Abstract

Glioblastoma multiforme contains a subpopulation of cancer stem–like cells (CSC) believed to underlie tumorigenesis and therapeutic resistance. Recent studies have localized CSCs in this disease adjacent to endothelial cells (EC) in what has been termed a perivascular niche, spurring investigation into the role of EC–CSC interactions in glioblastoma multiforme pathobiology. However, these studies have been limited by a lack of in vitro models of three-dimensional disease that can recapitulate the relevant conditions of the niche. In this study, we engineered a scaffold-based culture system enabling brain endothelial cells to form vascular networks. Using this system, we showed that vascular assembly induces CSC maintenance and growth in vitro and accelerates tumor growth in vivo through paracrine interleukin (IL)-8 signaling. Relative to conventional monolayers, endothelial cells cultured in this three-dimensional system not only secreted enhanced levels of IL-8 but also induced CSCs to upregulate the IL-8 cognate receptors CXCR1 and CXCR2, which collectively enhanced CSC migration, growth, and stemness properties. CXCR2 silencing in CSCs abolished the tumor-promoting effects of endothelial cells in vivo, confirming a critical role for this signaling pathway in GMB pathogenesis. Together, our results reveal synergistic interactions between endothelial cells and CSCs that promote the malignant properties of CSCs in an IL-8–dependent manner. Furthermore, our findings underscore the relevance of tissue-engineered cell culture platforms to fully analyze signaling mechanisms in the tumor microenvironment. Cancer Res; 73(23); 7079–89. ©2013 AACR.

Introduction

Glioblastoma multiforme, a high-grade (IV) astrocytoma, remains the most common and lethal primary brain tumor in adults with a median survival of 12 to 15 months (1). Clinical management of glioblastoma is largely palliative, due to poor efficacy of conventional therapeutics and reflexive secondary tumor formation following resection. The identification of glioblastoma–associated cancer stem cells (CSC) has fueled research into the contributing role of this cell type in glioblastoma pathogenesis and therapeutic resilience (2). Glioblastoma CSCs are capable of self-renewal and multilineage differentiation, enable tumorigenesis upon intracranial implantation within immunocompromised rodents (3), and exhibit elevated therapeutic resistance relative to bulk glioma cells (4). Therefore, elucidation of the molecular mechanisms underlying CSC maintenance and proliferation in the tumor microenvironment offers new focus toward the development of improved anti-glioblastoma therapies.

Cell–microenvironment interactions modulate glioblastoma pathogenesis, whereby perivascular localities are particularly important as they support the malignant behavior of CSCs (5, 6). Specifically, association with endothelial cells (EC) or capillary structures supports maintenance of Nestin+ and CD133+ CSCs by sustaining their undifferentiated state and proliferation (5). Likewise, paracrine signaling by CSCs enhances the apoptotic threshold of endothelial cells (7), thereby preserving the perivascular microenvironment necessary for downstream processes involved in glioblastoma pathology. Nevertheless, despite accumulating evidence confirming the importance of CSC–EC signaling in glioblastoma pathogenesis, the precise molecular mechanisms involved in CSC localization to—and function within—the perivascular niche remain poorly defined.

Interleukin (IL)-8 has received significant attention as a promigratory and proangiogenic stimulus in multiple cancers but may regulate glioblastoma CSC functions as well (8). The effects of the ~8-kDa chemokine are mediated via binding to either of 2 related G-protein–coupled receptors, CXCR1 and CXCR2, whose expression varies dramatically by cell type and throughout pathogenesis (9). While initially
identified as a monocyte-secreted chemotactic factor for neutro/basophils and T lymphocytes, IL-8 can similarly facilitate invasion of bulk glioma cells and has been associated with increased tumor grade in astrocytic neoplasms (10, 11). In addition to its direct proangiogenic effects, IL-8 can promote glioblastoma tumor vascularization indirectly by elevating the apoptotic threshold of endothelial cells as well as promoting expression of matrix-remodeling enzymes necessary for endothelial sprouting (9, 12). Although it has been recently revealed that autocrine IL-8 signaling contributes to CSC self-renewal within other tumors of the breast and liver (13, 14), its impacts on the behavior of glioblastoma CSCs, signaling with endothelial cells of the perivascular niche, and participation in glioblastoma tumor growth have yet to be elucidated.

IL-8 expression and secretion are heavily influenced by 3-dimensional (3D) cell–microenvironment interactions (15, 16), underscoring the importance of physiologically relevant, 3D culture models to study IL-8 signaling as it relates to human CSCs and endothelial cells throughout glioblastoma pathogenesis. We have previously shown that tissue-engineered 3D culture systems mimic in vivo–like IL-8 expression by recapitulating appropriate microenvironmental conditions including cell–cell and cell–extracellular matrix (ECM) interactions (15, 16). Here, we used porous, polymeric scaffolds as platforms to investigate the role of paracrine endothelial cell signaling on patient-derived glioblastoma CSC pathogenesis in vitro and in vivo. Our findings indicate that IL-8 serves as a critical mediator supporting CSC growth and migration toward endothelial cells, which may partially explain their perivascular colocalization in the glioblastoma microenvironment, thereby offering promise as a novel therapeutic target for the clinical management of the disease. Furthermore, these studies underscore the importance of appropriate culture systems in studying tumor–stroma interactions and support that tissue-engineered models are suitable to evaluate microenvironmentally regulated paracrine signaling in vitro and in vivo.

