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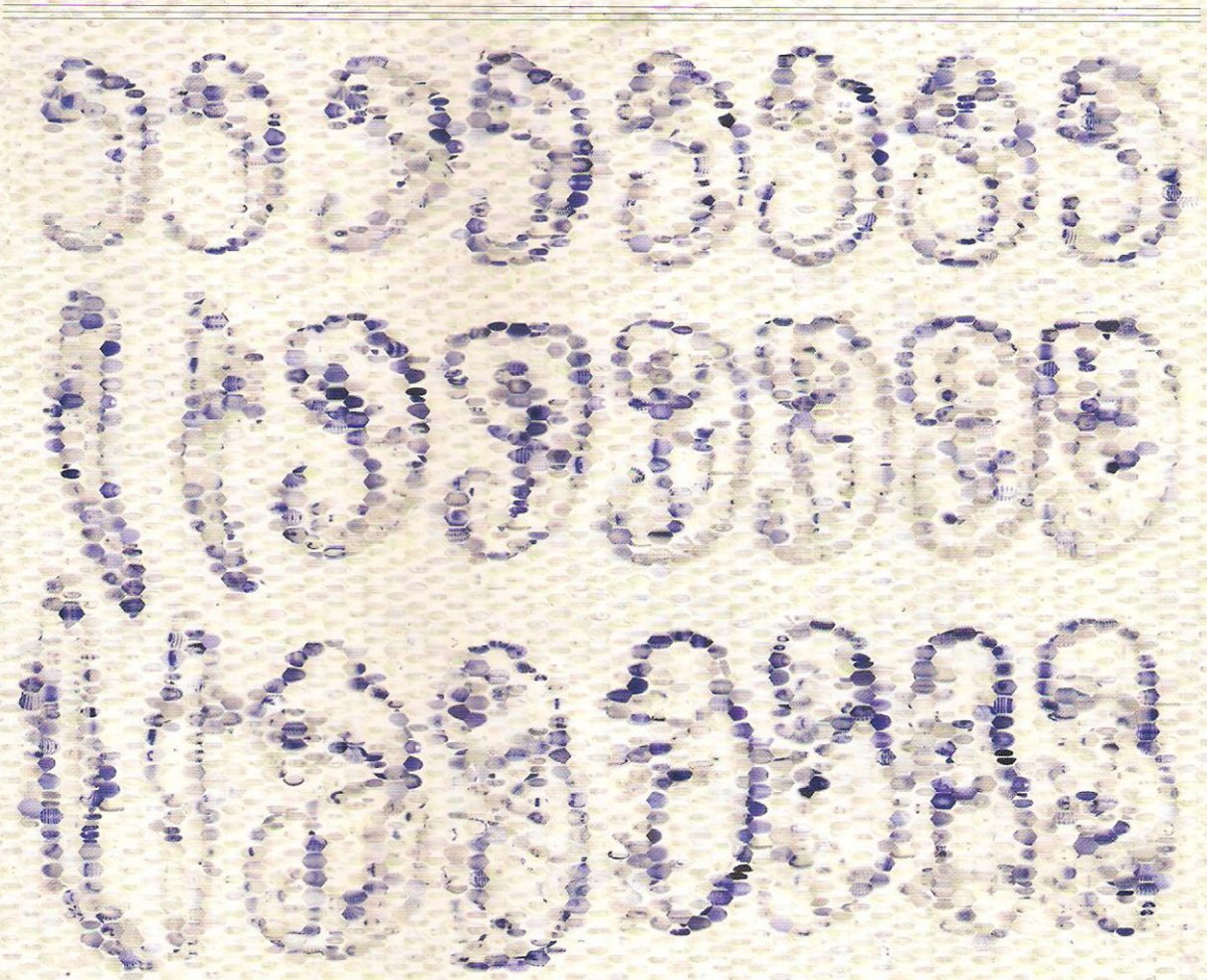

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LETTER

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Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells

Lucia Ricci-Vitiani[1]*, Roberto Pallini[2]*, Mauro Biffoni[1], Matilde Todaro[3], Gloria Invernici[4], Tonia Cenci[5], Giulio Maira[2], Eugenio Agostino Parati[4], Giorgio Stassi[3,6], Luigi Maria Larocca[5] & Ruggero De Maria[1,7]

