Supplementary Figure Legends

Figure S1. GBM Stem Cells Are Able to Differentiate into Pericytes in vitro, related to Figure 1

(**A**) Immunofluorescent (ImF) staining of the GSC marker (SOX2) and pericyte marker (α-SMA or NG2) in the freshly isolated GSCs from a primary GBM (CW1326). The sorted GSCs were immunostained with specific antibodies against the GSC marker SOX2 (in red) and a pericyte marker (α-SMA or NG2, in green), and then counterstained with DAPI. The sorted GSCs express SOX2 but do not contain any cell positive for the pericyte markers.

(**B**) ImF staining of pericyte marker (α-SMA or NG2) and the GSC marker (SOX2) in GSC tumorspheres. Frozen sections of GSC tumorspheres derived from GSCs from a primary GBM (CW1217) were immunostained with specific antibodies against a pericyte marker (α-SMA or NG2, in green) and a GSC marker (SOX2, in red), and then counterstained with DAPI to mark nuclei (in blue). The majority (91-96%) of cells in GSC tumorspheres expressed SOX2 but no cell in the tumorspheres expressed the pericyte markers.

(**C**) ImF staining of pericyte markers (α-SMA, NG2, CD146 and CD248) in the differentiated cells derived from the freshly isolated GSCs from a human primary GBM (CCF2049). The sorted GSCs were cultured in DMEM with 10% FBS for 6 days, then immunostained with specific antibodies against the indicated pericyte markers (in green) and counterstained with DAPI to mark nuclei (in blue). Quantification on right side shows fractions of the differentiated cells expressing the pericyte markers. The

differentiation experiments were done four times and 300 cells were counted each time for each pericyte marker.

(**D** and **E**) ImF staining of α-SMA and NuMA (a human cell-specific nuclear antigen) in the differentiated cells derived from GSC tumorspheres. The cells from single tumorsphere derived from GSC (CCF1468) were induced for differentiation in DMEM with 10% FBS for 6 days. The differentiated cells were immuno-stained with specific antibodies against α-SMA (in green) and the human cell-specific nuclear antigen NuMA (in red) to mark human cells, and counterstained with DAPI (in blue). Quantification (E) shows that the differentiated cells contain a fraction (4-11%) of cells expressing the pericyte marker.

(**F**) ImF staining of α-SMA (in green) and SOX2 (in red) in the single GSC-generated tumorspheres derived from the GSCs without host-derived pericyte. The freshly sorted GSCs from a GBM xenograft (T3691) were subjected for secondary selection positive for TRA-1-85 (a human cell-specific surface antigen) and negative for CD146 by using the FITC-conjugated anti-TRA-1-85 and the PE-conjugated anti-CD146. Frozen sections of the single GSC-derived tumorspheres from the GSCs (TRA185+CD146-) after the double sorting were immunostained with specific antibodies against the pericyte marker (α-SMA, in green) and a GSC marker (SOX2, in red), and then counterstained with DAPI (in blue). All cells in the tumorspheres are negative for the pericyte marker α-SMA but most cells show SOX2 expression.

(**G**) ImF staining of α-SMA (in green) and NuMA (a human cell-specific nuclear antigen, in red) in the differentiated cells from the single GSC-generated tumorsphere described in (F). Cells were counterstained with DAPI (in blue). The differentiated cells

are all NuMA positive and contain a fraction (4-11%) of cells expressing the pericyte marker α-SMA.

(**H**) FACS sorting of pericyte population (CD146+CD248+) from a GBM xenograft. The pericytes were isolated from total cells derived from GBM xenografts (T3359) through cell sorting with the PE-conjugated anti-CD146 and the FITC-conjugated anti-248 specific antibodies.

(**I**) ImF staining of NuMA (in red) in the freshly sorted pericyte population (CD146+CD248+) described in (H). Cells were counterstained with DAPI to show nuclei (in blue). The human cell-specific nuclear antigen NuMA (in red) was expressed in the majority of sorted pericytes from the GBM xenografts.

(**J**) FACS sorting of endothelial cell (EC) population (CD31+CD105+) from a GBM xenograft. The ECs were isolated from total cells derived from GBM xenografts (T3359) through cell sorting with PE-conjugated anti-CD31 and the FITC-conjugated anti-105 antibodies.

(**K**) ImF staining of NuMA (in red) in the freshly sorted EC population (CD31+CD105+) described in (J). Cells were counterstained with DAPI to show nuclei (in blue). The sorted ECs from the GBM xenografts do not contain any cell expressing the human cell-specific nuclear antigen NuMA.

(**L**) Quantification of the fractions of the human GBM cell- or mouse-derived pericytes and ECs in the freshly sorted pericyte population and EC population from the GBM xenografts. More than 70% of the freshly sorted pericytes (CD146+CD248+) from GBM xenografts are positive for the human cell-specific antigens NuMA, while the freshly sorted ECs (CD31+CD105+) from the same xenografts are completely negative for the

human cell-specific antigen NuMA, indicating that ECs are mouse-derived and rarely derived from human cancer cell in the GBM xenografts.

