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A Three-Dimensional (3D) Organotypic Microfluidic Model for Glioma Stem Cells – Vascular Interactions

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Abstract

Glioblastoma (GBM) is one of the deadliest forms of cancer. Despite many treatment options, prognosis of GBM remains dismal with a 5-year survival rate of 4.7%. Even then, tumors often recur after treatment. Tumor recurrence is hypothesized to be driven by glioma stem cell (GSC) populations which are highly tumorigenic, invasive, and resistant to several forms of therapy. GSCs are often concentrated around the tumor vasculature, referred to as the vascular niche, which are known to provide microenvironmental cues to maintain GSC stemness, promote invasion, and resistance to therapies. In this work, we developed a 3D organotypic microfluidic platform, integrated with hydrogel-based biomaterials, to mimic the GSC vascular niche and study the influence of endothelial cells (ECs) on patient-derived GSC behavior and identify signaling cues that mediate their invasion and phenotype. The established microvascular network enhanced GSC migration within a 3D hydrogel, promoted invasive morphology as well as maintained GSC proliferation rates and phenotype (Nestin, SOX2, CD44). Notably, we compared migration behavior to *in vivo* mice model and found similar invasive morphology suggesting that our microfluidic system could represent a physiologically relevant *in vivo* microenvironment. Moreover, we confirmed that CXCL12-CXCR4 signaling is involved in promoting GSC invasion in a 3D vascular microenvironment by utilizing a CXCR4 antagonist (AMD3100), while also demonstrating the effectiveness of the microfluidic as a drug screening assay. Our model presents a potential *ex vivo* platform for studying the interplay of GSCs with its surrounding microenvironment as well as development of future therapeutic strategies tailored toward disrupting key molecular pathways involved in GSC regulatory mechanisms.

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INTRODUCTION

Glioblastoma (GBM) is one of the most common and deadliest forms of brain cancer in adults. Despite aggressive treatment, including surgery, radiation and chemotherapy, prognosis for GBM patients remains dismal with a 5-year survival rate of 4.7% [1]. These tumors almost always recur within a few months after treatment. Tumor recurrence is hypothesized to be driven in part by glioma stem cell (GSC) populations within the primary brain tumor [2, 3]. GSCs have similar properties to neural stem cells – they can self-renew and differentiate into several neuro-glial lineages leading to intratumoral heterogeneity [4]. Importantly, GSCs are also highly tumorigenic, invasive, and resistant to several forms of therapy [5]. As such, GSCs evade conventional therapies that target bulk populations of cancer cells allowing these cells to survive and regenerate the tumor [6].

GSCs are often concentrated near specific niches around pre-existing blood vessels, referred to as the perivascular niche and around necrotic areas known as the peri-necrotic or hypoxic niches [7–9]. These specialized niches shelter, maintain, and regulate GSC stem phenotypes, invasion, and therapeutic resistance [10]. Previous studies have shown that cell-cell interactions within the perivascular niches, which involve soluble factors released by endothelial cells (ECs) of the nearby blood vessels, lead to self-renewal of GSCs and propagation of the brain tumor *in vivo* [8]. Different signaling pathways have been implicated in GSC-EC interactions, such as Notch, TGF β , nitric oxide, and sonic hedgehog (SHH) [7]. Targeting some of these signaling pathways have resulted in reduced tumor growth [11, 12]. Therefore, disruption of vascular niches could serve as a potential therapeutic strategy for GBM. In that regard, it is important to identify specific signaling pathways within the niches that support GSC self-renewal and propagation.

The CXCL12-CXCR4 signaling axis regulates cell migration and proliferation during normal development in several tissues including the brain [13]. However, high levels of the chemokine receptor CXCR4 is overexpressed in gliomas and its expression level is correlated with tumor grade and poor prognosis [14]. Expression of CXCL12 was also found to be highly expressed in the endothelium surrounding GBM whereas normal brain tissue lacked CXCL12 expression [15]. As such, CXCL12-CXCR4 signaling axis has been described to play a central role in tumorigenesis of GBM along with several other human cancers, such as breast, colon, pancreatic, ovarian, and prostate [16–21]. Importantly, inhibition of the CXCL12-CXCR4 signaling using AMD3100 has resulted with some success in tumor regression *in vivo* [22]. However, it is still unclear if GSC-EC interaction enhances GSC migration within the vascular niche and if this effect is mediated by CXCL12-CXCR4 interaction.

To better understand the role of the vascular niche and cell-cell signaling, such as CXCL12-CXCR4, that govern GBM tumorigenesis, an appropriate model with well-controlled architecture is needed to recapitulate the components of the *in vivo* tumor microenvironment. Conventional *in vitro* platforms, such as transwell assays and spheroid models, have been widely utilized to perform biological studies and drug screening [23]. However, transwell systems are end-point assays that do not allow real-time observations of dynamic cellular interactions [24, 25]. In addition, these conventional assays often require a

large population of cells, which may be problematic for patient-derived cells. Most importantly, these models do not fully recapitulate the complex *in vivo* tumor microenvironment leading to non-physiologically relevant cell behavior [26]. Therefore, there have been significant innovations in the use of microscale tissue-engineered technologies to develop complex 3D tumor models across different types of cancer, with precise control over cell-cell, cell-matrix and cell-soluble factor interaction [23, 27–34]. While these 3D models have had great success in interrogating tumor responses to the microenvironment, few have studied GBM and even less in the context of the vascular niches (i.e. tumor-vascular interactions) within a 3D platform [35, 36]. For instance, a 3D *in vitro* model of the perivascular niche was developed by Infanger et al. to study GSC behavior using 3D hydrogels. However, in this model, GSCs and ECs were not co-cultured together simultaneously and EC-conditioned medium was used to simulate paracrine signaling [37]. On the other hand, a 3D microfluidic device was used to study glioma invasion influenced by ECs. The GSCs and ECs were seeded as 2D monolayers separated by a 3D collagen layer in between, which did not truly represent the 3D GBM microenvironment [27]. With recent attempts in modeling vasculature in 3D, there have also been notable advances in developing vascular networks with increasing physiological relevance within 3D hydrogels [29, 35, 38, 39]. However, much of the focus have not been on GBM or patient-derived GSCs. Additionally, those studying GBM were specifically investigating the influence of GBM on tumor-associated angiogenesis, such as the work from Cui et al, which highlighted the importance of macrophage-associated immunosuppression during GBM angiogenesis, and not the changes in GSC invasion and stem phenotype within the context of a vascular niche, which is the focus of our study [38, 40].

