-</sup> cells were significantly impaired in the ability to generate subsequent colonies (Figure 7C). To determine whether MET is directly involved in the clonal re-growth after radiation, we performed the similar clonogenic assays by MET knockdown or treatment with SU11274. Cells with MET inhibition were hardly able to generate spheres after radiation treatment, suggesting that MET inhibition significantly sensitizes GBM cells to radiation (Figure 7D and Figure S9).

The above data raise the possibility that MET upregulation after radiation can be a prognostic biomarker for the GBM patients who have received radiation therapy. We acquired 14 matched sets of pre- and post- irradiated (recurrent) tumor specimens from the same GBM patients (Table S3). All the patients were diagnosed as de novo primary GBMs and received radiation as a front line therapy after initial surgical resection. We determined the expression level of MET protein by immunohistochemical analysis on
the sections from pre- and post-radiated GBM specimens. In four sets, MET expression in post-radiated GBM specimens was significantly increased (>2 fold increase in the number of MET positive cells) compared to the untreated specimens. The remaining ten sets showed either unchanged or modest changes in MET expression. Notably, the patients showing the significant MET induction had worse prognosis (median survival of 55 days after recurrence) than the other group (median survival of 189 days; p < 0.01; Figure 7F), suggesting that MET up-regulation after radiation is associated with aggressive growth of recurrent tumors.
DISCUSSION

GSCs remains controversial due to unresolved questions regarding the frequency of these cells, the surface markers by which they can be identified, and the nature of the cell(s) of origin (31, 36). Previous reports have identified a series of GSC enrichment markers including CD133, CD15, CD44, and A2B5 (7, 28, 37, 38). Although useful for the prospective isolation of putative GSCs, it is unclear whether these markers have distinct functions pertinent to GSC phenotypes (39, 40). Therefore, our screening approach in search for a GSC regulator was focused on the protein expression pattern in patient-derived GBM specimens, based on the hypothesis that GSCs are highly enriched in perivascular regions of human GBMs in situ (41).

In this study, we identified a distinct fraction of cells expressing high level of MET in various human primary GBMs and demonstrated that these subpopulations have key characteristics of GSCs. Through extensive in vivo limiting dilution tumor formation assays, we have shown that $\text{MET}^{\text{high}}$ GBM cells are highly enriched with tumor stem/propagating populations, supporting that MET is a key regulator of GSCs. We showed the importance of MET in GSC biology by demonstrating that MET inhibition disrupts the clonogenicity, radioresistance, and tumorigenicity of GSCs. Co-option with angiogenesis and tumor cell survival in hypoxia are critical for tumor growth and MET signaling is known to play important roles on these processes. Therefore, high MET expression in GSCs can be viewed as an adaptive response of cancer cells to their microenvironment (42, 43).
Numerous small molecule inhibitors and antagonistic antibodies targeting the HGF/MET pathway are at advanced stages of clinical development (44-48). Understanding of the molecular determinant(s) of response and resistance to HGF/MET targeting therapeutic agents is one of the most critical unmet needs in clinical research. A few of biomarkers to predict therapeutic response have been proposed, including genomic amplification of \textit{MET}, HGF levels or MET expression (total and phosphorylated MET), and PTEN status (33, 49, 50). Robustness of these biomarkers in GBM needs further validation. For example, genomic \textit{MET} amplification appears to predict sensitivity to MET inhibition in gastric cancers but less likely in GBMs (33). A recent paper reported that HGF autocrine status may correlate with MET activity in GBM and predict sensitivity to MET inhibitors (33). We have determined the correlation between tumor-derived HGF and sensitivity to MET inhibition. Our ongoing studies so far indicated that high HGF-producing GBM cells appear to be more sensitive to MET inhibition compared to the little or no HGF-expressing GBM cells, consistent with the previous report (data not shown) (33). Further studies are warranted to determine the role of HGF in GSC biology, the effects of HGF targeting on GSC self-renewal and tumorigenicity, and the correlation between HGF and GSC sensitivity to MET inhibition \textit{in vivo}.

In conclusion, we demonstrate that MET is an enrichment marker for GSCs and a functional requisite for cancer stem phenotype. Not only will these data provide a clue for a more thorough understanding of cancer stem cell biology, but also they further implicate MET as a promising therapeutic target.
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FIGURE LEGENDS

Figure 1: Expression of MET in primary human GBM specimens

(A and B) Kaplan-Meier progression free (A) and overall (B) survival graphs of patients with high (red) and low expression of MET (blue). GBM sections from 93 patients were stained by using MET antibodies and categorized by the frequency of MET stained cells (> 10% of tumor cells as high expression group). P values were determined by log rank test: p < 0.007 for (A) and p < 0.015 for (B).