Materials and Methods

Cell culture

Immortalized human brain endothelial cells (hCMEC, provided by Dr. Babette Weksler, Weill Cornell Medical College, New York, NY; ref. 17) were cultured on collagen-coated (1%) flasks in Clonetics EGM-2 media (Lonza). Patient-derived glioblastoma multiforme stem cells (CSCs, provided by Dr. John Boockvar, Weill Cornell Medical College) were isolated from glioblastoma tumor specimens (WHO grade IV) as described previously (18). Briefly, glioblastoma tissue was digested in PBS containing papain (Worthington) and DNase 1 (Sigma-Aldrich), then triturated via pipette, and filtered through a 70-μm sterile cell filter (BD Biosciences). Cells were resuspended in stem cell media containing 1:1 Dulbecco’s Modified Eagle’s Medium:F12 (Gibco) plus basic fibroblast growth factor and EGF (each 20 ng/mL; Invitrogen) and 1× antibiotic/antimycotic (Gibco). An isolated cell was clonally expanded in nonadherent culture flasks and media changed every 48 to 72 hours, resulting in neurosphere aggregates. Two glioblastoma multiforme CSC cell populations, labeled CSC 248 (EGFR+/PTEN+) and CSC 974 (EGFR+/PTEN−), were used. Expression of stem cell markers Nestin, Sox2, Oct4, Musashi-1, GFAP, and Bim-1 and absence of Olig-1, Tuji-1, and NeuN were verified via immunocytochemistry and tumorigenicity confirmed via implantation into athymic mice (18).

Polymeric scaffold fabrication and cell seeding

Porous, polymeric scaffolds were fabricated using a gas-foaming, particulate leaching method (19). Briefly, poly(lactide-co-glycolide) (PLG) particles (ground and sieved to ~250 μm diameter; Lakeshore Biomaterials), PLG microspheres (formed via double emulsion, ~5–50 μm diameter), and sodium chloride (sieved to ~250–400 μm diameter; J.T. Baker) were mixed and pressed into matrices (8.5 mm diameter, 1 mm thick) at room temperature (Carver Press, Fred S. Carver). Polymer foaming was achieved by exposing matrices to high-pressure (800 psi) CO2 and releasing gas quickly using a pressure vessel (Parr Instruments). Subsequently, sodium chloride porogen was leached out with de-ionized water. Scaffolds were sanitized in 70% ethanol and washed in sterile PBS. 1.5E6 hCMECs, 1.0E5 CSCs, or both cell types (aforementioned cell densities) were suspended in 30 μL of 1:1 (media: Matrigel; BD Biosciences) and slowly pipetted onto the scaffold. Following 30 minutes of incubation at 37°C (to allow cell permeation), scaffolds were placed on ice until implantation. Alternatively, media were to scaffolds under dynamic conditions using an orbital shaker for in vitro experiments.

Animal studies

Animal studies were conducted according to approved protocols by the Cornell University Animal Care and Use Committee. Male, 6- to 8-week-old, CB17 SCID mice (Charles River Labs) were anesthetized and incisions made to the dorsal infrascapular skin. A subcutaneous pocket was created, irrigated with sterile PBS, cell-seeded PLG scaffolds (described above) inserted, and then sutured with 5-0 Ethilon (Ethicon). Studies investigating in vivo polymer degradation used blank sanitized scaffolds. High-resolution ultrasound imaging was conducted weekly using the VEVO 770 Imaging system and RMV 706 single-element transducer (Visualsonics). Mice were anesthetized (1.5% isoflurane) and implantation site hair removed by chemical debridement (Nair, Church & Dwight Co.). Mice were placed prone on a heated stage and scaffolds imaged with semiautomated 3D, B-mode imaging at 40 MHz frequency. To calculate tumor volume, cross-sectional areas of PLG scaffold + tumor were determined and then integrated to measure total volume, using VEVO software (v. 3.0.0).

Immunostaining and histology

CSC neurospheres cultured in nonadherent flasks were collected by centrifugation and embedded in OCT (Tissue-Tek) in minimal PBS following washing, fixation with 4% paraformaldehyde (PFA), and incubation in 20% sucrose/PBS. After cryosectioning (14 μm), immunostaining was conducted on Triton-X (VWR, 0.5%) permeabilized cells with antibodies
against human Sox-2 (Sigma), Oct-4 (Millipore), Nestin (Millipore), or control rabbit/mouse IgG (Invitrogen) at 1:200 dilution. Secondary antibodies (1:500, anti-rabbit Alexafluor 488 or anti-mouse Alexafluor 546; Invitrogen) were diluted in PBS containing 4′,6-diamidino-2-phenylindole (DAPI: 1:5,000) for nuclear counterstain; imaging was conducted on a Zeiss LSM 710 confocal microscope.