Glioblastoma is a highly angiogenetic malignancy, the neoformed vessels of which are thought to arise by sprouting of pre-existing brain capillaries. The recent demonstration that a population of glioblastoma stem-like cells (GSCs) maintains glioblastomas[1,2] indicates that the progeny of these cells may not be confined to the neural lineage[3]. Normal neural stem cells are able to differentiate into functional endothelial cells[4]. The connection between neural stem cells and the endothelial compartment seems to be critical in glioblastoma, where cancer stem cells closely interact with the vascular niche and promote angiogenesis through the release of vascular endothelial growth factor (VEGF) and stromal-derived factor 1 (refs 5–9). Here we show that a variable number (range 20–90%, mean 60.7%) of endothelial cells in glioblastoma carry the same genomic alteration as tumour cells, indicating that a significant portion of the vascular endothelium has a neoplastic origin. The vascular endothelium contained a subset of tumorigenic cells that produced highly vascularized anaplastic tumours with areas of vasculogenic mimicry in immunocompromised mice. *In vitro* culture of GSCs in endothelial conditions generated progeny with phenotypic and functional features of endothelial cells. Likewise, orthotopic or subcutaneous injection of GSCs in immunocompromised mice produced tumour xenografts, the vessels of which were primarily composed of human endothelial cells. Selective targeting of endothelial cells generated by GSCs in mouse xenografts resulted *in tumour reduction and degeneration, indicating the functional relevance of the GSC-derived endothelial vessels. These findings* describe a new mechanism for tumour vasculogenesis and may explain the presence of cancer-derived endothelial-like cells in several malignancies.

From archival material, we selected a group of glioblastomas showing both remarkable angiogenesis and nuclear accumulation of mutant p53 in tumour cells (Supplementary Table 1). In 83.3% (20/24) of these tumours, we found cells with nuclear accumulation of mutant p53 that lined the lumens of capillaries and/or vascular glomeruli (Supplementary Fig. 1a and Supplementary Table 1). Double immunohistochemistry analysis of p53 and CD31 demostrated the endothelial phenotype of the p53-positive cells facing the lumen of the vessels (Supplementary Fig. 1b). Mouse and human tumour-associated endothelial cells can harbour chromosomal alterations[10–12]. To assess whether a subset of endothelial cells showed glioblastoma-specific chromosomal aberrations, we analysed the tumour vasculature in 15 glioblastomas by combined CD31 immunofluorescence and fluorescence *in situ* hybridization (FISH) using probes for the centromere of chromosome 10 (Cep10), for the telomere of chromosome 19 (Tel19q), and a locus-specific probe on chromosome 22 (breakpoint cluster region locus q11.2; LSI22). In all the tumours carrying aneuploidy for one or more of these chromosomes, we detected a substantial fraction of endothelial cells bearing the same chromosomal aberrations (Supplementary Fig. 1c). Interestingly, double immunostaining of vascular glomeruli in glioblastoma revealed a significant number of $GFAP^+$ microvascular cells showing an aberrant endothelial/glial phenotype (Supplementary Fig. 1d). Thus, a variable number of endothelial cells in glioblastoma seem to originate from the tumour. To quantify the contribution of tumour-derived endothelial cells to glioblastoma vasculature, we used FISH to analyse purified $CD31^+/CD144^+$ (VE-Cadherin$^+$) endothelial cells from freshly dissociated glioblastoma specimens (Fig. 1a). Again, we detected $CD31^+/CD144^+$ endothelial cells that shared the same chromosomal alterations as the tumour cells in any given glioblastoma harbouring aberrations of chromosomes 10, 19 and 22 (Fig. 1b and Supplementary Table 2). The amount of endothelial cells with tumour-specific chromosomal changes ranged between 20 and 90% of the sorted cells (mean 60.7 ± 28.1 standard deviations (s.d.)).

