Human Glioma Endothelial Cells With Stem cell properties

Aman Sharma (amanstemcell@gmail.com)
ExoCan Healthcare Technologies Pvt Ltd
Dattatraya Muzumdar
Seth G.S. Medical College, KEM Hospital
Anjali Shiras
National Centre for Cell Science (NCCS), SP Pune University Campus

Research Article

Keywords: Glioblastoma, Endothelial Cells, Glioma Stem Cells, Heterogeneity, Primary culture

Posted Date: December 28th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2279000/v1

Abstract

Glioblastoma (GBM) tumor relapse is attributed to presence of therapy defying Glioma stem cells (GSCs). GSCs have been shown to trans-differentiate into endothelial-like cells. However, the influence of tumor micro-environment on endothelial cells is not clearly understood. Here, we investigated whether tumor micro-environment conditioning can alter endothelial cell phenotype and endow them with stem cell-like properties. For this, we establishing a battery of primary human glioma endothelial cell cultures (hGECs) and characterized them for purity in multiple in vitro, in vivo assays. Our data shows that hGECs harbored stemness and multi-lineage differentiation potential as assessed in serum free growth assay, sphere forming assay, limiting dilution assay, and in a serum-induced differentiation assay where Nestin and CD31 co-expressing hGECs could spontaneously differentiation into GFAP positive cells. Moreover, immunohistochemistry analysis of human GBM tumors showed that tumor vessel regions expressed two key stem cell specific markers Nestin and Mushashi. Together, our data shows that tumor-specific endothelial cells are enriched with stem cell properties in GBM.

Introduction

Glioblastoma (GBM) classified as grade IV gliomas are one of the most aggressive and lethal tumors of the Central Nervous System (CNS) with median survival of less than 15 months[1–4]. Cancer stem cell hypothesis in GBM suggest that these tumors are driven by a small subpopulation of cells or glioma stem cell (GSCs) which are endowed with self-renewal properties and elevated DNA damage response pathways to prevent therapeutic insult [2, 5–13]. Interestingly, it was reported that GSCs can switch their phenotype and acquire endothelial cell (EC) functions to protect GBM tumors from antiangiogenic therapies[14, 15]. Considering that fact that GBM is a highly angiogenic tumor, multiple antiangiogenic approaches have been developed however, their successful clinical translation has been limited owing to heterogeneity of GBM tumors [16–21]. Of note, phenotypic functionality of GSCs is maintained in a closed interaction with tumor specific ECs and these localized regions are called perivascular niche. Taken together, it is evident that GBM aggressiveness and angiogenesis is dependent on tight association between GSCs and ECs.[5, 7, 8, 22–24]. While it is reported that cellular plasticity of GSCs allow them to self-create a niche if required by transdifferentiating into endothelial-like cells [15, 25], not much is known if the same is true for GBM-specific endothelial cells as well. Although, endothelial cells are considered as highly specialized, terminally differentiated cell types with tissue specific heterogeneity [26–30], their cellular plasticity cannot be ruled under specific conditions [31, 32]. In this paper, for the first time we provide evidences that human glioma endothelial cells (hGECs) are multipotent cell types which can sustain serum free growth, display stemness features, and spontaneously differentiate into GFAP positive astrocytic linage under specific growth conditions. Using a novel cell culture strategy, we selectively enriched tumor vascular fragments (VFs) from GBM tumor biopsies and expanded them *in vitro* to obtain hGEC lines which were grown under chemically defined culture system. These hGEC cultures over multiple generations efficiently mimicked GSC phenotype. Our report suggest a new axis of cellular

plasticity in GBM tumors which can re-define GSC-niche concept and allow development of new therapeutic approaches.

Material And Methods

Glioblastoma Derived Endothelial Cultures

The study was approved by the Ethics Committee for Research on Human Subjects (ECHRS) of King Edward Memorial (KEM) Hospital & Seth G S Medical College, Mumbai (approval no: EC/GOVT-2/2012) and a written consent from patients was obtained as a part of this study. Briefly, tumor biopsies were minced finely and mixed with dextran (30%) followed by centrifugation at 10,000 rpm to obtain microvascular fragments (MVFs). MVFs were enzymatically digested in 2% dispase solution for 1–2 hours at 37^0C and enzyme was neutralized in RPMI medium with 10% FCS followed by centrifugation at 2000 rpm. The cells were then seeded on to fibronectin (Sigma, USA) (5 μg/ml) coated plates in RPMI-1640 medium supplemented with 2% FCS and 50 μg/ml Endothelial Cell Growth Supplement (ECGS) (Millipore; Burlington, MA, USA).

Flow cytometry

Confluent hGEC cultures were harvested and washed twice with PBS. Cells were blocked with 5% BSA followed by incubation with respective primary antibodies “e.g. VE-Cadherin (1:100), CD31 (1:50) and Tie2 (1:50)” for 1 hour at room temperature. Cells were washed in PBS buffer thrice. Next, cells were incubated with Alexa-flour 488 tagged anti-mouse (for VE-cadherin), anti-rabbit (for CD31) and anti-goat (for Tie2) secondary antibodies for 40 minutes and washed in PBS. Stained cells were acquired on BD FACS Calibur (San Jose, CA, USA). . For cell cycle analysis, 1.5 x 10^6 hGECs were fixed in 70% ethanol and pelleted at 1200 rpm. Fixed hGECs were incubated with 50 µl of Propidium Iodide (PI)(1mg/ml stock concentration) for 30’ in dark and were acquired on a FACS Calibur instrument using a 488 nm excitation laser. Cell cycle analysis was performed by BD CellQuest Pro software. For sorting, hGEC 1 culture was harvested and stained for CD31 (1:50 dilution). BD FACS Aria was used to sort 1000 CD31 positive cells in each well of a 96 well plate for sphere formation assay. Bulk CD31+ hGEC1 cells were sorted in a 15ml falcon tube and used in other assays. Analysis was performed in FACS Diva software

Immunocytochemistry

1X10^4 hGECs were grown in 8 well glass chamber slides and spheroids were cytospun for analysis. Both preparations were fixed with 4% formaldehyde for 10 min at RT and permeabilized with Triton X-100 (0.25% v/v) for 2 min followed by blocking in BSA for 1 hour. Samples were incubated with respective primary antibodies- Ki67 (abcam, UK) Nestin, CD31, GFAP (Sigma, USA) at RT for 1 hour followed by PBS washing and incubation with Alexa Fluor 594 goat anti-rabbit-IgG (Invitrogen, Germany) in PBS for 1 hour. Nuclei were counterstained with DAPI and examined under a confocal microscope (Carl Zeiss AG, Germany).