For in vivo studies, tumors were removed and fixed overnight in 4% PFA, then bifurcated and half stained for paraffin sectioning (4 μm) and subsequent hematoxylin and eosin (H&E) staining; returning half was immersed in 20% sucrose/PBS overnight, embedded in optimum cutting temperature (OCT), and cryosectioned (14 μm). Immunostaining was conducted as above to detect stem cell marker levels; in addition, species-specific EC marker CD31 was probed (mouse anti-human, Invitrogen; rat anti-mouse, BD Pharmingen) at 1:200 dilution, followed by secondary Alexafluor 546 (goat anti-mouse) or Alexafluor 467 (goat anti-rat) antibody at 1:500 (both from Invitrogen). Sections were counterstained with DAPI (1:5,000) and imaged on a Zeiss LSM 710 confocal microscope.

Conditioned media preparation
hCMEC-seeded PLG scaffolds were cultured for 3 days, after which EGM-2 media were removed, scaffolds washed in sterile PBS, and basal EBM-2 media (sans growth supplements, with 0.25% FBS and 0.1% penicillin/streptomycin) added. Media were collected at 24 hours and IL-8 ELISA (R&D Systems) conducted per manufacturer’s instructions. Subsequently, media were concentrated 10× at 4°C using Amicon Ultrafree 15 centrifugal filter units (3,000 MWCO; Millipore). Concentrated media (termed “3D conditioned endothelial cell medium”) was normalized to DNA content, as determined by fluorimetric DNA assay (QuantiFluo Assay; Promega) of scaffold lysates in Caron’s buffer. To generate 2D conditioned endothelial cell medium, hCMECs were cultured as subconfluent monolayers and media collected, concentrated, and normalized to like DNA concentrations as above described for 3D conditioned media. Basal control medium was generated by incubating basal EBM-2 media for 24 hours at 37°C and concentrating 10-fold as above described. Before use, conditioned media were diluted to 2× final concentration in stem cell medium and supplemented to CSC cultures for 3 days of preconditioning before subsequent analyses.

Conditioned CSC medium was created by culturing CSCs in nonadherent flasks until neurospheres of ~100 μm formed, at which point media were replaced with fresh stem cell medium lacking antibiotics. Media was then concentrated and normalized to DNA content as above described.

Transwell migration assay
Transwell inserts (8 μm pore size; Falcon HTS FluoroBlok Inserts) were coated with 1% collagen I (BD Biosciences), washed, and placed in 24-well plates. Dissociated CSCs (10,000, with or without prior preconditioning in 3D conditioned endothelial cell media) were added to each Transwell in 300 μL stem cell media and migration assessed following addition of recombinant human IL-8 or IgG (50 or 5 ng/mL; Genescript) to stem cell media in the lower compartment. Alternatively, migration toward endothelial cell-secreted factors was determined by adding 300 μL medium composed of equal parts stem cell medium and either 10× 3D endothelial cell medium or 10× basal EC medium. To confirm the role of IL-8 in CSC chemotaxis, antagonist antibodies to human IL-8 (7.5 μg/mL), CXCR1 (2 μg/mL), or CXCR2 (2 μg/mL, all from R&D Systems) were added before the start of the experiment (anti-IL-8 added to medium; anti-CXCR1/2 added to cells + medium for 1 hour at room temperature before experiment). After 16 hours, Transwells were fixed in 4% PFA and DAPI stained (1:5,000). Transwell underside images were collected at 10× on a Zeiss Observer Z.1 microscope fitted with an AxioCam MRN camera. For each of triplicate experiments, the average number of migrated cells from 7 random images per well from 4 wells was calculated using ImageJ software (NIH).

Cell growth in response to IL-8 stimulation
Freshly dissociated CSCs were placed in nonadherent plates (30,000/12-well) in stem cell medium. IL-8 supplementation (50 ng/mL; Genescript) alone or with antibody antagonists to IL-8, CXCR1, or CXCR2 (dosages and pre-incubation as above for Transwell studies) was provided at the start of the experiment and at 24 hours. After 48 hours, cells were collected by brief centrifugation and DNA assay conducted on Caron’s buffer–treated cell lysates as above described. Data are represented as normalized values relative to CSC cultures without IL-8 treatment.

Real-time reverse transcription PCR and short hairpin RNA–mediated CXCR2 gene knockdown
Following 3 days of preconditioning in 3D conditioned or basa media, total RNA from CSC neurospheres was harvested with TRIzol (Invitrogen) according to manufacturer’s instructions and 1 μg reverse-transcribed to cDNA (qScript cDNA supermix; Quanta BioSciences) using random hexamer and oligo(dt) primers. Real-time reverse transcription (RT)-PCR was carried out on 25 ng template using SYBR green detection (Quanta Biosciences) and an Applied Biosystems 7500 System. Primer sequences (300 nmol/L; IDT Technologies) were as follows: human CXCR1 (forward: 5′tcagactcctagttgctt3′, reverse: 5′gggtgtgatctttctgc3′); human CXCR2 (forward: 5′agaatttcgatgcctctctatc3′, reverse: 5′aggtgaagcttcggcctgaa3′) and β-actin loading control (forward: 5′aattctgctggagctaa3′, reverse: 5′acaggtggaaaggctctctgtaa3′). Relative quantification was conducted using the ΔΔCT method (20).