We assessed further the phenotype of sorted $CD31^+/CD144^+$ glioblastoma cells by immunofluorescence, which showed that the vast majority of these cells (83.9 ± 4.2%; range 79–90%) expressed the mature endothelial cell marker von Willebrand factor (vWF), although a substantial proportion of them (30.9 ± 21.3%; range 10–76%) co-expressed vWF and GFAP (Fig. 1c, d). Thus, it seems that the $CD31^+/CD144^+$ cells harbouring chromosomal aberrations are glioblastoma-derived endothelial cells that either differentiated towards the canonical endothelial lineage ($GFAP^-$) or showed a mixed endothelial/glial phenotype ($GFAP^+$), whereas the euploid fraction is likely to represent endothelial cells derived from normal brain vessels. *In vitro* experiments using a microvascular culture of fresh $CD31^+$ cells isolated by magnetic microbeads from glioblastoma samples confirmed the existence of endothelial cells with aberrant GFAP expression (Supplementary Fig. 2a–c), as well as the presence of a substantial number of aneuploid endothelial cells (Supplementary Fig. 2d, e). Grafting of freshly purified $CD31^+/CD144^+$ cells showed that three of five glioblastomas contained tumorigenic endothelial cells that produced highly vascularized anaplastic tumours (Supplementary Fig. 3a–c). These cells, however, lost their tumorigenic activity on *in vitro* culture with endothelial medium (Supplementary Fig. 3a).

Although there is no general agreement on the definiton and markers identifying so-called cancer stem cells, there is good evidence that GSCs can be enriched by the use of anti-CD133 antibodies or through the generation of clusters of undifferentiated cells (neurospheres) in serum-free media containing epidermal growth factor (EGF) and basic fibroblast growth factor (FGF)[1,5,7,13,14]. We demonstrated recently that GSCs can differentiate into mesenchymal cells, giving rise to osteoblastic and chondrocytic cells[3]. To determine the potential contribution to the angiogenic process of GSCs, we cultivated glioblastoma neurospheres and primary glioblastoma differentiated cells under endothelial conditions, or $CD133^+/CD31^-$ and $CD133^-/CD31^-$ cells derived from the

[1]Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena 299, Rome 00161, Italy. [2]Department of Neurosurgery, Catholic University of Rome, Largo Francesco Vito 1, Rome 00168, Italy. [3]Department of Surgical and Oncological Sciences, University of Palermo, Via Liborio Giuffrè 5, Palermo 90127, Italy. [4]U. O. Cerebrovascular Disease Cellular Neurobiology Laboratory, Fondazione IRCCS, Neurological Institute "Carlo Besta", Via Celoria 11, 20133 Milan, Italy. [5]Institute of Pathology, Catholic University of Rome, Largo Francesco Vito 1, Rome 00168, Italy. [6]Cellular and Molecular Oncology, Istituto di Ricovero e Cura a Carattere Scientifico Fondazione Salvatore Maugeri, Via S. Maugeri 6, Pavia 27100, Italy. [7]Mediterranean Institute of Oncology, Via Penninazzo 7, 95029, Viagrande, Catania, Italy.
*These two authors contributed equally to the manuscript.