All scale bars represent 25 μm. The error bars represent SD.

Figure S2. GSC-derived Pericytes in Xenografts Express Similar Pericyte Markers as Vascular Pericytes in Normal Brain and Primary GBMs, related to Figure 2

(**A**) ImF staining of pericyte markers (NG2, Desmin, PDGFRβ, CD248 and α-SMA) in normal brain. Frozen sections of normal brain tissue adjacent to a GBM tumor were immuno-stained with anti-CD31 antibody to mark ECs (in red) and a specific antibody against one of the pericyte markers (in green). Sections were counterstained with DAPI to mark nuclei (in blue). A portion of the vessel in each panel was enlarged and displayed at corner of the picture. Vascular pericytes in normal brain tissue show abundant expression of the pericyte markers.

(**B** and **C**) ImF staining of pericyte markers (NG2, Desmin, α-SMA, PDGFRβ, CD248, Ang1 and CD13) in primary GBM tumors. Frozen sections of primary GBMs (B: CCF2509; C: CCF2445 and CW1336) were immuno-stained with anti-CD31 antibody to mark ECs (in red) and a specific antibody against one the pericyte markers (in green). Sections were counterstained with DAPI to mark nuclei (in blue). Although pericytes surrounding the tumor vessels appear abnormal, these tumor pericytes also express the specific pericyte markers that are expressed by pericytes in normal brain.

(**D** and **E**) In vivo cell lineage tracing of GSCs with GFP constitutive expression and ImF staining of pericyte markers in the GSC-derived GBM xenografts. GSCs from a primary GBM (CW738) were transduced with GFP stable expression and then

transplanted into mouse brains to establish GBM xenografts. Frozen sections of the GBM xenografts were immuno-stained with specific antibodies against the pericyte markers (NG2, CD146, PDGFRβ or CD248, in red) and counterstained with DAPI to show nuclei (in blue). Representative images of tumor vessels with GFP+ pericytes expressing NG2, PDGFRβ, CD146 or CD248 are shown in (D). Quantifications (E) shows that the majority (>78%) of the pericyte marker-expressing cells are GFP positive (in green), confirming that GSCs generate the majority of vascular pericytes in the GBM xenografts.

All scale bars represent 25 μm. The error bars represent SD.

Figure S3. In Vivo Cell Lineage Tracing of GSCs with Pericyte or EC Specific Promoter-driven Fluorescent Reporters, related to Figure 3

(A) Cell type-specific expression of the Desmin promoter-driven GFP, α-SMA promoter-driven mCherry, CD31 or CD105 promoter-driven GFP constructs in the in vitro functional test. Endothelial cells (HBMECs), pericytes (HBVPs) and neural progenitor cells (NPCs) were transduced with the indicated expression constructs through lentiviral infection, and then immunostained with a specific antibody against a cell type marker (CD31 for ECs, α-SMA for pericytes, and SOX2 for NPCs). Nuclei were counterstained with DAPI (in blue). The Desmin promoter-driven GFP expression (green) or α-SMA promoter-driven mCherry (in red) was detected in pericytes but not in ECs and NPCs (left panels), while the CD31 or CD105 promoter-driven GFP expression (green) was detected in ECs but not in pericytes and NPCs (right panels), validating that

the reporter expression systems controlled by the lineage-specific promoters are functional and cell type-specific.

(**B** and **C**) In vivo cell lineage tracing of GSCs with different TCGA subtype by using the Desmin promoter-driven GFP expression in the xenografts. The TCGA subtypes of GBMs were determined by RT-PCR analysis of molecular signature of EGFR, PTEN, NF1, IDH1 and PDGFRα in the tumor cells (Supplemental table S1). Mesenchymal GBMs: EGFR wild-type and PTEN or NF1 deletion/mutations; Classic GBMs: EGFR amplification or expression of $EGFR^{VIII}$; Proneural/neural GBMs: PDGFRα amplification but EGFR wild-type. The GSCs isolated from primary GBMs with mesenchymal, classic or proneural subtype were transduced with DesPro-GFP through lentiviral infection and then transplanted into mouse brains to establish GBM xenografts. Frozen sections of the GBM xenografts were immuno-stained with the specific antibody against the pericyte marker (NG2, in red) and counterstained with DAPI to show nuclei (in blue). Quantification (C) shows that the fraction (%) of the GSC-derived pericytes in the GBM xenografts derived from mesenchymal GSCs is significantly higher than that in the GBM xenografts derived from classic or proneural GSCs, indicating that mesenchymal GSCs have significantly greater ability to generate vascular pericytes than classic and proneural GSCs in vivo. *, $p<0.002$; **, $p<0.003$; ***, $p>0.5$.

(**D**) In vivo cell lineage tracing of GSCs with the CD31 promoter-driven GFP in GBM xenografts. The freshly sorted GSCs (CW777) were transduced with CD31Pro-GFP and transplanted into mouse brains to establish GBM xenografts. Tumor sections from the GBM tumors were immuno-stained with the specific antibody against CD31 or Glut1 (EC markers, in red) and counterstained with DAPI to show nuclei (in blue). No GFP-

positive EC was detectable in the GBM xenografts, indicating that GSCs did not give rise to ECs in vivo.

