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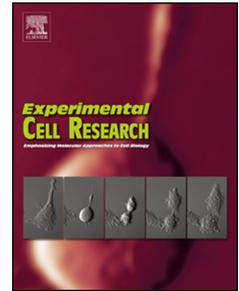
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1 **Title:** A tumorsphere model of glioblastoma multiforme with intratumoral heterogeneity for quantitative
2 analysis of cellular migration and drug response

3 **Running title:** Intratumoral heterogeneity in a glioblastoma model

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26 **ABSTRACT**

27 Glioblastoma multiforme (GBM) is the most common and malignant type of primary brain tumor and is
28 characterized by its sudden onset and invasive growth into the brain parenchyma. The invasive tumor cells
29 evade conventional treatments and are thought to be responsible for the ubiquitous tumor regrowth.
30 Understanding the behavior of these invasive tumor cells and their response to therapeutic agents could
31 help improve patient outcome. In this study, we present a GBM tumorsphere migration model with high
32 biological complexity to study migrating GBM cells in a quantitative and qualitative manner. We
33 demonstrated that the *in vitro* migration model could be used to investigate both inhibition and stimulation
34 of cell migration with oxaliplatin and GBM-derived extracellular vesicles, respectively. The intercellular
35 heterogeneity within the GBM tumorspheres was examined by immunofluorescent staining of
36 nestin/vimentin and GFAP, which showed nestin and vimentin being highly expressed in the periphery of
37 tumorspheres and GFAP mostly in cells in the tumorsphere core. We further showed that this phenotypic
38 gradient was present *in vivo* after implanting dissociated GBM tumorspheres, with the cells migrating away
39 from the tumor being nestin-positive and GFAP-negative. These results indicate that GBM tumorsphere
40 migration models, such as the one presented here, could provide a more detailed insight into GBM cell
41 biology and prove highly relevant as a pre-clinical platform for drug screening and assessing drug response
42 in the treatment of GBM.

43 **Keywords:** glioblastoma; GBM; migration; invasion; nestin; GFAP; tumorsphere; extracellular vesicles;
44 oxaliplatin

45

46 INTRODUCTION

47 Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor with a median
48 overall survival of only 15 months with the present standard of care [1]. Current best practice for treating
49 these tumors consists of maximal surgical resection followed by concomitant radio- and chemotherapy, but
50 recurrence of the tumor still remains ubiquitous [2]. GBM is characterized by its rapid growth and invasion
51 into the surrounding brain parenchyma, high vascularization, and hypoxic niches harboring cancer stem-like
52 cells within the tumor milieu [3]. Therefore, the complexity in the study of GBM resides in the
53 heterogenous nature at the molecular and cellular level, which hinders the derivation of representative *in*
54 *vitro* and *in vivo* GBM models. The study of GBM's ability to invade the brain parenchyma could potentially
55 reveal new targets for treatment by helping researchers understand the mechanisms driving cell invasion.
56 To facilitate this understanding, *in vitro* migration or invasion assays are commonly used [4]. Identification
57 of drugs or factors that can inhibit or stimulate cancer cell migration also rely on the use of *in vitro* studies
58 to select promising candidates for further assessment *in vivo*.

59 In vitro, invasion and migration assays are typically defined by separate parameters: Invasion
60 assays are characterized by embedding cells in a 3D milieu where a restructuring of the extracellular matrix
61 (ECM) takes place, whereas migration is defined by cells moving on a 2D ECM, i.e. Matrigel or collagen
62 matrices [4]. Many migration assays today rely on the use of adherent monolayer cell cultures (2D cultures)
63 that typically are dependent on the addition of serum to the growth medium for cell propagation [4]. In
64 recent years, more focus has been drawn to the use of cancer cell lines that are cultured as non-adherent
65 tumorsphere cultures without the addition of serum (3D cultures) [5]. Such cells have usually been cultured
66 in medium favoring stem-like properties that enable the formation of tumorspheres from single cancer
67 cells. When reaching a certain size, tumorspheres can display different cellular phenotypes generating a
68 more complex tumor-like composition [6]. The invasive potential of cancer cells has been directly
69 correlated to their degree of malignancy, and often the cells found to facilitate the process of tissue

70 invasion and metastasis have been identified as cancer cells with stem-like properties [7,8]. The
71 extracellular matrix in the tissue harboring the tumor plays an important role in cancer cell invasion,
72 modulating which cells move and which cellular pathways are utilized during the event [9]. This complex
73 microenvironment can, to some extent, be mimicked in *in vitro* migration assays where different matrix
74 components applied can affect the cells in different ways. For example, some studies apply a matrix
75 constituted of only a single type of ECM protein such as collagens or fibronectin, and others apply more
76 complex mixtures such as Matrigel [9–12].

77 In this study, we provide a more biologically relevant model with respect to cell migration by
78 combining primary tumorsphere cell cultures and complex ECM to create a more relevant milieu with
79 respect to cancer cell migration. We refine an established tumorsphere migration model to include both
80 real time quantification and the possibility to do subsequent high-resolution microscopy to assess
81 tumorsphere characteristics. The model uses a primary GBM cell line grown on Geltrex. A characterization
82 of intra-tumorsphere cellular heterogeneity was done by visualizing a gradient in nestin/vimentin and Glial
83 Fibrillary Acidic Protein (GFAP) expression between the tumorsphere periphery and core. The *in vitro* study
84 was supported by *ex vivo* examination of such phenotypic gradient in an orthotopic mouse GBM xenograft
85 generated with the same GBM tumorspheres. To illustrate that this model can be used to both inhibit and
86 stimulate GBM cell migration, we used oxaliplatin and extracellular vesicles (EVs) derived from GBM cells,
87 respectively, hereby underscoring its potential as an assay of therapeutic efficacy.

88

89 **MATERIALS AND METHODS**

90 **Ethical approval**

91 All experimental procedures were approved by the Danish Animal Welfare Council, the Danish Ministry of
92 Justice (license no. 2019-15-0201-00920). NMRI nude mice (Taconic Biosciences, Denmark) were housed in

93 IVC rack in Type III SPF cages with a maximum of 8 mice in each cage. Food and water were available ad
94 libitum.

95 **Cell culture**

96 Primary GBM tumorsphere cultures T78 were generated as previously described and cultured in
97 Neurobasal A medium supplemented with 1 % B27 supplement, 2 mM L-glutamine and 20 ng/mL EGF and
98 bFGF and penicillin-streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin) [13,14]. T78 cells were
99 used at passages 18-20 throughout all experiments. For EV isolation, a secondary GBM cell line was
100 cultured in DMEM-F12 supplemented with 10 % EV-depleted FCS and penicillin-streptomycin (100 U/mL
101 penicillin and 100 µg/mL streptomycin). EV-depleted FCS was generated by ultracentrifugation of FCS at
102 120,000 RCF for 16 hours, where the supernatant was further used for culturing cells for EV production.

103 **Geltrex coating**

104 Geltrex (ThermoFisher, MA, USA; #A1413302) was thawed on ice at 4°C prior to use. After thawing, Geltrex
105 matrix was diluted 1:50 in growth medium and seeded in a volume of 700 µL per well into the middle eight
106 wells of a 24 well plate. Everything was kept cool on ice while resuspending and coating the wells. The
107 plates were then incubated at 37°C for minimum 4 hours to let the Geltrex matrix solidify.

108 **Isolation of single tumorspheres and treatment**

109 Prior to GBM tumorsphere isolation, the plates were cooled to room temperature (usually 10-20 min), and
110 the medium was then removed from the wells. Tumorspheres were selected according to their size
111 (approximately 100-200 µm in diameter) and isolated with a pipette in a volume of 0.5 µL under a phase-
112 contrast microscope. One tumorsphere was spotted into the middle of each well. The surrounding wells
113 were filled with 500 µL mL PBS to avoid evaporation and drying of the tumorspheres. After spotting the
114 tumorspheres, the plates were incubated at 37°C for 30-45 min to allow adherence to the gel, and then 700
115 µL of pre-heated growth medium was carefully added to each tumorsphere-containing well.

