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Spontaneous glioblastoma spheroid infiltration of early-stage cerebral organoids models brain tumor invasion

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Abstract

Organoid methodology provides a platform for the *ex vivo* investigation of the cellular and molecular mechanisms underlying brain development and disease. High-grade brain tumor glioblastoma multiforme (GBM) is considered a cancer of unmet clinical need, in part due to GBM cell infiltration into healthy brain parenchyma making complete surgical resection improbable. Modelling the process of GBM invasion in real time is challenging as it requires both tumor and neural tissue compartments. Here, we demonstrate that human GBM spheroids possess the ability to spontaneously infiltrate early-stage cerebral organoids (eCO). The resulting formation of hybrid organoids demonstrated an invasive tumor phenotype that was distinct from non-cancerous adult neural progenitor (NP) spheroid incorporation into eCOs. These findings provide a basis for the modelling and quantification of the GBM infiltration process using a stem cell-based organoid approach, and may be used for the identification of anti-GBM invasion strategies.

Introduction

GBM is the most aggressive type of brain cancer and is associated with a median survival of ≤15 months from diagnosis.[1] Therapeutic intervention (including surgical debulking, radiotherapy, and chemotherapy) fails to effectively eliminate the entirety of the tumor and the rate of tumor recurrence remains high.[2] One aspect of GBM biology that poses a major therapeutic challenge is the diffuse GBM cell invasion into normal surrounding brain.[3-5] Emerging experimental and histological evidence indicates that GBM cell migration is accompanied by the expression of stem cell markers and can be predictive of patient outcomes.[6-8] Therefore, interventions that specifically target the invasive GBM phenotype are highly desirable. However, the molecular mechanisms underlying the infiltrative nature of GBM cells are not well understood. Furthermore, it is challenging to develop experimental strategies that enable the *ex vivo* investigation of GBM infiltration into brain-like/neural tissue.

Previous studies have shown invasiveness of xenografted GBM stem-like cells in the rodent brain.[9-11] Moreover, confocal microscopy has been used to visualize glioma cell migration in rodent brain aggregates and organotypic brain slice models.[12-15] This body of work, and recent advances in organoid technology,[16] motivated the *ex vivo* development of a throughput-compatible biological assay. We based our methodology on neural tissue infiltration by free-floating human GBM and NP spheroids in co-culture with mouse embryonic stem cell (mESC)-derived eCOs. Interestingly, we observed a pronounced eCO infiltration by GBM compared with NP spheroids, thus providing an exclusively cell-based experimental approach for the analysis of invasive GBM cellular tissue compartments in 3 dimensions and real time.

Materials and Methods

Cell culture

The generation and characterization of the GBM1 and NP1 cell models has been previously described.[17] GBM1 cells were cultured in Neurobasal medium (Gibco; 21103-049), supplemented with 0.5x B-27 (Gibco; 17504-044), 0.5x N-2 (Gibco; 17502-048), recombinant human basic fibroblast growth factor (bFGF; 40ng/ml; Gibco; PHG0024) and recombinant human epidermal growth factor (rhEGF; 40 ng/mL; R&D; 236-EG). Cells were maintained in a humidified incubator with 5% CO_2 at 37°C on poly-L-ornithine (5 µg/mL; Sigma P3655) and laminin-coated (5 µg/mL; Invitrogen; 23017-015) cell culture flasks. NP1 cells were cultured in DMEM/F-12 medium (Gibco; 2133-020) supplemented with bFGF (40 ng/mL), rhEGF (40 ng/mL), 0.5x B-27, 0.5x N-2, 1x GlutaMAX (Gibco; 35050-038) and 5% (v/v) fetal bovine serum (FBS, Gibco; 10270) at 37°C with 5% CO2.

GFP-expressing lentiviral particles were produced with pFUGW plasmid using a third-generation lentiviral system and HEK 293T cells. Viral particles were added to the cells at an MOI of 3.07. Spheres were generated through seeding and aggregation of 500 single GBM1 or NP1 cells (each cell line cultured in its respective media as stated above) in ultra-low attachment (ULA) 96-well plates (Corning; 7007).

Cerebral organoid development and eCO/GBM and eCO/NP assays

Mouse cerebral organoids were developed using R1 mESCs as described in[18] with minor modifications starting from differentiation day 6. R1 mESCs were cultured on mitotically inactivated mouse embryonic fibroblasts (MEF) in media consisting of Dulbecco's modified eagle medium high glucose (DMEM-HG; Gibco;11960-044) supplemented with 15% (v/v) knockout serum replacement (KSR; Gibco; 10828-028), 0.1 mM minimum essential medium non-essential amino acids solution (MEM-NEAA; Gibco;11140-035), 2 mM glutamine (Gibco; 10378-016), 1 mM sodium pyruvate (Gibco; 11360-039), 0.1 mM β-mercaptoethanol (Sigma; M3148) and leukaemia inhibitory factor (LIF; 1000U/mL; Millipore;ESG1106) at 37°C with 5% CO_2. Cells were trypsinized once they reached 70-80% confluency and separated from the MEF cells by differential adherence of these to gelatinized plates. Subsequently, 2000 dissociated R1 mESCs were plated in ULA 96-well plates. The cell suspension was cultured in differentiation media consisting of Glasgow minimum essential medium (GMEM; Sigma; G5154) supplemented with 10% KSR, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM MEM-NEAA, 0.1 mM β-mercaptoethanol and 10 µM SB431542 (Sigma; S4317) for 4 days as previously described.

