Supporting Information

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SI Materials and Methods

Antibodies, Peptides, and Chemicals. Anti-p-ERK-1/2 and anti-ERK1/2 antibodies were from Cell Signaling Technology; anti-CD31, CD81, CD63, calnexin, β-actin, α-tubulin, and αVβ3 mouse monoclonal antibodies as well as goat polyclonal anti-HB-EGF blocking antibody (no. 16783) were from Abcam. Anti-PAR-2 (no. 13504), PAR-1 (no. 13503), and TF (no. 80952) monoclonal mouse antibodies were from Santa Cruz Biotechnology. Rabbit polyclonal anti–PAR-2 antibody was from the same source as described (1). Goat polyclonal anti-TF antibody (no. AF2339), human phosphokinase antibody array (no. ARY003), and human angiogenesis array (no. ARY007) kits were from R&D Systems. NAPc2 was the same as previously described (2). Phalloidin-TRITC, Hoechst 33342, and fluorophore-conjugated secondary antibodies were from Invitrogen; and Ac-LDL was from Harbor Bio-Products. Specific agonist peptides for PAR-1 (TFLLRNPNDK) and PAR-2 (SLIGKV) were from Innovagen. SiRNA for PAR-2 (F2RL1) and a nonspecific, scrambled siRNA (silencer negative control) were from Ambion. IMUBIND TF ELISA kit and Actichrome TF activity assay kit, as well as recombinant human factors VIIa and Xa, were from American Diagnostica. MEK/ERK1/2 inhibitor UO126 was from Selleck Chemicals and [^{3}H]thymidine was from Amersham. Growth factor reduced Matrigel and transwell 8-μm pore size cell culture inserts were from BD. LDH assay kit, fine-grade chemicals, cell media, and supplements were from Sigma Chemical.

qRT-PCR. Extraction of RNA was achieved by using a GenElute Mammalian total RNA kit (Sigma) and cDNA synthesis was performed with RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). cDNA was used for quantitative PCR based on SYBR green I chemistry (Sigma) in an ABI Prism 7900 HT machine (Applied Biosystems). The following primers were used: PAR-1, forward, 5′- GTTTGGGTCTGAATTGTGTCGC-3′; reverse, 5′-GGACTGCATGGGATACACCA-3′; PAR-2, forward, 5′-GGGTTTGCCAAGTAACGGC-3′; reverse, 5′-GGGAACC-AGATGACAGAGAGG-3′; HB-EGF, forward, 5′-CATCTGT-CTGTCTGCTGGTCA-3′; reverse, 5′-CAATCCTAGACGGC-AACTGG-3′; TF, forward, 5′-TTTTAAGAGGATAGAATAC-ATGGAAACG-3′; reverse, 5′-AACAGGTCATATCAAGAG-TTTTTTGAAC-3′. Reference was as follows: EDF-1, forward, 5′-TTCAAGAAATGGGCTGCT-3′; reverse, 5′-CCGTCCGC-TCTCATAGTCC-3′, which was stable at hypoxic conditions and used for normalization. The comparative Ct method was used for relative quantification of gene expression on triplicates of each reaction.

Immunoblot Analysis. Cells were washed with ice-cold PBS solution and lysed in a reducing RIPA buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Nonidet P-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and Complete Mini Protease Inhibitor Mixture (Roche Diagnostics). For antibodies requiring nonreduced conditions (CD63 and CD81), samples were lysed in 1% Triton-X buffer supplemented with Complete Mini Protease Inhibitor Mixture. Protein concentration was determined by using the BCA Protein Assay Kit (Pierce), and equal amounts of protein were fractionated by electrophoresis in a 4% to 12% polyacrylamide-SDS gel. Proteins were blotted to PVDF membrane and probed with the indicated antibodies followed by HRP-conjugated secondary IgG antibodies for 1 h at room temperature. Visualization was performed by using the ECL Western blotting substrate (Pierce), and the membranes were exposed to X-ray film (Hyperfilm MP; Amersham). Blots were analyzed and quantified by using ImageJ software (National Institutes of Health) with β-actin or α-tubulin as loading controls.