To establish long-term knockdown of CXCR2 expression, lentiviruses encoding short hairpin RNA (shRNA) sequences targeted against human CXCR2 or random shRNA sequence (MISSION shRNA lentiviral transduction particles; Sigma-Aldrich) were transduced at 5 multiplicity of infection to 1E1 glioblastoma CSCs in a 12-well dish overnight, rinsed with sterile PBS, and transferred to stem cell medium. CXCR2 knockdown of 70% (vs. control transduced) was confirmed by real-time RT-PCR when 3 CXCR2-specific shRNA lentiviruses were used concurrently (TRCN0000009136, TRCN0000009137, and TRCN0000009138; Sigma-Aldrich). In addition, CXCR2 knockdown was confirmed at the protein level using Western blot analysis. CSCs previously transduced with shRNA-control
or siRNA-expressing lentiviruses were centrifuged and harvested at 4°C in radioimmunoprecipitation buffer containing protease and phosphatase inhibitors (Roche). Protein concentrations were assessed by bicinchoninic acid assay (Pierce). 50 µg separated on a pre-cast 10% PAGE (Bio-Rad), and then transferred to a polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked with 5% non-fat dry milk for 1 hour, then probed with mouse anti-human CXCR-2 (1:200; R&D Systems) and α-tubulin (1:5000; Genscript) antibodies followed by goat anti-mouse horseradish peroxidase (HRP) secondary antibodies. Densitometric analysis of bands was conducted with ImageJ (NIH).

Statistical analysis
All experiments were carried out 3 separate times and data reported as mean ± SD. One-way ANOVA followed by the Tukey post-test was conducted to conclude statistical significance, with α = 0.05, using Prism 5 software (GraphPad).

Results
In vivo tumor formation by CSC-seeded 3D polymeric scaffolds recapitulates clinical features of human glioblastoma multiforme and is accelerated by endothelial cell signaling

To study the contributions of the 3D microenvironment and endothelial cell signaling on CSC behavior, CSCs from patient glioblastoma resections were used. These cells were cultured in nonadherent flasks under typical stem cell culture conditions over multiple passages and formed characteristic neurospheres that robustly expressed stem cell markers Sox2, Nestin, and Oct4 (Fig. 1A) and were capable of multilineage differentiation under serum-containing conditions (Supplementary Fig. S1). Using this undifferentiated CSC population, we first wanted to confirm that coimplantation of human CSCs with human brain endothelial cells (hCMECs) within highly porous PLG scaffolds enhances glioblastoma formation relative to that of CSCs alone. Indeed, longitudinal measurements of tumor and scaffold volumes via high-resolution ultrasound showed that CSCs implanted together with hCMECs exhibited significantly accelerated tumor formation as compared with CSCs alone by 8 to 11 weeks (Fig. 1B). As blank control scaffolds were largely degraded (~80%) by 6 weeks, final tumor volumes were due to differences in cell number rather than scaffold contributions (Fig. 1C). Furthermore, ultrasound analysis revealed multiple regions of blood flow throughout the mass, suggesting that neoplasms derived from coimplantation were highly vascularized (Supplementary Fig. S2A), which is consistent with human glioblastoma multiforme.

Following explanation, tumors were subjected to histologic analysis to confirm that the subcutaneous PLG implantation model mimicked bona fide glioblastoma multiforme. Evaluation of H&E-stained cross-sections showed that tumors contained extensive necrotic cores, which were circumscribed by large pseudopalisading nuclei (Fig. 1D), both considered clinical markers of human glioblastoma (21). Furthermore, frequent microvascular hyperplasia (Supplementary Fig. S2B), along with common detection of mitotic figures (Supplementary Fig. S2C) and diffuse tumor cell infiltration outside of the primary tumor mass (Supplementary Fig. S2D), further supported the diagnosis of a grade IV malignant glioma (22). To further identify the fate of the implanted endothelial cells and assess whether CSCs maintained preferential perivascular colocalization as reported in clinical specimens (5), we conducted immunohistochemical analysis of endothelial and CSC markers. Similar to previous results with human dermal microvascular endothelial cells (23), hCMECs formed capillary structures that anastomosed with the mouse vasculature (Fig. 1E, Supplementary Fig. S3A). Similarly, these vessels borne from coimplantation appeared larger than those observed in tumors formed from by CSC implantation alone; however, the extent of tumor vascularization was similar between both groups (Supplementary Fig. S3B). Interestingly, Sox2⁺ (Fig. 1F) and Oct4⁺ (Supplementary Fig. S3C) CSCs were detected largely in close or direct proximity to these capillary structures and associated more frequently with portions of the vessels that contained human endothelial cells (Fig. 1F), suggesting the establishment of perivascular niches supportive of CSC maintenance in our model.