We previously developed microfluidic technologies comprising of 3D hydrogel-based layers for multi-culturing of tumor and stromal cells side-by-side to mimic the architecture of the native tumor microenvironment [28, 32]. We have investigated breast cancer cell invasion in response to signaling cues and stromal cells, such as cancer-associated fibroblasts and ECs [28, 32]. In this study, we describe the design, biological validation, and use of our microfluidic platform as a new *in vitro* 3D organotypic model for GSC invasion in the context of a vascular niche (Fig. 1). We formed a spontaneously assembled 3D vasculature within our platform, and then incorporated patient-derived GSCs within the core of the tumor region. The novelties of our model enabled the interaction of patient-derived GSCs and a 3D microvascular network which has not been described in previous studies. We specifically investigated the effect of GSC-EC interactions within our microfluidic on GSC proliferation, invasion, and stem phenotype. Importantly, in parallel to our *in vitro* experiments, we utilized a patient-derived xenograft (PDX) *in vivo* model to evaluate the physiological relevance of our microfluidic platform. Indeed, we observed similar GSC invasive behaviors between the two systems in terms of GSC migration, morphology, and proximity to the vasculature. Lastly, to determine the influence of CXCL12-CXCR4 signaling on EC-mediated GSC invasion, we tested the effect of AMD3100, a CXCR4 antagonist, within our *in vitro* microfluidic model. As such, our study presents a physiological relevant 3D organotypic model of GSC invasion for investigating the interaction between GSC and the surrounding microenvironment.

MATERIALS AND METHODS

Microfluidic device fabrication

The microfluidic platform was designed using CAD software, and transferred onto a transparent mask. The design consisted of three concentric semicircles comprising of tri-layer tumor, stroma, and vasculature regions with diameters of 1, 2, and 3 mm with heights of 200 μm . The intraregional distance between the concentric semicircles was 1 mm. Trapezoidal microposts were spaced evenly at 100 μm bound each region. The microposts were configured to separate the regions, while also enabling interactions between the regions by allowing exchange of media, biomolecules, and cells throughout the platform. To create a master mold of the devices, SU8-2075 (MicroChem) was spun to height of 200 μm onto a silicon wafer after which, the wafer was exposed to UV through the mask placed on the wafer forming the primary mold. The surfaces of the silicon wafer were made hydrophilic by treatment with methyltrichlorosilane (MTCS, Sigma-Aldrich). Next, polydimethylsiloxane (PDMS, Sylgard 184 Silicon Elastomer Kit, Dow Corning) was poured over the wafer and cured for 1.5–2 h at 80 °C. The PDMS cast was then peeled from the micro-patterned mold. Individual devices were then cut from the cast using a razor, and inlet and outlet holes were hollowed out using biopsy punch. PDMS device casts were then bonded to 18 mm² glass slides to form the channels of the microfluidic. Before bonding, devices and glass slides were cleaned with ethanol and pressurized nitrogen gas and subjected to oxygen plasma (PDC-32G, Harrick Plasma) to produce hydrophilic surfaces on both the devices and the glass. PDMS device casts were then joined to glass slides with the channel side facing down and placed in oven at 80 °C overnight to secure bonding. Next, device sterilization was performed by one treatment in the liquid autoclave and a subsequent treatment by dry autoclave. Finally, devices were placed in the oven at 80 °C overnight to dry completely.

To enhance attachment of the hydrogels (i.e. Matrigel and Fibrin) to the 3D cell culture regions, poly-d-lysine (1 mg mL⁻¹) (PDL, Sigma-Aldrich) was injected into the cell culture region and incubated at 37 °C for 1 h, then washed with deionized (DI) water. Glutaraldehyde (1% (v/v)) (GA, Sigma-Aldrich) was then added to the culture region and incubated for 1.5–2 h at room temperature, then washed 4–5 times with DI water to remove excess GA. The devices were then placed in the oven at 80 °C overnight to restore the hydrophobicity of the channels. Hydrophobicity enabled microposts to contain a hydrogel within the culture regions before it can polymerize and attach to the inner surface [41].

Cell culture

Patient samples used for this research were provided by the Biobank Core Facility at St. Joseph's Hospital and Medical Center and Barrow Neurological Institute (BNI), Phoenix, Arizona. The samples were de-identified and conformed to the Biobank Institutional Review Board (IRB) protocol. Patient-derived cell line GB3 was established from resected primary GBM tumor tissue at BNI. Briefly, tumor tissue was processed using the Gentle MACS Dissociator and Tumor Tissue Dissociation kit (Miltenyi Biotec Inc.). Cells were expanded as neurospheres in tissue culture dishes coated with poly-(2-hydroxyethyl methacrylate) (Sigma-Aldrich) or grown adherent on laminin (Fisher Science), in neural stem cell (NSC)

medium consisting of DMEM and F12-Glutamax supplemented with B27 and N2 (Fisher Science), in the presence of 20 ng ml⁻¹ EGF and 20 ng ml⁻¹ FGF2 (EMD Millipore). To generate GB3-RFP cell line, GB3 cells were transduced with pre-made lentiviral particles (Amsbio) expressing RFP-Luc (GB3-RFP) and were selected using blasticidin (2 µg ml⁻¹). Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza and cultured in EGM-2 (Endothelial Growth Medium, Lonza) and passaged at 70–80%.

Vasculogenesis in the microfluidic device

To model vasculogenesis within our microfluidic platform, HUVECs were dislodged from culture flasks using trypsin-EDTA and centrifuged. Next, 5 mg mL⁻¹ bovine fibrinogen (Sigma-Aldrich) dissolved in Dulbecco's phosphate buffered saline (DPBS, Gibco) was used to make the fibrinogen solution. Bovine thrombin (Sigma-Aldrich) was dissolved in DPBS to create 4 U ml⁻¹ thrombin solution. Fibrinogen and thrombin solutions were then filter sterilized (Denville scientific). HUVECs were added to the fibrin solution (5 mg ml⁻¹ fibrinogen plus 4U ml⁻¹ thrombin) to form a hydrogel with the final cell density of 20 million cells ml⁻¹. The mixture was then immediately injected into the vascular region (third layer, Fig. 1B) of the device and the devices were incubated at room temperature for 10 min to promote fibrin polymerization. After hydrogel polymerization, EGM-2 (endothelial growth medium supplemented with additional 50 ng ml⁻¹ VEGF) was added to each device and the platforms were kept in the incubator (humidified, 37 °C, 5% CO₂). Cell culture medium was exchanged every 24 h throughout vasculogenesis (3 d).