(**E**) In vivo cell lineage tracing of GSCs with CD105 promoter-driven GFP in GBM xenografts. The freshly sorted GSCs from a primary GBM (CCF2049) were transduced with CD105Pro-GFP and then transplanted into mouse brains to establish GBM xenografts. Frozen sections of the GBM xenografts were immunostained with the anti-CD31 (in red) and counterstained with DAPI (in blue). No GFP+ EC was detectable in the GBM xenografts, confirming that GSCs did not generate ECs in vivo.

The scale bars represent 10 μm (A) and 25 μm (B, D and E). The error bars represent SD.

Figure S4. GSC-derived Pericytes Express Similar Pericyte Markers as Normal Brain Vascular Pericytes but rarely Express GSC or Astrocyte Markers, related to Figure 3

(**A**) RT-PCR analysis of pericyte marker expression in GSC-derived pericytes and human brain vascular pericytes (HBVPs). GSC-derived pericytes were obtained by sorting GFP+CD146+ cells from the xenografts derived from the DesPro-GFP GSCs. GFP-CD146- cells sorted from the same xenografts were used for negative control. Normal vascular pericytes (HBVP line, from ScienCell) were used for positive control. RNA samples isolated from these cells were subjected to RT-PCR analyses to examine the expression of several pericyte markers (α-SMA, Desmin, CD146, NG2, PDGFRβ and CD248), an astrocyte marker (GFAP) and a GSC maker (OLIG2) in these cells. GFP serves as a positive marker for the GSC-derived pericytes isolated from the DesPro-GFP

GSC xenografts. GSC-derived pericytes and HBVP pericytes show similar but not identical expression of the pericyte markers.

(**B**) RT-PCR analysis of GSC marker expression and pericyte marker expression in GSCs and GSC-derived pericytes (G-pericytes). The G-pericytes (GFP+CD146+) were isolated from DesPro-GFP GSC xenografts. The freshly sorted G-pericytes barely express the NSC or GSC markers (SOX2, OLIG2, CD133 and Nestin), while the GSCs do not express the pericyte markers (Desmin and NG2). The expression of GFP in the sorted G-pericytes (GFP+CD146+) serves as a positive control for this population.

(**C-F**) Immunofluorescent staining of GSC markers (SOX2, OLIG2 or Nestin, in red) and astrocyte markers (GFAP or S100β, in red) on tumor sections of GBM xenografts derived from the DesPro-GFP-transduced GSCs. The GSCs from a primary GBM (CCF2509) or a GBM xenograft (T4121) were transduced with the Desmin promoter-driven GFP through lentiviral infection, and then transplanted into mouse brain to establish GBM xenografts. Frozen sections of the xenografts were immunostained with the specific antibody against SOX2, OLIG2, Nestin, GFAP or S100β (in red), and counterstained with DAPI to show nuclei (in blue). The GSC-derived pericytes (GFP+, in green) rarely express the GSC marker SOX2, OLIG2 or Nestin, while GSCs expressing SOX2, OLIG2 or Nestin (in red) are distributed in the perivascular niches but rarely overlap with GFP+ cells (G-pericytes) in the GBM xenografts (C and D). GSC-derived pericytes (GFP+) do not express any astrocyte marker GFAP or S100β (in red) (E). Quantification (F) shows that a small fraction (0.9-1.6%) of GSC-derived pericytes (GFP+) are positive for the GSC markers (SOX2, OLIG2 or Nestin) but no GSC-derived pericyte showed positive for the astrocyte markers.

(**G**) Immunofluorescent staining of the astrocyte marker (GFAP) and the pericyte markers (Ang1 and CD13) in primary GBMs. Frozen sections of GBMs (CCF2445 and CW1336) were immunostained with specific antibodies against GFAP (in green) and Ang1 or CD13 (in red), and then counterstained with DAPI to show nuclei (in blue). Pericytes and astrocytes are two distinct populations without overlapping expression of their markers in the primary GBMs.

All scale bars represent 25 μm.

Figure S5. Endothelial Cells Are Rarely Derived from Cancer Cells in Human Primary GBMs and the Genetically Engineered Mouse GBMs, related to Figure 4

(**A**) Fluorescence in situ hybridization (FISH) analyses of genetic alterations with FITC-conjugated probes of CEP-7, CEP-10, EGFR and PTEN (in green) in vascular ECs marked by CD31 staining (in red) and in cancer cells in primary GBMs. Representative images of ECs positive for CD31 are indicated by white arrows. ECs did not carry the same genetic alterations as cancer cells. * marks the vessel lumen.

(**B**) Quantification shows that tumor ECs rarely carry the cancer genetic alterations (CEP-7 polysomy, 28 cases; EGFR trisomy or amplification, 25 cases; CEP-10 loss, 19 cases; or PTEN loss, 21 cases) in 56 cases of primary GBMs in tissue arrays. TCs: tumor cells; ECs: Endothelial cells.