Immunohistochemistry

The 5-micron paraffin sections of human GBM were de-paraffinized in xylene and rehydrated in graded alcohol series starting with 100%, 95%, and 75% for 5’ each. The sections were then washed and heated in an oven in citrate buffer (10 mM, pH 6) for CD31, Nestin, Ki67, and Mushashi for 15 min for antigen retrieval. Next, slides were incubated with 5% BSA in PBS for 1 hour followed by incubation at room temperature for 2 hours with primary antibodies to CD31 (1:50 dilution), Ki67 (1:100 dilution), Nestin (1:100 dilution), and Mushashi (1:100 dilution) followed by three washes with TBST buffer (Tris buffered saline, pH 7.4, with 1% Tween-20). For CD31 and Mushashi staining slides were incubated for 40 minutes with anti-mouse Alexa Flour 488 (1:100) and for Nestin, Ki67 with anti-rabbit Alex-flour 488, 594 (1:100 each) respectively. Sections were washed thrice with TBST buffer 5’ each followed by counterstaining of nuclei with DAPI for 2 minutes. Slides were imaged in confocal microscope (Carl Zeiss AG, Germany).

RT-PCR

For expression analysis of mesenchymal genes, 100ng of hGEC cDNA was amplified using Taq DNA polymerase enzyme in a 10 µl reaction volume comprising of RT buffer (1X, 1 µl), Nuclease free water (6.5 µl), dNTPs (.25 µl), primers (.5 µl each), and 1 µl cDNA. The RT-PCR products were analysed using in agarose gel electrophoresis. The list of primers used are provided in Table I.

Quantitative Real-time PCR

RNA was harvested from hGECs using Trizol reagent (Invitrogen, USA). cDNA from was prepared from 2 µg of RNA using AMV RT system (Promega, USA) in a total of 20 µl. *Real-Time* qRT-*PCR* was performed in 7500 Fast Real Time PCR system using SYBR Green PCR Master Mix (Applied Biosystems, USA) in a 10 µl reaction containing 5 µl of SYBR Green Master Mix, 0.25 µl forward and reverse primers, and 100 ng cDNA. Relative fold changes in gene expression were quantified using comparative Ct method.

Sphere formation Assay

Three hGEC cultures were seeded on to low attachment plates at cell densities of 100, 200, 300, 400, 500, and 1000 cells per well in a 96 well plate in GSC medium DMEM/HF12, bFGF 10ng/ml, FGF 20 ng/ml, B27 1X (Invitrogen, USA) and Penicillin Streptomycin (1X). After 7 days, neurospheres were imaged, and analyzed under phase contrast microscope. Statistical analysis was performed in GraphPad PrismTM software (San Diego, USA)

Serum Induced Differentiation of Spheres and adherent hGEC culture

hGEC spheres were harvested by centrifugation at 800 rpm for 2 min. Spheres were seeded in a chamber slide in total of 100 µl serum containing medium (DMEM/H12 with 10%FBS) and incubated for 5 days to allow differentiation. After 5 days, differentiated spheres were fixed in 4% para-formaldehyde and stained for differentiation marker GFAP as described earlier. Flow cytometry sorted CD31+ hGEC cells (hGEC1) were grown for 5 days in 10% FBS containing medium and stained for GFAP.

Matrigel Tube Formation Assay

Tube formation assay was performed as described. Briefly, in a 96 well plate 100 µl of growth factor reduced Matrigel (BD Biosciences) was added. $1X10^5$ cells were re-suspended in 100 µl endothelial cell culture medium (RPMI-1640+ 2%FCS+ 50 µg/ml ECGS) and placed on top of solidified Matrigel. After 14-16 h images were acquired. Analysis was performed in ImageJ (NIH, USA) software

Matrigel Plug Assay

A total of $1X10^4$ hGECs were re-suspended in 200 µl of DMEM and growth factor reduced Matrigel™ (1:1 ratio). The cell suspension was injected subcutaneously into right flank of SCID mice as a plug. The plugs were collected after 7 days, fixed, stained with H&E and imaged.

Statistical Analysis

The statistical analysis was performed using GraphPad Prism 5 software. Multiple comparison analysis was performed using two-way ANOVA test. *P* values are calculated from three independent experiments (p=****, <0.0001; p=***, <0.001, p=**, <0.01; p=*, <0.05).

Results

Enrichment, characterization of hGECs:

Generally, hGECs are enriched from digested tumor tissues either using CD31 coated magnetic beads or by flow cytometry [33, 34]. However, non-vascular cell types may also express CD31 protein which can potentially contaminate hGEC preparations leading to impure cultures [15, 35] (Supp. Fig 1). Here, we describe a novel strategy for establishing hGEC cultures which is based on selective enrichment of vascular fragments (VFs) from digested GBM tissues (Fig 1A). hGEC colonies were established from sprouting VFs and expanded in monolayer (Fig 1B). hGECs displayed cobblestone morphology and contact inhibited growth pattern in monolayer culture confirming their purity and retention of EC phenotype. hGEC characterization in a flow cytometry assay showed expression of classical endothelial markers including VE-cadherin,Tie2, and CD31 (Fig.1C). Further, we performed quantitative real time PCR analysis of four representative hGEC cultures (hGEC1, hEC2, hGEC3, hGEC4) to checked the expression of endothelial specific genes NRP1/2 and Tie2 w.r.t. normal brain endothelial cells (NBEC; established from epileptic human brain tissues). Indeed, we could detect transcripts of these genes in all established hGEC cultures (Fig. 1D). Interestingly, the data indicated that there was a decrease in EC marker expression in all hGEC cultures w.r.t. NBEC culture. Subsequently, we checked whether tumor EC specific genes (TEM1, TEM8) were also expressed in hGEC cultures [36] (Supp Table 1). Our data showed that both were up regulated in hGEC cultures (Fig. 1E). Collectively, our data demonstrated that we could establish long term hGEC cultures which expressed tumor EC specific genes.