Phosphokinase and Angiogenesis Arrays. HUVECs were seeded in 80-cm^2 dishes and incubated in normoxia or hypoxia for 24 h, followed by starvation for 16 h in serum-free medium. Cells were untreated or stimulated with PAR-2AP for 5 min for phosphokinase array and 6 h for angiogenesis array before lysate collection. In MAPK inhibition experiments, cells were treated with 50 μM UO126 or with DMSO in controls for 3 h before stimulation with PAR-2AP. Levels of phosphorylated or nonphosphorylated proteins (200 μg per sample) were analyzed in cell lysates according to the protocol provided by the manufacturer. The arrays were analyzed and quantified by using ImageJ software (National Institutes of Health).

Flow Cytometry. HUVECs were detached with concentrated PBS solution (2×)/0.5 mM EDTA, washed with PBS solution/BSA 1% (wt/vol), and incubated with mouse monoclonal anti–PAR-2 antibody (1:200; no. 13504; Santa Cruz). Cells were washed in PBS solution/BSA and incubated with Alexa Fluor-488–conjugated goat anti-mouse antibody (1:200; Invitrogen). All antibody incubations were performed in PBS solution/BSA for 30 min on ice. Finally, HUVECs were washed in PBS solution/BSA and analyzed by flow cytometry on a FACSCalibur instrument integrated with Cell-Quest software (BD Biosciences). Controls without PAR-2 primary antibody were included in all experiments.

TF ELISA. TF levels were analyzed in cell media according to the protocol provided by the manufacturer. Analysis of human TF levels was performed in plasma from mice inoculated with U-87 MG s.c. xenografts as described previously (3).

TF Actichrome Assay. TF activity was analyzed in cell media or in purified MV fractions according to the protocol provided by the manufacturer. Samples were incubated at 37 °C with or without 10 nM VIIa and 50 nM X. Aliquots of the incubation medium were quenched with 100 mM EDTA, and the Xa activity was determined by hydrolysis of the chromogenic substrate Spectrozyme FXa in a plate reader.

RNA Interference. Subconfluent cells in serum-free medium were transfected with 100 nM predesigned PAR-2 siRNA (F2RL1; ID 1960; Ambion) or control, scrambled siRNA (silencer negative control siRNA no. 1; Ambion) with Lipofectamine 2000 reagent (Invitrogen) as recommended by the manufacturer. After 5 h of transfection, cells were washed twice with 1 M NaCl, and medium was changed to growth medium, followed by another incubation period of 16 h before the tube formation and LDH assays as described later.

Matrigel Tube Formation Assay. HUVECs were untransfected or transfected with siRNA for PAR-2, or a scrambled siRNA sequence, followed by the tube formation assay by using growth factor-reduced Matrigel (4). Briefly, transfected HUVECs were seeded on polymerized Matrigel, and tubes were allowed to form during 16 h at normoxia, followed by incubation at normoxia or hypoxia for another 24 h. HUVEC tubes were captured using a Leitz Fluovert FS microscope equipped with a 4× objective and a digital camera.

LDH Release Assay. HUVECs were seeded in 24-well plates at 30,000 cells per well and cultured in growth medium for 24 h. Medium was changed to EBM supplemented with 2% heat-inactivated FBS, and the cells were kept at normoxic or hypoxic conditions for 24 h. LDH activity was analyzed in cell media according to the protocol provided by the manufacturer (Sigma).

Immunofluorescence Microscopy. For immunofluorescence analysis of specimens from patients with GBM, cryosections (5 μm) were fixed in ethanol, blocked for 1 h with PBS solution and 5% goat serum, and incubated with rabbit anti–PAR-2 (1:200), mouse anti-CD31 (1:100), or mouse anti-αVβ3 (1:200) antibody at 4 °C overnight, followed by incubation with fluorophore-conjugated secondary antibody (1:300) for 1 h at room temperature. Negative controls were stained in parallel with secondary antibody alone. Sections were counterstained with Hoechst 33342 nuclear stain, mounted with PermaFluor (Beckman Coulter), and analyzed using a Zeiss HBO 100 fluorescence microscope.

