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Original Citation:
The MET oncogene is a functional marker of a glioblastoma stem cell subtype. / De Bacco F; Casanova E; Medico E; Pellegatta S; Orzan F; Albano R; Luraghi P; Reato G; D' Ambrosio A; Porrati P; Patane M; Maderna E; Pollo B; Comoglio PM; Finocchiaro G; Boccaccio C. - In: CANCER RESEARCH. - ISSN 0008-5472. - STAMPA. - 72:17(2012), pp. 4537-4550.

Availability:
This version is available http://hdl.handle.net/2318/127216 since 2016-01-13T12:40:17Z

Published version:
DOI: 10.1158/0008-5472.CAN-11-3490

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(Article begins on next page)
The MET Oncogene Is a Functional Marker of a Glioblastoma Stem Cell Subtype

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Abstract
The existence of treatment-resistant cancer stem cells contributes to the aggressive phenotype of glioblastoma. However, the molecular alterations that drive stem cell proliferation in these tumors remain unknown. In this study, we found that expression of the MET oncogene was associated with neurospheres expressing the gene signature of mesenchymal and proneural subtypes of glioblastoma. Met expression was almost absent from neurospheres expressing the signature of the classical subtype and was mutually exclusive with amplification and expression of the EGF receptor (EGFR) gene. Met-positive and Met-negative neurospheres displayed distinct growth factor requirements, differentiated along divergent pathways, and generated tumors with distinctive features. The Methigh subpopulation within Met-pos neurospheres displayed clonogenic potential and long-term self-renewal ability in vitro and enhanced growth kinetics in vivo. In Methigh cells, the Met ligand HGF further sustained proliferation, clonogenicity, expression of self-renewal markers, migration, and invasion in vitro. Together, our findings suggest that Met is a functional marker of glioblastoma stem cells and a candidate target for identification and therapy of a subset of glioblastomas.

Introduction
Glioblastoma, the highest grade glioma variant, is a relatively rare (yearly incidence of 4–5/100,000 people) but very aggressive tumor, associated with high morbidity, mortality, and recurrence (median survival of 12–15 months; ref. 1). These meager treatment options prompted a huge effort to achieve comprehensive profiling of gene expression patterns and genetic alterations (2–6), in order to identify molecular targets for innovative—possibly personalized—therapies. By expression profiling, 3 main glioblastoma subtypes have been recognized: classical, mesenchymal, and neural/proneural (here proneural; ref. 6). The classical subtype mostly displays alterations of the EGF receptor (EGFR) gene, such as amplification or deletion of the extracellular domain (EGFRvIII; ref. 6). The mesenchymal subtype often harbors a normal EGFR gene and deletion of NF1 or PTEN tumor suppressor genes (6). The proneural subtype preferentially associates with mutations of isocitrate dehydrogenase 1 (IDH1) or 2 (IDH2), or aberrant activation of PDGFRα, resulting from gene amplification/mutation, or occurrence of autocrine loops. This subtype, often evolving from lower grade gliomas, may associate with a more favorable prognosis, but does not benefit from current therapies (6, 7).
To understand glioblastoma pathogenesis, it is crucial to identify the driving genetic lesions and to recognize that glioblastoma onset and progression depend on a (small) subpopulation of cancer stem cells (CSC), which, according to an operational definition, hold replicative immortality in
vitro and tumor-initiating potential when transplanted in vivo\(^{(8, 9)}\). Despite the controversy arising on the ultimate, elusive nature of these cells, convincing evidence indicates that CSCs possess inherent radio- and chemoresistance, a major cause of treatment failure and disease recurrence \(^{(10, 11)}\). To envisage new therapeutical strategies, genetic and molecular alterations occurring in glioblastoma stem cells must be identified. Therefore, we investigated whether the molecular alterations detectable in the clinically manifest glioblastoma were present in the tumor-initiating subset, allowing classification in subtypes already at the CSC level. From primary glioblastoma tissues, we isolated and propagated extensively self-renewing neurospheres, that is, cultures enriched in stem and progenitor cells \(^{(12)}\). These neurospheres displayed mutational profiles largely overlapping with those of the original tumors and could be classified as classical, mesenchymal, or proneural, according to their gene expression profile. We then specifically associated expression of the MET oncogene with mesenchymal and proneural neurospheres, and we showed that Met signaling actively supported the stem-like and invasive phenotype.

**Materials and Methods**

**Neurospheres derivation and culture**

Neurospheres were derived from glioblastoma specimens diagnosed according to WHO criteria \(^{(13)}\) and cultured in standard medium containing EGF and bFGF \(^{(12)}\). HGF (20 ng/mL) was added where indicated.

**Evaluation of tumorigenicity**

Cells were injected orthotopically (2 \(\times 10^5\) cells) or subcutaneously (10\(^5\) cells in v/v PBS/Matrigel) into 6 weeks old male NOD.CB17-Prkdcscid/J mice.