hGEC cultures are proangiogenic

Next, we functionality characterized hGEC cultures by performing Matrigel tube formation assay. hGEC cultures rapidly assembled into tube-like networks of differentiated endothelial cells (Fig 2A). While all the hGEC cultures formed tube networks without the addition of exogenous VEGF growth factor, NBEC cultures made tubes only when VEGF was added into the growth medium. Analyses of tube structures revealed that hGECs made more number of tubes and branch points w.r.t. NBECs confirming their proangiogenic nature (Fig 2B).The data indicated that the GBM microenvironment conditioning of hGECs could perhaps render them with enhanced angiogenic potential. Moreover, it also indicates that a VEGF redundancy in GBM microenvironment programmed endothelial cells is possible. Next, we checked for *in vivo* angiogenic potential of hGECs by performing a Matrigel plug assay. Analysis of H&E stained *in vivo* plugs obtained from SCID mice showed glomeruloid assembly of endothelial cells which is a characteristic feature GBM vasculature (Fig 2C, magnified region, black & yellow arrow). Thus, our data confirmed that hGEC cultures were proangiogenic and functionally active both under *in vitro* and *in vivo conditions*.

hGEC cultures show stemness properties

It is known that GSCs can trans differentiate into endothelial cells to support tumor vasculature [37]. However, it is not known if hGECs could also manifest GSCs like properties. One of the fundamental properties of GSCs is their slow cycling nature. Interestingly, cell cycle profiling and Ki67 staining of hGEC culture showed that they are slow cycling cell types (Supp Fig 2). The data was substantiated by co-staining GBM tumor sections for Ki67 and CD31 which further confirmed that tumor vasculature cells were indeed slow cycling and had reduced Ki67 expression (Supp Fig 2). Similar to GSCs, hGECs were also able to survive and proliferate under serum-free conditions in Neurobasal medium, when cultured on fibronectin coated surfaces (Fig. 3A, suppl Fig 3). Of note, under similar conditions NBECs underwent apoptosis indicating GSC like properties in hGECs (Fig 5A). Importantly, hGECs monolayers appeared flat with tight cell-cell contacts, and expanded to form colony-like growth pattern even in absence of serum while retaining expression of CD31 protein (Fig 3B). Next, we investigated whether hGEC cultures also displayed colonogenic properties of GSCs under SF conditions. Endothelial cells are adherent cell types and till date have not been reported to grow in suspension conditions. Surprisingly, hGECs formed round, non-necrotic sphere-like floating structures which did not attach to culture vessels (Fig 3C). Again, NBECs did not survive and died in few hours (Fig 3C). To confirm whether such growth pattern was related to stemness features, we performed limiting dilution assay. For this, hGECs were grown for 5 days at various cell densities (100-1000 cell/well) in a 96 well plate and analysed for their colonogenic ability. Our data shows that hGEC cultures are heterogeneous in their colonogenic potential. Comparatively, hGEC1 culture was most clonogenic (31.66 spheres per 1000 cell/well) (Fig 3D) whereas the other two cultures - hGEC2 and hGEC3 did not make spheres at low cell seeding density (100 cell/well). In line, endothelial heterogeneity is a well-known feature of vasculature [29]. Likewise, we believe that hGEC cultures may be heterogeneous due to tumor-specific conditioning. Moreover, hGEC spheres also expressed stemness marker Nestin, however the expression was non-filamentous and less in intensity in all hGEC cultures tested (Fig 3E). It is to be noted that hGEC spheres retained CD31 expression under sphere culture conditions which further confirms their original EC nature. GSCs can undergo serum induced

differentiation by allowing expression of astrocytic marker GFAP. Similarly, we evaluated the multi-lineage differentiation potential of the hGEC spheres under serum induced differentiation condition. Indeed, hGEC spheres readily differentiated into GFAP positive cells when cultures for 5 days in serum conditions (Fig 3F). Finally, to check if stemness properties are inherent to hGECs and not *in vitro* culture induced due to the process of endothelial-mesenchymal transition, we checked expression of three known mesenchymal markers-Twist, Zeb1 and Zeb2 in hGEC cultures. Our data showed that compared to control (KW10, a mesenchymal glioma cell line), hGEC cultures showed only basal level expression of these genes (Fig 3G) ruling out this possibility.

Human GBM vasculature express stemness markers

It is shown that proliferative, but not mature vasculature expresses Nestin protein [38]. To test if human GBM (hGBM) vasculature also expresses stemness proteins, we performed immunohistochemistry for two known stemness markers- Nestin, and mushashi. Interestingly, we found more Nestin staining in vasculature (Fig 4A, arrow) compared to surrounding tumor cells (Fig 4A, encircled area). In all hGBM tissue studied, Nestin expression was predominantly restricted to tumor vasculature. Likewise, we found intense staining of Mushashi in vasculature (Fig 4A, arrow). However, unlike Nestin, neighbouring tumor cells also expressed Mushashi (Fig 4A). Together, our data indicated that tumor derived endothelial cells in GBM express stemness markers.

Discussion

Hyper-vascularization is a prominent feature of GBM tumors and hence anti-angiogenic therapies offer some treatment benefits to patients [39, 40]. Importantly, there is lack of in vitro tumor endothelial models to study novel anti-angiogenic molecules and human umbilical vein endothelial cells (HUVEC) are conventionally used for this purpose. However, the ECs in tumor endothelium have unique phenotypes and they greatly differ from HUVEC [41]. Here, we describe a novel EC isolation strategy which does not use any specific antibody-based assay. Using this novel method, we were able to successfully develop multiple (> 8) hGEC cultures from GBM biopsy tissue. Importantly, the hGEC lines were stable in culture and retained the EC phenotype. The expression of tumor endothelial markers (TEM1, TEM8) was evident in hGEC cultures which ensured their tumor origin. Moreover, hGECs were highly angiogenic and showed elevated migration potential and VEGF redundancy. It is reported that doubling time of brain tumor endothelial cells in culture is less than normal brain endothelial cells [34]. Similarly, we find that even in serum supplemented growth medium hGEC cultures are not fast growing. However, in GBM tumors, frequent endothelial cell proliferation is required for tumor growth [42]. To understand this paradox, we performed flow cytometry, confocal microscopy and GBM tissue section analysis. Unexpectedly, the hGEC cultures are slow dividing in nature. In line, vessel regions of human GBM tumor sections show weaker Ki67 reactivity in vessel-specific ECs which was confirmed by our findings. Under normal physiological conditions, ECs remain in a slow dividing or quiescent state, however upon activation of ‘angiogenic switch’, they tend to divide rapidly to promote angiogenesis [43]. Hence, in our analysis, GBM vessel ECs that stained positive for Ki67 may represent actively dividing ECs of neoangiogenic vessel regions.