1. Dorfleutner A, Ruf W (2003) Regulation of tissue factor cytoplasmic domain phosphorylation by palmitoylation. *Blood* 102:3998–4005.
2. Lee AY, Vlasuk GP (2003) Recombinant nematode anticoagulant protein c2 and other inhibitors targeting blood coagulation factor VIIa/tissue factor. *J Intern Med* 254:313–321.
3. Svensson KJ, et al. (2008) Hypoxia-mediated induction of the polyamine system provides opportunities for tumor growth inhibition by combined targeting of vascular endothelial growth factor and ornithine decarboxylase. *Cancer Res* 68: 9291–9301.
4. Kucharzewska P, Welch JE, Svensson KJ, Belting M (2009) The polyamines regulate endothelial cell survival during hypoxic stress through PI3K/AKT and MCL-1. *Biochem Biophys Res Commun* 380:413–418.

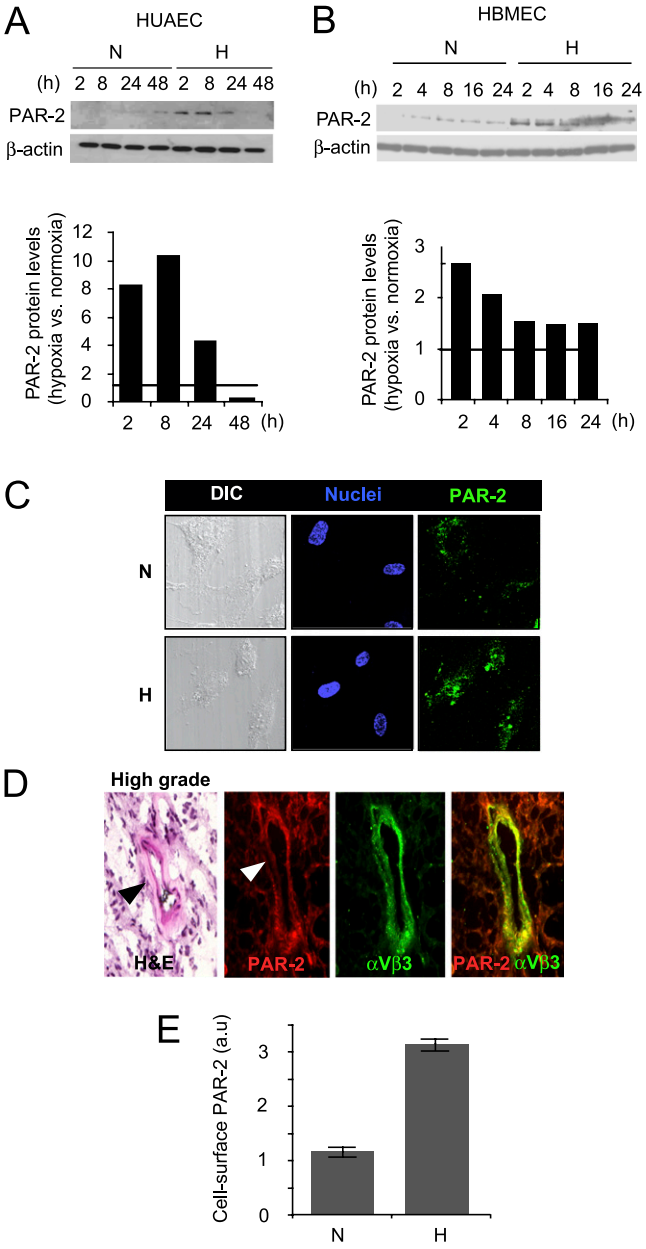

Fig. S1. PAR-2 induction by hypoxia in ECs. HUAECs (*A*) and HBMECs (*B*) were cultured at normoxia (N) or hypoxia (H) for the indicated time periods, and PAR-2 and β-actin protein levels were determined in cell lysates by immunoblotting. *Lower*: Relative hypoxic PAR-2 levels [hypoxic (PAR-2/β-actin)/normoxic (PAR-2/β-actin)] shown as representative data of at least three independent experiments. (*C*) HBMECs were cultured at normoxia or hypoxia for 4 h, fixed and stained for PAR-2 (green) and nuclei (DAPI; blue), and then analyzed by confocal fluorescence microscopy. (*D*) Induction of PAR-2 in pathological vasculature of tumors from patients with GBM. Serial cross-sections of fresh frozen patient tumor specimens were stained with H&E (*Left*) or analyzed by immunofluorescence microscopy using PAR-2 (red) and integrin αVβ3 (green; marker of activated endothelium) antibodies. PAR-2 coassociates with integrin αVβ3 in typical dilated tumor vessels (arrowheads). Images are representative of six cross-sections obtained at 40× magnification. (*E*) Increased cell surface-associated PAR-2 by hypoxia. HUVECs were cultured at normoxia (N) or hypoxia (H) for 24 h, and cell-surface PAR-2 expression was determined by flow cytometry.