(**C** and **D**) FISH analysis with CEP-7 probe in the freshly sorted ECs and GSCs from primary GBMs. The sorted ECs (CD31+CD105+) and GSCs were subjected to FISH analysis with FITC-conjugated CEP-7 probe (in green) and ImF staining of Glut1 (another EC marker, in red), and counterstained with DAPI to show nuclei (in blue).

Representative images of FISH analysis with CEP-7 (in green) and Glut1 staining (in red) in sorted ECs and GSCs from a primary GBM (CW837) are shown in (C). Quantification (D) shows that the sorted ECs from six primary GBMs rarely carry the same genetic alterations (CEP-7 polysomy or CEP-10 loss) as GSCs.

(**E**) ImF staining of EC markers in the genetically engineered mouse GBMs (Nestin-tva/Ink4a/Arf-/-/HA-PDGFB models). Frozen sections of the mouse GBM tumors were immunostained with specific antibodies against EC markers (CD31 or Glut1, in green) and HA-tag to detect cancer cells expressing HA-PDGFB (in red), and then counterstained with DAPI to show nuclei (in blue). Typical cells showing positive for the EC marker are enlarged and displayed at corners of the panels. Vascular ECs in the mouse GBMs do not express HA-PDGFB (cancer cell marker in this tumor model), indicating ECs are rarely derived from the cancer cells.

The scale bars represent 10 μm (A and C) and 25 μm (E). The error bars represent SD.

Figure S6. Selective Targeting of GSC-derived Pericytes Disrupts Vascular Structure and Function in GBM Xenografts, related to Figure 5

(**A**) Schematic illustrations of lentiviral constructs for the Desmin promoter-driven expression of HsvTK (herpes simplex virus thymidine kinase) or co-expression of HsvTK and GFP (DesPro-TK or DesPro-TK-GFP). Desmin promoter (312 bp) with an enhancer (284 bp) was cloned from human genomic DNA. The Desmin promoter-driven expression of HsvTK or/and GFP should occur specifically in GSC-derived pericytes after GSCs are transduced with the construct.

(**B**) In vitro cell lineage tracing of GSCs with Desmin promoter-driven GFP expression. The isolated GSCs (CCF2045) were transduced with DesPro-TK-GFP through lentiviral infection, and then induced to differentiate. The expression of GFP was turned on in some of differentiated cells derived from the GSCs transduced with DesPro-TK-GFP (right panel).

(**C**) Detection of apoptotic cells by the PE-conjugated anti-Annexin V antibody (PE-Anx V) in differentiated cells derived from the DesPro-TK-GFP transduced GSCs after GCV treatment. GSCs were transduced with DesPro-TK-GFP through lentiviral infection and then induced to differentiate in DMEM with 10% FBS. The differentiated cells were treated with GCV or vehicle control for 48 hours and then immuno-stained with PE-conjugated anti-Annexin V antibody (in red) to detect apoptotic cells. Nuclei were counterstained with DAPI (blue). GCV treatment selectively induced apoptosis in the GSC-derived pericytes (GFP+).

(**D** and **E**) Immunofluorescent staining of CD31 showing effects of selective targeting of GSC-derived pericytes on vessel structure in GBM xenografts. GSCs (D456) were transduced with DesPro-TK-GFP through lentiviral infection, and then transplanted into mouse brains to establish GBM xenografts. Mice bearing the GBM tumors were treated with ganciclovir (GCV) or vehicle control daily for one week. Frozen sections from the GBM tumors were immuno-stained with anti-CD31 antibody to mark ECs (in red) and analyzed for GSC-derived pericytes (GFP+, in green). GCV treatment resulted in depletion of GSC-derived pericytes (loss of GFP+ cells surrounding the vessels), closure of vessel lumens and disruption of vascular walls in GBM xenografts (right panels).

Quantification (E) shows that GCV treatment significantly reduced vessel pericyte coverage by the GSC-derived pericytes (GFP+). *, $p<0.001$.

(**F** and **G**) Assessment of vascular function with FITC-conjugated mega-dextran (in green) in the GBM xenografts after selective elimination of GSC-derived pericytes by GCV treatment. Mice bearing the GBM tumors derived from Des-TK-transduced GSCs were treated with GCV or vehicle control for one week, perfused with FITC-mega-dextran for 60 minutes, and then harvested for fluorescent analysis of FITC-mega-dextran perfusion in tumors. Frozen sections of the GBM xenografts were counterstained with DAPI (blue) and then examined for intensity of perfused FITC-mega-dextran. Quantification (G) shows that selective elimination of GSC-derived pericytes by GCV treatment significantly reduced FITC-mega-dextran perfusion into tumors. *, $p < 0.001$.