Tumor ECs are known to acquire stemness markers and display multilineage differentiation potential [32, 41, 44, 45]. Similarly, our data indicated that hGECs have GSC-like features including stemness marker expression, slow cycling nature, and colonogenic abilities. Importantly, hGECs were also immune to apoptosis and continued to expand when grown in serum free conditions. Of note, hGEC colonies in these conditions retained CD31 expression and did not require exogenous VEGF for their growth. This indicates that hGECs are tumor conditioned cell types which can take up a GSC-like phenotype while retaining their own EC identity. Hence, the study reveals multipotent nature of GBM ECs and cellular plasticity of these tumors. Finally, we believe that understanding of hGEC complexity in GBM is critical for designing new anti-angiogenic drugs and our study could be the starting point in that pursuit.

Declarations

Authors' disclosures of potential conflict of Interest: The authors declare no conflict of interest.

Authorship: Conceptualization, data analyses, writing: AShiras, ASharma. Experimental work, data analyses, draft preparation: ASharma. Clinical grading of tissues: NG, DM.

Funding: This work was supported by the Department of Biotechnology, India Project Grant BT/PR4271/MED/31/145/2012.

Acknowledgements:

The authors acknowledge fellowship support of Council of Scientific and Industrial Research, New Delhi, India to ASharma. This work was supported through funding support from the Department of Biotechnology, Govt of India, India. Project Grant BT/PR4271/MED/31/145/2012 to A Shiras.

Data Availability

The published data in this manuscript is available from corresponding author upon request

References

1. T. Gupta, V. Nair, S.N. Paul, S. Kannan, A. Moiyadi, S. Epari, R. Jalali, Can irradiation of potential cancer stem-cell niche in the subventricular zone influence survival in patients with newly diagnosed glioblastoma?, Journal of neuro-oncology, 109 (2012) 195-203.
2. M. Jackson, F. Hassiotou, A. Nowak, Glioblastoma stem-like cells: at the root of tumor recurrence and a therapeutic target, Carcinogenesis, 36 (2015) 177-185.
3. C.R. Chokshi, N. Savage, C. Venugopal, S.K. Singh, A Patient-Derived Xenograft Model of Glioblastoma, STAR protocols, 1 (2020) 100179.
4. A. Turpin, A. Sharif, L. Stoven, S. Blond, C.A. Maurage, E. Le Rhun, [The stem cell niche in glioblastoma: from fundamental aspects to targeted therapies], Bulletin du cancer, 102 (2015) 24-33.

5. S. Alcantara Llaguno, L.F. Parada, Cancer stem cells in gliomas: evolving concepts and therapeutic implications, Current opinion in neurology, 34 (2021) 868-874.
6. I. Nakano, H. Saya, [Cancer stem cells in malignant glioma-the mechanism of cancer initiation and the therapeutic development], No shinkei geka. Neurological surgery, 38 (2010) 879-889.
7. K. Biserova, A. Jakovlevs, R. Uljanovs, I. Strumfa, Cancer Stem Cells: Significance in Origin, Pathogenesis and Treatment of Glioblastoma, Cells, 10 (2021).
8. D. Schiffer, L. Annovazzi, C. Casalone, C. Corona, M. Mellai, Glioblastoma: Microenvironment and Niche Concept, Cancers, 11 (2018).
9. Y. Shirakawa, K. Ohta, S. Miyake, A. Kanemaru, A. Kuwano, K. Yonemaru, S. Uchino, M. Yamaoka, Y. Ito, N. Ito, T. Hide, N. Shinojima, A. Mukasa, H. Saito, H. Jono, Glioma Cells Acquire Stem-like Characters by Extrinsic Ribosome Stimuli, Cells, 10 (2021).
10. N. Koike, R. Kota, Y. Naito, N. Hayakawa, T. Matsuura, T. Hishiki, N. Onishi, J. Fukada, M. Suematsu, N. Shigematsu, H. Saya, O. Sampetrean, 2-Nitroimidazoles induce mitochondrial stress and ferroptosis in glioma stem cells residing in a hypoxic niche, Communications biology, 3 (2020) 450.
11. C.M. Howard, J. Valluri, A. Alberico, T. Julien, R. Mazagri, R. Marsh, H. Alastair, A. Cortese, M. Griswold, W. Wang, K. Denning, L. Brown, P.P. Claudio, Analysis of Chemopredictive Assay for Targeting Cancer Stem Cells in Glioblastoma Patients, Translational oncology, 10 (2017) 241-254.
12. Y.C. Lim, T.L. Roberts, B.W. Day, B.W. Stringer, S. Kozlov, S. Fazry, Z.C. Bruce, K.S. Ensbey, D.G. Walker, A.W. Boyd, M.F. Lavin, Increased sensitivity to ionizing radiation by targeting the homologous recombination pathway in glioma initiating cells, Molecular oncology, 8 (2014) 1603-1615.
13. J. Wang, T.P. Wakeman, J.D. Lathia, A.B. Hjelmeland, X.F. Wang, R.R. White, J.N. Rich, B.A. Sullenger, Notch promotes radioresistance of glioma stem cells, Stem cells, 28 (2010) 17-28.
14. J. Dong, Y. Zhao, Q. Huang, X. Fei, Y. Diao, Y. Shen, H. Xiao, T. Zhang, Q. Lan, X. Gu, Glioma stem/progenitor cells contribute to neovascularization via transdifferentiation, Stem cell reviews and reports, 7 (2011) 141-152.
15. Y. Soda, T. Marumoto, D. Friedmann-Morvinski, M. Soda, F. Liu, H. Michiue, S. Pastorino, M. Yang, R.M. Hoffman, S. Kesari, I.M. Verma, Transdifferentiation of glioblastoma cells into vascular endothelial cells, Proceedings of the National Academy of Sciences of the United States of America, 108 (2011) 4274-4280.
16. K. Beal, L.E. Abrey, P.H. Gutin, Antiangiogenic agents in the treatment of recurrent or newly diagnosed glioblastoma: analysis of single-agent and combined modality approaches, Radiation oncology, 6 (2011) 2.
17. M.C. Chamberlain, Antiangiogenic blockage: a new treatment for glioblastoma, Expert opinion on biological therapy, 8 (2008) 1449-1453.
18. T.J. Yun, H.R. Cho, S.H. Choi, H. Kim, J.K. Won, S.W. Park, J.H. Kim, C.H. Sohn, M.H. Han, Antiangiogenic Effect of Bevacizumab: Application of Arterial Spin-Labeling Perfusion MR Imaging in a Rat Glioblastoma Model, AJNR. American journal of neuroradiology, 37 (2016) 1650-1656.