116 The day after spotting the tumorspheres (referred to as day 1 or D1), treatment groups were randomly
117 assigned. Tumorspheres received a single-dose of oxaliplatin on day 1 at a concentration of 5 μ M, similar to
118 the concentration used in other studies [15]. EVs were added in a concentration of approximately 6.5×10^7
119 particles per well in triplicates. EVs isolated from non-conditioned medium were included in triplicates as a
120 control to account for the potential effects of EVs or other factors remaining in the medium. No treatment
121 controls (NTC) were done in five replicates. TGF- β 1 was added to the cells in a concentration of 4 ng/mL in
122 triplicates.

123 **Quantitative data acquisition and analysis**

124 Phase-contrast images were acquired each day for a total of 5 days (D0 – D4) with a Zeiss Axio Observer Z.1
125 (DE). Area of the growing spheres was estimated with Zeiss ZEN2 Blue Edition. All graphs were generated in
126 GraphPad Prism 6.

127 **Fluorescence microscopy and time-lapse imaging on tumorspheres *in vitro***

128 Sterile coverslips were placed in each well, and Geltrex coating was done as previously described. Bulk GBM
129 tumorspheres were seeded (10-30 per well) in growth medium and incubated overnight. Tumorspheres
130 were washed in PBS and fixed in 4 % formaldehyde for 15 min. at room temperature. Tumorspheres were
131 then washed again and blocked in 5 % BSA PBS for 30 min. Primary antibodies Ms anti-human nestin
132 (Abcam, Cambridge, UK; #ab22035; 1:1000), Ms anti-vimentin (Abcam; #ab92547; 1:1000), Rb anti-GFAP
133 (Dako, DK; #Z0334; 1:1000), were added to cells in 0.5 % BSA PBS and incubated on a rocking table
134 overnight at 4°C. Tumorspheres were then washed and secondary antibodies Dnk-anti-Ms-Alexa-488
135 (ThermoFisher; #R37114; 1:1000), Dnk-anti-Rb-Alexa-555, (ThermoFisher; #A-31572; 1:1000) were added
136 to cells and incubated on a rocking table for 2 hours at room temperature. Tumorspheres were then
137 washed and stained with Hoechst33342 (ThermoFisher; #H3570; 1:3000) for 10 min on a rocking table at
138 room temperature. Coverslips were transferred to SuperFrost (Menzel Gläser, ThermoFisher) slides with a
139 drop of fluorescent mounting medium (Dako; #S3023) and stored in a fridge at 4 – 6°C overnight to harden.

140 Images were obtained on Zeiss Observer Z.1 with Apotome-2 structured illumination microscopy with a 40x
141 NA1.30 objective (Zeiss). Quantification of GFAP:nestin ratio between core and periphery was done by
142 threshold analyses of 4 images and presented as a bar chart with mean + SD in GraphPad Prism 6. Time-
143 lapse imaging was done on tumorspheres directly after seeding onto Geltrex and imaged on a Zeiss
144 Observer Z.1 with a mounted Pecon Incubator P S compact (Pecon, Erbach, DE). Images were acquired with
145 Zeiss ZEN2 Blue software every 10 min. with automated focus over the course of 24 hours. Images and
146 time-lapse series were processed and analyzed in Fiji [16].

147 **Paraffin embedding and immunostaining of free-floating GBM tumorspheres *in vitro***

148 Tumorspheres were fixed free-floating in methanol for five minutes before embedding in paraffin for
149 sectioning. 5 μ m sections of embedded spheroids were cut on a Leica RM 2255 microtome (Nussloch, DE)
150 and fixated on glass slides by melting of paraffin residue at 60°C for one hour. Sections were stained using
151 primary antibodies Rb anti-GFAP (Dako; #Z0334; 1:200) and Ms anti-human nestin (Abcam; #ab22035;
152 1:200). Secondary antibodies were Dnk-anti-Ms-Alexa-488 (Invitrogen; #A-21202; 1:500) and Gt-anti-Rb-
153 Alexa-594 (Invitrogen, CA, USA; #A-11037; 1:500). Antigen retrieval was performed using a 10 mM sodium
154 citrate buffer (pH 6.0) with 0.05% Tween. Cells were additionally immunostained with 4,6-diamidino-2-
155 phenylindole (DAPI) (Sigma; #000000010236276001; 1:500) for nuclear staining. Slides were mounted using
156 fluorescent mounting medium (Dako; #S3023) and images were acquired on a Zeiss Observer Z.1 using the
157 Colibri light source (Zeiss) and Orca-Flash4.0 V2 (Hamamatsu) as the detector. To quantify the area that
158 nestin and GFAP signal covers, the manual threshold tool in Fiji was used. Five images of five different
159 tumorspheres were used for thresholding and the area coverage in percent was normalized for each
160 tumorsphere to the respective tumorsphere size determined by area of the nuclear stain (DAPI) when over-
161 saturated. Data was plotted and analyzed in GraphPad Prism 6.

162 **GBM mouse xenograft model**

163 T78 GBM tumorspheres were grown until 100-200 μm in diameter and dissociated with TrypLE
164 (ThermoFisher; #12604013). Cells were then washed twice, counted, and cell numbers adjusted to 20,000
165 cells/ μL . A total of 10 μL (200,000 cells) was resuspended in growth medium and injected into the striatum
166 (0.5 mm below Bregma, 1.5 mm lateral) of nude NMRI mice using a syringe pump running at 30 nL/s. To
167 avoid cells being dragged back up with the removal of the needle, the needle was left in the injection site
168 for 3 minutes prior to removal. Tumor size and growth was monitored with MRI (BioSpec 7T, Bruker,
169 Mannheim, DE) using T2-weighted sequence of the mouse brain obtained in axial and coronal directions
170 (Figure S1). Mice were anesthetized with Sevoflurane when the tumor size reached 10 – 20 mm^3 and
171 transcardially perfused with PBS followed by perfusion of 4 % methanol-free paraformaldehyde. Brains
172 were removed from the skull and post-fixed overnight at 4°C.

173 **Fluorescence immunohistochemistry on GBM tumors**

174 Brains were immersed in cryoprotection with 10%, 20% and 30% sucrose (each step overnight), embedded
175 in OCT (Micro and Nano; #16-004004) and frozen in isopentane on dry ice. 30 μm coronal sections were
176 obtained with a cryostat (Leica CM 1850 UV). Sections were blocked with blocking solution containing 5 %
177 donkey serum (Millipore, Darmstadt, DE; #S30-100ML) and 0.2 % saponin (VWR, DK; #27534.187) in TBS for
178 1 hour. The sections were then blocked with mouse on mouse blocking reagent (Vector Laboratories, CA,
179 USA; Cat. #MKB-2213) and after 2 hours, the solution was changed to mouse on mouse blocking reagent in
180 0.2 % saponin in TBS for 1.5 hours. The sections were incubated with primary antibodies: Rb anti-human
181 GFAP (Abcam; #ab33922; 1:500) and Ms anti-human nestin (Abcam; #ab22035; 1:400) overnight at 4°C.
182 After washing in TBS, sections were stained with Hoechst 33342 (ThermoFisher; #62249; 1:1000) and
183 secondary antibodies: Dnk-anti-Rb-Alexa-568 (Invitrogen; Cat. #A10042; 1:1000) Dnk-anti-Ms-Alexa-647
184 (Invitrogen; Cat. #A-31571; 1:1000) for 3 hours at room temperature. Sections were washed and mounted
185 using ProLong™ Diamond Antifade Mountant mounting media (Invitrogen; #P36970). Samples were imaged
186 with a confocal laser scanning microscope (Zeiss LSM 710) and fluorescence slide scanner (Zeiss Axio

187 Scan.Z1). For the images obtained with fluorescent slide scanning shading correction was applied using
188 Zeiss ZEN Blue 2.3 software. Secondary antibody controls are presented in Figure S2.

189 **EV isolation**

190 EVs were isolated from GBM cells grown in DMEM-F12 supplemented with 10 % FCS and 1 % penicillin-
191 streptomycin. To produce conditioned medium (CM), EV-depleted FCS was made by ultracentrifugation of
192 FCS at 120,000 RCF for > 16 hours. The EV-depleted FCS was then diluted to 10 % in DMEM-F12 and added
193 to the cells in T175 flasks (30 mL) and incubated for 24 hours at 37°C. CM was harvested and centrifuged
194 for 20 min at 2000 RCF and either stored at -20°C until further processing (for maximum two weeks) or
195 processed directly. The supernatant was transferred to a new tube and centrifuged at 9000 RCF for 30 min.
196 The supernatant was filtered through 0.2 µm filters and centrifuged at 120,000 RCF for 2.5 hours. The
197 resulting supernatant was discarded, and the pellet resuspended in growth medium or Trehalose-PBS, for
198 either Tumorsphere migration assay or NTA and TEM validation (see below), respectively. The EV CTRL was
199 made by running non-conditioned medium through the exact same EV isolation protocol.