(**H** and **I**) Immunohistochemical (IHC) staining of CD31 to examine vessel density in GBM xenografts after selective elimination of GSC-derived pericytes by GCV treatment. Mice bearing the GBM tumors derived from GSCs (CCF2170) transduced with DesPro-TK or DesPro-GFP (control) were treated with GCV for two weeks, and the tumor sections were analyzed by IHC staining of CD31 to mark blood vessels. Sections were counterstained with hemotoxylin for nuclei. GCV treatment disrupted vessels in the GBM tumors derived from GSCs transduced with DesPro-TK but not in control tumors derived from GSCs transduced with DesPro-GFP. Quantification (I) shows that selective targeting of GSC-derived pericytes by GCV treatment significantly reduced vessel density in GBM xenografts derived from GSCs with DesPro-TK but not in the control xenografts derived from GSCs with DesPro-GFP. *, $p < 0.001$.

(**J**) Representative images of cross sections (H&E stained) of mouse brains bearing the GBM xenografts derived from GSCs transduced with Desmin promoter-driven TK after GCV or vehicle control treatment for two weeks. GSCs (CCF2170) were transduced with DesPro-TK and then transplanted into mouse brains to establish GBM xenografts. Mice bearing the GBM tumors were treated with ganciclovir (GCV) or vehicle control daily for two week. Cross sections of mouse brains bearing the GBM xenografts were analyzed by H&E staining. GCV treatment depleting the GSC-derived pericytes expressing HsvTK potently inhibited tumor growth of GBM xenografts derived from GSCs with DesPro-HsvTK.

The scale bars represent 25 μm (B and C) and 100 μm (D, F and H). The error bars represent SD.

Figure S7. Glioblastoma Stem Cells Are Recruited toward Endothelial Cells via the SDF-1/CXCR4 Axis to Generate Vascular Pericytes, related to Figure 6

(**A** and **B**) In vitro matrigel invasion assay showing that SDF-1α (CXCL12) is a dominant cytokine to attract GSC migration. GSCs (CW619) were added to upper chambers and the cytokines were added to lower chambers of the transwells in the matrigel invasion assay. Cells migrated through the matrigel were stained and the representative images are shown in (A). Quantification (B) indicates that SDF-1 potently promotes GSC migration through the matrigel. *, $p < 0.04$; **, $p < 0.09$; ***, $p < 0.001$.

(**C** and **D**) In vitro matrigel invasion assay showing that HBMECs attract GSC toward the ECs and this effect was attenuated by the SDF-1 blocking antibody. GSCs (CW702) were added to upper chamber and HBMECs were cultured on bottom chamber without or

with SDF-1 blocking antibody (SDF1 mAb) or IgG control in the Boydern chambers. GSCs migrated through the matrigel were stained and representative images from the indicated conditions are shown in (C). Quantification (D) shows that HBMECs promote GSC migration through the matrigel, and this effect was significantly inhibited by the SDF-1 blocking antibody (D). *, $p < 0.001$; **, $p < 0.002$.

(**E**) Immunofluorescent staining of SDF-1 (in green) and CD31 (in red) in normal brain tissue and a primary GBM tumor. Sections of a human primary GBM tumor (CCF2045) and the adjacent normal brain tissue were immuno-stained with specific antibodies against SDF-1 (in green) and the EC marker CD31 (in red). Nuclei were counterstained with DAPI (blue). Blood vessels in both brain tissue and GBM tumor produce abundant SDF-1 that forms chemo-attractant gradient around the vessels.

(**F** and **G**) In vitro endothelial complex formation assay showing that recruitment of GSCs toward HBMEC complex was inhibited by the SDF-1 blocking antibody. GFP-labeled GSCs (CCF2170) were added to the established HBMEC complex in the presence of the anti-SDF-1 mAb or IgG control. The integration of GSC-derived cells (in green) into the endothelial complex was monitored. The representative merged images of HBMEC complex with GSC-derived cells at 36 hours after cell mixing are shown (F). Quantification (D) shows the fractions of GSC-derived cells (GFP+) integrated into the EC complex in the presence of SDF-1 mAb or IgG at 36 hours. The SDF-1 blocking antibody significantly inhibited the recruitment of GSCs (D456 and CCF2170) to the endothelial complex. *, $p < 0.001$.

(**H** and **I**) The Effect of CXCR4 inhibition by AMD3100 on recruitment and integration of GSCs to HBMEC complex. HBMECs labeled with the green fluorescent cell tracer

CFSE were allowed to form endothelial complexes (in green). The GSCs (D456MG or CCF1468) labeled with the red fluorescent cell tracer CMTRX (in red) were added to the HBMEC complex in the presence of AMD3100 or vehicle control. The representative images of the EC complex at indicated time points are shown (H). Quantification (I) shows the fractions of GSC-derived cells integrated into HBMEC complex in the presence of AMD3100 or vehicle control at indicated time points. AMD3100 significant inhibited the recruitment and integration of GSC-derived cells into HBMEC complex. *, $p < 0.001$.