19. I. Arrillaga-Romany, A.D. Norden, Antiangiogenic therapies for glioblastoma, CNS oncology, 3 (2014) 349-358.
20. M.R. Gilbert, Antiangiogenic Therapy for Glioblastoma: Complex Biology and Complicated Results, Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 34 (2016) 1567-1569.
21. J. de Groot, D.A. Reardon, T.T. Batchelor, Antiangiogenic therapy for glioblastoma: the challenge of translating response rate into efficacy, American Society of Clinical Oncology educational book. American Society of Clinical Oncology. Annual Meeting, (2013).
22. A. Filatova, T. Acker, B.K. Garvalov, The cancer stem cell niche(s): the crosstalk between glioma stem cells and their microenvironment, Biochim Biophys Acta, 1830 (2013) 2496-2508.
23. G.N. Yan, L. Yang, Y.F. Lv, Y. Shi, L.L. Shen, X.H. Yao, Q.N. Guo, P. Zhang, Y.H. Cui, X. Zhang, X.W. Bian, D.Y. Guo, Endothelial cells promote stem-like phenotype of glioma cells through activating the Hedgehog pathway, J Pathol, 234 (2014) 11-22.
24. D. Schiffer, M. Mellai, E. Bovio, I. Bisogno, C. Casalone, L. Annovazzi, Glioblastoma niches: from the concept to the phenotypical reality, Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology, 39 (2018) 1161-1168.
25. A. Hormigo, B.S. Ding, S. Rafii, A target for antiangiogenic therapy: vascular endothelium derived from glioblastoma, Proceedings of the National Academy of Sciences of the United States of America, 108 (2011) 4271-4272.
26. K. Jiang, C. Pichol-Thievend, Z. Neufeld, M. Francois, Assessment of heterogeneity in collective endothelial cell behavior with multicolor clonal cell tracking to predict arteriovenous remodeling, Cell reports, 36 (2021) 109395.
27. L.M. Becker, S.H. Chen, J. Rodor, L. de Rooij, A.H. Baker, P. Carmeliet, Deciphering Endothelial Heterogeneity in Health and Disease at Single Cell Resolution: Progress and Perspectives, Cardiovascular research, (2022).
28. C. Wilson, X. Zhang, M.D. Lee, M. MacDonald, H.R. Heathcote, N.M.N. Alorfi, C. Buckley, S. Dolan, J.G. McCarron, Disrupted endothelial cell heterogeneity and network organization impair vascular function in prediabetic obesity, Metabolism: clinical and experimental, 111 (2020) 154340.
29. W.C. Aird, Endothelial cell heterogeneity, Critical care medicine, 31 (2003) S221-230.
30. B.J. Ballermann, Endothelial Cell Identity, Heterogeneity and Plasticity in the Kidney, Journal of the American Society of Nephrology : JASN, 31 (2020) 1-2.
31. D. Medici, E.M. Shore, V.Y. Lounev, F.S. Kaplan, R. Kalluri, B.R. Olsen, Conversion of vascular endothelial cells into multipotent stem-like cells, Nature medicine, 16 (2010) 1400-1406.
32. A.C. Dudley, Z.A. Khan, S.C. Shih, S.Y. Kang, B.M. Zwaans, J. Bischoff, M. Klagsbrun, Calcification of multipotent prostate tumor endothelium, Cancer cell, 14 (2008) 201-211.
33. S. Miebach, S. Grau, V. Hummel, P. Rieckmann, J.C. Tonn, R.H. Goldbrunner, Isolation and culture of microvascular endothelial cells from gliomas of different WHO grades, Journal of neuro-oncology, 76 (2006) 39-48.

34. C. Charalambous, F.M. Hofman, T.C. Chen, Functional and phenotypic differences between glioblastoma multiforme-derived and normal human brain endothelial cells, Journal of neurosurgery, 102 (2005) 699-705.
35. X. Mei, Y.S. Chen, F.R. Chen, S.Y. Xi, Z.P. Chen, Glioblastoma stem cell differentiation into endothelial cells evidenced through live-cell imaging, Neuro-oncology, 19 (2017) 1109-1118.
36. B. St Croix, C. Rago, V. Velculescu, G. Traverso, K.E. Romans, E. Montgomery, A. Lal, G.J. Riggins, C. Lengauer, B. Vogelstein, K.W. Kinzler, Genes expressed in human tumor endothelium, Science, 289 (2000) 1197-1202.
37. L. Ricci-Vitiani, R. Pallini, M. Biffoni, M. Todaro, G. Invernici, T. Cenci, G. Maira, E.A. Parati, G. Stassi, L.M. Larocca, R. De Maria, Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells, Nature, 468 (2010) 824-828.
38. K. Sugawara, H. Kurihara, M. Negishi, N. Saito, Y. Nakazato, T. Sasaki, T. Takeuchi, Nestin as a marker for proliferative endothelium in gliomas, Laboratory investigation; a journal of technical methods and pathology, 82 (2002) 345-351.
39. P. Fu, Y.S. He, Q. Huang, T. Ding, Y.C. Cen, H.Y. Zhao, X. Wei, Bevacizumab treatment for newly diagnosed glioblastoma: Systematic review and meta-analysis of clinical trials, Molecular and clinical oncology, 4 (2016) 833-838.
40. E. Galanis, S.K. Anderson, J.M. Lafky, J.H. Uhm, C. Giannini, S.K. Kumar, T.K. Kimlinger, D.W. Northfelt, P.J. Flynn, K.A. Jaeckle, T.J. Kaufmann, J.C. Buckner, Phase II study of bevacizumab in combination with sorafenib in recurrent glioblastoma (N0776): a north central cancer treatment group trial, Clinical cancer research : an official journal of the American Association for Cancer Research, 19 (2013) 4816-4823.
41. A.C. Dudley, Tumor endothelial cells, Cold Spring Harbor perspectives in medicine, 2 (2012) a006536.
42. K.E. Hovinga, F. Shimizu, R. Wang, G. Panagiotakos, M. Van Der Heijden, H. Moayedpardazi, A.S. Correia, D. Soulet, T. Major, J. Menon, V. Tabar, Inhibition of notch signaling in glioblastoma targets cancer stem cells via an endothelial cell intermediate, Stem cells, 28 (2010) 1019-1029.
43. S.M. Weis, D.A. Cheresh, Tumor angiogenesis: molecular pathways and therapeutic targets, Nature medicine, 17 (2011) 1359-1370.
44. A.C. Dudley, M. Klagsbrun, Tumor endothelial cells have features of adult stem cells, Cell cycle, 8 (2009) 236-238.
45. T. Shimizu, K. Sugawara, M. Tosaka, H. Imai, K. Hoya, T. Takeuchi, T. Sasaki, N. Saito, Nestin expression in vascular malformations: a novel marker for proliferative endothelium, Neurologia medico-chirurgica, 46 (2006) 111-117.