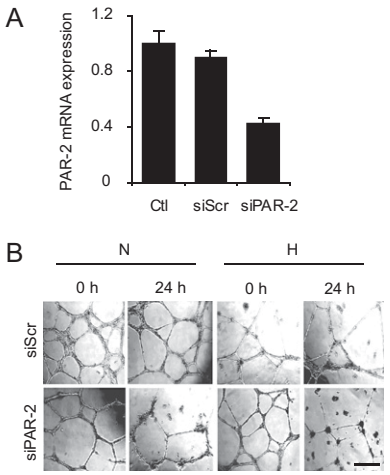

Fig. S2. PAR-2 deficiency accelerates EC tube disintegration in hypoxia. Subconfluent HUVECs were transfected with siRNA specific for PAR-2 (siPAR-2) or with scrambled siRNA (siScr). (*A*) Knockdown of PAR-2 mRNA by siPAR-2 compared with control (Ctl) and siScr-transfected HUVECs, as determined by qRT-PCR. (*B*) Transfected HUVECs were detached and seeded on Matrigel ECM for 24 h to allow tube formation (0 h). EC tubes were then subjected to normoxia (N) or hypoxia (H) for another 24 h, and EC tube morphology was captured by using an inverted microscope equipped with a 4× phase-contrast objective lens and a digital camera. Shown are representative images from two independent experiments. (Scale bar, 500 μm.)

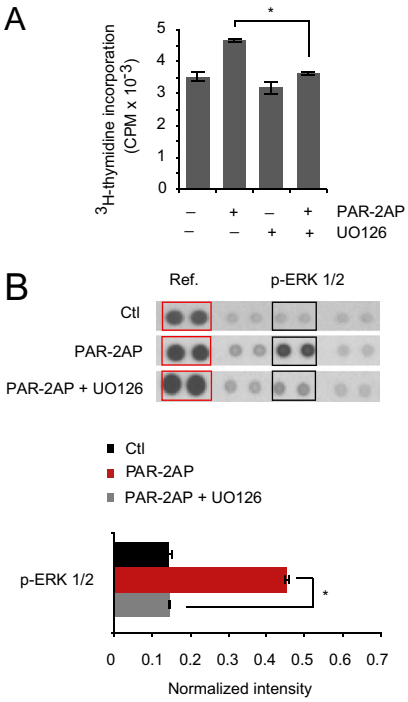

Fig. S3. PAR-2–mediated stimulation of hypoxic ECs involves p-ERK1/2. (*A*) Hypoxic HUVECs were untreated or pretreated with the p-ERK1/2 inhibitor UO126 (50 μM) for 3 h, followed by another incubation period of 24 h in the presence or absence of PAR-2AP (100 μM) as indicated. Cell proliferation was assessed by [^{3}H]thymidine incorporation (*$P < 0.05$). (*B*) Efficient inhibition of PAR-2–induced p-ERK1/2 by UO126. Hypoxic HUVECs were untreated or pretreated with UO126 (50 μM) for 3 h, and were then left untreated (Ctl) or treated with PAR-2AP SLIGKV (100 μM) for 5 min, followed by immunoblotting with a phosphokinase array. *Upper*: Representative immunoblot from three independent experiments. *Lower*: Quantification of p-ERK1/2 levels at the various conditions, presented as relative intensities versus array reference (red box). (*$P < 0.05$.)