(C and D) Representative microphotographs of immunohistochemical staining of MET protein in paraffin sections of GBM patient specimens. Immuno-positive cells were visualized by brown DAB staining. Arrows indicate the stained cells. Ve indicates blood vessels, and Nec indicates the area of necrosis. Scale bar represents 20 micron.

(E and F) Representative microphotographs showing dual immunofluorescence analysis using MET antibody (green) and CD31 (red) in frozen sections of GBM patient specimens. DAPI was used to visualize nuclei. Scale bar represents 100 micron.

(G) Measurement of the average distance between MET-positive cells and blood vessels.

Figure 2: Expression of MET in primary human GBM specimens and GBM-derived GSCs

(A) Flow cytometry data of MET-, CD133-, and CD15-positive cells in freshly isolated patient GBM cells.

(B and C) Immunoblot analysis using CD133 and/or CD15 +/- cell lysates from short-term cultured GSCs (B) and GSC-derived xenograft tumors (C). The numbers represent
the designated name for each GSC. Actin was used as a loading control.

**Figure 3: Characterization of MET<sup>high</sup> and MET<sup>low/-</sup> cells from freshly isolated GBM specimens and GBM-derived GSCs**

(A) Representative microphotographs of MET<sup>high</sup> and MET<sup>low/-</sup> GBM cells cultured for a week.

(B) Cell cycle analysis of MET<sup>high</sup> and MET<sup>low/-</sup> populations directly isolated from 448, the freshly isolated patient GBM cells. Each subpopulation was seeded at the density of 5000 cells/ml media and cultured for two weeks. After two weeks of *in vitro* culture, cells were harvested for cell cycle analysis. Error bars represent standard deviation (performed in triplicates). *p < 0.05.

(C) Cell cycle analysis of MET<sup>high</sup> and MET<sup>low/-</sup> GBM cells isolated from short-term cultured GSC 905. Each subpopulation was sorted on day 0 and cultured for additional 20 days. At the indicated time points, we performed cell cycle analysis of these populations.

(D) Limiting dilution sphere forming assays to determine the clonogenic potentials of MET<sup>high</sup> and MET<sup>low/-</sup> subpopulations from 448 GBM cells. Cells were plated into 96 well plates with various seeding densities (2 to 50 cells per well, 30 wells per each condition).

(E) Competitive proliferation assay to determine relative proliferation potentials of MET<sup>high</sup> and MET<sup>low/-</sup> cells isolated from the freshly isolated patient GBM cells (903). GFP-transduced MET<sup>high</sup> cells and RFP-transduced MET<sup>low/-</sup> GBM cells were mixed at various ratios (% of GFP input cells are shown in x axis) and cultured. Two weeks later, the number of total cells was counted and the ratio of GFP and RFP positive cells was
Figure 4: Tumorigenic potential of MET$^{\text{high}}$ and MET$^{\text{low/-}}$ cells from freshly isolated GBM specimens and GBM-derived GSCs

(A) Representative microphotographs of the brain sections of mice injected with either MET$^{\text{high}}$ and MET$^{\text{low/-}}$ cells derived from short-term cultured GSC 822. Bar represents 2 mm. Ten thousand cells of either MET$^{\text{high}}$ or MET$^{\text{low/-}}$ cells were injected into the brains of NOD/SCID Il2rg$^{/-}$ mice. Mice were sacrificed two months after injection, and their brains were sectioned and stained with Hematoxylin and Eosin (H&E).

(B) Tumor formation assay of MET$^{\text{high}}$ and MET$^{\text{low/-}}$ subpopulations from freshly isolated GBM cells (448 and 464) and short-term cultured GSCs (822 and 206). Tumor size was measured from the brain sections of tumor cell injected mice (three mice per each group). *p < 0.01.

(C) In vivo tumorigenicity titration assay of MET$^{\text{high}}$ and MET$^{\text{low/-}}$ subpopulations from freshly isolated GBM cells (559) and short-term cultured GSCs (905). The number of tumor-bearing mice and tumor cell injected mice are shown. Median survival of each group is shown in parenthesis.