Figures

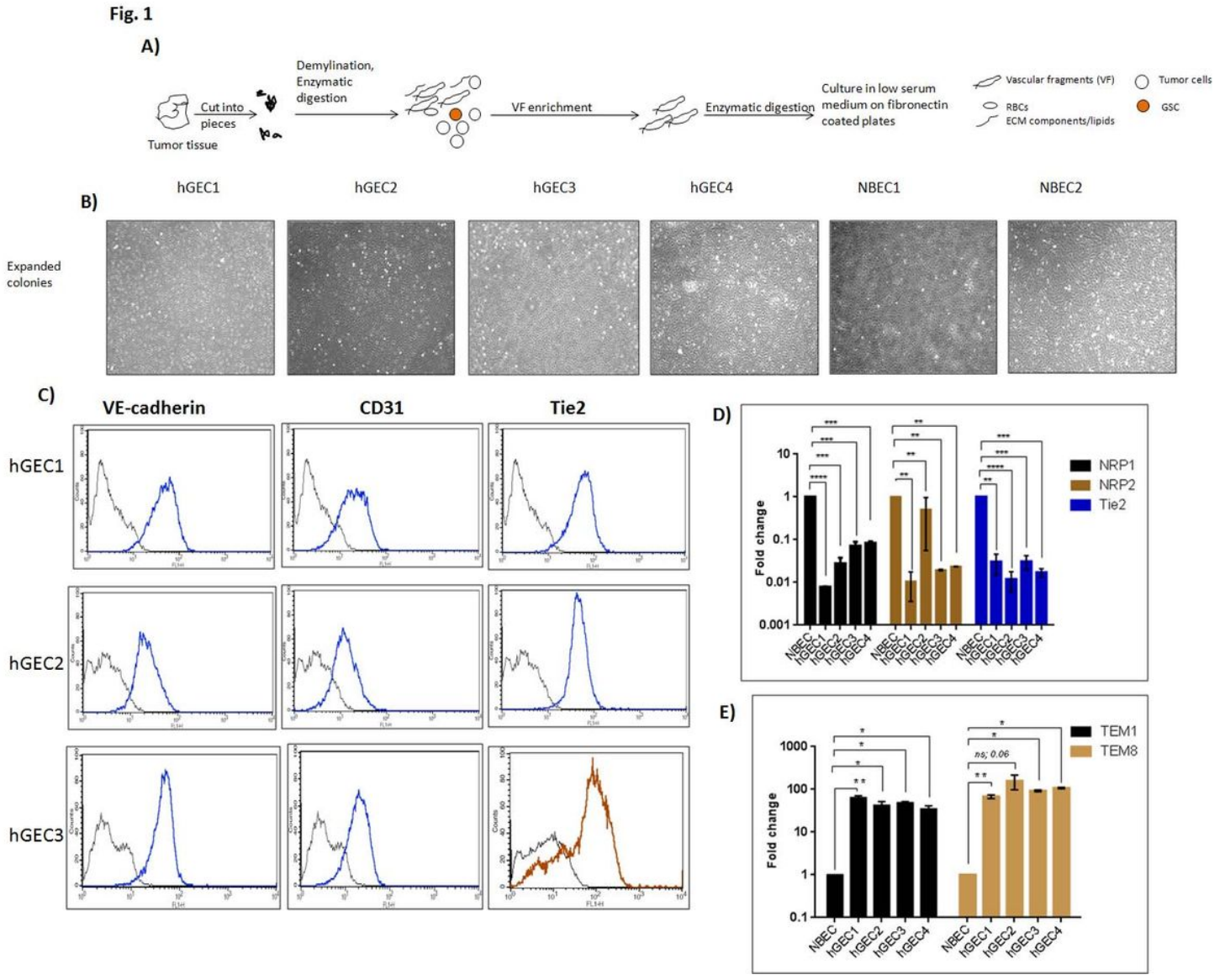

Figure 1

Isolation and characterization of human glioma derived endothelial (hGEC) cultures.

Schematic of hGECs isolation from GBM tissue (A). Phase contrast of expanded hGEC clones (B). Flow cytometry characterization of early passage (hGEC1-3) cultures for EC markers (VE-cadherin, CD31, Tie2) (C). Primary IgG was taken as control. Real time PCR expression analysis of EC specific transcripts (NRP1, NRP2, Tie2) in hGECs. NBEC is taken as control (D). Data represents three independent experiments as mean±SEM (t test). Real time PCR analysis of TEM1, TEM8 in hGECs, and NBEC cells (E). Data obtained from three experiments is expressed as fold change w.r.t. to NBEC and shown as mean±SEM (t test).