200 **Nanoparticle Tracking Analysis**

201 All NTA analyses were done on a NanoSight LM-10 (Malvern, UK). A dilution of the EVs was made to include
202 around 50 – 100 particles at once in the field of view. For video recording, shutter was between 700 and
203 800, gain was between 550 and 620, and the capture time for each recording was 30 s. For each sample, a
204 total of five videos were recorded. Prior to NTA, screen gain was adjusted to 2, blur was set to 3x3, and
205 detection threshold set between 16 and 28. Tracks were exported to Microsoft Excel and imported into
206 GraphPad Prism 6 (GraphPad, CA, USA) for further analysis.

207 **Immunoelectron microscopy of immunogold-labelled EVs**

208 Immunolabelling was performed by mounting 5 µL concentrated samples on carbon- coated, glow
209 discharged 400 mesh Ni grids for 30 s and washed 3 times with PBS. Grids were blocked with 0.5%

210 ovalbumin in PBS and then incubated with a cocktail of primary anti-CD9 (Ansell, MN, USA; #SN4/C3-3A2;
211 1:50), anti-CD63 (Ansell; #AHN16.1/46-4-5; 1:50) and anti-CD81 (Ansell; #1.3.3.22; 1:50) monoclonal
212 antibodies in 0.5% ovalbumin in PBS for 30 min at 37°C. After incubation grids were washed 3 times with
213 PBS and incubated with secondary antibody goat anti-mouse conjugated with 10 nm colloidal gold (British
214 BioCell, Cardiff, UK) 1:25 in 0.5% ovalbumin in PBS for 30 min at 37°C. The grids were then washed with 3
215 drops of PBS, before incubation on 3 drops of 1% cold fish gelatin for 10 min each. The grids were finally
216 washed with 3 drops of PBS before staining with 1 drop of 1% (w/v) phosphotungstic acid at pH 7.0 and
217 blotted dry. Images were obtained with a transmission electron microscope (JEM-1010, JEOL, Eching,
218 Germany) operated at 60 keV coupled to an electron- sensitive CCD camera (KeenView, Olympus, Center
219 Valley, PA, USA). For size determination of visible EVs a grid-size replica (2,160 lines/mm) was used. See
220 Table S1 for full antibody list.

221 **Production of oxaliplatin-loaded stealth liposomes**

222 Stealth liposomes were produced from a lipid formulation containing hydrogenated soybean
223 phosphatidylcholine (HSPC), DSPE-PEG₂₀₀₀, and cholesterol (Lipoid GmbH, Ludwigshafen, DE) in a molar
224 ratio of 56.8:38.2:5 mol%. Hydration of the lipid powder was done for 1 hour at 65°C 10 mM HEPES and 5 %
225 glucose (pH 7.4) containing oxaliplatin (Lianyungang Guiyuan Chempharm Co., LTD, Jiangsu, PRC). Extrusion
226 of the liposomes and determination of phospholipid and oxaliplatin concentration were performed as
227 described in *Johnsen et al.* (2019) [17]. The hydrodynamic diameter and ζ -potential of the resulting
228 oxaliplatin-loaded stealth liposomes were measured with a Zetasizer (ZetaPALS, Brookhaven Instruments
229 Ltd., NY, USA), showing a diameter of approximately 120 nm and a net negative surface charge.

230 **RESULTS**

231 **Intra-tumorsphere cellular heterogeneity display *in vitro***

232 Since GBM tumorspheres grow in a non-adherent 3D fashion, we hypothesized that a cellular heterogeneity
233 could arise within each tumorsphere. First, time-lapse image series were acquired of tumorspheres to
234 visualize attachment to Geltrex and migration for the first 24 hours (see Supplementary Video 1). The time-
235 lapse gave us an idea of how the tumorspheres transition from a free-floating state to becoming attached
236 to the Geltrex (illustrated in Figure S3A). To examine the intercellular heterogeneity within the
237 tumorspheres, tumorspheres were seeded in wells containing a Geltrex-coated coverslips and incubated
238 those overnight for subsequent immunostaining and high-resolution fluorescence microscopy. In the first
239 instance, smaller tumorspheres were stained for nestin expression to allow for high-resolution imaging of
240 whole tumorspheres. On visual inspection, the lower slices of the microscopy Z-stack, showed that the
241 tumorsphere core was nestin-negative, contrary to the positive nestin staining in the periphery (Figure
242 S3B). This pattern of nestin expression in the cells prompted us to look for more differentiated cells in the
243 tumorspheres. This was done by co-staining for GFAP and nestin/vimentin. Nestin and vimentin are known
244 to associate with invasive cancer cells and cancer stem-like cells in GBM, whereas GFAP expression was
245 indicative of a less invasive phenotype and is expressed in opposition to nestin, perhaps allowing for a
246 phenotypical distinction [18–21]. Images obtained in the periphery of tumorspheres showed cells highly
247 positive for vimentin and nestin and less positive for GFAP (Figure 1A, Figure S4). Interestingly, long
248 projections were shown to stretch from the core of tumorspheres towards the periphery, possibly
249 resembling astrocytic end-feet or tumor microtubes [22].

250 ***INSERT FIGURE 1***

251 To further illustrate the phenotypical gradient from the core to the periphery, images were taken close to
252 the core with an overlapping image towards the periphery. Here, less nestin-positive and more GFAP-
253 positive cells were observed by the core (Figure 1B), whereas the peripheral cells were all nestin and GFAP-
254 positive. However, despite being present, the GFAP displayed a fragmented (or non-filamentous) structure
255 in the periphery, which might indicate an ongoing degradation of GFAP at the time of acquisition (less than

256 24 hours after seeding the tumorspheres) (Figure 1C, Figure S5). Close to the core, GFAP expression was
257 predominantly displayed as filamentous structures (Figure 1B-C, Figure S5). Orthogonal views showed a
258 double-layer of cells close to the core with the top cell-layer mainly being nestin-positive/GFAP-negative
259 and the bottom layer mainly nestin-negative/GFAP-positive. In the tumorsphere periphery, orthogonal
260 views confirm these observations of fragmented GFAP expression, which also appeared to localize inside
261 the nucleus (Figure 1C, Figure S5). Quantification of the GFAP and nestin expression revealed significant
262 differences in the GFAP-to-nestin ratios when comparing the core and peripheral regions of the
263 tumorspheres (Figure 1D). This underscored the observation that GFAP expression is decreased with the
264 increase in migratory capacity of the tumorsphere cells.

265 To examine whether a heterogenous expression of GFAP/nestin was also evident in whole non-adherent
266 tumorspheres (free-floating), we fixed and paraffin-embedded whole tumorspheres and cut them in 4 μ m
267 sections for immunofluorescence staining (Figure 2A). Most cells in the tumorspheres were nestin-positive
268 with the nestin staining covering 66 % of the tumorspheres, and only 10 % appearing to be GFAP-positive
269 (Figure 2B). The GFAP pattern appeared quite diffuse, but the cells in the outermost periphery were GFAP-
270 negative and nestin-positive, confirming the heterogenous gradient shown in the Geltrex setup.