The resulting embryoid bodies were transferred to ULA 24-well plates and cultured in neural induction media consisting of DMEM/F12 medium, N2 supplement (1:100), 1x Glutamax, 1x MEM-NEAA and 1 µg/mL heparin (Sigma; H3149). On day 6, contrary to the Lancaster *et al*. protocol, organoids were not added to matrigel droplets but continuously grown in ULA 24-well plates. Neural induction media was removed (at day 6) and replaced with differentiation media consisting of a mixture of Neurobasal medium and DMEM/F12 medium (1:1) supplemented with N2 supplement (1:200), B27 supplement without vitamin A (1:100; Invitrogen;12587010), 3.5 µl/L of β-mercaptoethanol, Insulin (1:100; Sigma;I9278-5mL), Glutamax (1:100) and MEM-NEEA (1:200). On day 9, the differentiation media was exchanged for differentiation media containing B-27 supplemented with vitamin A (1:100; Gibco; 17504-044). The eCOs were incubated for 2 days before being transferred to ULA 96-well plates containing either GBM1 or NP1 pre-made spheroids which were generated 24 hours before t_0. eCO/GBM1 and eCO/NP1 were co-cultured in either GBM or NP media, respectively, for 48 hours. Following that time point, differentiation media containing B-27 supplemented with vitamin A was added in a proportion of 1:1.

For real time imaging, ULA 96-well plates containing eCOs and GBM1 or NP1 spheroids were transferred to an IncuCyte ZOOM® live cell imaging system (Essen Bioscience) at t_0. Phase contrast images were acquired for all conditions every 30 minutes over a period of 3 days using a 10x objective. Real time movies were compiled using the IncuCyte ZOOM® software package.

Immunohistochemistry

The eCO/GBM1 and eCO/NP1 fusion organoids were fixed with 4% (w/v) paraformaldehyde for 15 minutes at 4°C. The tissues were subsequently washed three times with PBS and re-suspended in 30% (w/v) sucrose solution, and allowed to sink for 24 hours at 4°C. Once the organoids had sunk, they were embedded in OCT (VWR; 361603E) in cryo-molds and sectioned on a cryostat (20 µm thickness). Images of every section of each individual organoid were taken and quantification of the GBM1 or NP1 compartments was achieved through colour thresholding of the GFP positive cells using ImageJ (standard settings). Migration distances were measured using ImageJ (freehand lines tool).

For immunohistochemistry, the sections were equilibrated in PBS, permeabilized with PBS containing 0.2% (v/v) Triton X-100 for 10 minutes and incubated for 1 hour at room temperature with a PBS blocking/staining solution containing 0.03% (v/v) Triton X-100 and 10% (v/v) FBS. Next, the sections were incubated at 4°C overnight with the following primary antibodies: anti-TuJ1 (1:1000; Cambridge Bioscience; 801202), anti-GFAP (1:200; DAKO; Z0334), anti-KI67 (1:200; Abcam; Ab16667), anti-SOX2 (1:150; Cell signalling; 35795), anti-VIMENTIN (1:200; DAKO; M0725), anti-NESTIN (1:200; Millipore; MAB5326), anti-MMP2 (1:50, Sigma, HPA001939), anti-MMP9 (1:200, Abcam, ab38898). VIMENTIN and NESTIN primary antibodies were human-specific, showing no cross-reactivity with mouse cells. The cells were then incubated for 1 hour at room temperature in the dark with the following secondary antibodies: Alexa-fluor-488 goat anti-mouse (1:200; Molecular Probes; A11029), and Alexa-fluor-Cy3 (1:400; Jackson ImmunoResearch; 711-165-152). Nuclei were stained with (DAPI; 1µg/mL; Sigma; D9542) and imaging was carried out using an EVOS digital inverted fluorescence microscope (Life Technologies) and a Nikon A1R confocal microscope.

Statistical analysis

Data was analyzed by two-sided unpaired Student's *t*-test and expressed as mean ± standard deviation (SD). N represents independent eCO/GBM and eCO/NP hybrids. P values of ≤ 0.01 were considered highly significant.