Figure 2

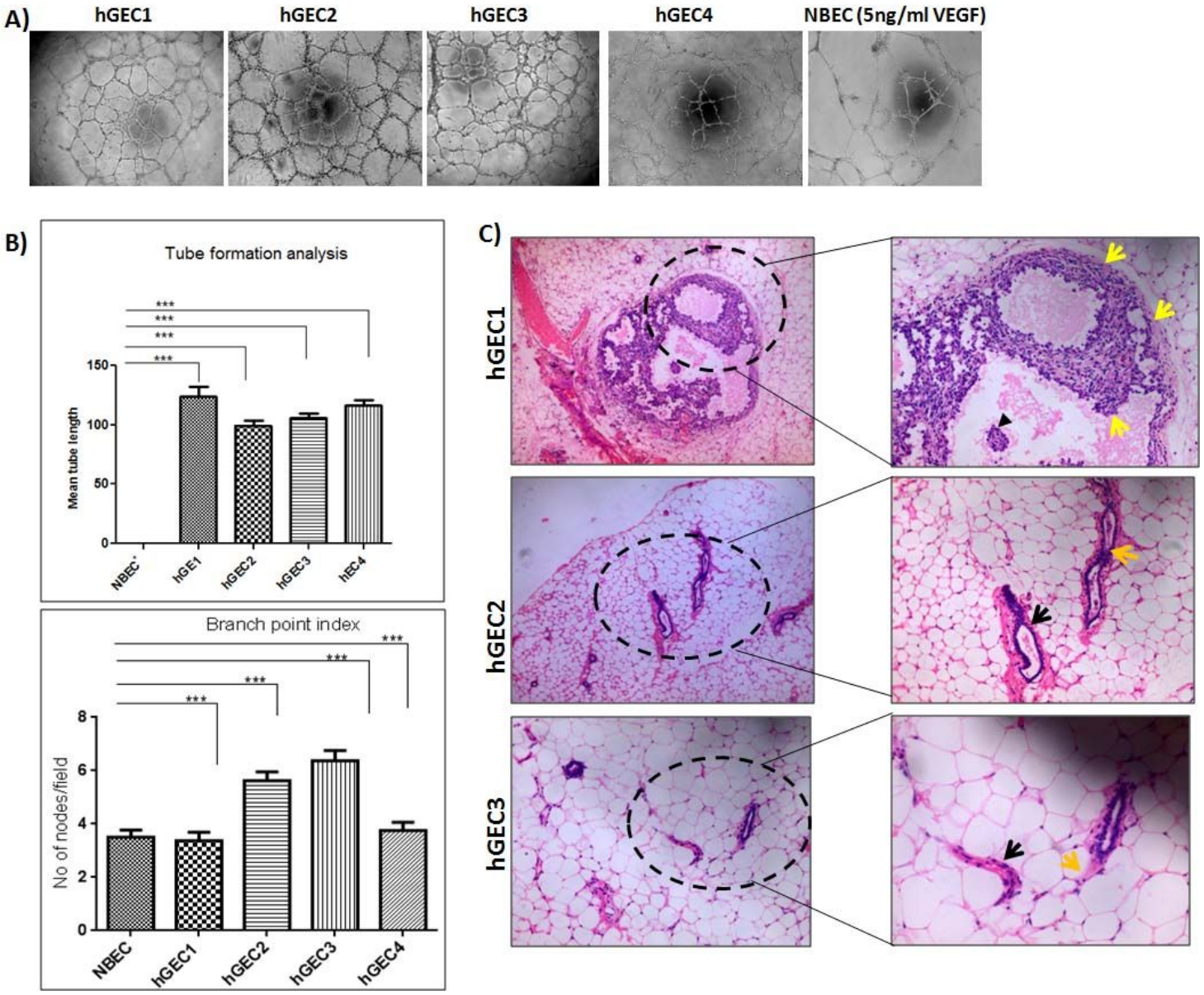

Figure 2

In vivo characterization of hGEC cultures for angiogenic potential. Matrigel tube formation assay in hGEC1-4 cultures and, NBEC culture (treated with 5ng/ml VEGF) (A). Quantification of tube length, and branch point index. Data represents mean ± SEM from three independent experiments (t test) (B). H&E staining of plugs obtained from in vivo Martigel plug assay showing neo-angiogenesis. Encircled areas showing angiogenesis regions and projected separately (C). Magnification X20. Arrows showing new vessels formation.