3D microenvironmental conditions stimulate endothelial cell secretion of paracrine factors that support CSC functions in vitro

To specifically isolate the paracrine signaling effects of hCMECs on CSC-mediated gliomagenesis in our model, we next conducted in vitro studies in which hCMECs were cultured in PLG scaffolds to recapitulate their 3D interactions within the vasculature. Cells adhered readily within the pores of the scaffold and arranged into capillary tube–like structures that matured over sustained culture periods (Fig. 2A), showing the ability of our model to support 3D vasculogenic processes and microenvironmental conditions in vitro. To evaluate whether scaffold culture and, thus, 3D microenvironmental context, affected the ability of hCMECs to modulate CSC functions, we carried out conditioned media experiments. To this end, conditioned media from hCMECs cultured in conventional 2D culture or 3D PLG scaffolds were collected and supplemented to CSC cultures (Fig. 2B). Paracrine factors from 3D hCMECs were significantly more efficacious in retaining expression of CSC stem cell markers Nestin and Sox2 than conditioned media from 2D hCMEC cultures or basal medium, where Sox2 expression was minimal and Nestin expression nearly absent (Fig. 2C and D). Furthermore, 3D-derived hCMEC signaling factors also accelerated CSC growth in vitro. This was evidenced by larger neurosphere size in cultures supplemented with media from 3D-cultured hCMECs, which was further corroborated by an almost 3-fold increase in total DNA relative to similar cultures fortified with conditioned media from 2D hCMEC cultures (Fig. 2E). In light of the heterogeneous stem cell marker expression, cultures supplemented with 2D conditioned media likely contain a mixed population of CSCs (Fig. 2C) making the differences in neurosphere area and DNA content seem even more dramatic. This was substantiated by the presence of frequent plate adhesion of glial-like cells in CSC cultures receiving 2D conditioned media, which was nearly absent in similar cultures fortified with 3D factors.
Collectively, these data suggest that 3D microenvironmental conditions play a pivotal role in mediating paracrine signaling by hCMECs, which support CSC maintenance and growth.

**IL-8 regulates the migration and growth of glioblastoma CSCs**

Three-dimensional culture conditions can increase IL-8 expression and secretion in various 3D culture formats (15, 19, 24). Furthermore, IL-8 reportedly plays important roles in glioblastoma angiogenesis (9), glioma, and tumor-associated endothelial cell motility (11, 25), and non-glioblastoma CSCs self-renewal and pluripotency (13, 14). Therefore, we next hypothesized that greater CSC growth and maintenance in response to 3D hCMEC-conditioned media may be related to elevated IL-8 availability. Indeed, 3D cultured hCMECs secreted significantly more IL-8 relative to the same number of cells maintained in 2D monolayers (Fig. 3A). Interestingly, exposure of 3D hCMEC cultures to soluble factors secreted by CSCs further enhanced IL-8 secretion by these cells. Together, these data show a supportive role for 3D cell–microenvironment interactions on endothelial cell–derived IL-8 concentrations within the perivascular microenvironment (Fig. 3A).

Figure 1. Brain endothelial cells accelerate tumor formation by CSC-seeded 3D polymeric scaffolds in vivo. A, patient-derived CSCs form neurospheres when grown in stem cell culture conditions and robustly express stem cell markers Sox2, Nestin, and Oct4. Nuclear counterstain with DAPI. Scale bars, 50 μm. B, coimplantation of hCMECs with CSCs in porous PLG scaffolds accelerated tumor growth relative to CSC implantation alone as calculated from 3D B-mode ultrasound images (inset) collected at weekly time points following implantation. Images show representative cross-sections of implanted and control groups 8 weeks following subcutaneous implantation. ***, P < 0.001 versus hCMEC alone; #, P < 0.01 versus CSC alone; †, P < 0.05 versus CSC + hCMEC at 6 weeks. Scale bars, 1 mm. C, because of hydrolytic degradation, the volume of blank control scaffolds was reduced markedly by 6 weeks as determined via longitudinal ultrasound measurements and confirmed following scaffold explanation. Scale bars, 1 cm. D, H&E-stained cross-sections of tumors explanted after 8 weeks revealed clinical features of human glioblastoma multiforme, including large necrotic (N) regions surrounded by pseudopalisades (**; left) and vascular hypertrophy (VH; right). Asterisk in right panel denotes non-hypertrophic vasculature for comparison. Scale bars, 500 μm (left) and 100 μm (right). E, immunohistochemical analysis of coimplanted tumors using species-specific antibodies to the endothelial cell marker CD31 and DAPI counterstain as well as detection of blood cells within vessel lumens suggests that implanted hCMECs formed capillary structures that anastomosed with the mouse vasculature. Scale bar, 20 μm. F, undifferentiated CSCs colocalized with hCMECs as detected by coimmunostaining for Sox2 and human CD31. Scale bar, 50 μm.
To better define the direct contributions of IL-8 to CSC migration and growth (i.e., 2 parameters regulating CSC recruitment to and maintenance within the perivascular niche), we conducted studies with recombinant human IL-8. The chemotactic ability of IL-8 on CSCs was assessed with a Transwell migration assay using 2 separate patient-derived CSC populations. In both cases, IL-8 increased migration of CSCs 5- to 10-fold from control groups challenged with control media (Fig. 3B), which was preserved to a lesser, yet significant, extent with a 10-fold reduction in IL-8 concentration (Supplementary Fig. S5A). To analyze whether IL-8 stimulation similarly accelerates CSC growth, patient-derived glioblastoma CSCs were cultured in typical stem cell medium in the presence or absence of human IL-8. Addition of IL-8 elicited a dose-dependent increase in neurosphere size of CSCs, which plateaued at 50 ng/mL (data not shown; Fig. 3C). To confirm that this effect was mediated via binding to the cognate IL-8 receptors CXCR1 and/or CXCR2 (9), CSCs were incubated with the respective antagonist antibodies before IL-8 treatment. CXCR1 blockade abolished IL-8-induced proliferation and resulted in CSC growth, which matched that of control cultures, suggesting that this receptor is partly responsible for the proliferative effects of IL-8 on CSCs (Fig. 3C). Interestingly, selective impairment of CXCR2 binding more dramatically impacted CSC growth: cell numbers dropped below controls and even below cell numbers at the start of the experiment (data not shown). This indicates that (i) signaling via this receptor is necessary for normal CSC viability and (ii) CSC functions may partly depend on autocrine IL-8 signaling (Fig. 3C). To confirm the functionalization of IL-8 on CSC growth, saturating levels of an antagonist IL-8 antibody were introduced into cultures that were concomitantly stimulated with IL-8. Again, CSC numbers decreased to levels observed with CXCR2 blockade, further supporting that IL-8 signaling is
necessary for both maintenance and proliferation of glioblastoma multiforme CSCs (Fig. 3C).