Co-culture invasion assay

To study GSC-EC interactions, GB3-RFP cells, referred to as GB3 hereafter, were added to the devices after vasculogenesis. GB3 cells were dissociated, centrifuged, and then the cells were directly suspended in pure Matrigel[®] with a final cell density of 15 million cells ml⁻¹. The cell–hydrogel solution was mixed and injected into the tumor region of the device (Fig. 1B). The devices were then incubated (humidified, 37 °C, 5% CO₂) for 3 min. Subsequently, Matrigel[®] was injected into the stroma region followed by 5 min of incubation allowing hydrogel polymerization. Cell culture medium was added afterwards and exchanged daily. Invasion distance was measured by drawing lines from the microposts to the edge of leading cells and then averaging at least 10 lines. Extensions from the cell body were measured by drawing a line from the front edge of nucleus to the end of the extension. Click-iT EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher) was used to assess proliferation in invasion assay.

In vivo animal Experiments

Animal husbandry was performed according to the guidelines of St. Joseph Hospital and Medical Center and Barrow Neurological Institute under the Institutional Animal Care and Use Committee–approved protocol. Five- to six-week-old IcrTac:ICR-Prkdcscid mice were used for in vivo orthotopic transplant of fluorescently-tagged GB3 cells. For orthotopic transplants, 2 µL of dissociated cells at a density of 50,000 cells µL⁻¹ were injected in the right hemisphere (stereotactic coordinates AP 0, ML -2, DV -2.5), as described previously [42, 43]. 8 weeks after injection, tumor-bearing mice were euthanized with a lethal intraperitoneal injection of 2.5% Avertin (2,2,2-Tribromoethanol, Sigma-Aldrich; T48402;

tert-Amyl alcohol, Sigma-Aldrich, A1685). Tissues were fixed through intracardial perfusion with Ringer's solution (Electron Microscopy Sciences; 11763–10) supplemented with 40 mM NaNO₂, 2 mM NaCHO₃, and 50 IU mL⁻¹ heparin, followed by ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Brains were subsequently cryoprotected with incubation in a solution of 30% sucrose in PB for 48 hours before being frozen and cut into 40 µm coronal sections using a cryostat (Microm HM550, Thermo Scientific).

Immunostaining within the microfluidic device

Cells within microfluidics were fixed in 4% PFA following 3 days after co-culture by removing cell culture medium and exchanging for 4% PFA before applying a gentle negative pressure to other well to create flow. Devices were then incubated for 30 min at room temperature (RT). After incubation, the samples were rinsed twice with PBS-glycine (100 mM glycine in PBS) incubating for 10 min at RT and washed with PBS-Tween (0.05% (v/v) Tween-20 in PBS) for another 10 min at room temperature to permeabilize the cells. Following permeabilization, the samples were blocked with immunofluorescence buffer and 10% goat serum (GS) (IF buffer: (0.2% (v/v) Triton X-100, 0.1% (v/v) BSA (radioimmunoassay grade), 0.05% Tween 20, 7.7 mM NaN₃ in PBS) for 2 h to inhibit nonspecific binding of antibodies. Primary antibody was diluted in PBS-tween and then added to the blocked samples and kept at 4 °C overnight. Devices were kept in petri dishes and wrapped in parafilm to avoid evaporation. Then, devices were washed with IF buffer three times each at 20 min intervals at room temperature. Species-matching Alexa-conjugated secondary antibodies were used at 1:500 dilution (Invitrogen). After adding the mixture to each sample, the devices were incubated at room temperature for 1 h. The devices were then washed once with IF buffer for 20 min followed by two times washing with PBS-Tween-20 for 10 min. 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) (1:1000) was added to the devices to counterstain nucleus. Following staining, the devices were finally washed with PBS-Tween-20 three times each at 10 min intervals. The following primary antibodies were used: mouse anti-Ki-67 (1:100, Dako), rabbit anti-Sox2 (Cell Signaling, 1:100), mouse CD-31 (10 µg mL⁻¹, P2B1, Developmental Studies Hybridoma Bank, DHSB), mouse anti-Nestin (1:200, Abcam), rabbit anti-CD44 (Cell signaling, 1:400), rabbit anti-GFAP (Millipore, 1:500), rabbit anti-pCXCR4 (Cell Signaling, 1:100).

Immunohistochemistry of in vivo tissue sections

Immunohistochemistry was performed on free-floating sections. When antigen retrieval was necessary, tissues were treated for 45 minutes in citrate buffer (pH 6) at 80°C. To block unspecific staining when mouse-raised primary antibodies were used, we initially incubated sections with goat anti-mouse and goat anti-rabbit IgG (Jackson ImmunoResearch) 1:50 in 0.1 M PB 0.4% Triton-X (PBTx). After a permeabilization and blocking incubation for 2 hours at RT in PBTx supplemented with 10% heat-inactivated GS, the sections were incubated with the primary antibodies for 12 hours at 4°C in PBTx, 2% GS. Incubation with secondary antibodies was performed for 2 hours at 4°C in PBTx, 2% GS. Nuclear counterstaining was achieved with DAPI (0.5 µg/mL). The following primary antibodies and dilutions were used: mouse anti-Ki67 (1:100, Dako), rabbit anti-RFP (1:100, Abcam), rabbit anti-Sox2 (Cell Signaling, 1:100). For blood vessel staining, sections were incubated with

DyLight-488 Tomato Lectin (Vector Lab) for 1hr RT at 1:1000. Coverslips were mounted using Pro-long Gold (Vector Lab).

Imaging

Microfluidic devices were imaged using fluorescence microscopy (Zeiss Axio Observer Z1 with the Zen Pro software suite) equipped with Apotome.2 (Zeiss) at 20× and 40× magnification. Fluorescent brain tissue sections were imaged using a Leica SPE Confocal microscope at 20× magnification (1.2 micrometer stacks). Analysis and 3D reconstructions were performed with Imaris (Bitplane).

Statistical Analysis

For all analyses, values were obtained from at least three independent experiments ($n > 3$) with 2–3 technical replicates each. Data are expressed as average $\pm$ standard deviation or average $\pm$ standard error of mean. When only two conditions, we utilized an unpaired two-tailed Student's t-test. Two-way ANOVA with Sidak multiple comparison tests were used when comparing more than two conditions and statistical significance defined as $p < 0.05$. Statistical analyses were performed within the GraphPad Prism software (GraphPad Prism 6).

RESULTS

Formation of vasculature within the microfluidic model

We first established and tested the formation of a 3D microvascular network within our microfluidic model prior to incorporating GSCs within the tumor region (Fig. 1B). To establish the microvascular network, we utilized fibrin as the candidate ECM for encapsulating the HUVECs. Fibrin has been widely used to model the tumor vasculature as it promotes physiologic vasculogenesis [29, 38]. Importantly, fibrin has been well-documented to be deposited near tumor-associated vasculature for different cancers including those of the brain functioning as a source of growth factors for angiogenesis and cell recruitment [44, 45]. Additionally, in our previous extensive characterization, we demonstrated the suitability of fibrin for physiologic vasculogenesis [39]. So, we embedded HUVECs in a fibrin gel and injected them within the outer layer of the microfluidic to enclose the tumor and stroma layers. The process of microvascular network formation occurred over the course of 3 days in culture. As seen in Fig. 2A, the phase-contrast images demonstrated the formation of branched microvascular network within the microfluidic platform. The HUVECs began to elongate and connect within the first day of culture. By the third day, vessel-like structures could be seen similar to our prior publication [39]. Notably, fluorescent 70-kDa dextran flowed into the vasculature, demonstrating open lumens of the microvascular network (Fig. 2B). CD31 staining confirmed the microvascular phenotype and the cross-sectional view clearly showed the formation of lumen (Fig. 2C).