271 ***INSERT FIGURE 2***

272 **Tumorsphere phenotypic gradient is reflected *in vivo***

273 Given the observations and phenotypic distinctions in the *in vitro* tumorsphere migration model, an *in vivo*
274 experiment was set up using the same GBM tumorsphere culture to see if the *in vitro* model recapitulated
275 the situation observed *in vivo*. Dissociated T78 GBM tumorspheres were stereotactically implanted into the
276 striatum of nude mice and tumor growth monitored weekly with MRI (Figure S1). When the tumor reached
277 a sufficient size (10 – 20 mm³), mice were sacrificed and whole brains were removed and stained for human
278 GFAP and human nestin (Figure 3). Fluorescence slide scans of whole brain slices were correlated to the last
279 MRI sequence obtained just before the mice were sacrificed and showed that fluorescence imaging was

280 done approximately in the center of the tumor (Figure 3A-B, Figure S1C). Confocal microscopy of the same
281 slides showed that the tumor core contained both nestin and GFAP-positive cells, and the tumor periphery
282 showed a change towards nestin-positive and GFAP-negative cells with increased distance from the tumor
283 core (Figure 3C-D). In the area between the more distant tumor cells and the tumor core, tumor cells
284 positive for both nestin and GFAP were observed (Figure 3D). This could both indicate a transition zone
285 towards a more nestin-positive phenotype or that the cells expressing both intermediate filaments possess
286 migratory potential. The most distant tumor cells identified had migrated to the frontal superficial
287 hippocampal formation and were nestin-positive and GFAP-negative (Figure 3E).

288 ***INSERT FIGURE 3***

289 **Oxaliplatin reduces primary GBM tumorsphere migration *in vitro***

290 After having established that the intratumoral heterogeneity was recapitulated in our *in vitro* model, we
291 next wanted to study the potential of using the model as an assay of therapeutic efficacy. For this purpose,
292 we utilized the platinum-based chemotherapeutic drug, oxaliplatin [23]. Single GBM tumorspheres were
293 seeded onto Geltrex matrix on day 0, and treatment groups were assigned on day 1, followed by the
294 addition of 5 μ M oxaliplatin. Phase-contrast images were acquired daily and the area of migration was
295 measured (Figure 4A). On day 1, total area between groups was similar, however, a large reduction in
296 migration was observed the following days after oxaliplatin treatment compared to controls (Figure 4B).
297 During the experiment, the tumorspheres in the control group increased five-fold in size whereas the
298 tumorspheres that received oxaliplatin increased only two-fold in size (Figure 4C). This indicated that the
299 treatment had reduced the growth more than two-fold compared to that of the control after a single dose
300 of oxaliplatin. When encapsulating oxaliplatin into stealth liposomes with low capacity for associating and
301 endocytosing into the cells due to their polymer surface coating, the effects of oxaliplatin were markedly
302 reduced (Figure 4C). Thus, the growth-inhibiting effects of oxaliplatin were successfully modelled and could
303 be diminished by interfering with the interaction potential between the drug and GBM cells.

304 ***INSERT FIGURE 4***

305 **GBM-derived EVs stimulate GBM tumorsphere growth *in vitro***

306 To illustrate that the Geltrex migration model can also be used to evaluate potential stimulatory effects on
307 GBM cell migration, EVs isolated from a GBM-derived secondary cell line were applied to the system. The
308 EVs were characterized by NTA and immunogold TEM, and subsequently added to the tumorspheres on day
309 1. The administrated EVs ranged in size from ~50 – 350 nm with most of the EVs being around 150 nm
310 (Figure 5A). NTA measurements on EV CTRL (EVs isolated from non-conditioned medium) did not yield
311 enough events for analysis, thus were regarded as being EV-depleted. The tetraspanin proteins CD9, CD63
312 and CD81 are among the most widely used EV markers, and to validate that the isolated EVs used in this
313 study were in fact EVs, we performed immunogold staining with a cocktail of monoclonal antibodies against
314 these three types of tetraspanins followed by morphological assessment using TEM. The antibody-gold
315 nanoparticle complexes showed an association to the outer membrane of the particles, indicating that the
316 particles were positive for one or more of the tetraspanins and thus confirming that they were EVs (Figure
317 5B, Figure S6). The tumorspheres that received GBM EVs showed a significant increase in area compared to
318 all the controls (Figure 5C-D). The GBM EVs increased GBM cell migration by more than 30 % compared to
319 both NTC and EV CTRL (EVs isolated from non-conditioned medium). TGF- β 1 was included as a simple
320 positive control but did not enhance the migration of the cells in our setup. These results indicate that EVs
321 isolated from a secondary GBM cell line could significantly stimulate GBM tumorsphere migration *in vitro*.
322 Thus, it was demonstrated that both stimulatory and inhibitory effects on GBM cell migration could be
323 measured using this model.

324 ***INSERT FIGURE 5***

325 **DISCUSSION**

326 The highly invasive behavior of GBM limits therapeutic efficacy of current treatment strategies, which is
327 substantiated by the almost ubiquitous occurrence of relapse [24]. Only subtle progress in patient
328 prognosis has been made during the past two decades with a two-month increase in median survival by the
329 addition of temozolomide to the treatment regimen [25]. The cells that most frequently invade the
330 surrounding brain parenchyma and migrate far away from the tumor core (or primary tumor) have been
331 shown to possess stem-like properties [26–28]. Understanding the invading and migrating GBM cells
332 potentially harbors an avenue for improving treatment and therefore patient prognosis. Here, we
333 presented a quantitative migration model based on GBM tumorspheres for assessment of cancer inhibiting
334 or stimulating substances.

335 Generally, the study of GBM invasion and migration dynamics and the effects of different
336 treatments on this property *in vitro* is limited by the model cell line and the assay of choice. Many different
337 quantitative cell invasion and migration assays exist, including the wound-healing assay, transwell assay,
338 cell exclusion assay, and fence (or ring) assay [4]. One feature is common for these assays; the cells are
339 often conveniently grown as an adherent cell monolayer, typically with the addition of serum to the culture
340 medium. Tumorspheres on the other hand are grown in absence of serum and preferentially in stem cell-
341 promoting medium, which can induce and maintain a cellular heterogeneity within tumorspheres [29].
342 Tumorspheres can be generated from established cell cultures that are usually grown as a monolayer after
343 a period of weaning or from primary cell lines directly isolated from tumor tissue [30,31]. The drawback of
344 using monolayer cells in such an assay is that the cells might already have gone through a harsh selection
345 process immediately after isolation, i.e. the selection of mesenchymal-like cells based on adherence, and
346 might therefore not represent intercellular heterogeneity as well as tumorspheres from primary cells would
347 do [29]. For example, drug resistance is different in cells grown either in 2D or 3D cultures, where the 3D-
348 cultured cells appeared to be more resistant in the study by *Imamura et al.* [30]. Here, they used adherent
349 cells as a 2D culture and induced non-adherent tumorspheres from the same cells to produce a 3D culture,
350 which indicates that the 3D organization of the cells could play a role in drug response [30]. In this study,

351 we presented a GBM tumorsphere migration model using primary GBM cells isolated under tumorsphere-
352 inducing conditions. We used the Matrigel-derived ECM Geltrex as the migration matrix of choice due to its
353 complex composition, the fact that it is hESC-qualified and has a reduced concentration of growth factors.

354 The cellular heterogeneity in our tumorsphere model showed a crude differential phenotypic
355 gradient of cells based on their location in the tumorsphere. Nestin and vimentin were found to be highly
356 expressed in the tumorsphere periphery, whereas GFAP was expressed both in the core and periphery.
357 However, the structure of GFAP in the periphery appeared fragmented, which could indicate an ongoing
358 degradation of GFAP and hence a cellular phenotype shift from GFAP-positive towards nestin/vimentin-
359 positive [32]. This reduction in GFAP expression was also reflected, when quantitatively comparing the core
360 and peripheral regions of the tumor. We further showed a similar distribution of nestin/GFAP staining in
361 free-floating tumorspheres *in vitro* and *in vivo* in a mouse intracranial xenograft setup using the same
362 primary GBM cells. Nestin has for a couple of decades been known as a multi-lineage progenitor marker
363 and was in embryonic stem cells shown to be expressed in the progenitor 'transition' period and then
364 turned off when cells fully differentiated [33,34]. Similarly, glial progenitor cells were nestin-positive and
365 GFAP-negative, but at the end of cellular differentiation, GFAP had replaced nestin [35]. Nestin has further
366 been associated with a migratory phenotype, where it facilitates migration of neural stem cells and directs
367 inflammatory cell migration in atherosclerosis [36,37]. In cancer, nestin expression is generally associated
368 with cancer stem-like cells, and more specifically in GBM, nestin has been shown to be useful for
369 identifying migrating tumor cells [38,39]. Downregulation of nestin demonstrated a reduction of
370 tumorsphere formation and tumor size *in vivo*, and overexpression results in increased cell growth,
371 tumorsphere formation and cell invasion [40]. However, the opposite has also been reported, where
372 downregulation of nestin increased matrix degradation and pFAK localization to focal adhesions for
373 increased prostate cancer cell invasion, which could indicate functional differences between different types
374 of cancer [41]. In the case of human GBM tumors, nestin is expressed in the tumor periphery and in the
375 invading tumor cells [42]. *Munthe et al.* reported nestin-positive cells both in the core and periphery of

376 human GBM tumors, and showed that the same distribution could be seen in a GBM xenograft model,
377 using the same cells (T78) as in our study [27,28]. This could indicate that our *in vitro* tumorsphere
378 migration model shows a crude similarity to human GBM tumors, thus demonstrating a biological relevance
379 for our tumorsphere model in drug screening and cellular responses to the drugs used.