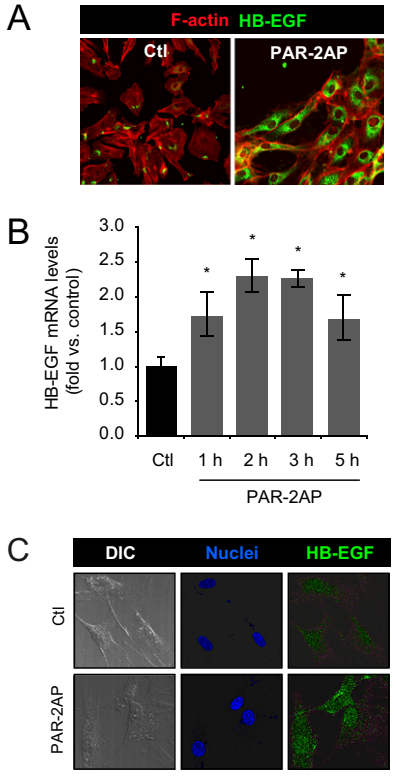

Fig. S4. Induction of HB-EGF by PAR-2AP in hypoxic ECs. (*A*) Confocal fluorescence microscopy of hypoxic HUVECs untreated (*Left*) or treated (*Right*) with PAR-2AP (100 μM) for 6 h, and then stained for HB-EGF (green) and F-actin (phalloidin; red). (*B*) Hypoxic HBMECs were incubated without (Ctl) or with PAR2-AP (100 μM) for the indicated time periods; HB-EGF mRNA expression was determined by qRT-PCR. (*Significant up-regulation vs. control, $*P < 0.05$.) (*C*) Confocal fluorescence microscopy of hypoxic HBMECs untreated (*Upper*) or treated (*Lower*) with PAR-2AP (100 μM) for 6 h, and then stained for HB-EGF (green) and nuclei (DAPI; blue).

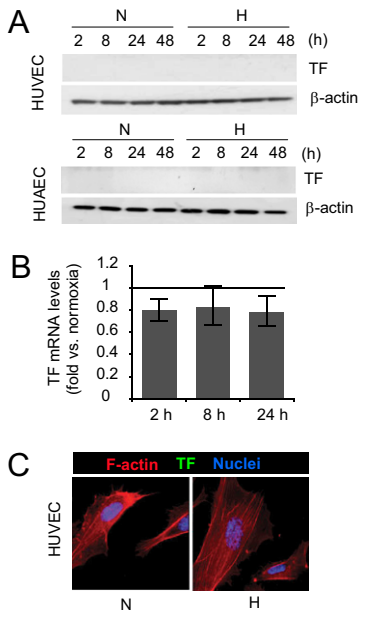

Fig. S5. TF is not induced by hypoxia in ECs. (*A*) HUVECs (*Upper*) and HUAECs (*Lower*) were cultured at normoxia (N) or hypoxia (H) for the indicated time periods, and TF and β-actin protein levels were determined in EC lysates by immunoblotting. Shown are representative blots of at least three independent experiments. (*B*) HUVECs were cultured at normoxia or hypoxia for the indicated time periods, and TF mRNA expression was determined by qRT-PCR and presented as fold expression at hypoxia versus normoxia averaged from three separate experiments ± SD. (*C*) Confocal fluorescence microscopy shows negative TF staining in normoxic and hypoxic HUVECs.