Phenotype of GSCs in the microfluidic model

We utilized GB3 GSCs, a patient-derived cell line established from resected primary GBM tumor at the Barrow Neurological Institute (BNI). GB3 GSCs have previously been shown to be highly invasive within 2D *in vitro* transwell assays as well as *in vivo* mice models [46].

When cultured within 3D hydrogels, these cells demonstrated expression of Nestin, SOX2, and CD44, and the ability to maintain self-renewal which are classical GSC markers [47]. However, the phenotype of patient-derived GB3 cells within the 3D microenvironment in presence of vasculature has not been assessed. Therefore, prior to invasion analysis, we confirmed the expression of specific proteins related to stemness and different neural lineages of the GSCs within our microfluidic model through immunofluorescent staining. While GSCs are typically cultured in serum-free condition, the co-culture with HUVECs requires further optimization of medium conditions. HUVEC growth requires media containing 2% fetal bovine serum (FBS) along with additional growth factors which may influence invasion and phenotype of the GSCs in the co-culture condition. Therefore, we designed experiments with different media composition to conduct our biological studies. The culture media were comprised of: NSC medium, NSC medium supplemented with 5% FBS (NSC +), and EGM-2.

To establish working microfluidic model, we first formed the microvascular network within the model for 3 days. Next, we utilized Matrigel[®] as it is compatible with 3D culture and rich in laminin, which is important ECM component of the GSC microenvironment [48, 49]. Patient-derived primary GSCs were encapsulated within Matrigel[®] and introduced into the primary tumor region of the platform. Subsequently, Matrigel[®] was added into the second layer (i.e. stroma) of the device to surround and enclose the tumor region. Within different medium compositions, we examined the expression of Nestin, SOX2, GFAP, and CD44 markers in the GSCs embedded in Matrigel[®] within the second layer of the microfluidic platform in the presence of microvascular network. Nestin, which is an intermediate filament and a neural progenitor marker, was expressed in the GSCs (Fig. 3A). We did not detect expression of astrocytic marker, GFAP (Fig. 3B), while the transcription factor SOX2, a marker for pluripotency and self-renewal (Fig. 3C) [50], was widely expressed in GSCs. Moreover, CD44, a receptor for hyaluronic acid which is highly expressed in malignant GBM [51], was clearly shown on the membrane of the GSCs (Fig. 3D). These results from our 3D microfluidic model are consistent to prior publications which suggests that the GSCs maintain stemness and GBM phenotype markers in presence of the vasculature [46, 47].

Invasion of GSC in presence of the 3D microvascular network

We intended to find out if the formed vasculature influenced the invasion distances of GSCs within a 3D microenvironment. As previously described, we introduced different medium conditions in our microfluidic model. Within the first day of culture, we observed that GSCs did not form spheroids but demonstrated elongated and branching morphology under all the conditions. Additionally, GSCs exhibited propensity to migrate into the stroma as chains of cells (Fig. 4). We compared invasion of GSC between mono-culture (– HUVECs) and co-culture with HUVECs (+ HUVECs) for the three different media conditions (Fig. 4A). Co-culture with HUVECs led to significant invasion of GSC in the NSC medium (Fig. 4B; – HUVECs $52.8 \pm 11.3 \mu\text{m}$ and + HUVECs $262.4 \pm 39.2 \mu\text{m}$) and NSC + medium (Fig. 4B; – HUVECs $170.5 \pm 21.0 \mu\text{m}$ and + HUVECs $319.4 \pm 60.6 \mu\text{m}$). On the other hand, we found no difference between mono- and co-culture conditions in EGM-2 (Fig. 4B; – HUVECs $310.7 \pm 22.4 \mu\text{m}$ and + HUVECs $318.3 \pm 29.24 \mu\text{m}$). This suggests that EGM-2 may contain cytokines that elicit a strong invasion response in GSCs thereby shielding the influence of

HUVECs on invasion. Conversely, since there was a significant increase in GSC migration upon co-culture in both NSC and NSC + medium conditions, this suggests that HUVECs do play a role in enhancing invasion of GSCs within the 3D tumor-vascular microenvironment.

Based on the phase-contrast images of the invasion assay on day 3, we also noticed morphological differences in how GB3 GSCs invaded when co-cultured with HUVECs. Cell membrane protrusions or extensions from the cell body were observed within the GSCs (Fig. 5A), which have been attributed to invasive phenotype in GBM [52, 53]. We measured the extensions of the leading GSCs and compared within different medium conditions. As seen in Fig. 5A, the migrating GSC exhibited more elongated extensions from the cell body when co-cultured with HUVECs, which was correlated with their invasive response (Fig. 4). This is indeed also consistent with previous *in vivo* studies on invasive morphology of the GSCs [52]. Quantifications of the extensions demonstrated that GSCs had a significant increase in extension length upon co-culture with HUVECs within each medium condition (Fig. 5B; NSC: –HUVECs $70.8 \pm 16.5 \mu\text{m}$ and + HUVECs $106.5 \pm 20.6 \mu\text{m}$, NSC +: – HUVECs $60.0 \pm 5.4 \mu\text{m}$ and + HUVECs $104.5 \pm 19.0 \mu\text{m}$, and EGM-2: – HUVECs $84.2 \pm 14.9 \mu\text{m}$ and + HUVECs $129.8 \pm 26.8 \mu\text{m}$) corroborating the observations from phase-contrast images (Fig. 5A). We also speculated that, apart from the migration distance (Fig. 4), the presence of vascular network may influence the density of migrating cells within the stromal region. Therefore, we utilized two metrics, number of cells in a migrating chain as well as number of cells in a field of view (FOV) within the stroma, to quantitatively discern the density of migrating cells. For co-culture with HUVECs in the NSC medium condition, there were significantly increased numbers of nuclei per chain of migrating cells (Fig. 5C; – HUVECs 2.8 ± 0.9 and + HUVECs 5.6 ± 1.5) and within a FOV in the stroma (Fig. 5D; – HUVECs 9.6 ± 6.1 and + HUVECs 32.2 ± 9.2) as compared to mono-culture. On the other hand, no significant difference between mono- and co-culture conditions was found in either NSC + or EGM-2 media (Fig. 5C and D). As such, the microfluidic model was capable of monitoring GSC invasive behaviors in terms of migration distance and morphology under different medium conditions in the absence and presence of a functional 3D vasculature.