380 We demonstrated that oxaliplatin could reduce tumorsphere migration by more than two-
381 fold, and by encapsulating oxaliplatin in stealth liposome these therapeutic effects were reduced. The cell
382 repulsion effects of stealth liposome formulations can thus be reliably assessed in this model even after a
383 period of four days as shown here, which could indicate that this migration model could provide a useful
384 tool for researchers working on various drug delivery systems [43]. The model could also be used to
385 visualize stimulation of GBM tumorsphere migration by adding EVs harvested from a GBM cancer cell line.
386 The EV field is rapidly expanding with thousands of new publications each year ranging from basic biology
387 to drug delivery. Models, such as the one presented here, could potentially contribute to elucidating
388 functional effects of both engineered EVs for drug delivery and specific biological populations of EVs, since
389 several studies have shown that parts of the functional cell-cell communication in GBM happens via EVs
390 [44–47]. In addition to the quantitative assessment of EVs, the EVs secreted from the cells in such a setup
391 can be isolated and analyzed with a potential minimum of serum-derived contaminants as they are grown
392 under serum-free conditions, which might help with overcoming a technical barrier in EV analyses [48].

393

394 *METHODOLOGICAL CONSIDERATIONS*

395 Although we do not directly demonstrate a high-throughput model, a few protocol alterations could easily
396 make it high-throughput for GBM drug screening. We used 24-well plates and manually picked single
397 tumorspheres and seeded into the wells, but this process could be replaced by a limiting dilution of
398 tumorspheres into 96-well plates. We manually acquired images of the tumorspheres and this could be
399 optimized by acquiring an automated image station such as IncuCyte (Essen Bioscience) or Celigo
400 (Nexcelom Bioscience). *Vinci et al.* demonstrated a high-throughput 3D GBM tumorsphere invasion assay

401 using such an image station with automated quantitation of invasion [49]. To further enhance cellular
402 complexity of our model, GBM organoids could be used. Development in the field of organoid research is
403 accelerating and several techniques and models within the GBM field are emerging, showing much more
404 cellular complexity than tumorspheres [50,51]. However, generation of organoids takes longer time (up to
405 several months) and thus serves as rate-limiting for the use in high-throughput drug screens [50]. In
406 between the convenience of monolayer cultures and the lengthy process of organoid generation,
407 tumorspheres might present an acceptable middle ground with both convenient culturing and sufficient
408 complexity.

409

410 CONCLUSIONS

411 In conclusion, we presented a GBM tumorsphere migration model with intercellular heterogeneity, which
412 might provide a relevant *in vitro* model for drug response evaluation. The cellular organization and
413 complexity of cancers are hard to reproduce *in vitro* for high-throughput drug screening and drug response
414 evaluation, but we believe that tumorsphere migration models such as presented here could be an
415 important step towards more accurate drug screening prior to evaluation in expensive pre-clinical animal
416 models. The research in this field is fortunately accelerating with more advanced cell models and
417 equipment for better analysis.

418

419

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428 **CONFLICT OF INTEREST**

429 The authors declare no conflict of interest.

430 **SUPPLEMENTARY INFORMATION**

431 Supplementary information is available at the journal's website.

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552

553

ACCEPTED MANUSCRIPT

554 **FIGURE LEGENDS**

555 **Figure 1:** Intra-tumorsphere cellular heterogeneity. (A) Immunofluorescence stainings for nestin or
556 Vimentin (green) and GFAP (red). Images show nestin and Vimentin expression most prominent in the
557 tumorsphere periphery and GFAP expression mostly from the tumorsphere core with GFAP-positive
558 filaments stretching from core to periphery (arrows). Scale bars: 20 μm . (B) Overlapping
559 immunofluorescence images acquired to visualize differences in nestin/GFAP expression based on cellular
560 location. The periphery shows filamentous nestin distribution and non-filamentous (or fragmented) GFAP
561 distribution, which was also seen within the nucleus. Closer to the core, where a double cell layer was
562 observed, the bottom cells appeared GFAP-positive/nestin-negative and the top cells appeared nestin-
563 positive/GFAP-negative. Yellow stippled line approximately indicates the transition zone. Arrows indicate
564 examples of filamentous GFAP. Scale bars: 20 μm . (C) Zooms on orthogonal regions from both periphery and
565 core, which shows fragmented and intra-nuclear GFAP localization in the periphery and filamentous
566 cytosolic GFAP in the core. In the periphery, arrows indicate fragmented GFAP inside nuclei and in the core,
567 arrows show nuclei free of GFAP. (D) Quantification of GFAP:Nestin ratio between tumorsphere core and
568 periphery. Data is presented as Mean + SD from four separate images.