**Paracrine communication between brain endothelial cells and CSCs enhances IL-8 signaling and facilitates CSC migration and growth in vitro**

Next, we sought to determine the functional consequence of the abovementioned results by evaluating whether 3D-cultured hCMECs promote CSC migration and growth due to elevated IL-8 signaling. Toward this end, we first broadly compared CSC migration toward 3D hCMEC-conditioned media, IL-8-fortified, or control basal media using Transwell migration assays. Our results indicated that paracrine factors secreted by scaffold-cultured hCMECs enhanced CSC migration in a similar manner as recombinant IL-8 (2.7- vs. 1.9-fold relative to control conditions). To further determine whether chronic signaling by endothelial cells as present in the brain perivascular microenvironment impacts the migratory potential of CSCs, CSCs were preconditioned with 3D hCMEC-secreted factors before migration experiments. While this pre-exposure enhanced the overall migratory potential of CSCs only insignificantly, a much more pronounced effect was detected for directed migration toward either 3D hCMEC-conditioned media or IL-8 (Fig. 3D). As these results suggested that hCMEC-derived paracrine signaling factors elevated IL-8 responsiveness, we hypothesized that enhanced migration of preconditioned CSCs to IL-8 may be related to altered expression of the cognate receptors CXCR1 and/or CXCR2. Indeed, quantitative PCR (qPCR) analysis of receptor expression for IL-8 receptors CXCR1 and CXCR2 revealed that preconditioning with 3D hCMEC-conditioned media significantly upregulated both receptors. To determine whether the increased expression of these receptors is sufficient to enhance migration, we performedTranswell migration assays using anti-CXCR1 and anti-CXCR2 antibodies. These experiments demonstrated that the addition of antibodies to either CXCR1 or CXCR2 significantly inhibited migration of CSCs to IL-8 (Fig. 3E). These results suggest that paracrine factors secreted by 3D hCMECs enhance CSC migration and growth due to elevated IL-8 signaling, which in turn increases the expression of IL-8 receptors, thereby facilitating migration of CSCs to IL-8.

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IL-8 signaling via cell-specific upregulation of both ligand (in hCMECs) and its cognate receptors (in CSCs).

While these findings support endothelial cell paracrine signaling as an underlying cause of enhanced CSC invasion, they do not directly evaluate participatory roles of IL-8 or CXCR1/2 in this process. Therefore, to directly assess the contributions of IL-8 stimulation and/or CXCR1/2 binding on cell migration, antagonist antibodies to each were incorporated in repeat Transwell assays. Selective inhibition of IL-8 in 3D conditioned hCMEC media, strongly decreased migration of preconditioned glioblastoma CSCs, thereby showing that signaling cascades initiated by this factor are pivotal to CSC chemotaxis (Fig. 3F). Likewise, when cells were treated with antagonists to either CXCR1 or CXCR2, CSC migration was similarly attenuated, establishing that signaling through both receptors functions to facilitate CSC migration. Interestingly, CXCR2 inhibition appeared to have a greater impact on CSC invasion than did CXCR1 blockade, yet these results were not statistically significant. Importantly, the impact of these antagonist antibodies was specific to IL-8 signaling, as treatment with IgG control antibody elicited no effect on CSC migration (Supplementary Fig. S5C). These data implicate both receptors in CSC migration toward endothelial cell–secreted IL-8 within the perivascular microenvironment.

Lastly, the functional significance of 3D hCMEC-mediated CXCR1 and CXCR2 upregulation on CSC growth was evaluated. To this end, preconditioned CSCs were cultured in the presence or absence of selective antagonists to IL-8 or its cognate receptors. Similar to the migration findings above, blockade of either receptor significantly impaired the effects of preconditioning on CSC growth (Fig. 3F). Similarly, direct blockade of IL-8 also impaired the effects of preconditioning with 3D hCMEC-derived factors on CSC proliferation, further implicating that the stimulatory effects of paracrine endothelial cell signaling on CSC growth are mediated through IL-8 binding to both CXCR1 and CXCR2.