(**J** and **K**) Immunohistochemical (IHC) staining of CD31 to examine the effect of CXCR4 knockdown in GSCs on vessel density in the GBM xenografts. Tumor sections from the GBM xenografts derived from GSCs expressing non-targeting shRNA (shNT) or CXCR4 shRNA (shCXCR4) were analyzed by IHC staining of CD31 (in brown). Representative images of the vascular staining in the GBM tumors are shown in (J). Quantification (K) shows that the vessel density was significantly reduced in GBM xenografts derived from GSCs expressing shCXCR4 relative to control xenografts derived from GSCs expressing NT shRNA. *, $p < 0.001$.

The scale bars represent 25 μm (A and C) and 100 μm (E, F, H and J). The error bars represent SD.

Supplemental Table S1. Summary of genetic lesions in GBM tumors with molecular signature of different TCGA subtype, related to Figure 3

TCGA Subtype	GBM Case #	Genetic Alterations						
		$EGFR^{amp}$	$EGFR^{VIII}$	PTEN*	NF1*	$PDGFRA^{amp}$	$IDH1/2^{\$}$	P53*
Classic	CW739	+	-	-	-	-	-	+
	CW777	-	+	-	-	-	-	-
	CW1326	+	+	-	-	-	-	-
	CCF2128	-	+	-	-	-	-	+
	CCF2049	+	-	-	-	-	-	-
	CG253	+	-	-	-	-	-	+
Mesenchymal	CW702	-	-	+	-	-	-	+
	CW1231	-	-	+	+	-	-	-
	CCF2170	-	-	+	-	-	-	-
	CG269	-	-	+	-	-	-	+
	CG302	-	-	-	+	-	-	+
Proneural / Neural	CW738	-	-	-	-	+	-	-
	CW756	-	-	-	-	+	-	+
	CG272	-	-	-	-	+	+	-
	CCF2509	-	-	-	-	+	-	-

Note: (*), genetic mutation (s) or deletion; (^{amp}), gene amplifications; $EGFR^{VIII}$, expression of variant form (active) of EGFR; ($^{\$}$), mutation at hot spots of IDH1/2.

Supplemental Methods

Isolation of GSCs and Non-stem Tumor Cells from GBMs

GBM surgical specimens were collected for this study in accordance with a Cleveland Clinic Institutional Review Board-approved protocol. GSCs and non-stem tumor cells (non-stem TCs) were derived from GBM surgical specimens or xenografts and characterized as previously described (Bao et al., 2006a; 2008; Guayronva et al., 2011; Li et al., 2009) with minor modification. Briefly, GBM tumors were disaggregated using the Papain Dissociation System (Worthington Biochemical) according to the manufacturer's instructions. Isolated cells were recovered in stem cell medium (Neurobasal-A medium with B27 supplement, 10 ng/ml EGF and 10 ng/ml bFGF) for at least 6 hours to allow re-expression of surface markers, and then sorted by fluorescence-activated cell sorting (FACS) or magnetic cell sorting for GSCs using at least two surface markers (CD15/CD133, or CD15/L1CAM). The enriched GSCs were maintained in the stem cell medium and confirmed by SOX2 and OLIG2 expression and GSC ability to turn on the SOX2 promoter-driven GFP expression. The cancer stem cell phenotype of GSCs was validated by functional assays of self-renewal (serial neurosphere passage), in vitro differentiation and tumor propagation (in vivo limiting dilution assay) as previously described (Guayronva et al., 2011; Li et al., 2009). To avoid any pericyte contamination in GSCs, the sorted GSCs from primary GBMs were subjected to secondary sorting (negative selection for CD146 and CD248) using FITC-conjugated anti-CD248 and PE-conjugated anti-CD146 (BD Bioscience), and the isolated GSCs from GBM xenografts were subjected to the secondary selection positive for TRA-1-85 (a human cell-specific surface antigen) and negative for CD146 using FITC-conjugated anti-TRA-1-85 and PE-

conjugated anti-CD146. To generate single GSC-derived tumorspheres, the sorted GSCs were seeded in 96-well plates by serial dilution or by the FACS sorter. The single GSC-generated neurosphere from each well was transferred into new well in a 24 well plate for the in vitro differentiation assay. The freshly sorted GSCs from GBM surgical specimens or xenografts and the derived GSC tumorspheres were used for in vitro or in vivo experiments as indicated.

Cardiac Perfusion for Collection of Mouse Brains

Prior to the collection of mouse brains bearing the tumors, cardiac perfusion with PBS followed by perfusion with 4% PFA (Paraformaldehyde, Sigma-Aldrich) was performed. Tumors were fixed with 4% PFA overnight at 4^{o}C, post-fixed in 70% ethanol, cryopreserved in 30% sucrose and cryosectioned for staining and fluorescent analysis, or embedded with paraffin and cut for histological and IHC staining.