Results and Discussion

To establish an experimental approach that was amenable to real time imaging and medium throughput (in a 96-well format), we separately generated eCOs and GBM spheroids (Figure 1A). The latter were generated from an established patient-derived GBM cell model (GBM1) which is characterized by stem cell-like features, including NESTIN/SOX2 co-expression as well as clonal growth, and *in vivo* tumorigenicity/invasion capacity.[17, 19] Homogenous GBM spheroids were generated by aggregation of 500 GBM1 cells stably expressing green fluorescent protein (GFP). To maximize experimental throughput, we used a short mESCs differentiation period (12 days) that suffices to induce neural tissue identity in eCOs[18] prior to the co-culture assay (Figure 1A). Qualitative immunohistological analysis showed the presence of the neuronal marker TuJ1 and distinct GFAP-positive cells within the eCOs, thus confirming neural lineage commitment at day 12 (Supplementary image 1). Notably, we observed that the subsequent co-culture of GBM spheroids and eCOs for 48 hours resulted in the spontaneous incorporation of GBM1 spheres into eCOs in 100% of cases, based on a total of 114 specimens across 7 different experimental sets (Figure 1B). Hybrid organoid formation was preceded by GBM spheroid/eCO fusion (as visualized by confocal microscopy after a 16-hour co-culture period; Figure 1C). These results indicate a high reproducibility of GBM/eCO hybrid organoid formation.

Based on the spontaneous infiltration of the eCOs by GBM cellular compartments, we next sought to address the dynamics of this process, and whether the hybrid-organoid phenotype was dependent on the cellular context (i.e., GBM versus NP). To this end, we utilized an adult brain-derived NESTIN and GFP-expressing NP line (NP1; originating from epilepsy surgery excess tissue[17]) (Supplementary image 2) and generated 500-cell spheroid aggregates from GBM and NP cells, respectively. In agreement with reports describing fused cerebral organoids,[20-21] time lapse microscopy experiments revealed that both GBM and NP spheroids first attached to, then fused with, and ultimately became incorporated into eCOs (Figure 2A, Supplementary movies 1 to 4; *note that we refer to incorporated GBM and NP spheroids as GBM and NP compartments*). However, the GBM spheroids showed a rapid incorporation with eCOs (resulting in hybrid eCO/GBM compartments <18 hours), whereas NP incorporation required a prolonged period (≥24 hours; 96% ± 3.7, n=70).

Furthermore, histological analysis and image quantification showed a striking phenotypic difference between eCO/GBM and eCO/NP hybrid organoids (Figure 2B). When compared to their NP counterparts, the GBM compartments within eCO/GBM hybrid organoids were significantly larger (≥5-fold) after a 52-hour co-culture period (Figure 2C). In addition, within the organoids, GBM cells displayed a significantly increased migration capacity at the 24-hour time point (Figure 2D). GBM cells consistently infiltrated the inner layers (core) of eCOs more effectively compared with their NP counterparts at the experimental endpoint (Figure 2E). Further qualitative immunohistological analysis of inner-layer GBM-compartments (using human-specific antibodies for GBM compartment

visualization) showed that the infiltrative GBM cells are characterized by co-expression of VIMENTIN and matrix metalloproteinase 2 (MMP2) proteins, which have been previously implicated in glioma invasion (Figure 3A and B; MMP9 immunopositivity was rarely detected, Supplementary image 3).[22-23] In addition, the GBM cells expressed the known 'stemness' markers NESTIN and SOX2 (Figure 3C). Expression of the cell cycle marker Ki67 was low/absent in the invasive GBM cell population, indicating a migratory rather than proliferative GBM cell phenotype (Supplementary image 4).

In summary, our technical study demonstrates that co-culture of human GBM/NP spheroids and mESC-derived eCOs robustly resulted in the formation of hybrid cellular compartment organoids. We aimed to limit the experimental variability that is often associated with organoid methodology (e.g., reviewed in[24]). For example, long-term-cultured cerebral organoids have been reported to contain heterogeneous architectures and cellular diversity.[18, 25] Thus, we utilized a short 12-day mESC differentiation period generating eCOs and a well-characterized patient-derived GBM cell model[17, 19]. Accordingly, we developed a reproducible and self-aggregating *ex vivo* experimental model system that aims, in real-time, to recapitulate the infiltrative characteristic of GBM that makes current therapy so ineffective. The use of standard (inverse) microscopy, live cell imaging and a medium-throughput (96-well) assay format was made possible by eCO diameters of 300-800 µm. The resulting hybrid organoid sizes would allow for the use of 384-well (optical bottom) microtiter plates in medium/high-throughput screening applications, and automation of hybrid organoid dispension into microliter plates may be an option (note that a gel environment is currently not required for the assay). The protocol described herein may require significant modification(s) in regards with using the 1536-well screening format.