**Gene copy number and sequencing**

Gene amplification was assessed using commercially available TaqMan Copy Number Assays. For gene sequencing, specific primer pairs used are listed in Supplementary Table S7.

**Microarray data**

Data have been deposited in the GEO database, accession number GSE36426.

**Immunophenotypical analysis and fluorescence-activated cell sorting**

Cells were incubated with the antibodies listed in Supplementary Table S8.

**Proliferation assay**

Cells were plated at clonal density (10 cells/µL) in 96-well plates in a medium devoid of growth factors. Twenty-four hours after seeding (day 0), the indicated growth factors were added, and proliferation was measured by Cell Titer Glo.

**Clonogenic assay and long-term propagation**

Single cells were directly sorted into 96-well plates (1 cell/well). Neurospheres were counted 14 days after seeding. For long-term propagation, cells were plated at clonal density, and formed neurospheres were dissociated, counted, and replated once a week.

**Migration and invasion assays**

A total of 10\(^5\) cells were seeded in Transwell in the absence (migration) or in the presence (invasion) of Matrigel (10 µg/cm\(^2\); ref. \(^{(14)}\)).
Statistical analysis
Numerical results were expressed as means ± SEM. Statistical significance was evaluated using 2-tailed Student t tests, Fisher exact tests or $\chi^2$ tests. Multiple comparisons were carried out using Bonferroni correction. Values of $P$ less than 0.05 were considered statistically significant. For the other methods see Supplementary Experimental Procedure.

Results
Neurospheres harbor genetic lesions specific of glioblastoma subtypes
Eighteen neurospheres were randomly chosen from an ample panel of neurospheres derived from surgical specimens of primary glioblastomas (WHO grade IV; ref. 13; Table 1). Histologic sections of the corresponding tumors were analyzed for mitotic index (invariably high, data not shown), EGFR, and p53 expression (15), and traits associated with subtyping, including vascular stroma proliferation and YKL-40 expression (ref. 5; Table 1 and Supplementary Fig. S1A).

Table 1.
Clinical and neuropathologic data of primary glioblastoma (WHO grade IV)

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<th>Patient code</th>
<th>Gender</th>
<th>Age</th>
<th>OS (wks)</th>
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<th>EGFR</th>
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NOTE: Immunohistochemistry scores, 3+, intensive positive; 2+, positive; 1+, focally positive; 0, no staining.

Abbreviations: OS, overall survival (wks); n.a., not assessed.

• *AAlive January 2012.
Neurospheres and their corresponding tumors were analyzed for the presence of genetic alterations known occurring at high frequency in glioblastoma, such as amplification of EGFR (EGFR<sup>amp</sup>), or deletion of its extracellular domain (exon 2–7, EGFR<sup>vIII</sup>), amplification of PDGFRA, mutations of IDH1/2, TP53, PTEN, and NF1 (refs. 6, 16; Table 2; EGFR and PDGFRA gene copy number and expression of EGFR<sup>vIII</sup> in Supplementary Fig. S1B and C; TP53, PTEN, and NF1 mutations in Supplementary Table S1).

View this table:

Table 2.
Genetic lesions in neurospheres (NS) and the corresponding original glioblastoma tissue specimens (T)

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NOTE: +, presence of genetic alterations; –, absence of genetic alterations.

Abbreviation: n.a., not accessed due to lack of material.

- Analysis of EGFR<sup>amp/vIII</sup> is shown in Supplementary Fig. S1 and reported in Supplementary Table S2.
- Detailed list of mutations is reported in Supplementary Table S1
- chr7 polysomy
- Homozygous deletion

Table 2.
Genetic lesions in neurospheres (NS) and the corresponding original glioblastoma tissue specimens (T)

Eight of 18 neurospheres displayed EGFR amplification and/or deletion (EGFR<sup>amp/vIII</sup>), a genetic trait preferentially associated with the "classical" glioblastoma subtype, whereas the remaining harbored a normal EGFR gene (EGFR<sup>wt</sup>), usually found in either "mesenchymal" or "proneural" subtypes (ref. 6; Table 2). No neurosphere displayed the genetic landmarks of the "proneural" subtype, such as PDGFRA amplification or autocrine loop, or IDH1/2 mutations (ref. 6; Table 2 and
data not shown). TP53, PTEN, or NF1 alterations (mutations or deletions) were found in 9 of 18, 10 of 18, and 2 of 18 neurospheres, respectively (Table 2 and Supplementary Table S1). Mutations of TP53 and NF1 were shown to preferentially associate with EGFR wt glioblastomas (6). In our panel, however, only PTEN deletion/mutation was significantly associated with EGFR wt (for TP53, Fisher exact test, \(P = 0.637\); for PTEN, \(P = 0.003\); for NF1, \(P = 0.477\)).