(D and E) Kaplan-Meier survival plot of mice injected with MET$^{\text{high}}$ and MET$^{\text{low/-}}$ cells from freshly isolated GBM 559 cells (1000 cells injected) and short-term cultured GSC 905 (100,000 cells injected). P value was determined by log rank test. p < 0.01 in both 559 and 905 cells.

(F to H) Representative microphotographs of the brain sections of 448 MET$^{\text{high}}$ cell-derived xenograft tumors. Many tumor cells were found in corpus callosum (CC) in H&E.
section (F) and stained with anti-PCNA antibody (G) and anti-MET antibody (H). Immuno-positive cells were visualized by brown DAB staining. Arrows indicate strongly stained cells. Bars represents 100 micron.

**Figure 5: Clonogenicity and tumorigenicity of GBM subpopulations fractionated based on MET and CD133 expression**

**(A)** *In vivo* tumorigenicity titration assay of (CD133+/MET\textsuperscript{high}, CD133+/MET\textsuperscript{high}, CD133+/MET\textsuperscript{low/-}, and CD133-/MET\textsuperscript{low/-} subpopulations derived from freshly isolated GBM cells (060) and xenograft tumor-derived GSCs (559 and 464). The number of tumor-bearing mice and tumor cell injected mice are shown. Ten thousand tumor cells were intracranially injected into the brains of SCID mice and animal survival was monitored over 5 months.

**(B)** Limiting dilution sphere forming assays to determine the clonogenic potentials of each subpopulations from 060 and 090, the freshly isolated patient GBM cells. Cells were plated into 96 well plates with various seeding densities (1 to 500 cells per well, 24 wells per each condition).

**Figure 6: Effects of MET inhibition on *in vitro* clonogenicity and *in vivo* tumorigenicity of GSCs**

**(A)** Limiting dilution sphere forming assays to determine the clonogenic potentials of various GSCs after treatments with SU11274 (2 μM).

**(B)** Representative microphotographs of immunohistochemistry determining MET expression in the brains of control siRNA- or MET siRNA-treated mice. Immunopositive
cells were visualized by brown DAB staining. Arrows indicate strongly stained cells. Scale bar represents 100 micron.

(C and D) The brains from control siRNA or MET siRNA treated mice were examined by MRI images (C) and histology sections (D). White arrowheads indicate tumor. Bars represent 2 mm.

(E) Tumor volume in the brains was determined by measuring the size of the tumor in the serial H&E sections. Error bars represent SD.

Figure 7: Roles of MET in GBM radiation response

(A) Flow cytometry analysis of MET (FITC labeled, x-axis) in GSCs after irradiation. GSC 822 cells were irradiated in vitro either with 5 or 10 Gy, and the survived cells were examined for MET expression at two days after irradiation. The vertical line depicts the cut-off for MET<sup>high</sup> cells. Numbers indicate % of MET<sup>high</sup> cells in each condition.

(B) Immunoblot analysis of lysates from the cells used in (A).

(C) Limiting dilution sphere-forming assay to determine clonogenic potentials of MET<sup>high</sup> and MET<sup>low/-</sup> GBM 448 cells with 5 Gy or without (cont) irradiation. The sorted cells were mock treated or irradiated and immediately seeded with various seeding densities (2 to 50 cells per well, 30 wells per each condition).

(D) Limiting dilution sphere forming assay to determine clonogenic potentials of MET knockdown in GBM 822 cells after irradiation. Higher seeding densities (5 to 500 cells per well) were used. Three independent experiments were performed and the data from a representative experiment were shown.
(E) Kaplan-Meier survival plot of the patients with MET upregulation (shown as red) or without MET upregulation (shown as black) in post-radiated tumors compared to untreated GBM specimens. P value was determined by log rank test: $p < 0.01$. 
Figure 1

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Distance from the nearest vessel (μm ± S.D.)</th>
<th>T test</th>
</tr>
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<tr>
<td></td>
<td>MET\textsuperscript{high}</td>
<td>MET\textsuperscript{low/-}</td>
</tr>
<tr>
<td>541</td>
<td>18.2 (± 13.7)</td>
<td>67.3 (± 28.8)</td>
</tr>
<tr>
<td>609</td>
<td>28.5 (± 21.3)</td>
<td>77.0 (± 37.4)</td>
</tr>
<tr>
<td>586</td>
<td>18.2 (± 12.7)</td>
<td>35.9 (± 16.5)</td>
</tr>
<tr>
<td>381</td>
<td>19.2 (± 8.1)</td>
<td>27.1 (± 14.8)</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3
FIGURE 4

