Supplemental Methods

Cells

Both normal brain ECs and TECs were propagated in media recommended by Cell Systems for brain ECs (1). Normal brain ECs were used during the first 8 passages, and the cell type verified by positive staining for von Willebrand factor (vWf) and tube formation on Matrigel™ or collagen gels (1). TECs were also used during the first 8 passages, sorted every two passages for CD31 or CD105 positivity, and the cell type verified as described (1). $CD133^+$ GBM cells were propagated in suspension in Neurobasal A media with B27 and N2 supplements (referred to hereafter as NBM) (Life Technologies) and with EGF (10 ng/ml) and bFGF (5 ng/ml), as described (2). The matched non-$CD133^+$ (non-stem) tumor cells were propagated in DMEM media with 10% FBS, conditions that induce differentiation of the $CD133^+$ cells (2). MM.1R human myeloma cells were propagated as described (3); authentication of cell identity was performed using short tandem repeat (STR) profiling.

Reagents

The following reagents were purchased: 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) (Sigma-Aldrich #A3085); lysine-fixable tetramethylrhodamine(TMR)-70-kDa-Dextran (Molecular Probes #D1818); and biotinylated recombinant human-VEGF165 (ACRO Biosystems #VE5-H8210). The following antibodies were purchased: Alexa-Fluor®-488-phalloidin, Alexa-Fluor®-488- and Alexa-Fluor-633®-anti-human IgG, Alexa-Fluor®-594-anti-rabbit IgG, Alexa-Fluor®-594-anti-mouse IgG, and Alexa-Fluor®-647-Streptavidin (Molecular Probes); mouse or rabbit

anti-vWf (Abcam #ab68545, Millipore #AB7356), rabbit anti-Rab4 (Thermo Scientific #PA3-912), rabbit anti-Lamp1 (Abcam #ab24170), mouse anti-Sox2 (R&D Systems #MAB2018), rabbit-anti-Iba1 (Wako #019-19741), rabbit anti-human FcRn (Santa Cruz #sc-66892), goat anti-FcγRIIB (R&D Systems #AF1330), mouse anti-LC3 (Nanotools #0231-100/LC3-5F10), rabbit or mouse anti-LAMP2 (proteintech #10397-1-AP, Abcam #ab25631), mouse Alexa Fluor® 488-anti-human nuclear antibody (EMD Millipore #MAB1281A4), goat anti-actin (Santa Cruz #sc-1615), mouse mAb anti-VEGFR2 (Cell Signaling #9698 clone D5B1), rabbit anti-phosphotyrosine (Millipore #05-1050), rabbit mAb anti-cleaved caspase-3 (Cell Signaling #9664), rat anti-Ki67-Alexa-647 (BioLegend #151206), sheep anti-mouse CD44 (R&D Systems #AF6127), and goat anti-rat IgG-Alexa-488 (ThermoFisher Scientific #A-11006). Control human IgG was purchased (Sigma Aldrich #I4506). Bevacizumab was a gift (Cleveland Clinic pharmacy).

Animal Studies

For the $CD133^+$ orthotopic GBM model, female nude mice (4-6 weeks of age) were purchased (Taconic) and injected intracerebrally with 15,000 CD133+ GBM cells using an established procedure (2). At 14 days, monitoring of mouse brains for luciferase activity was initiated and continued every 3 days at the Cleveland Clinic Bioluminescent Imaging Facility. When a predetermined amount of luciferase activity was detected, daily injection of bevacizumab or hIgG was initiated and continued for 5 days, followed by euthanasia and harvesting of the brains. The patient-derived GBM xenograft (PDX) tumors (G39, G59, G44) were established intracerebrally in athymic nude mice as described (4). Treatment with bevacizumab was initiated on day 13 (PDX G39), day 20 (G44), or day 28 (PDX G59) post-injection and given twice weekly (5mg/kg) until euthanasia at the detection of neurologic signs, followed by brain harvest (4). Frozen blocks

randomly selected from three bevacizumab-treated and two or three vehicle-treated tumor-bearing brains from each of the three PDX tumors were sectioned. One vehicle-treated tumor from G39 was excluded due to differences in processing. For the somatic gene transfer GBM model, six to eight-week-old *Ntv-a/ink4a-arf*$^{-/-}$ mice were used to generate GBM tumors via introduction of RCAS-PDGF-B-HA, as described (5). Rat anti-mouse-VEGF-A IgG (BioLegend clone 2G11-2A05) (5 mg/kg i.p., 2X/week) was administered once the tumors were established (4 weeks post-introduction of RCAS-PDGF-B-HA) and continued for two weeks, followed by euthanasia, brain harvest and fixation as above.

Immunofluorescence

Double-label immunofluorescence was visualized and photographed using Leica laser confocal microscopes (Leica-SP5 and Leica-TCS-SP2-AOBS) and a Leica-DM5500B upright microscope. Immunofluorescent conditions were assayed in triplicate and 10 fields per replicate analyzed for co-localization. Analysis of co-localization was carried out as described using ImageJ software with the JACoP-plugin (6, 7). For each field, only p values indicating a $\geq$95% certainty of co-localization were utilized in determining the Mander's coefficient (6, 7). Controls included secondary antibody alone.

For immunofluorescence analysis of frozen sections, tissue sections (8-µm) were fixed in 4%-paraformaldehyde, blocked, reacted with the primary antibodies overnight, washed, reacted with the secondary fluorescent-conjugated antibodies, followed by cover slipping with mounting media

containing DAPI nuclear stain, as described (8). Sections were viewed and photographed using a Leica laser confocal microscope as described above.

Transcytosis Assay

A standard transcytosis assay for brain ECs was performed as described (9, 10). Briefly, ECs (isolate 422 or 376) or TECs were plated as a confluent monolayer on collagen coated 0.4-µm-pore filters in Boyden chambers in regular media. After 3 days, 70-kDa-FITC-Dextran (Molecular Probes, #D1823) or bevacizumab was added to the confluent monolayer of ECs or TECs in the wells, and the fluorescence in the bottom chamber quantitated over time.

ELISA Assay for Bevacizumab

Rec-human-VEGF-A_{165} (100 ng/ml in 50 µl of PBS; BioVision #4363) was coated on 96 well Immulon H2B plates (Thermo Fisher Scientific #3455) overnight at 4^{o}C. The following day the wells were washed 3X with PBS, blocked with 200 µl of 2% BSA/PBS (2 hours, 22^{o}C), followed by addition of 200 µl of various dilutions of media from the bottom chamber of the transcytosis assay containing bevacizumab and incubated overnight at 4^{o}C. The next day the wells were washed 3X with PBS, HRP-conjugated-anti-human IgG was added (200 µl of a 0.5 µg/ml solution in PBS) and incubated (1 hr, 22^{o}C), washed 3X with PBS, followed by addition of 100µl TMB substrate (Thermo Fisher Scientific #34028) (2 min, 22^{o}C) and 2M sulfuric acid to stop the reaction, as described (11). The absorbance was read at 450 nm in an ELISA Plate Reader. All samples were analyzed in replicates of three. The absorbance readings were compared to a standard curve of bevacizumab (0.24 to 15.6 ng/ml) that was immobilized on the plate in replicates of three.

Supplemental References

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