Immunofluorescence, Immumohistochemistry, and Immunoblot Analysis

Immunofluorescent (ImF) staining was performed as previously described (Guryanova et al., 2011; Huang et al., 2011). Briefly, cultured cells or frozen sections of tumorspheres, GBM xenografts, surgical specimens or the genetically engineered mouse GBMs were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 15 minutes then blocked with 10% normal goat serum (Vector) with or without 0.1% Triton X-100 (Bio-Rad) in PBS for 30 minutes at room temperature. Samples were incubated with primary antibodies overnight at +4°C followed by the appropriate secondary fluorescently labeled antibodies (Invitrogen Molecular Probes) for one hour at room temperature. Nuclei were

counterstained with DAPI. Images were taken with a wide-field fluorescence microscope (Leica) or Leica SP-5 confocal microscope. Immunohistochemical (IHC) staining to examine blood vessel density in GBM xenografts was performed with an ABC kit using DAB detection as described previously (Bao et al., 2006b; Guryanova et al., 2011). Immunoblot analysis was performed as previously described (Guryanova et al., 2011; Huang et al., 2011). Cells were lyzed in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche). Pre-cleared protein samples were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. After blocking in milk-based buffer, blots were incubated with primary antibodies overnight at 4°C followed by HRP-linked species-specific antibodies (Santa-Cruz). Specific antibodies against CD31/PECAM-1 (Bethyl laboratories or Dako), Glut1 (Millipore), Desmin (Dako or Santa Cruz), α-SMA (Dako), NG2 (CSPG4) (Millipore), PDGFRβ (Cell Signaling), CD248 (TEM1) (Millipore or Santa Cruz), CD146 (BD Bioscience), Connexin45 (Millipore), Ang1 (Abcam), CD13 (BD Bioscience), GFAP (Covance), S100β (BD Bioscience), SOX2 (Millipore or Santa Cruz), Nestin (BD Bioscience), OLIG2 (R&D System), HA (Roche) and NuMA (Santa Cruz) were used for the ImF staining, IHC or immunoblot analysis.

Analysis of Vessel Density, Pericyte Coverage and Vessel Function

Vessel density and pericyte coverage were analyzed as described (Bao et al., 2006b; Nolan-Stevaux et al., 2010). Vascular or pericyte area was measured and percentage of pixel overlap was analyzed. To test vessel function, mice bearing GBM xenografts were perfused with FITC-conjugated mega-dextran (MW=2,000 KDa, Invitrogen) for 30-60

minutes, and then harvested for fluorescent analysis and quantification of the perfused FITC-mega-dextran (in green) as described (Bell et al., 2010).

shRNA Lentiviruses and Lentiviral Infection

CXCR4 shRNA clones in lentiviral vector (Mission shRNA, Sigma-Aldrich) were used to reduce CXCR4 expression in GSCs through lentiviral infection. Lentiviruses expressing CXCR4 shRNA (shCXCR4) or non-targeting shRNA (shNT) were produced in 293FT cells with pPACK set of helper plasmids (System Biosciences) and then titered as described previously (Guryanova et al., 2011; Li et al., 2009). The viruses were concentrated by precipitation with polyethylene glycol 8000 (PEG8000) (Fisher Scientific). GSCs transduced with shCXCR4 or shNT were used for the in vitro EC complex formation or the in vivo pericyte lineage tracing experiments.

Endothelial Complex Formation and Coculture of ECs with GSCs

To examine the role of GSC-derived pericytes in supporting endothelial complexes, HBMECs were pre-labeled with red fluorescent cell tracer CMTPX (Invitrogen) and then allowed to form EC complexes. CFSE-labeled GSCs (green) or GFP-expressing GSCs were then added to the established EC complexes, and then monitored and photographed under the EVOS fluorescent microscope over time (day 0 to day 8). The expression of α-SMA (a pericyte marker) and N-cadherin in the EC complex with GSC-derived cells was detected by immunofluorescent staining with anti-α-SMA (Dako) and anti-N-cadherin (Santa Cruz) antibodies. To investigate the impact of CXCR4 knockdown on GSC recruitment to EC complexes, GFP-labeled GSCs expressing CXCR4-targeting shRNA

(shCXCR4) or non-targeting shRNA (shNT) were added to the established EC complexes, and the integration of GSC-derived cells (GFP+) into EC complexes was monitored, photographed and then quantified. To examine the effect of CXCR4 inhibitor AMD3100 on recruitment of GSCs to EC complexes, HBMECs were pre-labeled with the green fluorescent cell tracer CFSE (Invitrogen) and allowed to form complexes, and GSCs labeled with the red fluorescent cell tracer CMTPX (Invitrogen) were added to the HBMEC complexes without or with AMD3100 at 1 μg/ml concentration. The recruitment and integration of GSCs (in red) to the EC complexes (in green) was monitored and analyzed.

In vitro Matrigel Cell Migration Assay

The cell migration of GSCs toward cytokines or ECs through the Matrigel (BD Bioscience) in vitro was performed as described previously (Bao et al., 2006b). GSCs were added on upper chambers of the transwells, and cytokines (SDF-1, TGF-β or PDGFB) or HBMECs with/without anti-TGFβ mAb was put in the bottom chambers in the assay. Cells migrated through the BD Matrigel were stained and counted.