A

B

C

<table>
<thead>
<tr>
<th>Cell No. injected</th>
<th>MET high</th>
<th>MET low/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000</td>
<td>5/5 (8.0 wks)</td>
<td>4/5 (15.6 wks)</td>
</tr>
<tr>
<td>1,000</td>
<td>5/5 (12.6 wks)</td>
<td>1/5</td>
</tr>
<tr>
<td>100</td>
<td>2/5</td>
<td>0/5</td>
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</table>

D

E

F

G

H

FIGURE 4

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### Table A

<table>
<thead>
<tr>
<th>GB M</th>
<th>Cell No.</th>
<th>Subpopulations (No. of mice with tumor/total mice)</th>
<th>CD133+/MET\text{high}</th>
<th>CD133-/MET\text{high}</th>
<th>CD133+/MET\text{low/-}</th>
<th>CD133-/MET\text{low/-}</th>
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<tr>
<td>060</td>
<td>10,000</td>
<td>3/3</td>
<td>3/3</td>
<td>0/4</td>
<td>0/4</td>
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<tr>
<td>559</td>
<td>1,000</td>
<td>5/5</td>
<td>4/5</td>
<td>N.D.</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>464</td>
<td>10,000</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>464</td>
<td>1,000</td>
<td>4/4</td>
<td>4/4</td>
<td>0/4</td>
<td>0/4</td>
<td></td>
</tr>
</tbody>
</table>

### Diagram B

**FIGURE 5**

B

% of wells w/o spheres vs. No. of initial cells

- **060**
  - CD133+/MET\text{high}
  - CD133+/MET\text{low/-}
  - CD133-/MET\text{high}
  - CD133-/MET\text{low/-}

% of wells w/o spheres vs. No. of initial cells

- **090**

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FIGURE 6

A

\begin{center}
\begin{tabular}{ccc}
\hline
\noalign{\smallskip}
 protocol & 762 & 464 & 745 \\
\hline
\noalign{\smallskip}
\end{tabular}
\end{center}

\begin{itemize}
\item control
\item SU11274 2uM
\end{itemize}

\begin{center}
\begin{tabular}{ccc}
\hline
\noalign{\smallskip}
% of wells w/o spheres & 120 & 120 & 120 \\
\noalign{\smallskip}
\hline
\noalign{\smallskip}
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{ccc}
\hline
\noalign{\smallskip}
No. of initial cells/well & 0 & 0 & 0 \\
\noalign{\smallskip}
\hline
\noalign{\smallskip}
\end{tabular}
\end{center}

B

\begin{center}
\begin{tabular}{cc}
\noalign{\smallskip}
Control siRNA & MET siRNA \\
\noalign{\smallskip}
\hline
\end{tabular}
\end{center}

C

\begin{center}
\begin{tabular}{cc}
\noalign{\smallskip}
Control siRNA & MET siRNA \\
\noalign{\smallskip}
\hline
\end{tabular}
\end{center}

D

\begin{center}
\begin{tabular}{cc}
\noalign{\smallskip}
Control siRNA & MET siRNA \\
\noalign{\smallskip}
\hline
\end{tabular}
\end{center}

E

\begin{center}
\begin{tabular}{cc}
\noalign{\smallskip}
Control siRNA & MET siRNA \\
\noalign{\smallskip}
\hline
\end{tabular}
\end{center}

\begin{center}
\begin{tabular}{cc}
\noalign{\smallskip}
Control siRNA & MET siRNA \\
\noalign{\smallskip}
\hline
\end{tabular}
\end{center}
FIGURE 7

A. % of MET\textsuperscript{high} cells after radiation

- Non-radiated: 14%
- 5 Gy: 22%
- 10 Gy: 43%

B. 822

- Cont
- 5 Gy
- 10 Gy

- MET
- pAKT
- AKT
- β-Actin

C. % of wells w/o spheres

- MET\textsuperscript{high} cont
- MET\textsuperscript{high} 5 Gy
- MET\textsuperscript{low} cont
- MET\textsuperscript{low} 5 Gy

D. 822

- 5 Gy
- 10 Gy

- ShCont
- ShMET345
- ShMET3310

E. Fraction survival

- Progression-free survival (days)

$P < 0.01$
MET Signaling Regulates Glioblastoma Stem Cells.

Kyeung Min Joo, Juyoun Jin, Eunhee Kim, et al.

_Cancer Res_ Published OnlineFirst May 22, 2012.

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<td>Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.</td>
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