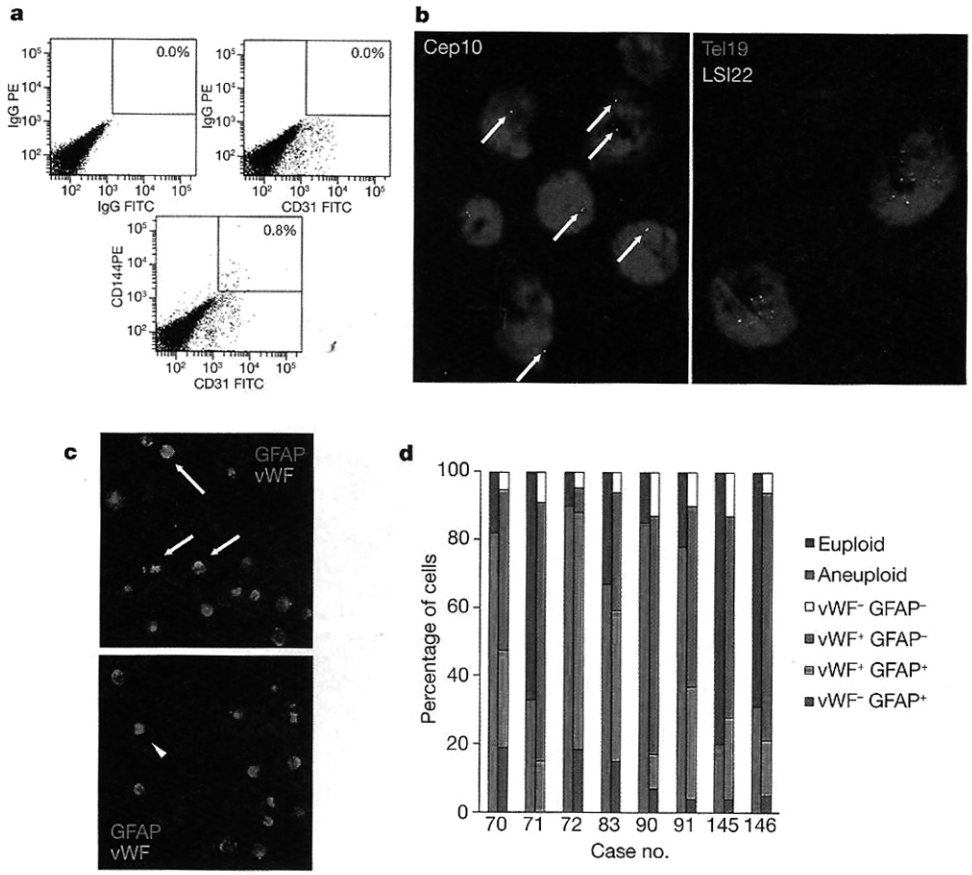

Figure 1 | Microvascular endothelial cells isolated from glioblastoma harbour tumour-specific chromosomal aberrations. **a**, $CD31^+/CD144^+$ cells were isolated from surgical glioblastoma specimens ($n = 15$). FITC, fluorescein isothiocyanate; PE, phycoerythrin. **b**, Sorted cells were analysed by interphase FISH assay for tumour-specific chromosomal changes, such as monosomy of Cep10 (left, arrows) or polisomy of Tel19 and LSI22 (right). **c**, The phenotype of the $CD31^+/CD144^+$ sorted cells was further analysed by anti-GFAP and anti-vWF immunofluorescence. A fraction of the $CD31^+/CD144^+$ cells coexpressed GFAP and vWF (arrows), indicating an aberrant endothelial/glial phenotype. A minority of sorted cells were $GFAP^+/vWF^-$. **d**, Quantification of results from FISH and immunofluorescence analysis.

same tumours. Whereas cells enriched in GSCs generated microvascular cultures of $CD31^+$ and $Tie2^+$ cells, neither differentiated cells nor the U87MG cell line were able to produce endothelial-like cells (Fig. 2a). Such GSC-derived endothelial cells showed considerable tube-forming ability, together with low-density lipoprotein (LDL) uptake and endothelial nitric oxide synthase (eNOS) expression, which were completely absent in differentiated tumour cells and in the U87MG cell line (Fig. 2b–d and Supplementary Fig. 4). Unsupervised gene-expression analysis of glioblastoma and endothelial cells showed that neural-differentiated glioblastoma cells and normal endothelial cells constitute the two more distant groups in a dendrogram in which tumour endothelial cells cluster between normal endothelial cells and glioblastoma neurospheres (Supplementary Fig. 5).

To investigate the ability of GSCs to form endothelial vessels *in vivo*, we measured the relative amount of murine versus human endothelial cells within glioblastoma neurosphere xenografts (Fig. 3a). Flow cytometry analysis with human- and mouse-specific antibodies showed that about 70% of the $CD31^+$ cells from the inner portion of the tumour were of human origin, whereas nearly all the $CD31^+$ cells in the tumour capsule were murine (Fig. 3b). Likewise, human $CD144^+$ cells were detected only in the core and not in the tumour capsule (Fig. 3b). Immunohistochemistry of subcutaneous and intracranial xenografts showed that glioblastoma neurosphere-derived tumours contained human vessels labelled by human-specific anti-CD31, whereas xenografts generated with U87MG or other glioma cell lines grown in serum did not (Fig. 3c, Supplementary Fig. 6a and data not shown). The presence of human-derived endothelial cells was confirmed by labelling sections of tumour xenografts obtained with GFP^+ glioblastoma neurospheres with anti-GFP and anti-human CD31 antibodies (Supplementary Fig. 6b). Moreover, immunofluorescence staining with validated human-specific endothelial antibodies showed that these cells expressed consistently CD34, CD144 and VEGFR2 (Fig. 3d) but not the stem-cell markers SSEA-1 and CD133 (Supplementary Fig. 6c). Such human-specific endothelial antigens identified microvascular structures containing circulating erythrocytes (Fig. 3d and Supplementary Fig. 6a), indicating the functional relevance of human angiogenesis in the tumour xenografts. Of note, a similar formation of human endothelial cells was observed in subcutaneous xenografts obtained with the injection of freshly purified $CD133^+/CD31^-$ cells, whereas $CD133^-/CD31^-$ cell xenografts contained only mouse endothelial vessels (Supplementary Fig. 7).