In addition to assessing GSC invasion distance and morphology, we investigated the behavior of the formed vessels under different media compositions. Consistent to our prior publication [39], we measured the diameter of the vessels (Fig. S1A; NSC: $55 \pm 5 \mu\text{m}$, NSC +: $63 \pm 14 \mu\text{m}$, EGM-2: $48 \pm 9 \mu\text{m}$) and the length between junctions (Fig. S1B; NSC: $267 \pm 71 \mu\text{m}$, NSC +: $276 \pm 103 \mu\text{m}$, EGM-2: $247 \pm 42 \mu\text{m}$) [39]. We found slight differences between the different media compositions but none were statistically significant. Furthermore, we did not observe extensive angiogenesis over the three-day time-period of our assay.

Effect of vasculature on GSC proliferation

As the vascular microenvironment had been demonstrated to maintain proliferation of GSCs [8], we asked if the microvascular network within our model played a similar role on GB3 proliferation. In mono- and co-culture conditions, we utilized Ki-67, in addition to pulsing with EdU for 24 h, to assess the proliferation of GSCs. As shown in Fig. 6, we observed expression of Ki-67 and EdU suggesting that the GSCs divided within the microfluidic

platform. However, regardless of the medium conditions, we did not observe a significant difference in proliferative markers between mono- and co-culture conditions. This suggests that HUVECs within this model did not influence GSC proliferation. Moreover, this also suggests that the invasion responses of the GB3 cells were more likely due to migration speed and elongation of cells rather than proliferation (Fig. 4).

Comparison of the microfluidic platform of GSC invasion to *in vivo* PDX model

In parallel to our *in vitro* studies, we utilized a PDX model to investigate GSC invasive behavior *in vivo* to confirm the physiological relevance of our microfluidic platform. Notably, side-by-side validation of microfluidic models of GSC-EC interactions and *in vivo* PDX model had not been performed in prior microfluidic systems. [27, 38]. Fluorescently-tagged GB3 cells orthotopically transplanted in mice, demonstrated migration near and along blood vessels (Lectin, blue) within the mouse brain (Fig. 7Ai), and appeared comparable to the behavior of GSCs in our microfluidic model (Fig. 7Bi). Based on 3D and surface renderings of the tissue slices, we could clearly observe cells migrating along the vessels, consistent to other studies (Fig. 7Aii and iii) [54]. Similarly, within the microfluidic model, GSCs could be observed migrating in 3D space while also establishing contact with the vasculature (CD-31, green) through the 3D surface renderings of the cells (Fig. 7Bii and Supplementary Mov. 1). Additionally, we observed similar chain-like migration within the *in vivo* PDX model that resembled the migratory behavior of cells within the co-culture microfluidic model (Fig. 7C). We noticed long protrusion-like extensions in both systems consistent to other studies, which have been associated with invasive phenotype as well as resistance to radiotherapy of the GSCs [52, 55]. The expression of proliferation marker, Ki-67, was observed near vessels in both the microfluidic and *in vivo* model (Fig. 7D). The microfluidic model could also enable in-depth visualization of the 3D vasculature as shown in Fig. S2A. The surface renderings clearly depicted the inner membrane of the vasculature providing potential for high-resolution observation of cell-cell interaction at a single-cell level (Fig. S2B, C and Supplementary Mov. 2). Taken together, these evidences showed that our microfluidic model reliably represented GSC invasion *in vivo* in the context of a vascular niche. Importantly, our *in vitro* microfluidic assay only took 6 days to conduct our studies, which could provide faster and higher throughput for multiple analyses as well as high-resolution observation of cell-cell interactions prior to *in vivo* studies.

Effect of AMD3100 on GSC invasion within microfluidic model

High expression of chemokine receptor CXCR4 is a hallmark of malignant GBM [14]. As such, CXCL12-CXCR4 interaction has been previously implicated in malignant GBM invasion [21]. However, previous studies have not assessed the expression of this signaling axis in a 3D tumor-vascular niche model. We demonstrated in our model that the presence of the 3D microvascular networking consisting of HUVECs promoted migration of GSCs. Additionally, since HUVECs have previously been shown to be a source of CXCL12 [56,57], We hypothesized that CXCR4-CXCL12 signaling axis may be involved in the migration of GSCs when in presence of HUVECs. AMD3100 has been used to inhibit CXCR4 and disrupt neural stem cell and GBM cell migration [13, 58, 59]. Therefore, we utilized this drug to study the influence of CXCL12-CXCR4 on GSC invasion in the context of 3D *in vitro* vascular microenvironment.

We chose the NSC medium condition due to the strong invasion response of GSCs in presence of HUVECs (Fig. 3 and 4). HUVECs have previously been demonstrated to be a source of CXCL12 *in vitro* using ELISAs [56, 57]. Therefore, we primarily validated signaling of CXCL12- CXCR4 axis by staining for the phosphorylated active form of CXCR4 (pCXCR4) in GSCs [60]. In absence of the microvascular network, pCXCR4 showed a diffuse pattern and was not localized to the nucleus (white arrows in Fig. 8A). In presence of the microvascular network, pCXCR4 showed a punctate pattern and was localized to nucleus (white arrows in Fig. 8A), providing additional evidence for CXCR4 activation by CXCL12 in our model [61].

We introduced AMD3100 at a concentration range between 0 and 100 μ M after 24 h of establishing the model [59]. The cells were maintained in AMD3100 for 48 h while we assessed their invasion response (Fig. 8B). In mono-culture condition (– HUVECs) by day 3, the cells invaded in all drug treated concentrations but there were no significant differences in the invasion distances (Fig. 8C, blue line; 0 μ M: $98.1 \pm 20.1 \mu$ m, 1 μ M: $69.4 \pm 27.58 \mu$ m, 10 μ M: $52.9 \pm 13.1 \mu$ m, and 100 μ M: $59.0 \pm 0.5 \mu$ m). However, in the presence of HUVECs on day 3 (+ HUVECs), we observed that invasion distance for 0 μ M AMD3100 was significantly higher than all other concentrations with 1 μ M being significantly higher than 100 μ M (Fig. 8C, red line; 0 μ M: $285.1 \pm 65.4 \mu$ m, 1 μ M: $162.1 \pm 42.4 \mu$ m, 10 μ M: $137.5 \pm 65.3 \mu$ m, and 100 μ M: $69.9.0 \pm 15.9 \mu$ m). We observed a decreasing trend in invasion response with increasing AMD3100 concentration (Fig. 8C). The enhanced invasion response due to presence of the 3D microvascular network could be abrogated to mono-culture (– HUVECs) migration levels through treatment with AMD3100 by disrupting CXCL12-CXCR4 signaling (i.e. 100 μ m). Interestingly, we did not observe a significant difference between mono- and co-culture even in presence of AMD3100 within the EGM-2 condition (Fig. S3). This suggests that there may be other cytokines other within EGM-2 apart from CXCL12 that may influence GSC invasion. In that regard, when utilizing NSC medium condition, our findings demonstrated that CXCL12- CXCR4 is involved in EC-mediated invasion of GSC within the 3D tumor model.