Neurospheres displayed a mutational profile largely overlapping with the corresponding original tumors (Table 2). However, 5 of 13 neurospheres derived from EGFR amp/vIII tumors lacked EGFR alterations (Table 2). Moreover, in the remaining EGFR amp/vIII neurospheres, the number of EGFR gene copies was decreased with respect to the corresponding original tumor (Supplementary Table S2). These findings are consistent with previous reports, indicating that in vitro culture selects against EGFR genetic lesions (refs. 17, 18; see Discussion).

Neurospheres can be classified as classical, mesenchymal, or proneural according to gene expression profile

Next we carried out genome-wide gene expression profiling of the whole neurosphere panel and assessed whether they could be subdivided in classical, mesenchymal, or proneural subgroups by the transcriptional signature identified in an independent set of glioblastoma tissues by Verhaak (ref. 6; Fig. 1A and Supplementary Fig. S2). The classical, mesenchymal, and proneural centroids (i.e., 3 virtual samples displaying average expression of each signature gene in, respectively, classical, mesenchymal, and proneural glioblastomas) were extracted from the published datasets (https://tcga-data.nci.nih.gov/docs/publications/gbm_exp/) and mapped in the microarray, resulting in 549 probes, corresponding to 532 genes. The 3 centroids and the neurosphere samples were then hierarchically clustered by unsupervised analysis. The clustering sharply subdivided the neurospheres in 3 subgroups, including the classical, the mesenchymal, and the proneural centroid, respectively (Fig. 1A and Supplementary Fig. S2).
Neurospheres are classified according to gene expression profile and display subtype-specific EGFR or Met expression. Unsupervised hierarchical clustering of duplicate neurosphere samples (A and B) and classical (CLASS_cen), mesenchymal (MES_cen), or proneural (PRON_cen) centroids. Red cells, amplification/mutation of EGFR (EGFR<sup>amp/vIII</sup>); green cells, deletion/mutation of PTEN (PTEN<sup>del/mut</sup>); black cells, no lesion. *, derived from tumors with EGFR<sup>amp/vIII</sup>; BT273_B2, technical replicate. B, flow cytometric detection of EGFR or Met in neurospheres. C–E, immunophenotype of neurospheres representative of each subtype. F, variation of the average number of cells positive for the indicated markers in each subtype with respect to all neurospheres (absolute numbers in Supplementary Table S3). G–I, relative expression of 532 signature genes (https://tcga-data.nci.nih.gov/docs/publications/gbm_exp/; black dots). x-axis, log<sub>2</sub> ratio between glioblastoma (GB) samples of each subtype versus the other subtypes; y-axis, log<sub>2</sub> ratio between Met-pos-NS and Met-neg-NS.

By comparing neurosphere gene expression and mutational profiles, we observed that the majority (7 of 10) of neurospheres profiled as classical harbored EGFR gene amplification/deletion (EGFR<sup>amp/vIII</sup>; Fig. 1A). Interestingly, the 3 of 10 classical neurospheres without EGFR gene amplification displayed high chromosome 7 polisomy and, in 2 cases, derived from EGFR<sup>amp/vIII</sup> tumors (Table 2). On the contrary, 7 of 8 neurospheres profiled as mesenchymal or proneural harbored a normal EGFR gene (EGFR<sup>wt</sup>; Fig. 1A). Altogether, these data indicated a marked preferential association of EGFR<sup>amp/vIII</sup> with classical compared with mesenchymal/proneural neurospheres (C<sup>2</sup> test, P < 0.04). Vice versa, although not statistically significant, PTEN mutation/deletion was preferentially associated with mesenchymal/proneural compared with classical neurospheres (6 of 8 vs. 4 of 10 neurospheres, C<sup>2</sup> test, P = 0.31; Fig. 1A). Classical and mesenchymal/proneural neurospheres are discriminated by EGFR or Met expression.
Gene expression profiling and qPCR validation (Supplementary Fig. 3A–C) indicated that not only EGFR alteration but also transcription was preferentially associated with classical neurospheres (2-sided t test, P < 0.0001). Vice versa, transcription of the MET gene, known to be expressed in a fraction (≈30%) of unclassified human gliomas (19, 20), was preferentially associated with mesenchymal/proneural neurospheres (2-sided t test, P < 0.01).

To further investigate the role of EGFR and Met as markers of classical and mesenchymal/proneural neurosphere subgroups respectively, we assessed by flow cytometry the cell-surface expression of the 2 receptors in the whole neurosphere panel.

As expected, EGFR was detected in the majority (8 of 10) of classical neurospheres, but only in 2 of 8 mesenchymal/proneural neurospheres (Fig. 1B and Supplementary Table S3). Vice versa, Met was expressed by the majority (7 of 8) of mesenchymal/proneural neurospheres, with variable percentages of positive cells (5%–94%), but only in 2 of 10 classical neurospheres (Fig. 1B and Supplementary Table S3). Therefore, EGFR protein expression was strongly preferentially associated with the classical neurosphere subgroup (f2 test, P < 0.001), whereas Met protein was associated with the mesenchymal/proneural neurosphere subgroup (f2 test, P < 0.02).