To trace *in vivo* angiogenesis, we injected RFP-labelled glioblastoma neurospheres into transgenic NOD/SCID mice expressing GFP under the Tie2 promoter. Examination of a thick-section plane by confocal microscopy showed that GFP^+ mouse vessels were primarily outside the tumours (Supplementary Fig. 8a). To exclude the occurrence of fusion between tumour and mouse endothelial cells, we stained tumour xenograft sections with anti-human/mouse Tie2 and CD31 antibodies. Although CD31 staining showed the presence of vessels containing both mouse and human $CD31^+$ cells at the periphery of the tumour, the majority of endothelial cells inside the tumour mass did not express mouse Tie2 and were of human origin in the absence of fusion (Supplementary Fig. 8b, c). Moreover, FISH analysis of nuclei extracted from microdissected vascular structures of GSC xenografts confirmed the absence of murine chromosomes in human cells (Supplementary Fig. 9). Together, these findings demonstrate that the tumour xenografts obtained by injection of human glioblastoma neurospheres develop an intrinsic vascular network composed by

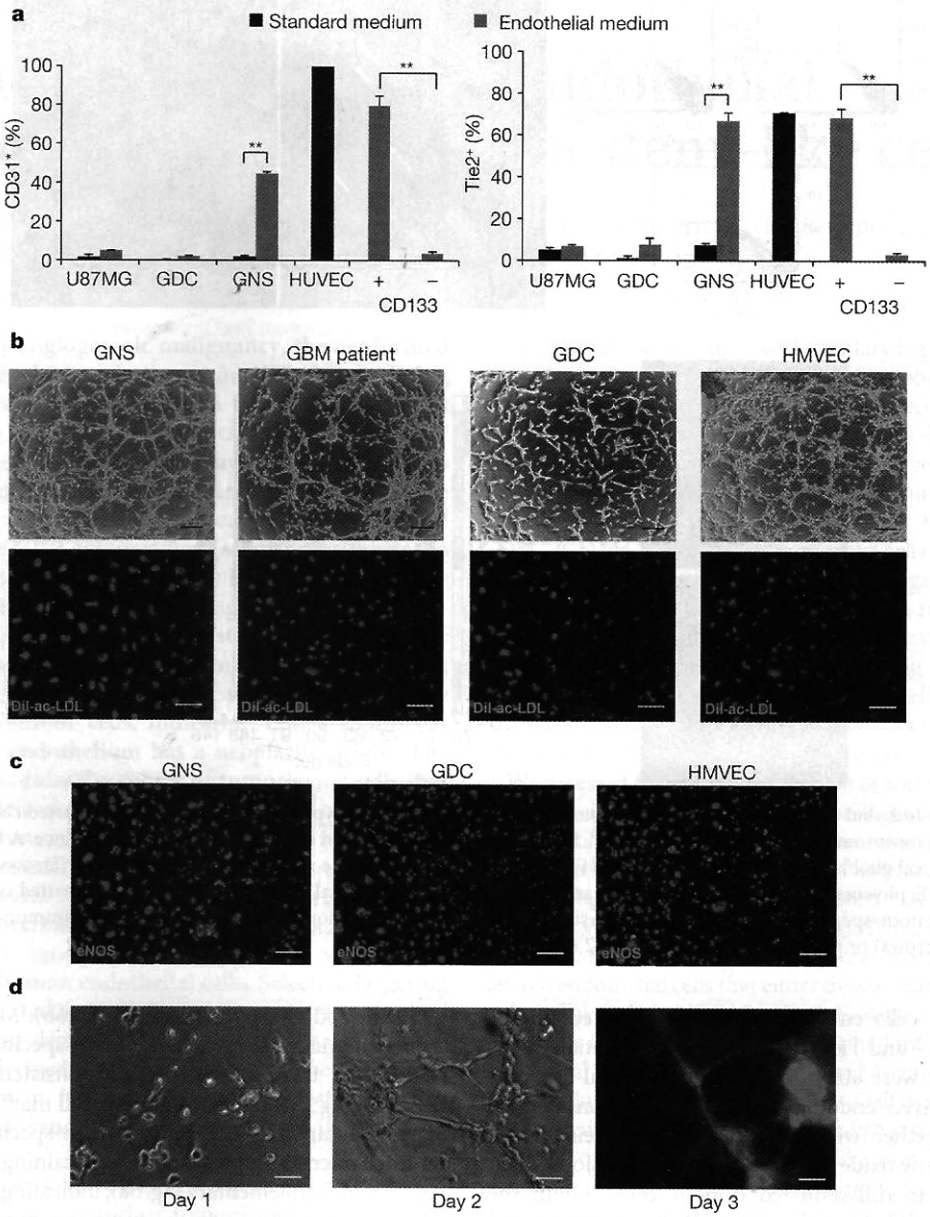

Figure 2 | GSCs cultured under endothelial differentiation conditions develop morphological, phenotypical and functional features of endothelial cells. **a**, Flow cytometry analysis of human umbilical vein endothelial cells (HUVEC), glioblastoma neurospheres (GNS), primary glioblastoma cells cultured in serum (GDC), U87MG, $CD31^-/CD133^+$ and $CD31^-/CD133^-$ cells from freshly dissociated glioblastomas. Cells were cultured under standard (black) or endothelial (grey) condition. Error bars represent the mean ± s.d. ($n = 4$). $^{**}P < 0.001$. **b**, Tube formation (top) and LDL-uptake (bottom) assay on cells under endothelial conditions as above (GNS and GDC), endothelial cells isolated from glioblastoma patients (GBM patients) and human dermal microvascular endothelial cells (HMVEC). DiI-ac-LDL, 1,1′-dioetadeeyl-3,3,3′,3′-tetramethylindocarboeyanine-perchlorate-acetylated LDL. Scale bars, 200 μm (top) and 50 μm (bottom). **c**, Immunofluorescence for eNOS in HMVEC, GNS and GDCs treated as above. Scale bar, 100 μm. **d**, *In vitro* perfusion assay on three-dimensional glioblastoma neurosphere-derived endothelial culture injected with fluorescein. Scale bar, 50 μm. One representative of four independent experiments performed in blind is shown for **b**, **c** and **d**.

tumour cells with an aberrant endothelial phenotype. To determine whether the GSC-derived endothelial cells contribute to tumour growth, we transduced glioblastoma neurospheres with a lentiviral vector containing the herpes simplex virus thymidine kinase gene (*tk*) under the control of the transcription-regulatory elements of Tie2 (Tie2-*tk*; Supplementary Fig. 10a), so that the tumour-derived endothelial cells would be sensitive to ganciclovir[15,16]. For this experiment, we selected glioblastoma neurospheres with no detectable expression of Tie2 (Supplementary Fig. 11). Control cells included glioblastoma neurospheres tranduced with an empty viral vector and U87MG cells transduced either with Tie2-*tk* or with a vector conferring constitutive expression of Tk (PGK-*tk*, Supplementary Fig. 10a). One week after ganciclovir administration, TdT-mediated dUTP nick end labelling (TUNEL) and double immunofluorescence labelling with anti-Tie2 antibodies in tumour subcutaneous xenografts showed selective apoptosis of the endothelial compartment only in animals injected with Tie2-*tk* neurospheres, whereas PGK-*tk* tumours contained a considerable number of apoptotic nuclei both in tumour and endothelial cells (Fig. 4a). Moreover, tumours generated by Tie2-*tk* neurospheres underwent a significant size reduction four weeks after ganciclovir administration, whereas control GSC xenografts increased their size over the same time interval (Fig. 4b).