DISCUSSION

In GBM, a sub-population of cells with stem-like properties, GSCs, have been shown to be one of the major drivers for tumor recurrence, invasion of normal brain parenchyma and resistance to therapies [3, 5]. Recent investigation on the tumorigenic influence of the microenvironment has added further layers of complexity to understanding GSC biology and behavior [10]. These specialized niche microenvironments provide safe harbor to GSCs making these cells difficult to therapeutically target. Importantly, the niches provide extrinsic cues that promote GSC growth and invasion and preserve the stem cell phenotype. In an elegant study by Calabrese *et al.*, GSC-EC interactions *in vivo* within the vascular niche was implicated as a key driver of tumor growth [7, 8]. Several signaling pathways with important roles in normal neural stem cell maintenance and growth, such as Notch, TGF β , hypoxia-inducible factors (HIFs), and SHH have also been shown in GSC-EC interactions [7, 11, 62, 63]. These studies highlight the complex intercellular interactions that occur within the components of the vascular niche and GSCs that regulate whether GSCs remain quiescent, proliferate, transdifferentiate into pericytes or endothelial cells, or invade along existing

blood vasculature [4]. Hence, further investigations are required into the GSC-niche interactions to unveil novel targets that could be used to disrupt the regulatory pathways that promote tumorigenesis.

Despite the significance of GSC-EC interactions within the niches, there are few *in vitro* models that could provide a 3D suitable biomimetic microenvironment for studying the influence of vascular niche components on GSC invasion. *In vivo* models are often too complex presenting challenges in determining the cause and effect relationships between the microenvironmental cues and tumor cell behavior [23]. In addition, reduced spatial-temporal resolution of real-time events such as invasion speed within *in vivo* models pose additional difficulties, resulting in end-point analyses [23]. More complex *in vitro* systems consisting of higher dimensionality (i.e. 3D), microfluidics, and cell-laden hydrogels, have been developed to bridge this gap to better recapitulate the GBM microenvironment while providing better control over the microenvironmental cues [23]. For instance, Infanger *et al.* developed a 3D *in vitro* model of the perivascular niche by utilizing 3D scaffolds and EC-conditioned medium. EC-conditioned medium was collected from a 3D scaffold and then was subsequently added to GSC cultures. They found that the EC-conditioned medium maintained SOX2 and Nestin expression in GSCs. However, simultaneous co-culture of GSCs and ECs required random mixing of the two cell-types within the 3D scaffold which limited an organotypic architecture and quantitative assessment of migration. Therefore, they utilized a transwell assay instead to study migration in response to EC-conditioned medium [37]. On the other hand, to overcome the limitations of transwell assay, Chonan *et al.* created a 3D microfluidic device to study glioma invasion influenced by ECs. The two cell types were seeded as monolayers in two adjacent channels separated by a 3D collagen layer in between. They observed that glioma cells demonstrated increased invasion capacity when near the monolayer of ECs. In this model, ECs were seeded as a monolayer, which did not represent a native-like 3D vasculature of the brain microenvironment [27]. In that regard, the relevance of our work lies in providing a 3D physiologically relevant *in vitro* model of GSC-EC interaction by spatially organizing GSCs near a 3D microvascular network with the possibility of adding additional cell types, such as astrocytes or pericytes. This enables us to engineer an organotypic architecture and to investigate the dynamic influence of the microvascular network on GSC invasion and phenotype. Importantly, we paired our *in vitro* study on GSC invasion with an *in vivo* PDX model to benchmark the physiological relevance of our microfluidic system. Therefore, aside from fundamental biological studies, the results of this study will also have implications for drug screening of therapeutics targeting GSC-EC interactions within niche microenvironments.

The microfluidic model, presented herein is based on a well-established 3D microvascular network that further enables detailed study of GSC invasion into a stroma-like region. This model also overcomes the limitation of prior work by introducing patient-derived GSCs after formation of the microvascular network instead of introducing both cell-types at the same time [39]. HUVECs require serum to develop into a 3D microvascular network which has been well-established by many groups [29, 35]. GSCs are cultured in serum-free conditions as addition of serum induces differentiation. To avoid prolonged exposure to serum, we leveraged the design of our microfluidic platform to first establish a microvascular network using EGM-2 as the culture medium. Then we introduced GSCs into the microfluidic

platform while exchanging EGM-2 for another medium, such as NSC, for the co-culture experiments.

We studied the influence of different medium compositions (i.e. NSC, NSC+, and EGM-2) on GSC phenotype within the co-culture assay. Expression levels of SOX2, Nestin, and CD44 were positive in GSCs across all medium conditions. In addition to these markers, we investigated expression of GFAP, which is an astrocytic maturation marker used to identify differentiated GSCs. As others have shown, GFAP expression is inversely correlated to SOX2 [64]. Consequentially, since the GSCs within our microfluidic device were positive for SOX2, we found negative staining for GFAP. Heffernan *et al.* utilized the same cell line, GB3, grown in serum-supplemented medium for 14 days which eventually resulted in expression of GFAP [47]. As the microfluidic assay was run for 3 days, it is possible that a longer time-period in either the EGM-2 or NSC + media (both containing serum) may result in positive expression of GFAP. Overall, the expression of these markers, (i.e. SOX2, Nestin, and CD44), suggested that the microfluidic model could recapitulate the classical hallmarks of GBM [65].

Utilizing our 3D organotypic microfluidic model, we tested the influence of the microvascular network on patient-derived GSC invasive behavior under different medium conditions. We observed that microvascular network promoted protrusive morphology in all medium conditions suggesting transition to an invasive phenotype. Osswald *et al.* described these long protrusions as infiltrating into normal brain tissue during invasion [52]. Interestingly, we also observed chain migration of GSCs into the stroma, which is often associated with neural stem cells migrating from the subventricular zone [66]. This type of migration pattern has been described previously for glioma but only in certain settings [67, 68]. For instance, U87 and U251N glioma cells were observed in a 2D *in vitro* ring migration assay, but instead of migrating as chains, the cells moved individually [69]. However, in separate study by Gritsenko *et al.*, when the same cells were cultured as multicellular spheroids on a basement membrane-like ECM, the cells exhibited the same chain-like migration, as we observed, suggesting the influence of ECM proteins on their migratory behavior [55]. Similarly, Grundy *et al.* cultured patient-derived GBM lines on 2D substrates demonstrating individual cell migration when the cells were cultured in 3D Matrigel® [70]. They observed chain migration consistent to our study. These evidences suggested that the chain-like migratory phenotype is dependent on the ECM as well as the 3D microenvironment, which we created in our microfluidic model. Importantly, we observed similar chain-like migration within the *in vivo* PDX model in brain tissue sections, which was also comparable to other studies in literature [55]. Taken together, this provides further evidence that our microfluidic platform is a suitable model to study the migratory behavior of GSCs in a 3D microenvironment.