Interestingly, Met expression (in the absence of any gene alteration) was detected in the majority of original tumors that generated neurospheres expressing Met, but was mostly absent from tumors that generated neurospheres not expressing Met, and that harbored EGFR amplification (Supplementary Table S4).

We then analyzed whether other cell-surface markers could be specifically associated with each neurosphere subgroup. CD133, previously used (21), and more recently questioned (22, 23), as glioblastoma stem cell marker, was inconstantly expressed (0%–90% of positive cells), without association with any subgroup (Fig. 1C–F and Supplementary Table S3). Also expression of Sox2, Nestin, and CD15/SSEA1, 3 markers associated with the neural stem cell phenotype (24), did not display any statistically significant difference among the subgroups (Fig. 1C–F and Supplementary Table S3; for Sox2, f2 test, P = 0.08; for Nestin, P = 0.09; for CD15, P = 0.314). Consistently, transcription of CD133 and Sox2 and other stem cell markers was comparable in all subgroups (Supplementary Fig. S3D). Finally, cell-surface expression of CD44 (a gene of the mesenchymal signature; refs. 5, 6), or CD271 and CD29 (2 markers of mesenchymal differentiation of neural progenitors; ref. 25), was not preferentially associated with any subgroup (for CD44, f2 test, P = 0.304; for CD271, P = 0.982; for CD29, P = 0.766; Fig. 1C–F and Supplementary Table S3).

Taken together, these data indicated that, in neurospheres, expression of EGFR and Met are almost mutually exclusive. By combined flow cytometric analysis, unlike other markers, the 2 receptors are sufficient to separate neurospheres in 2 subgroups: EGFR-pos/Met-neg-NS (hereafter indicated as Met-neg-NS), roughly corresponding to classical neurospheres, and EGFR-neg/Met-pos-NS (hereafter indicated as Met-pos-NS), roughly corresponding to mesenchymal/proneural neurospheres. Consistently, Met-pos-NS, unlike Met-neg-NS, were highly enriched in mesenchymal or proneural, but lacked classical signature genes (Fig. 1G–I). Strikingly, among all these signature genes, EGFR was, concomitantly, the most expressed in the classical subtype and the least expressed in Met-pos-NS (Fig. 1G).

Met-pos and Met-neg neurosphere subgroups are identified by multiple transcriptional signatures

Neurosphere gene expression profiles were also analyzed by applying additional transcriptional signatures, including 2 identified in glioblastoma neurospheres (26, 27), 2 in original tumors (5, 28), and 1 in mixed tissues, neurospheres, and cell lines (ref. 29; Supplementary Fig. S4 and Supplementary Table S5). The 5 signatures were almost fully mapped in the microarrays and hierarchically clustered neurospheres. In all cases, Met-pos-NS and Met-neg-NS were sharply separated into distinct and homogeneous clusters. By applying the 2-cluster signatures, the majority of Met-pos-NS fell into Cluster II (26), or Type II (27), or GSt/lines group (29), whereas the majority of Met-neg-NS fell into Cluster I (26), or Type I (27), or GSt/tumor group (29). By applying
the multicluster signatures generated from tumor tissues (5, 28), Met-pos-NS fell into the most aggressive subgroups (Supplementary Fig. S4 and Supplementary Table S5). Met-pos and Met-neg neurospheres display subtype-specific growth factor requirements and differentiation patterns. We observed that Met-pos-NS and Met-neg-NS displayed distinctive microscopic features: Met-neg-NS mostly displayed a compact, smooth surface, whereas Met-pos-NS appeared as aggregates of cells with loose intercellular adhesion (Fig. 2A). Moreover, in standard medium, Met-pos-NS displayed a proliferative rate significantly higher than Met-neg-NS (Fig. 2B). We then systematically analyzed the proliferative response to EGF or bFGF, alone or in combination (Fig. 2C–E and Supplementary Fig. S5A). Met-neg-NS were mostly quiescent in the absence of growth factors (data not shown), and, with one exception, were markedly stimulated by EGF, but weakly by bFGF alone; the 2 growth factors were not significantly additive (Fig. 2C and E; median fold increase with EGF = 4.54, with bFGF = 1.45, Supplementary Fig. S5A). Vice versa, Met-pos-NS grew in the absence of exogenous growth factors (data not shown), and, with some exceptions, were further stimulated by bFGF, but not by EGF; again, the 2 factors were not additive (Fig. 2D and E; median fold increase with EGF = 0.94, with bFGF = 2.73, Supplementary Fig. S5A).
The ability of Met-pos-NS to proliferate in the absence of exogenous growth factors could be explained by expression of autocrine loops (data not shown). The different sensitivity of Met-neg-NS and Met-pos-NS to EGF was correlated with the different levels of EGFR expression (Fig. 1B). Consistently, EGFR family members were found significantly phosphorylated, in the presence of EGF, only in Met-neg-NS (Supplementary Fig. S5C). The comparable sensitivity of both Met-neg and Met-pos neurospheres to bFGF correlated with similar expression and ligand-induced tyrosine phosphorylation of FGFR2, the main bFGF receptor (Supplementary Fig. S5B and C).