Importantly, the co-culture experiments indicated a significant difference in the infiltration capacity of GBM versus NP cells. Time-lapse microscopy identified the distinct phases of GBM/NP spheroid attachment and fusion, with tissue infiltration invasion exhibited exclusively by GBM cells. This hybrid organoid GBM invasion readout may complement limitations of mono-compartment assays, especially regarding identifying and distinguishing between molecular mechanisms that promote GBM cell migration and/or viability (e.g., utilizing chemical and/or genetic screens). The reported experimental approach is limited in being unable to observe migration of GBM cells along blood vessels and into the corpus callosum (evident in patient tumors[26]). Moreover, the results described are linked to the specific biological materials and protocols employed and hybrid organoid environment may substantially differ from the adult brain. Additional studies are required in terms of comparing stem cell-based organoids with other *ex vivo* glioma migration methodologies, including organotypic slice culture models.[13-15] However, eCO/GBM hybrid organoids (and their non-cancerous controls) provide the foundation for the future development of high-throughput assays to investigate the underlying molecular mechanisms of invasion and screen for drugs that abrogate this phenotype. Future modifications of the assay may be based on the replacement of R1 mESCs with other (e.g., human) ESCs, and/or the replacement of the GBM1 stem cell-like model with other brain tumor cell models and/or freshly-isolated primary cells. Likewise, future studies may investigate hybrid organoid maturation and the development of GBM versus NP compartment phenotypes (e.g., with respect to quiescence and self-renewal capacity) over a prolonged (>2 months) culture period.

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Declaration of Conflicting Interests

The authors declare no conflict of interest.

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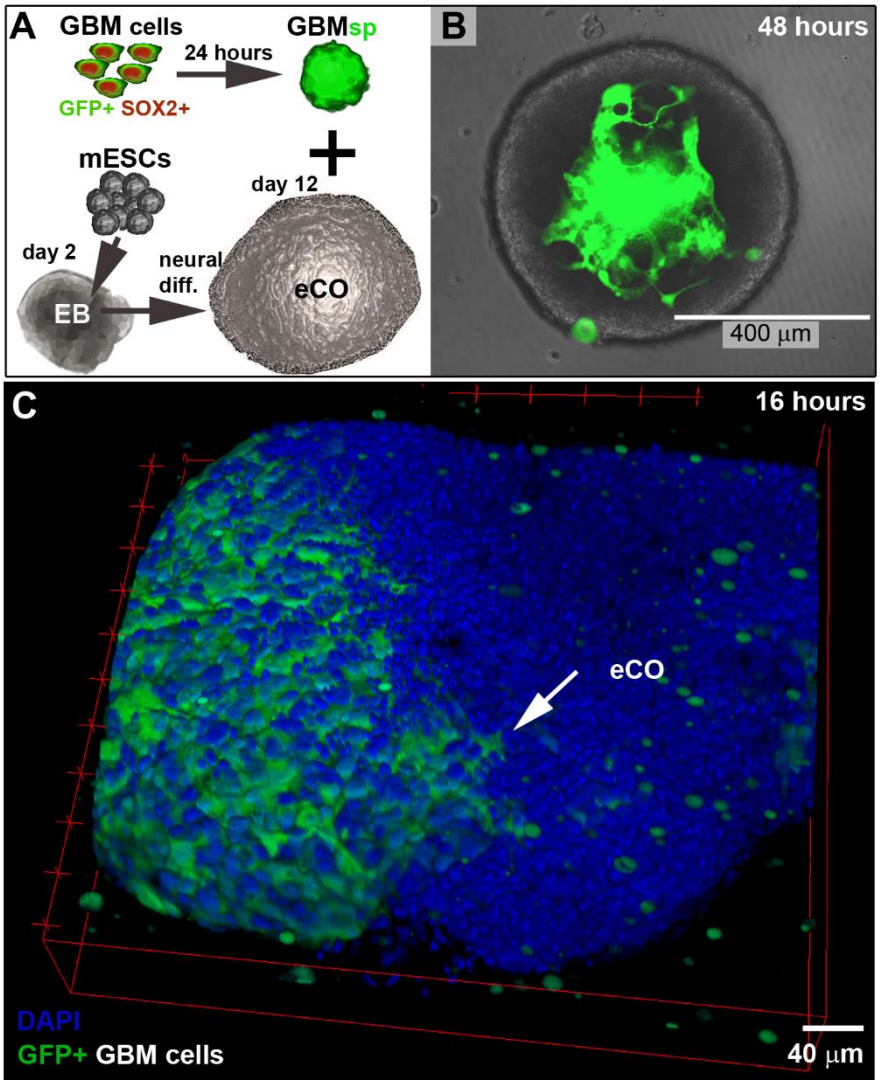


Figure 1. Investigation of neural tissue infiltration by free-floating GBM compared with NP spheroids through the co-culture with mouse embryonic stem cell (mESC)-derived eCOs. (**A**) Schematic overview of the experimental approach. The eCOs were formed from 2000 single mESCs, which were subjected to embryoid body formation, neural induction and differentiation. GBM (or NP) spheroid aggregates (GBMsp) consisted of 500 cells and were co-cultured with eCOs from day 12. (**B**) Representative immunofluorescence image of a well from a 96-well microtiter plate after a 48-hour GBMsp/eCO co-culture period showing the formation of a hybrid organoid consisting of eCO and an infiltrative/green fluorescent protein (GFP)-positive GBM cell compartment. (**C**) Representative confocal microscopy image (volume view) showing fusion of GBM1-GFP cells (green) with eCO resulting in a migratory GBM cell phenotype (arrowhead). Green color indicates positive signal for GFP and nuclei (blue) were stained with DAPI.