As we observed in our PDX model, the GSCs migrated near or along vasculature of the mouse brain. To investigate whether 3D vasculature influenced GSC invasion distance, we performed co-culture invasion experiments within our *in vitro* microfluidic model. Interestingly, NSC medium did not promote invasion in mono-culture. However, in the presence of the microvascular network, we observed significant invasion of GSCs using the NSC medium as opposed to EGM-2 that showed no difference between mono- and co-

culture. Firstly, this suggests that EGM-2, which contains both serum and additional growth factors, influenced GSC invasion regardless of the presence of the microvascular network. This essentially masks the influence of the microvascular network and confounds the experimental interpretation. On the other hand, using the NSC medium, we could identify that the microvascular network promoted invasion of GSC thereby allowing us to further investigate the signaling between ECs and GSCs. As such, our findings report the influence of medium composition on GSC-EC interaction.

In our experiments, we did not find a significant change in proliferation rate of GSCs in presence of vasculature. A similar study was performed by co-culture of neurospheres with human brain microvascular ECs (hBMECs), showing that there was no difference in proliferation measured through both PCNA and Ki-67 between the mono- and co-culture conditions. Expression of CD133, a marker for self-renewal, was increased in the presence of hBMECs, which suggested that there was only an increase in self-renewal capacity of the cells but not proliferation [11]. Similarly, Galan-Moya *et al.* compared the amount of proliferative and quiescent cells in control medium and EC-conditioned medium on GSCs and found no difference in the ratio of proliferative to quiescent cells [71]. However, like the study by Zhu *et al.*, they also found that the EC-conditioned medium maintained self-renewal of the GSCs confirmed by expression of Nestin and SOX2. Consistently, we also observed expression of Nestin and SOX2 when GSCs were in presence of the 3D microvascular network. Additionally, we demonstrated increased density of migrating GSCs. However, since we did not observe a statistical increase in proliferation, we propose that this increase is due to enhanced migration speed of GSC.

The CXCL12-CXCR4 signaling axis has also been shown to be important in GBM invasion and tumorigenesis [14, 21, 72]. Since CXCL12 has been also positively detected in ECs within GBM tumor tissue, it is suggested that GSC-EC interaction through CXCL12-CXCR4 may play a role in GBM invasion [15, 73]. To that end, we probed for pCXCR4, the phosphorylated form of CXCR4, as a measure of CXCR4 activation. We observed punctate staining for pCXCR4 in GSCs co-cultured with HUVECs but a diffuse staining in GSCs cultured alone, indicating more activated CXCR4 activation in co-culture. Moreover, we noted nuclear localization of pCXCR4 as additional evidence for activated CXCL12-CXCR4 signaling in the co-culture condition [61]. Treatment with AMD3100, a CXCR4 antagonist, we observed a decreasing trend in GSC invasion in co-culture as a function of drug concentration. In fact, this was not observed in mono-culture of GSCs. This distinction highlights the importance of multi-culture 3D microenvironment in drug responsiveness of the GSCs. However, the cells did not respond to AMD3100 treatment in EGM-2 condition. As such, we hypothesized that EGM-2 promoted GSC invasion in a CXCL12-CXCR4 independent manner due to the growth factors and cytokines, including insulin-like growth factor, in EGM2 which have been previously shown to be a strong mediator of GBM invasion *in vitro* [74, 75]. This behavior further highlights the critical role of medium composition in establishing a reliable model for molecular and cellular level analysis of GSC invasion.

As we experimentally confirmed, CXCL12-CXCR4 signaling was crucial to GSC invasion in a vascular microenvironment. GSC survival and growth have been also shown to be

dependent on the specialized niche microenvironments, such as hypoxic and perivascular [10]. These niche microenvironments can further protect GSCs from chemo- and radiotherapy. Therefore, GSC-EC interactions may also regulate resistance to radiotherapy. For instance, Brown *et al.* demonstrated that co-culture of HUVECs and U87 glioblastoma increased expression of ataxia telangiectasia mutated (ATM), a DNA damage response protein, which led to resistance to radiation-mediated apoptosis [76]. Therefore, based on our study and the findings of others, combination of AMD3100 ad chemo- or radiotherapy could be a potential basis for GBM treatment as it has been tested in U251 GBM immortalized cell line in nude mice [77]. However, further investigation is required to assess how patient-derived GSC growth and invasion is affected as compared to high passage, immortalized cell lines that are used in most of the previous studies [1, 2, 78]. Recently, Heffernan *et al.* studied the effects of radiotherapy on patient-derived GSCs, GB3 and GB7. The GSCs exhibited a dose response when grown as neurospheres, but demonstrated radioresistance when encapsulated in a 3D hydrogel [47]. Therefore, the proposed 3D co-culture model presented herein could be used to study the effect of the vascular niche on radioresistance as part of future work.

Investigating tumor-associated angiogenesis due to GSCs was one of the future goals of our study using the microfluidic assay. Based on the current experimental settings, we did not observe statistically significant differences in vascular morphology based on different media compositions. Furthermore, we did not observe extensive angiogenesis over the three-day time-period of our assay. We contend that it may take longer to observe extensive angiogenesis (i.e. > 7 days) as demonstrated by other works [38] or that addition of brain ECs (instead of HUVECs) or other stromal cells, such as pericytes may influence vascular sprouting [79]. In this regard, addition of these new elements is the subject of our future work based on the developed assays.

Targeting the tumor angiogenesis in GBM is another option that is being explored clinically [80, 81]. However, many studies have reported that tumors treated with only Bevacizumab (Avastin®), a VEGF inhibitor, all eventually relapse [82]. One caveat of disrupting angiogenesis is driving the tumors toward a hypoxic niche which could in turn promote invasiveness by co-opting native brain blood vessels [10, 83]. In that regard, our microfluidic model of GSC-EC interaction could provide a quick and economical method as a drug screening assay to study the consequences of disrupting the vasculature on GSC invasive behavior.