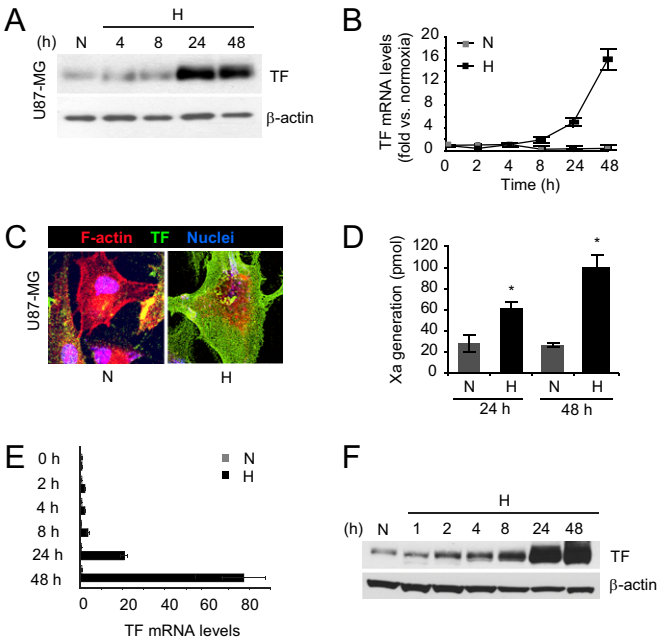

Fig. S6. Substantial induction of TF by hypoxia in GBM cells and lung cancer cells. (*A*) U87-MG cells were cultured at normoxia (N) or hypoxia (H) for the indicated time periods, and analyzed for TF and β-actin protein by immunoblotting. (*B*) U87-MG cells were cultured at normoxia (N) or hypoxia (H) for the indicated time periods, and TF mRNA expression was determined by qRT-PCR. Results are presented as fold increase at hypoxia versus normoxia averaged from three separate experiments ± SD. (*C*) Confocal fluorescence microscopy shows increased staining for TF in hypoxic compared with normoxic GBM cells. (*D*) GBM cells cultured at normoxia or hypoxia at the indicated time points were analyzed for procoagulative activity by the TF actichrome activity assay as described in *SI Materials and Methods*. Data are presented as the average ± SD. (*$P < 0.05$.) (*E*) A549 cells were cultured at normoxia or hypoxia for the indicated time periods, and TF mRNA levels were determined by qRT-PCR. (*F*) A549 cells were cultured at normoxia or hypoxia for the indicated time periods and analyzed for TF and β-actin protein by immunoblotting.

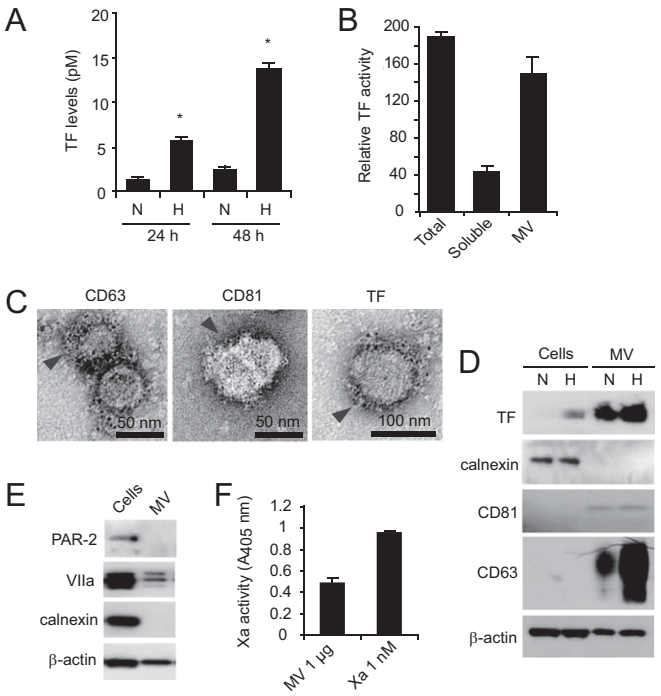

Fig. S7. Hypoxic GBM cells secrete TF/VIIa-bearing MVs with procoagulant activity. (*A* and *B*) Hypoxic induction of TF secretion in GBM cells. (*A*) GBM cells were cultured at normoxia (N) or hypoxia (H) for the indicated time periods, and TF levels in the cell culture conditioned medium were determined by TF ELISA. Data are presented as the average ± SD. (*B*) TF activity was determined by the TF actichrome assay in total cell culture medium (Total) isolated from hypoxic GBM cells or following fractionation into a soluble fraction (Soluble) and MV fraction as described in *Materials and Methods*. Data are presented as the average ± SD. (*C*) Immunogold staining (arrowheads) and EM of vesicles isolated from hypoxic GBM cells shows positive staining for CD63 and CD81 (typical markers of exosomes), as well as for TF. (*D*) Immunoblotting of cells and MVs from normoxic and hypoxic GBM cells shows the relative enrichment of TF, CD81, and CD63, and the relative depletion of the ER marker calnexin and the cytoskeletal protein β-actin in MVs compared with cells. Equal amounts of total protein were loaded in each sample. Data shown are representative of at least three independent experiments. (*E*) Similar experiment as in *D* shows significant levels of VIIa in GBM cells and in MVs. Notably, PAR-2 protein was undetectable in MVs. (*F*) MVs isolated from hypoxic GBM cells exhibited TF/VIIa protease activity as shown by the TF activity actichrome assay. Xa alone (1 nM) is shown as a positive control. Data are presented as the average ± SD.