**Inhibition of CXCR2 signaling by CSCs abolishes the stimulatory effects of endothelial cells on tumor growth in vivo**

Thus far, these results provide corroborating evidence that IL-8 signaling is a critical mediator of glioblastoma CSC migration, maintenance, and growth. Therefore, to directly assess whether IL-8 receptor signaling in glioblastoma CSCs underlies tumor formation siRNA-based inhibition strategies were used. Given the more pronounced effect of CXCR2 in IL-8–mediated CSC growth (Fig. 3C), siRNA sequences targeted against human CXCR2 were generated and incorporated into lentiviruses to stably knockdown expression of this receptor (Supplementary Fig. S6A). Cotransduction of 3 constructs in glioblastoma multiforme CSCs caused a robust (~80%, Supplementary Fig. S6B) knockdown of CXCR2 expression and protein levels (~65%, Supplementary Fig. S6C) with no impact on expression of CXCR1 (data not shown). Next, transduced or native cells were seeded alone or in combination with hCMECs in PLG scaffolds, which were then subcutaneously implanted in severe combined immunodeficient mice (SCID) mice. Coimplantation of native CSCs with hCMECs recapitulated prominent tumor formation by 8 weeks, whose volume dramatically exceeded that of tumors borne by CSC implantation alone (Fig. 4A and B). However, these effects were abolished with CXCR2 knockdown, whereby the volume of tumors formed by transduced glioblastoma CSCs was half that of those generated by native CSCs (Fig. 4B). Despite this difference in size, these tumors were vascularized to a similar extent as those resulting from native CSC implantation (Supplementary Fig. S7), supporting that differences in tumor growth were not merely the result of stunted tumor perfusion. Importantly, coimplantation of hCMECs with CXCR2 knocked-down CSCs failed to reverse these effects or accelerate tumor formation (Fig. 4B), thus providing strong evidence that the promoting effects of brain endothelial cells on glioblastoma multiforme CSC–dependent tumor growth are mediated, in large part, through CXCR2 signaling.

**Discussion**

Delineating the signaling pathways that regulate CSC maintenance, proliferation, and tumorigenicity in the perivascular niche has proved challenging due to a paucity of in vitro models, which faithfully recapitulate 3D microenvironmental conditions. In this study, we used a scaffold-based culture system that enables brain endothelial cells to form vascular networks, thereby initiating paracrine IL-8 signaling cascades,
which enhance CSC maintenance and growth in vitro and tumor formation in vivo.

The detected upregulation of IL-8 in our studies may have been caused by a variety of microenvironmental conditions including altered dimensionalities (15), direct cell–cell contact (26), and cell–ECM interactions (27). Furthermore, varied intraculture oxygen concentrations could have been involved (28), whereby this response is regulated by changes in cell morphology that may result from varied culture dimensionalities (29), as well as pathologic consequences of cell death (e.g., necrosis; ref. 30). Moreover, reciprocal interactions between various glioblastoma multiforme–associated cell types modulate this response. For example, glioblastoma–associated endothelial cells exhibit enhanced expression of IL-8 (31). Accordingly, hCMECs pre-exposed to conditioned media from glioblastoma CSCs increased IL-8 production in our studies (Fig. 3A), suggesting that CSC–derived signaling is partly responsible for the altered gene expression observed in glioblastoma–derived primary endothelial cells (31). It is unknown whether the concentrations of IL-8 secreted by endothelial cells in our study are sufficient to effect CSC functions as we observed, given that these latter experiments were carried out using a considerably higher IL-8 stimulus. However, ECM in the tumor microenvironment can function indirectly to enhance local IL-8 concentrations to levels that approach those used in our studies. For example, components of the ECM such as heparin and heparan sulfate can efficiently bind IL-8 via its heparin-binding domains (32), thereby creating signaling depots that contribute to enhanced cell chemotaxis (33). Future quantitative analysis of spatial differences in IL-8 from ex vivo tumor samples would help evaluate this possibility.

Culture context–driven endothelial upregulation of IL-8 directly affected CSC maintenance and growth, which was further enhanced by CSC preconditioning with media from 3D endothelial cell cultures. Specifically, chronic exposure of CSCs to endothelial cell signals as would occur in the perivascular microenvironment–upregulated IL-8 receptors CXCR1 and CXCR2, with functional consequences of enhanced migration and growth. Interestingly, other interleukin receptors have previously been associated with glioblastoma CSC malignancy. More specifically, glioblastoma CSCs express elevated levels of IL-6 receptors IL6Rα and glycoprotein 130 (gp130) relative to non–stem glioma cells, which promotes their self-renewal and tumorigenic capability (34). Given that the respective ligands for these receptors (i.e., IL-6 and IL-8) are regulated by the same transcription factors (i.e., NF-κB and NF-IL6; ref. 35), it is possible that IL-6 secretion by 3D cultured endothelial cells may partially contribute to altered CSC functions in our studies. Alternatively, IL-6 may serve as an upstream stimulus for the increased transcription of IL-8 (36). Hence, it is conceivable that CSC-derived IL-6 not only promotes gliomagenesis in an autocrine manner (34) but also by stimulating the transcription of IL-8 by brain endothelial cells (Fig. 3A).