Furthermore, future work will focus on improving our 3D organotypic *in vitro* microfluidic model by incorporating additional elements of the vascular niche. In this study, we utilized Matrigel® as the ECM component of model due to it containing 60% laminin, 30% collagen IV, and 8% entactin in addition to growth factors found in tumor vascular microenvironment [48, 49]. However, in our future studies, we aim to utilize and characterize other types of hydrogels, such as hyaluronic acid (HA), which is a main ECM component of the brain. Our current work was also the first step to establish and validate a successful model of GSC-vascular interactions for biological studies. However, we acknowledge that stromal cells play critical roles in the microenvironment [84]. For instance, pericytes are known to interact and mature the vasculature while astrocytes maintain hemostasis of the microenvironment,

however, the roles of both cells along with neurons and oligodendrocytes in GSC invasion behavior are still active areas of research [85]. Therefore, our future work, will involve fine-tuning and adding higher levels of complexities to our tumor microenvironment model by addition of stromal cells, including pericytes, smooth muscle cells, and additional glial cells.

CONCLUSIONS

We developed a 3D organotypic *in vitro* microfluidic model incorporated with a microvascular network to study the influence of patient-derived GSC and EC interaction on invasion and tumor behavior. Additionally, we have improved upon current *in vitro* platforms by first establishing a microvascular network prior to introducing the patient-derived GSCs. This avoids the confounding influence of vasculogenesis on the invading cancer cells allowing us to focus more on cell-cell interaction and role of the established vasculature on the migrating GSCs. Notably, we compared our microfluidic system to a PDX model using the same cells, finding similar invasive behaviors between the two models. In our experiments, we found evidence that the established microvascular network enhanced GSC invasion and promoted invasive morphology as well as maintained cell proliferation rates and classical GSC phenotype. Furthermore, we confirmed that CXCL12-CXCR4 signaling is involved in promoting GSC invasion within a vascular microenvironment while also demonstrating the effectiveness of this microfluidic model as a drug screening platform. Importantly, most of our experiments focused on utilizing NSC medium which did not contain serum and thereby allowed us study GSC-EC interactions in an unbiased experimental condition. As such, future studies using our model could incorporate collection of conditioned media from these experiments to probe for additional possible paracrine signaling cues mediating GSC invasion. Indeed, this would have proven difficult if serum were included in the medium, such as in EGM-2, and within *in vivo* models which could contain confounding factors from other cells and microenvironmental cues. Further work will also involve fine-tuning the microenvironment, such as by incorporation of brain-mimetic hydrogels, patient-derived brain microvascular ECs, pericytes, smooth muscle cells, and additional glial cells. Taken together, this model presents a potential platform for studying the interplay of GSCs with its surrounding microenvironment as well as development of future therapeutic strategies tailored toward disrupting key molecular pathways involved in GSC regulatory mechanisms.

DATA AVAILABILITY

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Schematic of GSC-EC interaction.

(A) Schematic of the vascular niche within the GBM tumor microenvironment demonstrating GSC (red) migration in response to ECs (green). (B) The 3D schematic and photograph of the microfluidic platform is showing the different regions for cell culture and migration. Blue represents the tumor region housing the GB3 and red represents the vasculature region containing the HUVECs. The green region in the middle enables GB3 migration toward the microvascular network.

Fig. 2. Successful formation of 3D microvascular network within the microfluidic platform. (A) Phase-contrast images of HUVECs forming microvascular network over 3 days of culture; Scale bar: 100 μm top image and 50 μm for bottom row. (B) Fluorescent dextran perfused into the media channels demonstrating open lumens within microvascular network. (C) CD-31 stain confirming the morphology of vessels within microfluidic device. Cross-sectional view (red lines) demonstrating hollow lumen of the vessels; Scale bar: 50 μm .

Fig. 3. GB3 GSCs demonstrated stem phenotype within the 3D microfluidic device.
 (A) GSCs demonstrated positive staining for Nestin and (B) negative for GFAP while (C and D) SOX2 and CD44 were positively expressed in GSCs within different medium compositions.

Fig. 4. GSC invasion in under different medium conditions within the 3D microfluidic model. (A) Phase-contrast images of GSC (red) invading in the presence of HUVECs in different medium conditions. Red dashed line delineates average migration boundary; Scale bar: 200 μm . (B) Quantification of invasion distance for each condition (* denotes $p < 0.05$; Student's T-test; $n > 3$ for each data set).

Fig. 5. GSCs demonstrated elongated morphology during invasion.

(A) Phase-contrast and fluorescent images of GSC invading into the stroma exhibiting extensions from cell bodies. (B) Quantification of the extensions in each medium condition. (C) Quantification of the nuclei per chain in each medium condition. (D) Quantification of the nuclei per FOV in each medium condition. (* denotes $p < 0.05$; 2-way ANOVA with Sidak post-hoc test; $n > 3$ for each data set).

Fig. 6. GSC proliferated within the 3D microfluidic model. (A) Immunofluorescence staining of Ki-67 proliferative marker; Scale bar: 50 μ m. (B) Quantification of Ki-67/Nuclei ratio of each medium condition. (C) Immunofluorescence staining of EdU incorporation into nuclei; Scale bar: 50 μ m. (D) Quantification of EdU/Nuclei ratio of each medium condition.

Fig. 7. GSC invasion in microfluidic and PDX models.

(A) i: GSCs (red) shown migrating toward vasculature (blue, Lectin) within the *in vivo* model; Scale bar: 50 μ m. ii: 3D view of tissue-slices of the *in vivo* model demonstrating GB3 migrating along vessel. Iii: surface rendering of migrating GSCs showing the cells establishing contact with the vasculature. (B) i: Immunofluorescence staining of invading GSCs (GB3, red) near vasculature (CD-31, green) in microfluidic model. The white dashed line demarcates the border between the stroma and the vascular regions. ii: Surface rendering with multiple views of migrating GSCs (red) within the microfluidic model; Scale bar: 40 μ m. (C) Comparison of chain-like migration between the two models; Scale bar: 25 μ m. (D) Comparison of proliferating GSC (green, Ki-67) near vessel in both models; Scale bar: 25 μ m.

Fig. 8. CXCL12-CXCR4 signaling in GSC-EC interaction.

(A) Immunofluorescence imaging of pCXCR4 in presence of microvascular network. White arrow denotes absence and presence of nuclear localization in mono- and co-culture respectively; Scale bar: 20 μm. (B) Phase-contrast image of GSC (red) invading in presence of HUVECs in different concentrations of AMD3100. Red dashed line delineates average migration boundary; Scale bar: 100 μm. (C) Quantification of invasion distance for each condition (* denotes a significantly different group for $p < 0.05$; # denotes a significant difference between 1 μM and 100 μM for $p < 0.05$; 2-way ANOVA with Sidak post-test; $n > 3$ for each data set).