By culturing dissociated neurospheres in prodifferentiating conditions, Met-neg and Met-pos neurospheres displayed divergent differentiation patterns (Fig. 2F and G, Supplementary Fig. S5D, and data not shown). Met-neg-NS differentiated into neuro-astroglial lineages, as shown by upregulation of the neural marker MAP2 and the astroglial marker GFAP. No oligodendroglial cells were detected after staining with the specific GalC marker. On the contrary, Met-pos-NS differentiated into neuro-oligodendroglial but not into the astroglial lineage.

Finally, a panel of representative Met-neg and Met-pos neurospheres were orthotopically transplanted into immunocompromised mice. Between the 2 subgroups, no significant differences were reported in mouse survival (Fig. 2H and Supplementary Table S6), tumor proliferative index (Supplementary Table S6), or vascularization (data not shown). In no case, invasion of the contralateral brain hemisphere could be observed (data not shown). However, consistent with the differentiation pattern observed in vitro, tumors derived from Met-neg-NS invariably expressed high levels of GFAP, but not GalC. Vice versa, those derived from Met-pos-NS invariably expressed high levels of GalC and traces of GFAP only at the tumor periphery (Fig. 2I, Supplementary Table S6, and data not shown).

The above data showed that, although selected and propagated in the same medium, Met-pos and Met-neg neurospheres have distinct, subtype-specific signaling requirements for proliferation and specific differentiation patterns, both in vitro and in vivo. Altogether, these observations suggested that the corresponding tumors may have different cells of origin (see Discussion).

In Met-pos-NS the Methigh subpopulation is enriched with clonogenic and tumorigenic cells. Having established that Met is a marker of a biologically distinct neurosphere subtype, we investigated its functional role. By flow cytometry (Fig. 1B–E and Supplementary Table S3) and immunofluorescence (Fig. 3A and data not shown), we observed that Met expression was restricted to cell subpopulations of various extents, which, in some cases, expressed also high levels of stem cell markers such as Sox2, Nestin, or CD133 (Fig. 3B and data not shown). We thus sorted the Methigh from the Methneg subpopulation (for sorting parameters see Supplementary Fig. S6) and carried out clonogenic assays by plating and culturing single cells in standard medium. In 9 of 9 Met-pos-NS, Methigh cells invariably displayed higher clonogenic ability as compared with Methneg cells (Fig. 3C and data not shown). Moreover, neurospheres derived from Methigh cells maintained their clonogenic ability and differentiative multipotentiality through more than 20 serial passages, whereas those derived from Methneg cells arrested their growth within 3 to 8 passages.
Cell-cycle analysis of the representative neurospheres BT308 showed that, immediately after sorting, the Met\textsuperscript{high} subpopulation contained a higher percentage of cells in the S phase (8.2% vs. 3.4%) and a lower percentage of apoptotic cells (3.4% vs. 20.7%), as compared with the Met\textsuperscript{neg} subpopulation (overlapping results were obtained with BT337, data not shown). In another set of experiments, Met\textsuperscript{high} and Met\textsuperscript{neg} cells were sorted, plated at clonal density, and cultured in standard medium. The secondary clones were analyzed by flow cytometry for Met expression after 7 and 14 days; the clones formed by Met\textsuperscript{high} cells progressively reacquired the same Met immunophenotypic profile (including Met\textsuperscript{high} and Met\textsuperscript{neg}) as the parental neurospheres (Fig. 3D, Supplementary Fig. S7C, and data not shown). In contrast, neurospheres formed by Met\textsuperscript{neg} cells remained entirely composed of Met\textsuperscript{neg} cells.