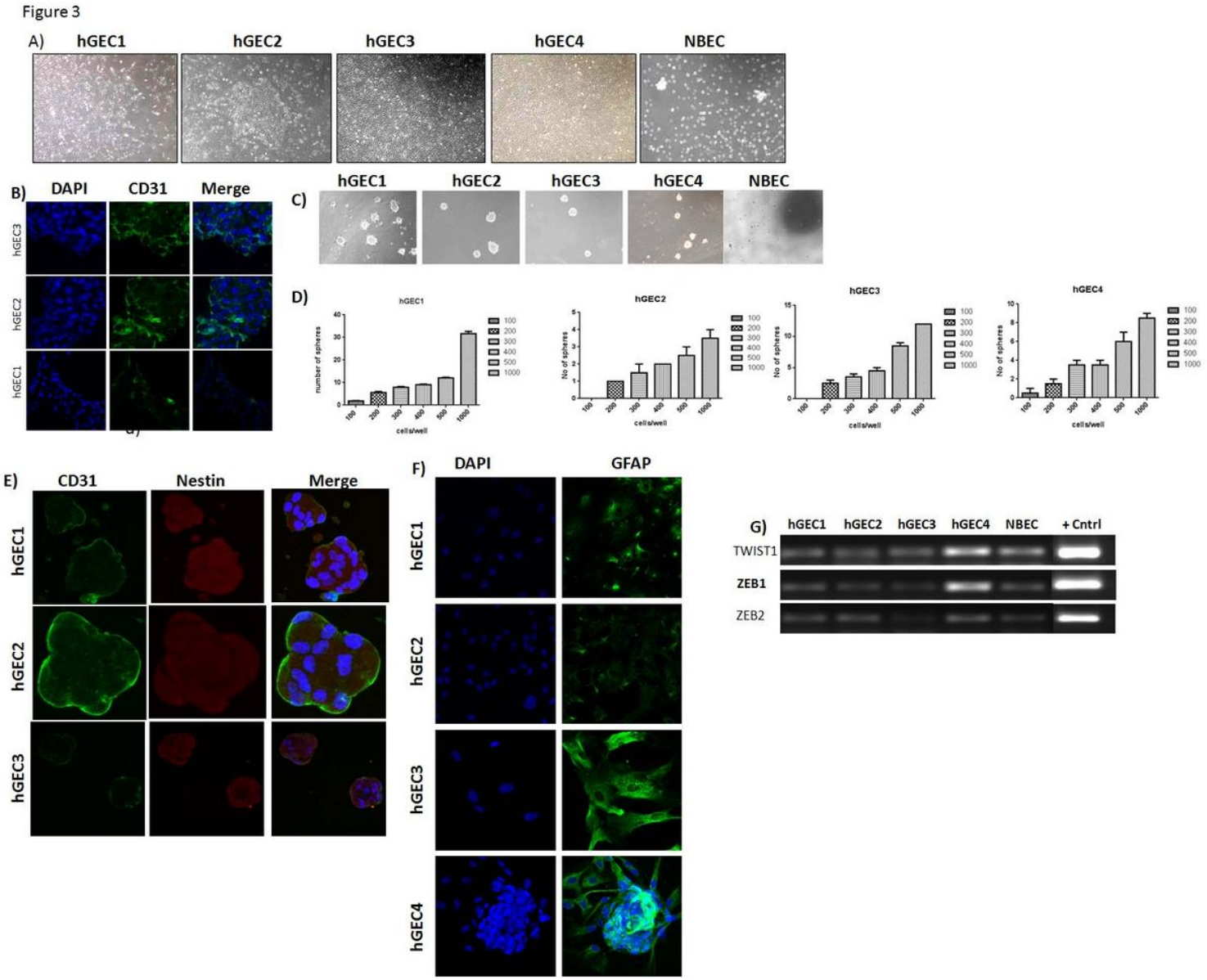

Figure 3

hGECs display stemness features. Phase contrast of hGEC cultures in serum free conditions (A). Immunostaining of endothelial marker CD31 in serum free cultures (B). Magnification X63. Sphere formation assay of hGEC cultures (C). Phase contrast images showing sphere-like growth of hGECs. Bar graphs show quantification of hGEC cultures for sphere forming frequency in four hGEC cultures (hGECs-1-4) (D). Immuno co-staining for CD31+Nestin in hGEC cultures grown under serum-free, suspension culture conditions (E). Magnification X63. Sphere differentiation assay showing hGEC sphere differentiation upon serum induction. Immunostaining of four hGEC cultures (hGEC1-4) for astrocytic marker GFAP (F). Magnification X63. RT-PCR analysis of mesenchymal markers (Twist1, Zeb1, Zeb2) in hGEC cultures w.r.t control (NBEC) (G).

Figure 4

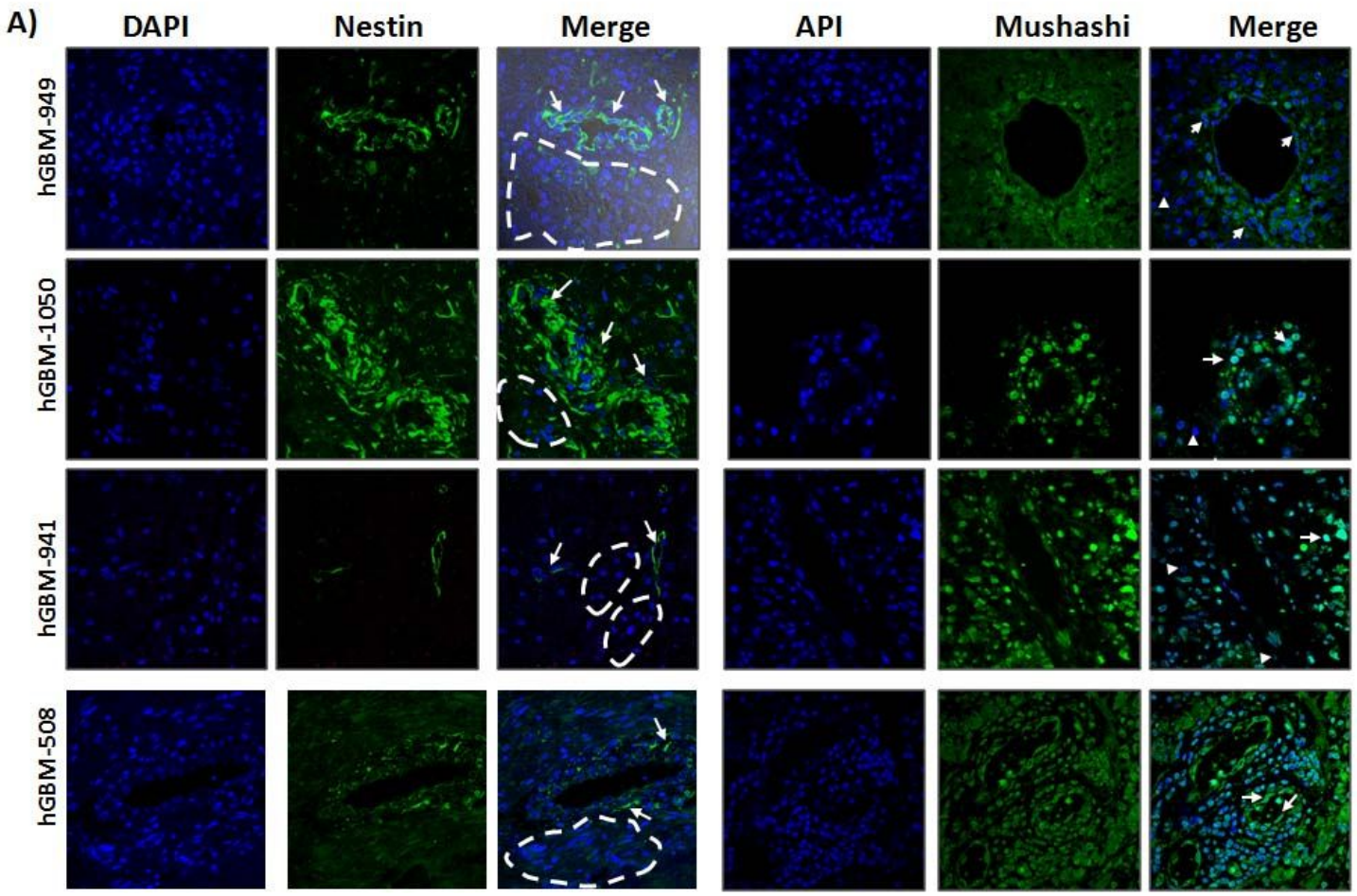

Figure 4

Human GBM (hGBM) endothelial cells express stemness markers. Confocal immunostaining for two stemness markers-NestinNestin and mushashiin four hGBM tumor tissue sections (A). Magnification X63. Arrows/arrow heads indicate tumor vessels showing prominent NestinNestin/mushashi expression w.r.t surrounding tumor cells. Encircled areas representing neighbouring tumor cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SuppTableListofprimers.docx
- SuppFig1.jpg
- SuppFig2.jpg
- SupplFig3.jpg