Fluorescence Immunophenotyping and Interphase Cytogenetics

Fluorescence in situ hybridization (FISH) analysis in combination with Imf staining (Fluorescence Immunophenotyping and Interphase Cytogenetics) was performed as described (Calabrese et al., 2007; Ricci-Vitiani et al., 2008). Sections of GBM specimens or tissue arrays (Duke Brain Tumor Center and US Biomax Inc.) were immunostained for □-SMA to mark pericytes or CD31 to label ECs, and then followed by FISH detection.

Locus-specific probes (FITC-labeled or PE-conjugated CEP-7, CEP-10, EGFR or PTEN; Vysis Inc.) were used according to the manufacturer's instructions.

Detection of Apoptosis in G-Pericytes Expressing HsvTK

In vitro apoptosis was detected with the PE-Annexin V staining kit (BD Bioscience). The detection of the GCV-induced apoptosis in G-pericytes expressing HsvTK in GBM xenografts by TUNEL assay was carried out with an in situ cell apoptosis kit (Cell Signaling).

Isolation of Pericytes and ECs from GBM Tumors

Pericytes and ECs were isolated from GBM tumors using fluorescence-activated cell sorting (FACS) (Crisan et al., 2008; Milner et al., 2008). The pericytes were sorted from total cells of primary GBMs by selecting CD146+CD248+CD34- cells through positive sorting with PE-conjugated anti-CD146 and FITC-conjugated anti-CD248 antibodies, and then negative sorting with PE-conjugated anti-CD34 antibody (BD Bioscience). The GSC-derived pericytes from the DesPro-GFP-GSC xenografts were sorted by selecting GFP+CD146+ cells using PE-conjugated anti-CD146 antibody and GFP fluorescence. ECs from GBM tumors were sorted by selecting CD31+CD105+ cells with FITC-conjugated anti-CD105 and PE-conjugated anti-CD31 antibodies (BD Bioscience).

Induction of GSC Differentiation in vitro

The freshly isolated GSCs or the single GSC-derived tumorspheres were induced for differentiation in DMEM with FBS (1-10%) as indicated. In some experiments, cytokine

TGF-β1 (R&D systems), PDGF-B (Cell Signaling) or SDF-1 (R&D systems) were added to the culture. The differentiated cells were harvested for Imf staining or immunoblot analysis to examine the expression of cell lineage makers.

RT-PCR Analysis of Pericyte and GSC Markers

To confirm the expression of pericyte markers in G-pericytes and examine whether G-pericytes express GSC or NSC markers, RNA samples were isolated from the sorted G-pericytes (GFP+CD146+) from DesPro-GFP GSC xenografts, normal human brain vascular pericytes (HBVPs) and GSCs using a RNA isolation kit (Qiagen), and then subjected to RT-PCR analyses with pairs of primers for pericyte markers and GSC or NSC markers. Primers for pericyte markers: α-SMA (forward: 5' TAG CAC CCA GCA CCA TGA AGA TCA 3', reverse: 5' GAA GCA TTT GCG GTG GAC AAT GGA 3'); Desmin (forward: 5' AAA TCC GGC ACC TCA AGG ATG AGA 3', reverse: 5' TTT CTC GGA AGT TGA GGG CAG AGT 3'); CD146 (forward: 5' TGG CAT TCA AGG AGA GGA AGG TGT 3', reverse: 5' ACT CGC TGT GGA TCT TGG TCT TGT 3'); NG2 (CSPG4) (forward: 5’ AGC TCT ACT CTG GAC GCC 3’, reverse: 5’ ATC GAC TGA CAA CGT GGC 3’); CD248 (forward: 5' AGA CCA CCA CTCA TTT GCC TGG AA 3', reverse: 5' AGT TGG GAT AAT GGG AAG CTG GGT 3'); PGDFR□ (forward: 5' ACG GCT CTA CAT CTT TGT GCC AGA 3', reverse: 5' TCG GCA TGG AAT GGT GAT CTC AGT 3'). Primers for GSC or NSC markers: OLIG2 (forward: 5' CAA GAA GCA AAT GAC AGA GCC GGA 3', reverse: 5' TGG TGA GCA TGA GGA TGT AGT TGC 3'); SOX2 (forward: 5’TCT TCG CCT GAT TTT CCT CG 3’, reverse: 5’TTG CTG ATC TCC GAG TTG TG 3’); CD133 (forward: 5’ CTT ACG GCA CTC

TTC ACC TG 3', reverse: 5' TCC CTG TGC GTT GAA GTA TC 3'); Nestin (forward: 5' TGC GGG CTA CTG AAA AGT TC 3', reverse: 5' TGA AAG CTG AGG GAA GTC TTG 3'). Primers for control proteins: GFP (forward: 5' AGA TTC GAG AAA CCA GCC TGG ACA 3', reverse: 5' TTG TGC TCC TGC TTG GAC TCC TTA 3'); and GAPDH (forward: 5' TGT TGC CAT CAA TGA CCC CTT 3', reverse: 5' CTC CAC GAC GTA CTC AGC G 3').

Supplemental References

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