Figure 3.
The Met\textsuperscript{high} subpopulation is enriched with clonogenic and tumorigenic cells. A, Met staining in a representative Met\textsuperscript{-pos}-NS (BT308; magnification, ×400). B, flow cytometry; coexpression of Met with Sox2, Nestin, or CD133 in a representative Met\textsuperscript{-pos}-NS (BT308). C, clonogenic assay; number of neurospheres formed by Met\textsuperscript{high} or Met\textsuperscript{neg} cells after 14 days in standard medium. §, 2-sided t test, $P < 0.05$. D, flow cytometry; Met expression analyzed in the unsorted (parental) BT308 neurosphere (black line) at day 0 presorting and in its sorted Met\textsuperscript{high} (red line) and Met\textsuperscript{neg} (blue line) subpopulations at the indicated days after sorting. E, volume of subcutaneous tumors formed by BT308 (left) and 302
(right) neurospheres (parental) and their sorted subpopulations (n = 3 in each group). §, 2-sided t test, P < 0.05. F, hematoxylin and eosin (H&E) and Met immunohistochemical staining (magnification, ×200) of the above tumors. Enlarged images in Supplementary Fig. S8. G, Met and GalC expression in BT308 neurosphere (parental) and its sorted subpopulations, 7 days after culture in standard medium (SM), or in prodifferentiating medium (2% serum). H, qPCR of miR-34a and miR-23b transcripts in BT308 neurosphere grown as in G. §, 2-sided t test, P < 0.05. I, qPCR of MET, miR-34a, and miR-23b transcripts in the Methigh and Metneg subpopulations immediately after sorting from BT308 neurosphere. §, 2-sided t test, P < 0.05.

To investigate the tumorigenicity of the 2 subpopulations, representative parental Met-pos-NS (BT308 and BT302) and their sorted Methigh or Metneg subpopulations were subcutaneously injected into immunocompromised mice. In the case of BT308, only the parental neurosphere and the Methigh subpopulation displayed the ability to form tumors, whereas in the case of BT302, even the Metneg subpopulation generated measurable tumors. However, in both cases, tumors formed by Methigh cells grew significantly more rapidly than those formed by the parental or its sorted Metneg subpopulation (Fig. 3E). Interestingly, tumors derived by Methigh or unsorted cells had a similar histopathologic aspect, featuring a mixture of small-rounded and spindle-shaped cells. By immunohistochemistry, the small-rounded cells were positive for Met expression, whereas spindle-shaped cells were negative. On the contrary, tumors derived by Metneg cells sorted from BT302 only contained uniform spindle-shaped cells negative for Met expression (Fig. 3F, Supplementary Fig. S8, and data not shown).

Taken together, these findings indicated that, unlike the Metneg subpopulation, the Methigh retained long-term clonogenic properties in vitro, enhanced growth kinetics in vivo, and generated a heterogeneous progeny, including Methigh and Metneg cells, both in vitro and in vivo. Whereas Methigh might correspond to stem-like cells, Metneg likely correspond to more differentiated cells that exhaust their proliferative potential. Consistently, we observed that Met expression was downregulated when neurospheres (or the sorted Methigh subpopulations) were cultured in prodifferentiating conditions (Fig. 3G and Supplementary Fig. S7D). Concomitantly, upregulation of miRNA-34a and miRNA-23b, both targeting the MET transcript (30, 31), was observed (Fig. 3H and data not shown). Interestingly, in neurospheres cultured in standard medium, the same miRNAs were more expressed in Metneg than Methigh cells (Fig. SI and Supplementary Fig. S7E).

HGF sustains clonogenicity, expression of self-renewal markers, and cell invasion in vitro. Next we investigated whether HGF stimulated proliferation of Met-pos-NS and their sorted Methigh and Metneg subpopulations. When supplied to parental neurospheres as the sole growth factor, HGF displayed a negligible proliferative effect, if compared with bFGF (Fig. 4A and Supplementary Fig. S9A). However, in the sorted Methigh subpopulation, HGF significantly increased proliferation, although less intensely than bFGF (2-fold versus 6-fold increase; Fig. 4A and Supplementary Fig. S9A). As expected, HGF did not stimulate proliferation of Metneg cells (Fig. 4A and Supplementary Fig. S9A) and Met-neg-NS (data not shown).
Figure 4.
HGF sustains clonogenicity and expression of self-renewal markers. A, proliferative effect of growth factors on the Met-pos-NS BT308 (parental) or its sorted subpopulations, with respect to control (no growth factor), after 8 days of culture. §, 2-sided t test, P < 0.05. B, clonogenic assay; number of neurospheres formed by Met$^{\text{high}}$ or Met$^{\text{neg}}$ cells after 14 days with (+) or without (−) HGF. §, 2-sided t test, P < 0.05. C, flow cytometry; expression of Sox2 or Nestin in BT308 neurosphere in standard medium (SM) with or without HGF. §, 2-sided t test, P < 0.05. D, qPCR; expression of Nanog and Sox2 in neurospheres treated with HGF with respect to standard medium (Ctrl).