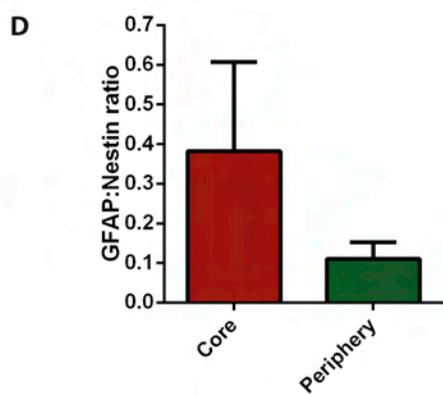
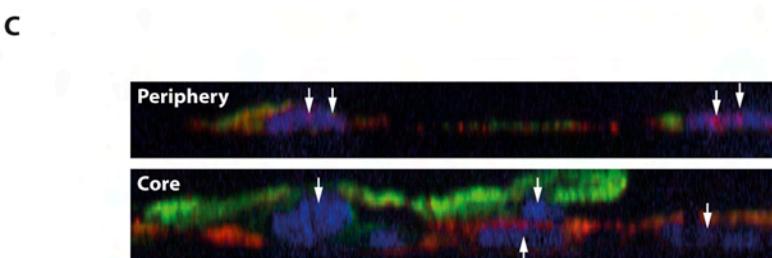
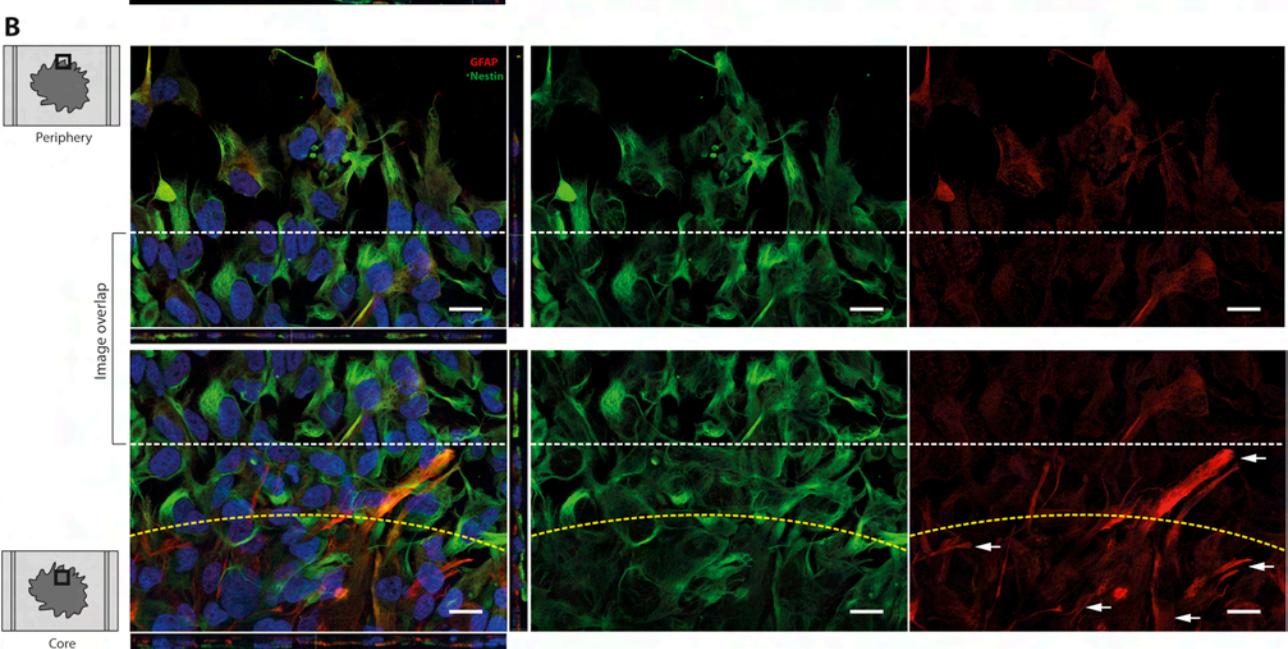
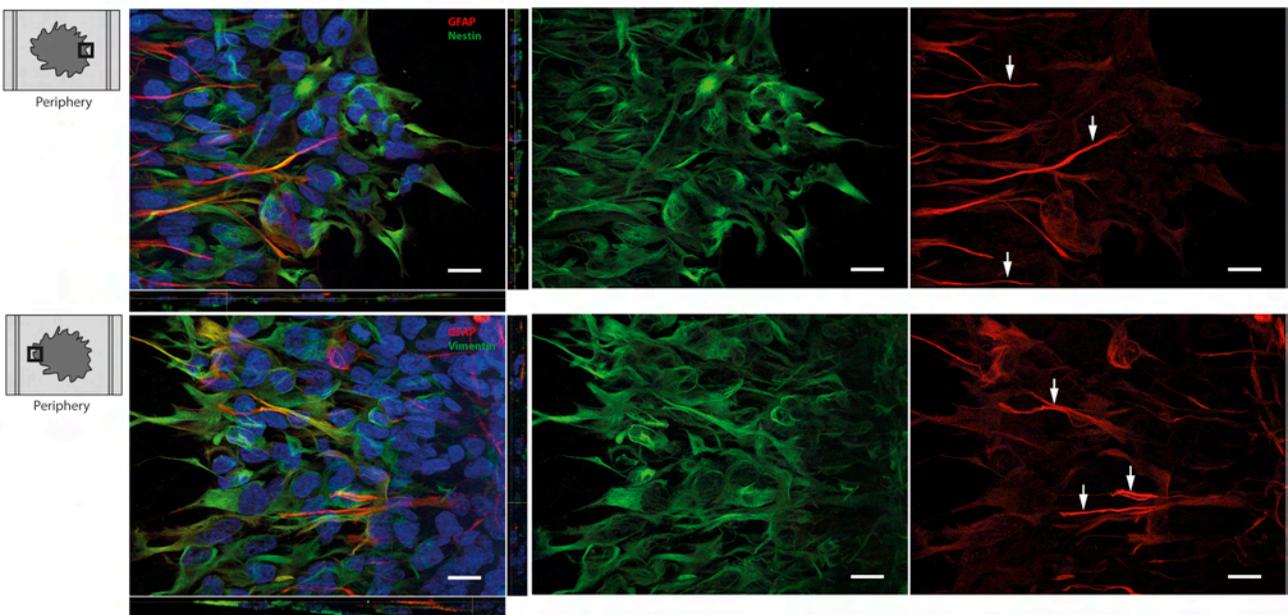
569 **Figure 2:** Nestin/GFAP distribution in free-floating tumorspheres. (A) Immunofluorescence of nestin (green)
570 and GFAP (red) showed most of the cells being nestin-positive and fewer cells GFAP-positive. Scale bar: 50
571 μm (B) Estimation of area coverage for each signal in percent using threshold analysis. Total nestin
572 coverage was around 66 % and GFAP total coverage around 10 %.

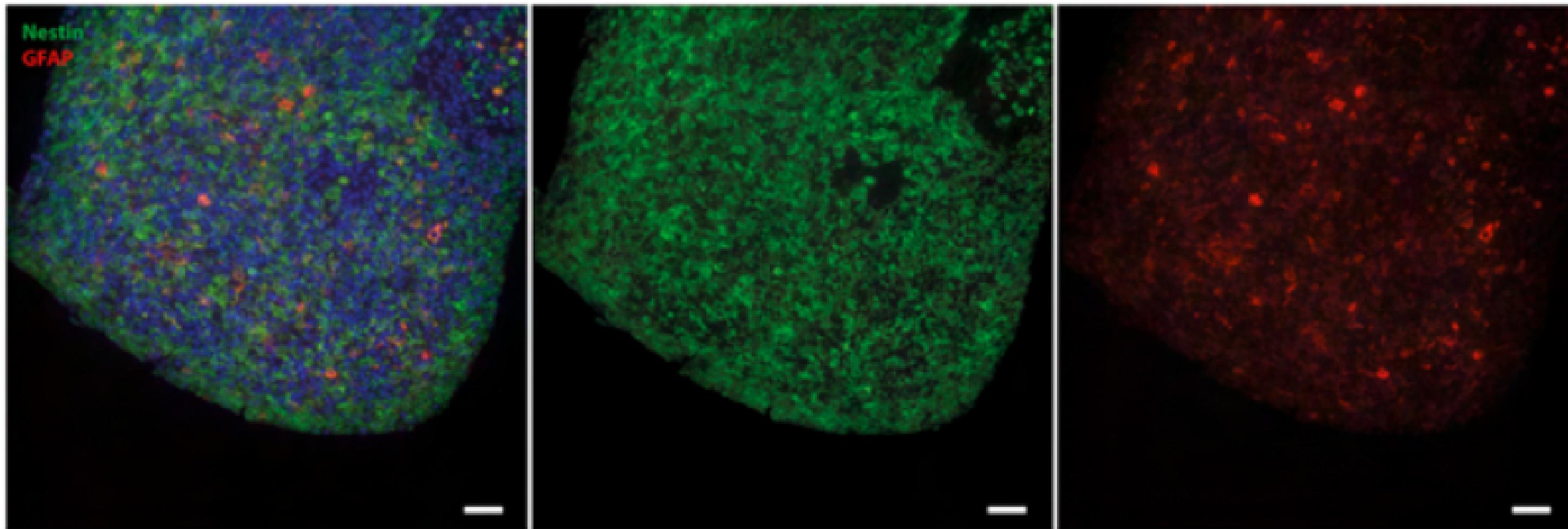
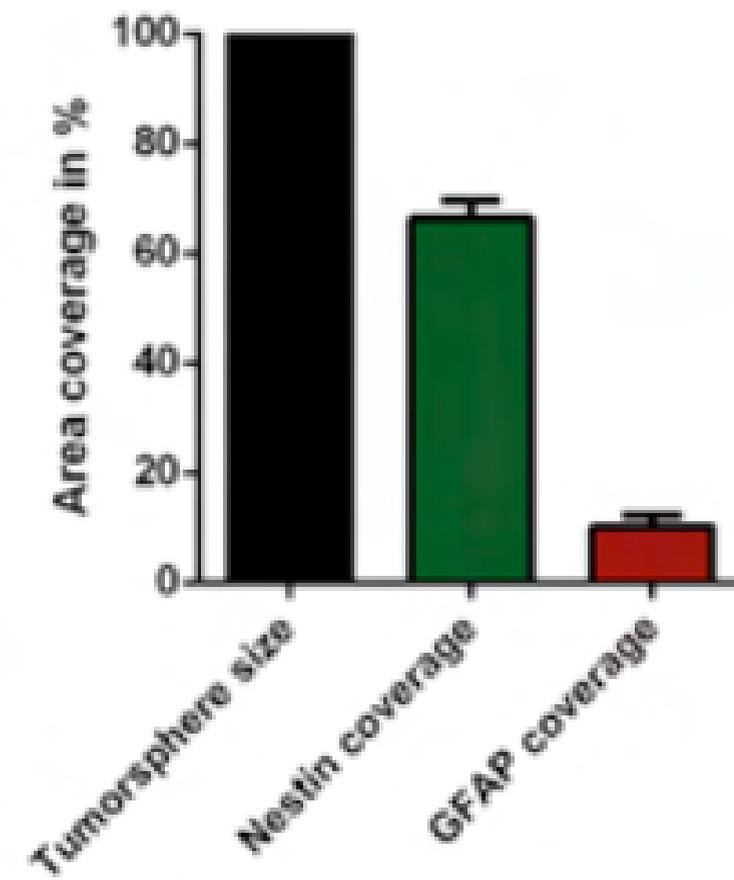
573 **Figure 3:** Distribution of nestin/GFAP in a GBM mouse xenograft model using the same cells. (A)
574 Fluorescence slide scanning of whole brain slices stained with GFAP (green) and nestin (red). Image show
575 both nestin and GFAP expression in the tumor core, but peripheral cells appear only nestin-positive (see
576 asterix). Scale bar: 1 mm. (B) MRI of mouse brain showing the tumor just prior to sacrificing the mouse.
577 Image shows that the fluorescent stainings were done on sections from the middle of the tumor (more