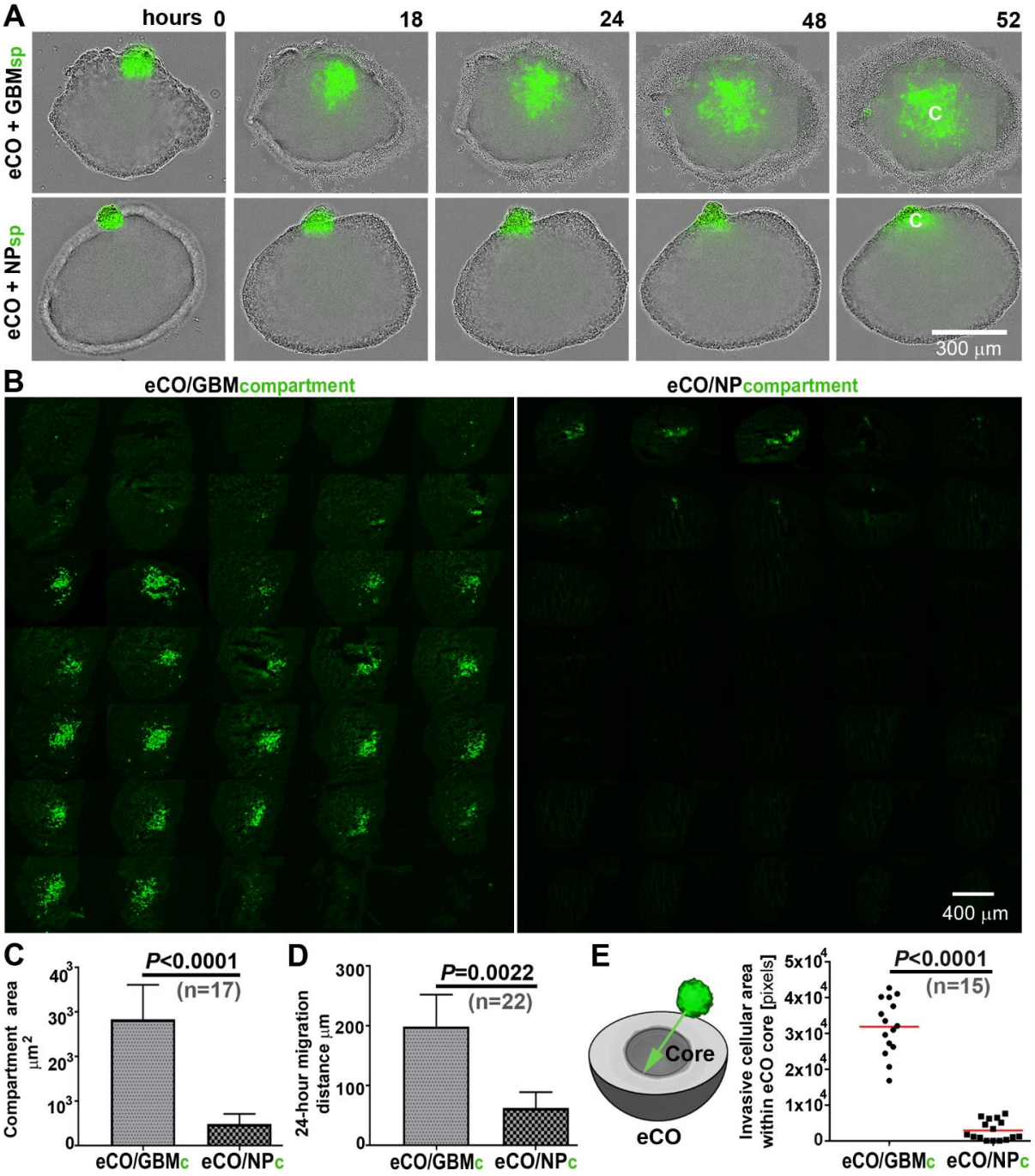


Figure 2. Comparison of GBM and NP spheroid infiltration of eCOs. (**A**) Still frame images of GBM1 and NP spheroids invading eCOs taken over a time-lapse microscopy period of 52 hours. GBM and NP fusion results in cellular compartments (c) within the hybrid organoid structures. (**B**) Representative histological section montage of representative (entire) eCO/GBM and eCO/NP hybrid organoids. Note the overt difference in GBM versus NP compartment spread. (**C**) Quantification of GBM and NP cell compartment (c) sizes within hybrid eCO. (**D**) Maximal migration distance of GBM and NP cells within eCOs. (**E**) Comparison of GBM and NP cellular compartments invading the eCO core, red bars represent the median.