Moreover, HGF supported the clonogenic ability of Met$^{\text{high}}$ cells, sorted as single cells from Met-pos-NS and cultured in the presence or in the absence of HGF as the sole growth factor (Fig. 4B). Conversely, HGF did not stimulate neurosphere formation by Met$^{\text{neg}}$ cells (Fig. 4B). Accordingly, in Met-pos-NS, addition of HGF significantly increased the number of Sox2$^{\text{pos}}$ or Nestin$^{\text{pos}}$ cells (Fig. 4C and Supplementary Fig. S9B) and transcription of self-renewal markers Sox2, Nanog, CD133, and EZH2 (Fig. 4D and Supplementary Fig. S9C), as compared with standard medium. Taken together, these data indicated that HGF sustains the stem-like phenotype of Met-pos-NS. Met signaling has been associated with induction of epithelial–mesenchymal transition and the “invasive growth” program (32–34). We thus investigated whether HGF supported the invasive properties of neurospheres. In transwell assays, addition of HGF to standard medium strikingly enhanced migration and invasion of Met-pos-NS (Fig. 5A and B and data not shown). This effect was completely abolished by specific Met inhibitors, including the Fab fragment of the anti-Met antibody DN30 (ref. 35; Fig. 5C) or the specific tyrosine kinase inhibitor JNJ-38877605 (ref. 14; Supplementary Fig. S9D). As expected, HGF did not increase migration or invasion of Met-neg-NS...
In these cells, transfection of Met did not promote invasiveness per se but conferred the ability to respond to HGF (Fig. 5D).

Figure 5.
HGF sustains invasive growth. A and B, transwell assay; neurospheres were dissociated and analyzed for migration (without Matrigel coating, A) or invasion (with Matrigel coating, B) in standard medium with or without HGF. §, 2-sided t test, \( P < 0.05 \). C, neurospheres assessed as in B, with (+) or without (−) anti-Met antibodies (DN30-Fab). §, 2-sided t test, \( P < 0.05 \). D, representative Met-neg-NS transfected (+) or not (−) with Met and assessed as in B. §, 2-sided t test, \( P < 0.05 \). E, phosphorylation of intracellular signal transducers in BT308 neurosphere cultured in standard medium with or without HGF for 24 hours. Statistically significantly modulated proteins were represented (2-sided t test, \( P < 0.05 \)).

Analysis of intracellular phosphoprotein arrays, showed that, in Met-pos-NS, HGF induced phosphorylation of signal transducers known to control cell invasion, such as JNK, MEK 1/2, several members of Src and STAT families, and p27 (ref. 34; Fig. 5E). Interestingly, the latter was phosphorylated at a residue (T157) that promotes cytoplasmic localization and activation of the cell migratory machinery (36). We also observed decreased phosphorylation of p53 at residues Ser15, Ser46, and Ser392, which results in p53 inhibition (37). Taken together, these results showed that Met activation by HGF concomitantly supports the stem-like and the invasive phenotype of Met-pos-NS in vitro and suggest that this mechanism may promote aggressiveness of a subset of glioblastomas.
A unifying model of tumor onset and progression that integrates the CSC model and the Darwinian model assumes that CSCs accumulate the driving genetic lesions and transmit them to the genetically and phenotypically heterogeneous progeny forming the tumor bulk (38). The comparative analysis reported in this article showed that, as a rule, the same mutations of primary glioblastomas are found in their matched neurospheres. This confirms that neurospheres are a faithful in vitro model of the original tumor, useful to dissect the relationship between genetics and biology, and to predict the therapeutical response.

Notably, neurospheres derived from EGFR^{ampl} tumors displayed a decreased—or in a few cases an even normal—number of EGFR gene copies, consistent with previous and recent data (17, 18). As EGFR amplification is usually detected only in a fraction of glioblastoma cells (data not shown and ref. 39), these findings can be explained by in vitro negative selection of clones harboring EGFR amplification and positive selection of clones with a normal EGFR gene, coexisting in the same tumor. Growth of clones with normal/low number of EGFR gene copies might be favored by concentrations of exogenous EGF (20 ng/mL), likely exceeding those in brain tissues (18).

Gene expression profiling allowed to classify the neurospheres into classical, mesenchymal, and proneural subtypes according to the signatures identified in glioblastoma tissues by Verhaak (6). Interestingly, the classical subgroup encompassed the vast majority of neurospheres harboring EGFR amplification (7 of 10), confirming an association between the classical expression profile and EGFR genetic alteration already observed in tumors (6). Vice versa, the mesenchymal/proneural subgroup included neurospheres mostly harboring a wild-type EGFR gene (7 of 8), together with deletion/mutation of PTEN tumor suppressor gene (6 of 8). This association—to our knowledge—was still unreported in tumors or neurospheres.