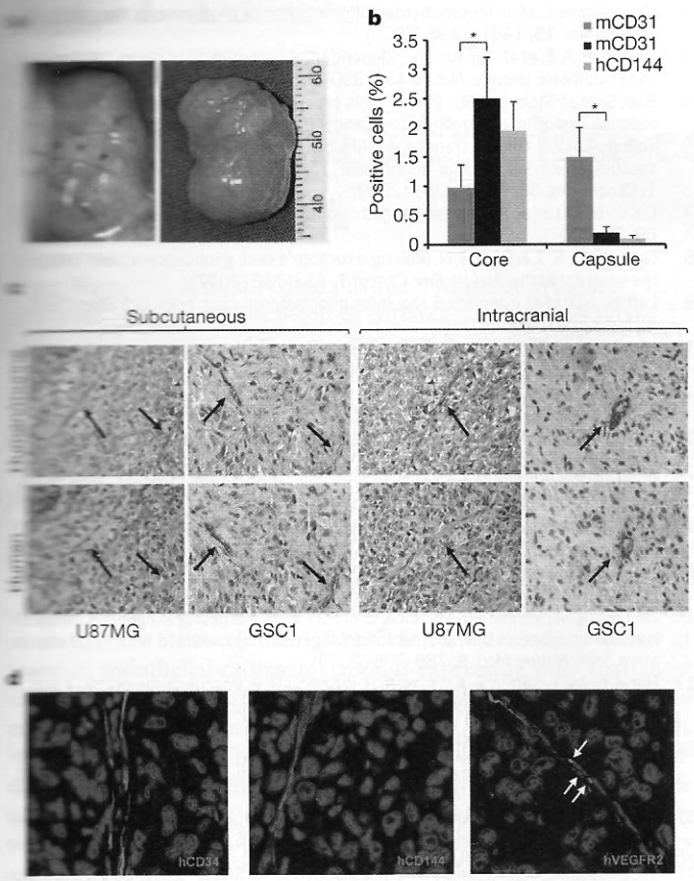

Figure 3 | Human origin of endothelial cells in glioblastoma neurosphere xenografts. **a**, Explanted subcutaneous xenograft obtained by injection of glioblastoma neurospheres. Detail of murine vessels on the surface of the xenograft (left, black arrowheads) and tumour after capsule removal (right). **b**, FACS evaluation of murine $CD31^+/CD45^-$ (mCD31), human CD31 (hCD31) and human CD144 (hCD144) in the capsule and core of the tumour (mean ± s.d., $n = 4$, $^*P < 0.05$). **c**, Immunohistochemistry of glioblastoma neurosphere (GSC1) and U87MG xenografts using either an anti-human CD31 or anti-human and murine CD31 (one out of four different glioblastoma neurosphere samples and serum-grown cell lines are shown). **d**, Immunofluorescence of tumour xenograft sections labelled with anti-human CD34 (left), anti-human CD144 (middle) or anti-human VEGFR2 (right). Arrows indicate circulating erythrocytes. Data represent one of four independent experiments obtained with different glioblastoma neurosphere samples.