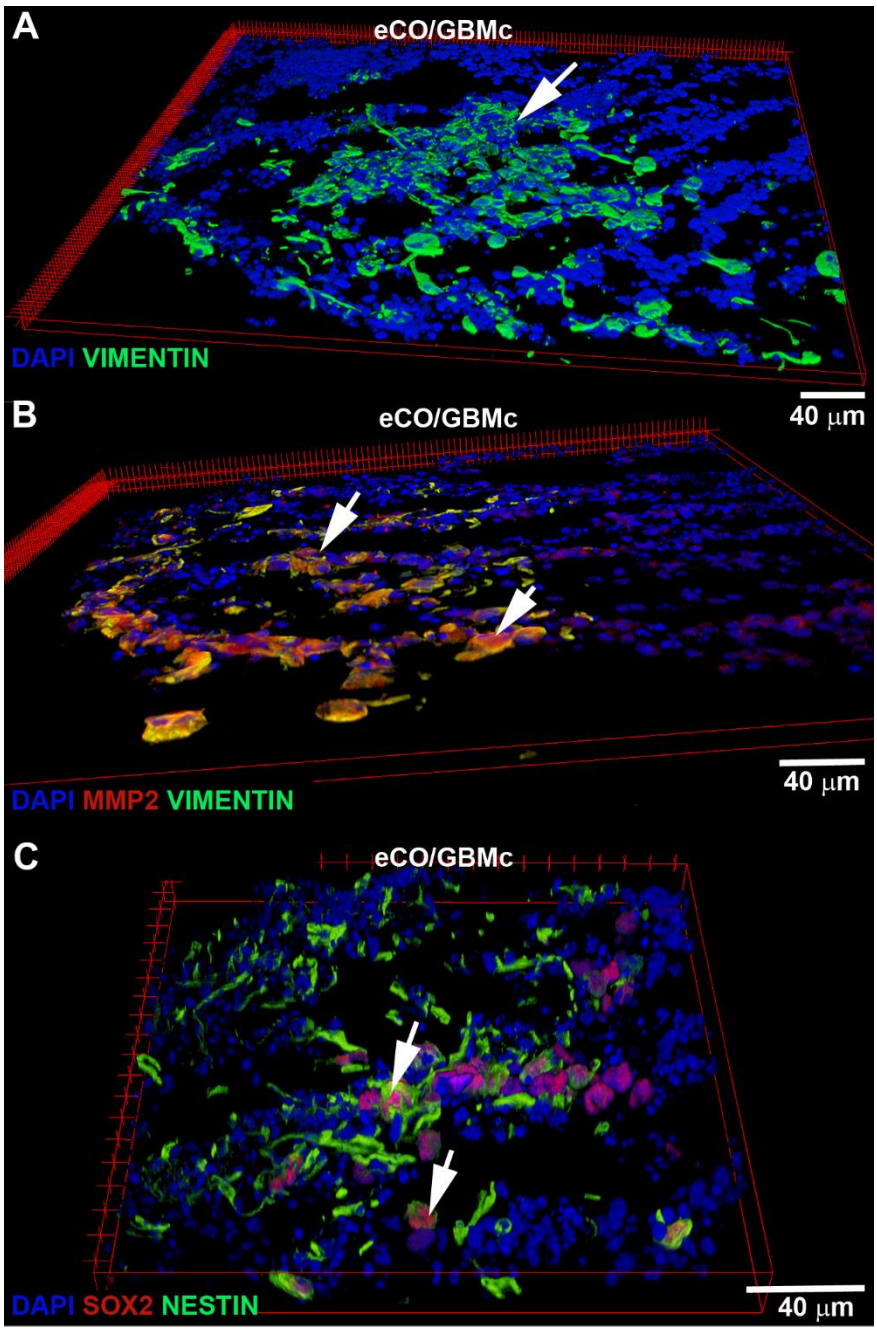
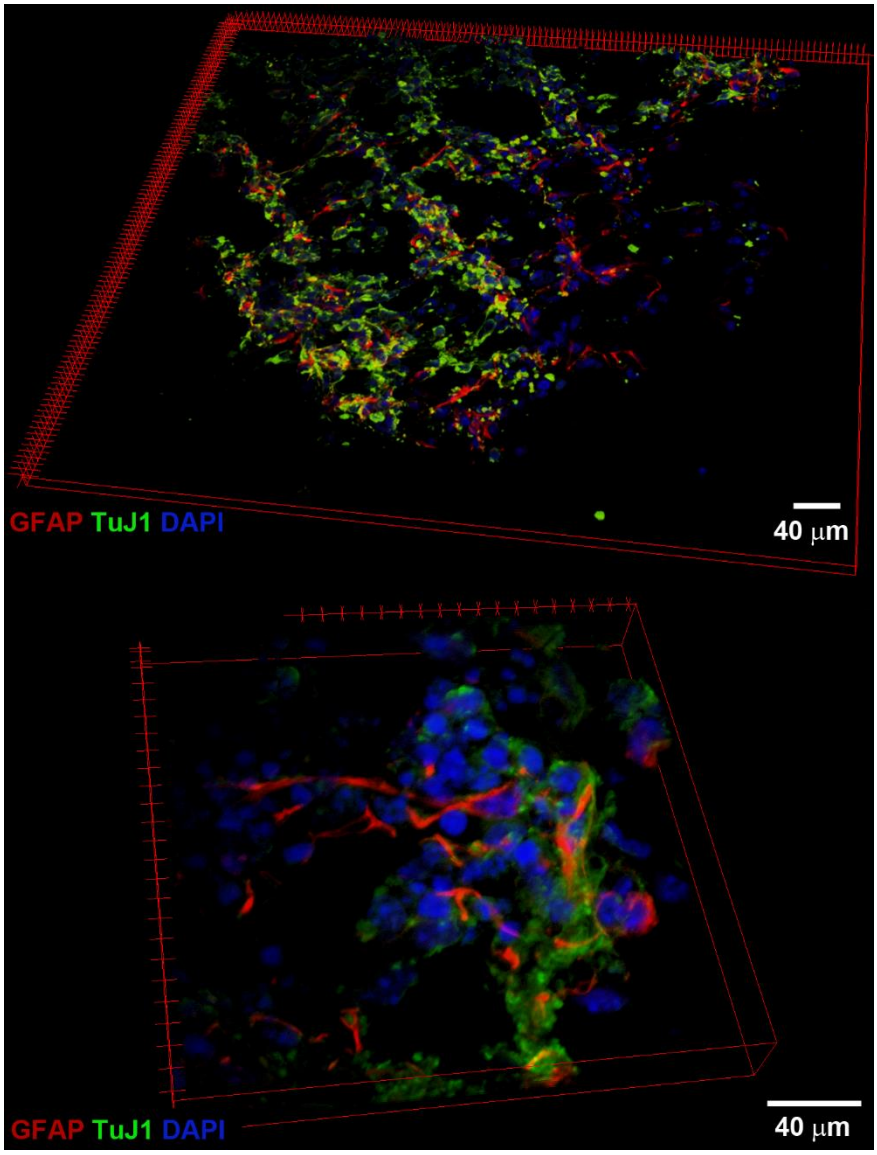