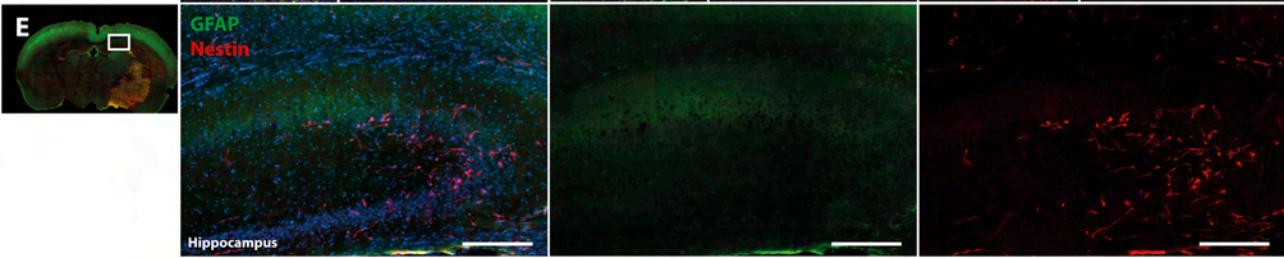
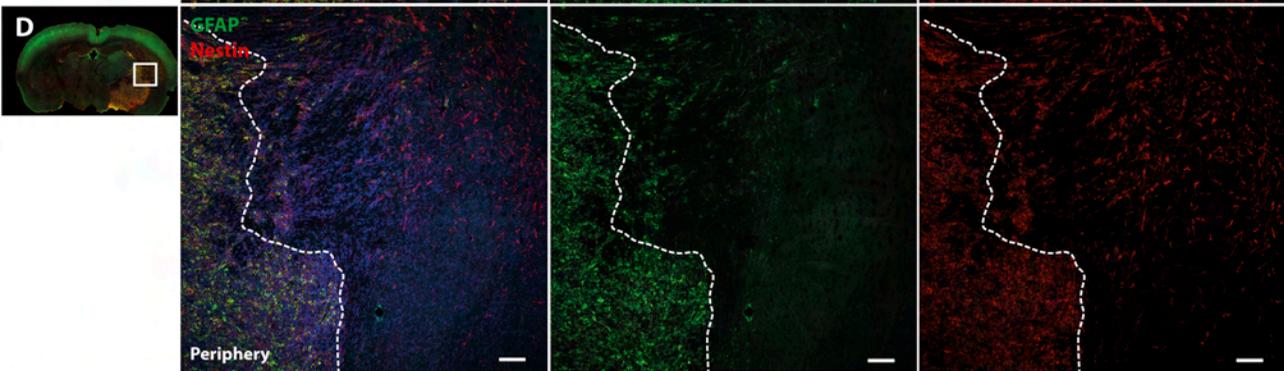
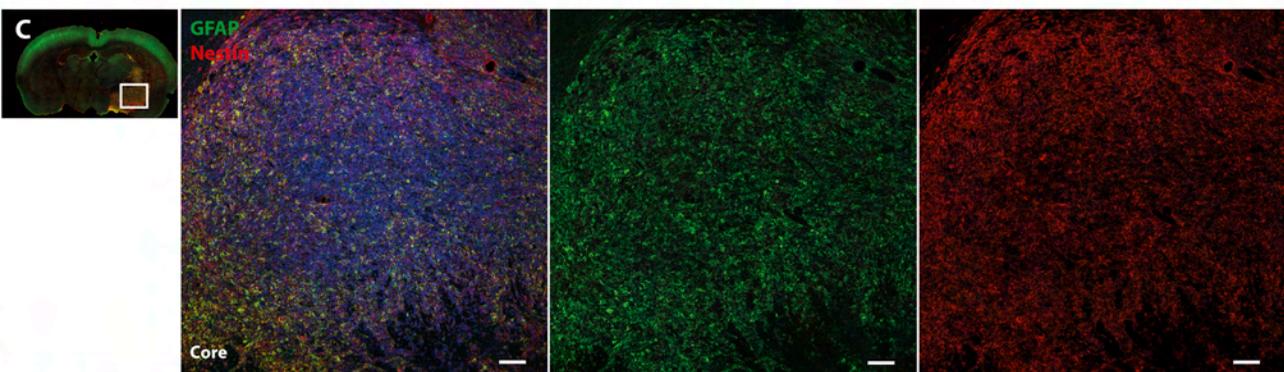
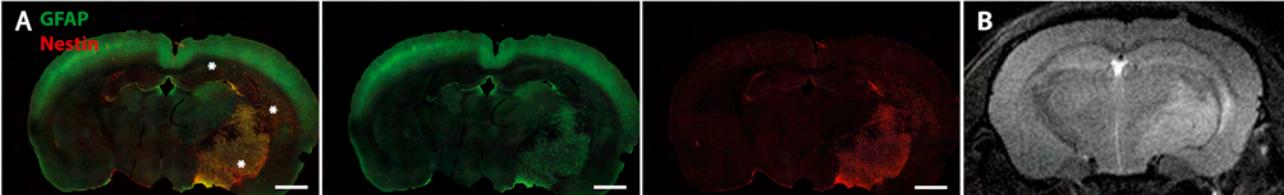
578 details in Figure S1C). (C) Fluorescence laser-scanning confocal image of from the tumor core showing both
579 nestin and GFAP-positive cells. Scale bar: 50 μm . (D) Fluorescence laser-scanning confocal image of the
580 tumor periphery showing the tumor cells farthest from the tumor being nestin-positive and GFAP-negative.
581 Scale bar: 50 μm . (E) Fluorescence slide scan zoom-in on frontal superior hippocampal formation showing
582 nestin-positive/GFAP-negative tumor cells. Scale bar: 200 μm .