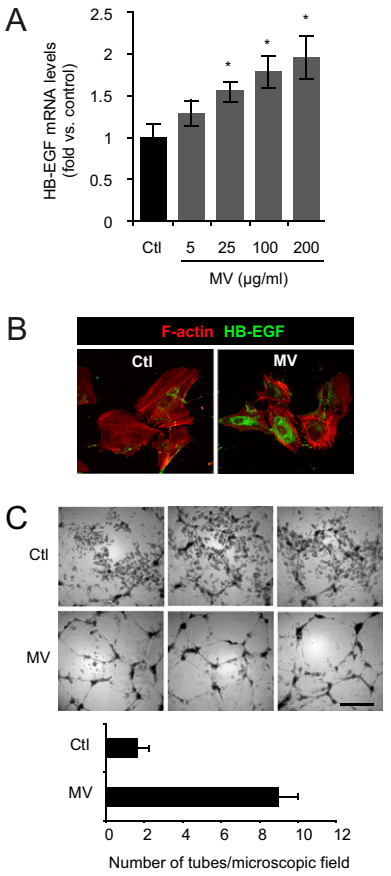

Fig. S8. MVs induce HB-EGF expression and promote EC tube stability in hypoxic ECs. (*A*) Hypoxic HUVECs were incubated without (Ctl) or with MVs at the indicated concentrations for 3 h; HB-EGF mRNA expression was determined by qRT-PCR. (*Significant up-regulation vs. control, $*P < 0.05$.) (*B*) Confocal fluorescence microscopy of hypoxic HUVECs untreated (Ctl) or treated with MVs (25 μg/mL) for 6 h, and then fixed and stained for HB-EGF (green) and F-actin (phalloidin; red). (*C*) HUVECs were seeded on Matrigel ECM in the absence (Ctl) or presence of MVs (20 μg/mL) from hypoxic GBM cells. Tube structures were captured after 36 h of incubation at hypoxic conditions by using an inverted microscope equipped with a 4× phase-contrast objective lens and a digital camera. *Upper*: Representative pictures from three separate wells. *Lower*: The number of EC tubes was quantified in three random microscopic fields from the Ctl and MV-treated group, respectively, by using ImageJ software. (Scale bar, 500 μm.)

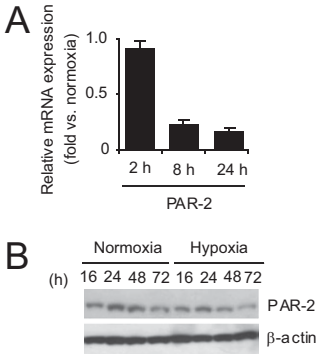

Fig. S9. Hypoxic reduction of PAR-2 in GBM cells. (*A*) U87-MG cells were cultured at normoxia or hypoxia for the indicated time points and PAR-2 mRNA expression was determined by qRT-PCR. Results are presented as fold expression at hypoxia versus normoxia averaged from three separate experiments ± SD. (*B*) U87-MG cells were cultured at normoxia or hypoxia for the indicated time periods, and PAR-2 and β-actin protein levels were determined in cell lysates by immunoblotting. Shown is a representative blot of three independent experiments.

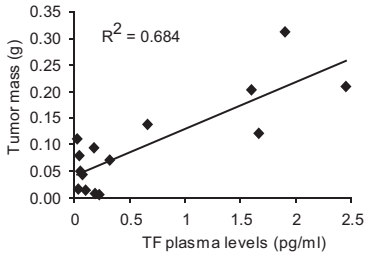

Fig. S10. Systemic release of TF in a GBM xenograft mouse model. U87-MG cells (2.5×10^6 in 200 μL PBS solution) were injected s.c. into the dorsal region of adult female SCID mice (n = 15). After various periods of inoculation, EDTA plasma was collected by cardiac puncture, and mice were killed for tumor mass measurement. Plasma TF levels were determined by an ELISA specific for human TF and correlated with tumor mass.