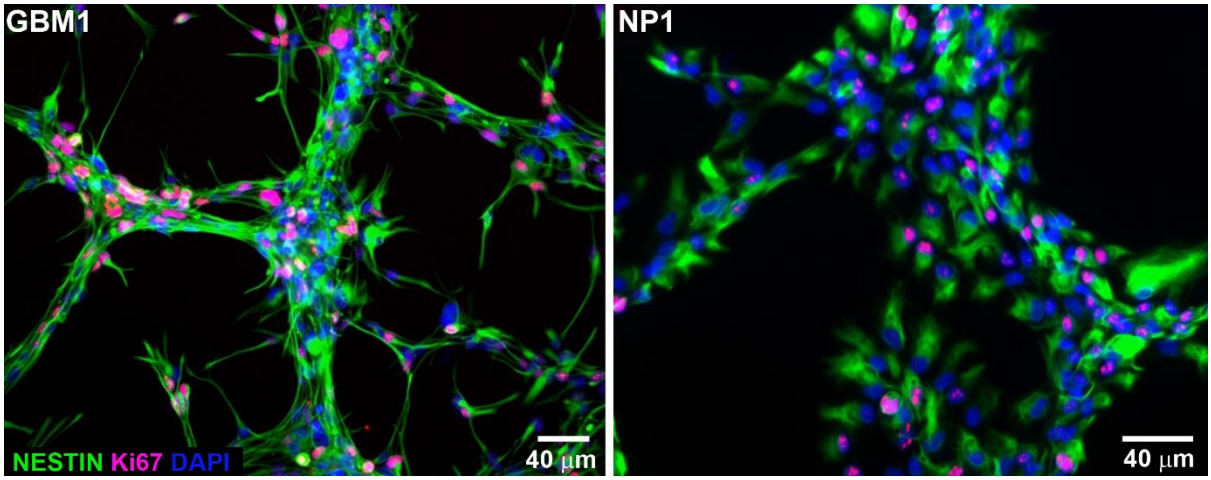