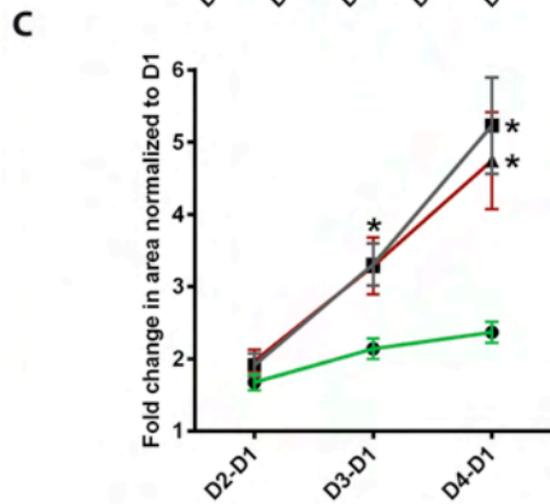
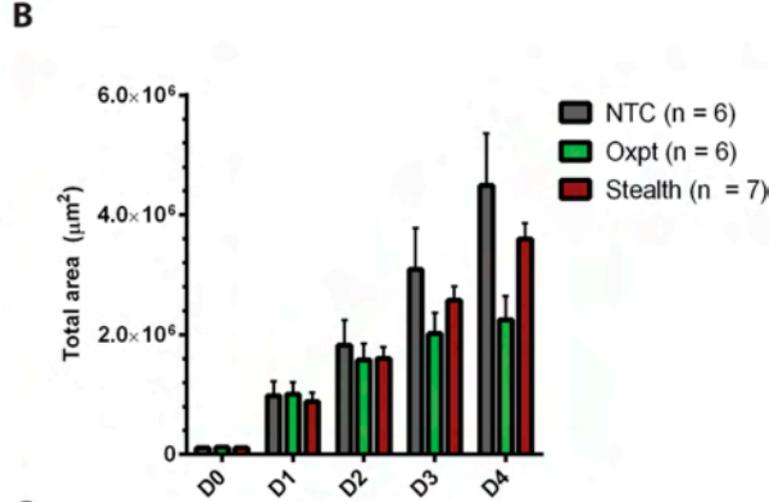
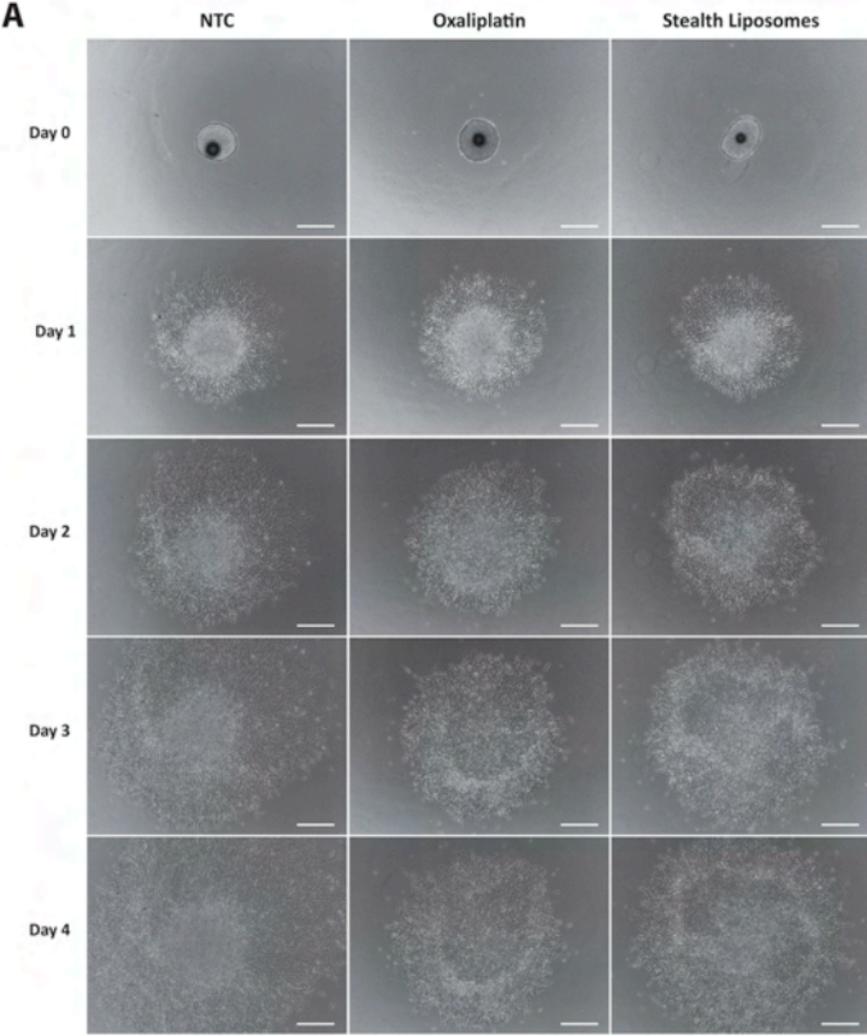
583 **Figure 4:** Inhibition of tumorsphere migration with Oxaliplatin. (A) Daily phase-contrast images of
584 representative tumorspheres from each group visualizing the difference in area of migration after
585 treatment initiated on D1. Scale bar: 400 μm . (B) Bar chart of total tumorsphere area measured on each
586 day. (C) Tumorsphere migration normalized by applying a fold-change from each day after treatment (D2-
587 D4) to the day of treatment (D1). Normalized data is presented as mean \pm SEM. $P = 0.03 - 0.05$ on D3-D1, P
588 $= 0.002$ on D4-D1.

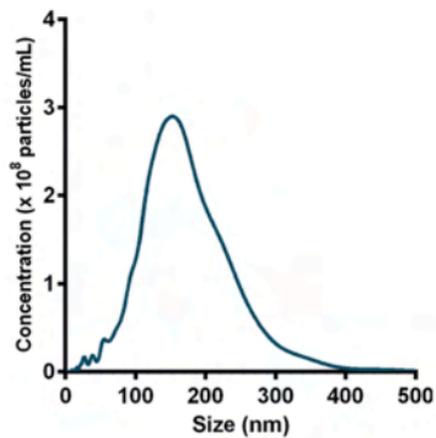
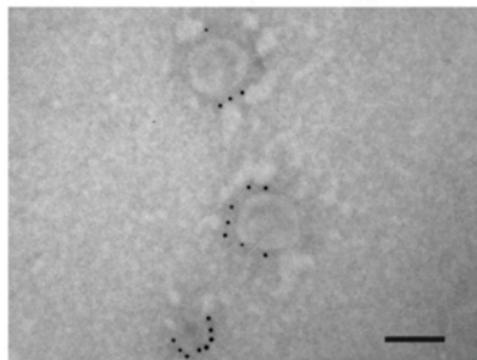
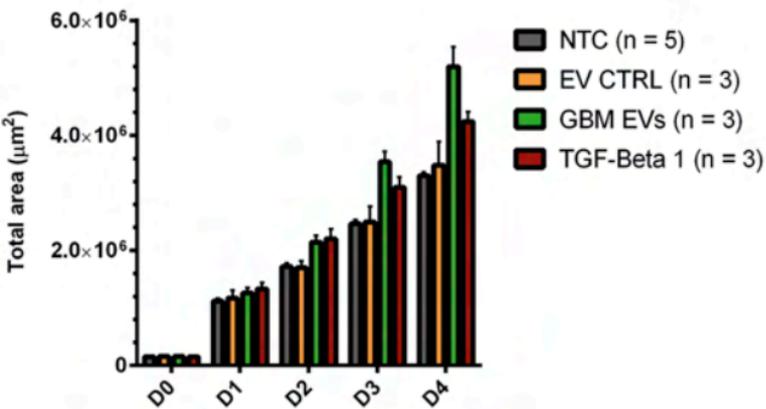
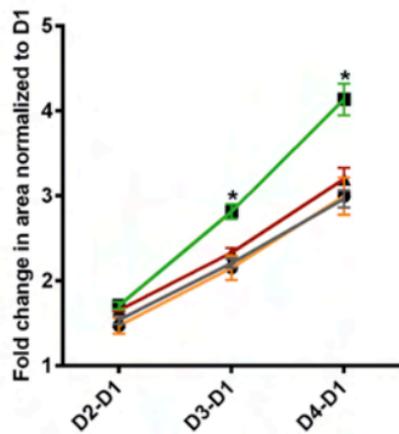
589 **Figure 5:** Stimulation of tumorsphere migration with GBM-derived extracellular vesicles. (A) Size
590 distribution of EVs measured with NTA. (B) characterization of EVs by immunogold TEM using a cocktail of
591 antibodies against the tetraspanins CD9, CD63 and CD81. Scale bar: 100 nm. (C) Bar chart of total
592 tumorsphere area measured on each day. (D) Tumorsphere migration normalized by applying a fold-
593 change from each day after treatment (D2-D4) to the day of treatment (D1). EV CTRL consisted of EVs
594 isolated from non-conditioned medium and TGF- β 1 was included as a positive migration control, however,
595 it did not induce any significant effects. Normalized data is presented as mean \pm SEM. $P = 0.002 - 0.02$ on
596 D3-D1, $P = 0.002 - 0.02$ on D4-D1.



A**B**





A**B****C****D**

HIGHLIGHTS

- Intratumoral heterogeneity is present in complex primary GBM tumorspheres *in vitro*
- Heterogeneity is visualized as a function of migration by differential distribution of nestin/vimentin and GFAP between core and periphery *in vitro* and *in vivo*
- Patient-derived GBM tumorspheres are promising for use in drug screens and studies of GBM biology *in vitro* and *in vivo*