We have shown that both cognate receptors CXCR1 and CXCR2 participate in the growth– and migratory-inducing effects of IL-8 on CSCs. These findings agree with observations in human microvascular endothelial cells, where blockade of either receptor attenuated chemotaxis toward IL-8 (37). However, with regard to cell growth, our data indicate a far greater impact of CXCR2 blockade, which resulted in declining cell counts below control culture levels (Fig. 3C). This may be partially explained by the broader range of signaling molecules, which can bind to this receptor (9). Alternatively, the specific downstream effectors of CXCR2 may be critical to IL-8–induced CSC growth. Included in these are activation of phosphoinositide 3-kinase (PI3K) and extracellular signal–regulated kinase (ERK)1/2 (38), which have been implicated in the proliferation and differentiation of neural stem cells (39), respectively. Interestingly, PTEN has also been shown to be a potent regulator of PI3K signaling and has been implicated in the maintenance of various CSC types (40, 41). However, in our studies, both PTEN+ and PTEN- CSCs responded similarly to 3D endothelial cell conditioned media (Fig. 2C), suggesting that CXCR2-mediated signaling may invoke similar signaling pathways as PTEN, possibly through PI3K activation.

While this study was specifically designed to better define the role of endothelial cell–derived soluble factors on CSC functions, the presented culture model may be broadly applicable to other questions of glioblastoma perivascular niche interactions. For example, CSCs may promote tumor growth by modulating blood vessel functions through differentiation into endothelial cells (42) or pericytes (43). Future studies involving direct coculture of both cell types within the presented scaffold system may help better define the underlying molecular and cellular mechanisms and assess whether altered IL-8 signaling may contribute to these changes. Furthermore, the composition and mechanical properties of endothelial cell–deposited ECM likely play key roles in guiding CSCs but were not considered in the current study. Endothelial cells assemble an ECM that is enriched in laminin α2, a promoter of CSC malignancy (44). As both laminin α2 and IL-8 inversely correlate with patient survival (44, 45) and are upregulated in glioblastoma–associated endothelial cells relative to normal brain endothelial cells (25, 44), it is provocative to consider that a functional link between these 2 factors exists. Finally, ECM mechanical properties critically not only impact glioma migration (46) and endothelial cell behavior (47) but also amplify cellular response to soluble signals (48). Modulating the presentation of biomaterials within these cell culture platforms would allow evaluation of the collective contributions of these parameters on CSC behavior, which would significantly advance our understanding of the physicochemical parameters influencing cell behavior in the perivascular niche. Finally, it is essential to realize that endothelial cells are not the sole source of IL-8 in the glioblastoma microenvironment. For example, additional cell types resident within the tumor parenchyma, including astrocytes (49) and bulk glioma cells themselves (50), can contribute to interstitial IL-8 levels. Future studies using the 3D culture platform with these additional cell types would help elucidate whether paracrine factors secreted by these cell types can similarly enhance CSC sensitivity to IL-8 via receptor upregulation as endothelial cells do.
It is critical to note that while subcutaneous coimplantation of CSCs with endothelial cells mimicked certain glioblastoma-like characteristics in vivo, CSCs implanted alone failed to recapitulate advanced tumors within the time span of the experiment (11 weeks). These findings appear in contrast with other animal models of glioblastoma, for example, intracranial CSC implantation, which show rapid tumor formation by these cells, and would thus suggest that additional signaling parameters not afforded by the scaffold model or subcutaneous environment are involved in CSC-mediated tumorigenesis (51). As it is possible that varied vascular density contribute to these differences (52), future studies will be required to evaluate the importance of IL-8 signaling in an orthotopic or transgenic model of glioblastoma.

The finding that inhibition of the CXCR2 signaling pathway in CSCs abolishes tumorigenesis in vivo has significant implications for the clinical management of human glioblastoma multiforme. It is important to note that additional cytokines—CXCL2, CXCL3, and CXCL5, among others (53)—also bind CXCR2 and, as such, may participate in CSC-driven gliomagenesis, which has not been investigated herein. Interestingly, the application of certain anticancer treatments such as photodynamic or temozolomide therapy in multiple glioma cell lines has been shown to stimulate transcription of many of the CXCR2 agonists (e.g., CXCL2, CXCL3, IL-8; refs. 54, 55) as well as IL-6 (54), which could, in theory, support subsequent CSC growth and recurrent tumor formation that is nearly reflexive for human glioblastoma. In light of this, the development and delivery of CXCR2 antagonists in combination with conventional therapeutic approaches may offer a significant advantage for clinical treatment of the disease.

In conclusion, we have developed a tissue-engineered culture model to resolve the impact of 3D microenvironmental conditions on endothelial cell–mediated paracrine signaling, which supports glioblastoma tumor initiation and progression. Our results revealed that perivascular niche–associated IL-8 signaling between endothelial and CSCs may contribute to poor clinical prognosis. These findings not only warrant the future study of anti-CXCR2 therapies for clinical management of glioblastoma multiforme but also underscore the value of physiologically relevant models for the study of patient cancer cell behavior.

Disclosure of Potential Conflicts of Interest
The content is solely the responsibility of the authors and does not necessarily represent the official views of NCI or NIH. No potential conflicts of interest were disclosed.

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