EGFR is renowned as a prominent player of glioma biology (40) and tumorigenic potential of glioblastoma stem cells (41). However, we found that EGFR was highly expressed in classical neurospheres, consistent with the presence of gene amplification, but barely detectable in most mesenchymal/proneural neurospheres. In search for a functional marker for glioblastoma stem cells lacking EGFR, we considered the MET oncogene. Indeed, we noticed that MET was listed among genes upregulated in microarrays of glioblastoma tissues, in association with the mesenchymal subtype (6). Moreover, recently, Met was shown to support the stem-like phenotype of unclassified glioblastoma neurospheres (42). The data presented in this article show, for the first time, that Met expression is preferentially associated with the mesenchymal/proneural subtype of glioblastoma stem cells, and that expression of EGFR and Met are mutually exclusive in neurospheres and, possibly, in original tumors. If further studies will confirm that the cellular distribution of Met and EGFR in patients reflects that observed in neurospheres, there will be far-reaching implications for the molecular diagnostics of glioblastoma. Flow cytometric or immunohistochemical analysis of the EGFR-Met pair could be proposed as a reliable test to discriminate between classical and mesenchymal/proneural glioblastoma, possibly in addition to previous criteria, such as YKL-40 expression (5).

Interestingly, the neurosphere subgroup expressing Met (Met-pos-NS), irrespective of their mesenchymal or proneural profile, and the subgroup lacking Met (Met-neg-NS) displayed significant biologic differences. They had a different proliferation rate, invariably higher in Met-pos-NS. In this respect, the 2 subgroups were reminiscent of those previously described (12). Interestingly, Met-pos-NS mostly proliferated even without growth factors, and, as expected, were insensitive to EGF, whereas Met-neg-NS depended on exogenous growth factor, mostly EGF. Moreover, the 2 neurosphere subgroups showed a divergent differentiation pattern, either in vitro, or in tumors formed by orthotopic transplantation: Met-pos-NS differentiated along the neuro-astroglial, whereas Met-neg-NS along the neuro-oligodendroglial pathway. These findings seem
consistent with observations in mouse model systems, in which brain progenitors inheriting high levels of EGFR give rise to astrocytes, whereas those inheriting low levels generate oligodendrocytes (43). Strikingly, the Met-pos and Met-neg neurosphere subgroups not only displayed distinct biologic features but were conserved according to 5 additional transcriptional classifiers, obtained in neurospheres or in original tumors (5, 26–29). When 2-cluster classifiers found in neurospheres by Gunther and colleagues (26) or Lottaz and colleagues (27) were applied, Met-neg-NS almost fully overlapped with Cluster I/Type I, whereas Met-pos-NS overlapped with Cluster II/Type II. Taken together, biologic and gene expression features of Met-pos and Met-neg neurospheres suggest that Met expression could associate with tumors deriving from different cells of origin. Met-neg-NS (and glioblastoma) could derive from stem or transit amplifying cells of the brain subventricular zone, which depend on EGFR signaling (8, 44). Conversely, Met-pos-NS (and glioblastoma) could originate either from the subventricular progenitors inheriting low levels of EGFR (43), or the diffuse astrocytes of the reactive glia. These cells reactivate their proliferative and regenerative potential in response to injuries (45). The reactive astrocytes are an appealing candidate as a glioblastoma cell-of-origin, also because they are intermingled within the blood–brain barrier and may be easily exposed to genotoxic agents.

Another novel finding presented in this study is that, in each Met-pos-NS, Met marks and functionally supports a cell subpopulation that retains long-term clonogenic and multipotential ability in vitro and enhanced growth kinetics in vivo and thus may retain cancer stem cell properties. Conversely, in Met-pos-NS, loss of Met expression characterizes a cell subpopulation that exhausts its clonogenic activity in vitro and in vivo. These data strongly suggest that Met is a glioblastoma stem cell marker, which can be proposed for cell isolation as an alternative to CD133. A further new finding presented in this study indicates that Met supports not only the stem-like but also the invasive phenotype, at least in vitro. Indeed, invasiveness of Met-pos-NS was significantly enhanced by the Met ligand HGF, a key driver of invasive growth (32), and counteracted by specific Met inhibitors. The Met ability to concomitantly support stemness and invasiveness shows that the two phenotypes are functionally associated and driven by the same signaling circuits and genetic programs, consistent with previous observations (46).

It has been noticed that “the existence of molecularly defined subgroups of glioblastoma raises the question of whether these categories actually represent separate disease entities rather than the expression of minor variability in a single tumor class (15).” Our study suggests that primary glioblastomas contain distinct types of CSCs, each possibly arising from distinct cells of origin, each endowed with specific molecular markers and signaling circuits responsible for stem and tumorigenic properties. These findings contribute to identify separate glioblastoma entities and to define criteria that might be exploited to guide therapeutic decision making.

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

Grant Support
The study was supported by Italian Association for Cancer Research (Investigator Grants and “Special Program Molecular Clinical Oncology 5xMille, N. 9970”), Regione Piemonte (PI-STEM), European Union Framework Programs 7 (N. 201279 and 201640).

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Acknowledgments
The authors thank Andrea Bertotti, Livio Trusolino, and Claudio Isella for critical discussion; Stefania Giove for histopathology; Daniela Gramaglia, Antonella Cignetto, and Michela Bruno for secretarial assistance.

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