Figure 3. Representative confocal microscopy immunofluorescence images (volume view) of the invasive GBM cellular compartment in histological sections of self-aggregated hybrid organoids (eCO/GBM). (**A**) False colors indicate VIMENTIN expression in GBM cells. Arrowhead highlights the migratory edge of GBM cells within the tissue. (**B**) False colors indicate Vimentin and MMP2 immunopositivity and co-localization in GBM cells (highlighted with arrowheads). (**C**). False colors indicate positive staining for SOX2 and NESTIN in GBM cells (marker co-localization is highlighted with white arrowheads). Nuclei were stained with DAPI.

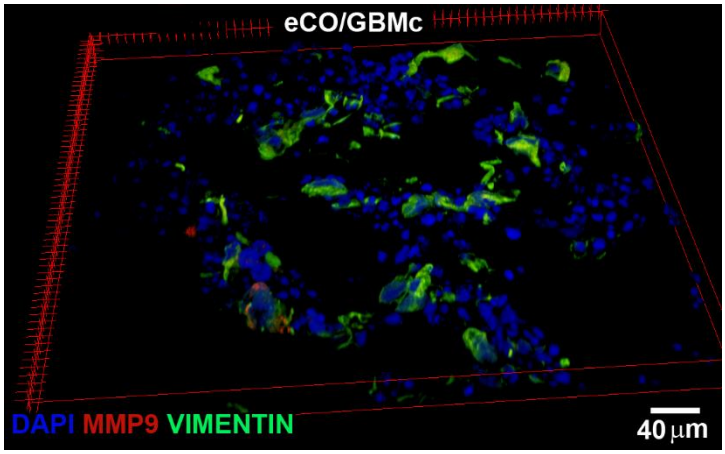
Supplementary images



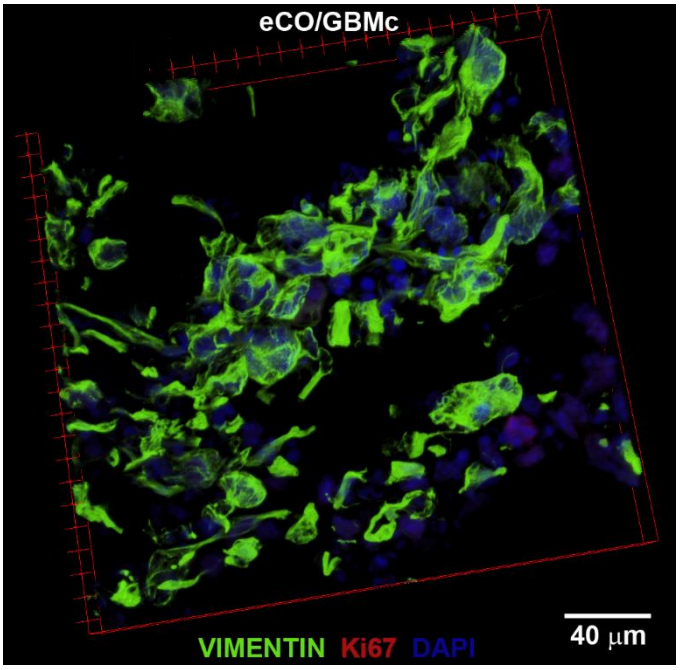
Supplementary image 1. Confocal microscopy immunofluorescence images of eCOs at day 12 of the neural induction protocol. False colors indicate positive signal for astrocytic marker GFAP (red) and neuronal marker TuJ1 (green). Nuclei (blue) were stained with DAPI. Upper panel: 20x objective. Lower panel: 100x objective.



Supplementary image 2. Immunofluorescence images of the GBM1 (left) and NP (right) cell models. Cells express NESTIN (false color: green) and proliferation marker Ki67 (false color: red). Nuclei (blue) were visualized with DAPI.



Supplementary image 3. Representative confocal microscopy immunofluorescence image of the invasive GBM cellular compartment (eCO/GBM, volume view). False colors indicate positive signal for VIMENTIN (green) and rare signal for MMP9 (red). Nuclei (blue) were stained with DAPI.



Supplementary image 4. Representative confocal microscopy immunofluorescence image of the invasive GBM cellular compartment (eCO/GBM, volume view). False colors indicate positive signal for VIMENTIN (green) and negative signal for Ki67 (red). Nuclei (blue) were stained with DAPI.

Supplementary Movie captions:

Supplementary Movie 1 and 2: Representative time lapse microscopy imaging of GBM spheroid incorporation into eCOs.

Supplementary Movie 3 and 4: Representative time lapse microscopy imaging of NP spheroid incorporation into eCOs.

Throughout the GBM and NP spheroid infiltration experiments, varying degrees of cellular debris (stemming from eCO generation) and minor cellular necrosis (at the eCO peripheries) were observed.