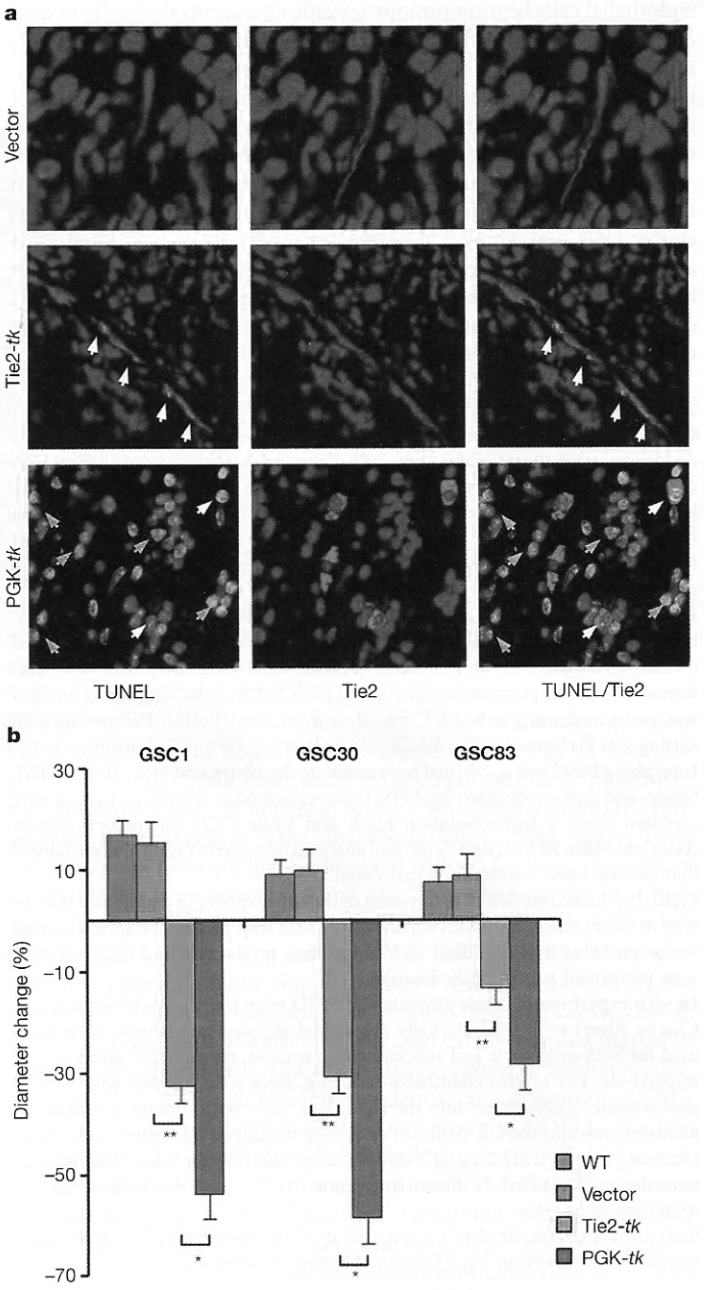

Figure 4 | Selective targeting of glioblastoma neurosphere-derived endothelial cells impairs the growth of subcutaneous tumour xenografts. **a**, Double immunofluorescence using anti-TUNEL and anti-Tie2 in xenografts from Tie2-*tk*, PGK-*tk* and vector glioblastoma neurosphere cells one week after ganciclovir administration. Arrows indicate apoptotic $Tie2^+$ (white) and $Tie2^-$ (yellow) cells. **b**, Tumour size measured four weeks after ganciclovir administration in xenograft obtained from three different glioblastoma neurosphere samples either untransduced (wild type (WT)) or transduced with vector, Tie2-*tk* or PGK-*tk*. Error bars are mean ± s.d. of three different experiments. $^*P < 0.005$, $^{**}P < 0.001$.

Histological examination revealed massive degeneration in the tumour xenografts developed by injection of Tie2-*tk* neurospheres. Four weeks after ganciclovir treatment, these tumours were completely devoid of vascular glomeruli, tiny capillaries with ongoing phenomena of endothelial disruption being the only residual vascular structures (Supplementary Fig. 10b). Although all PGK-*tk* tumours degenerated massively, U87MG Tie2-*tk* xenografts were not affected by ganciclovir treatment (Supplementary Fig. 10c, d), confirming that this cell line was unable to generate endothelial cells. These findings indicate that GSC-derived angiogenesis is essential for tumour survival. Moreover, mouse models based on adherent cell lines grown in serum do not seem suitable for the study of glioblastoma angiogenesis.

Here we demonstrated that GSCs are able to differentiate in functional endothelial cells. Such angiogenic potential could be inherited from normal neural stem cells, which have been shown to differentiate in endothelial cells both *in vitro* and *in vivo*[4]. The formation of fluid-conducting networks by nonendothelial cells has been described for melanomas, sarcomas, breast, ovary, lung and prostate carcinomas[17,18] as a result of vasculogenic mimicry, which is a feature associated with a pluripotent gene expression pattern in aggressive tumour cells[19].

The ability of cancer stem-like cells to directly contribute to the tumour vasculature by endothelial cell differentiation represents a new mechanism of angiogenesis that might not be restricted to glioblastoma. A similar endothelial potential may be shared by $CD44^+$ cells purified from ovarian cancer[20]. However, the existence of tumour-derived endothelial cells in ovarian cancer has not been demonstrated yet. Endothelial-like cells with cancer-specific genomic alterations have been described in other tumour types, such as lymphoma and neuroblastoma[11,12]. Although the angiogenic activity of cancer stem-like cells has not been investigated in other tumours, it is likely that the

endothelial cells bearing tumour-specific alterations derive from cancer cells endowed with stem-cell plasticity. Likewise, the vasculogenic mimicry might represent an incomplete differentiation of cancer stem-like cells towards the endothelial lineage, as indicated by the aberrant mixed phenotype of glioblastoma xenografts generated by the subset of $CD31^+/CD144^+$ cells that retain tumorigenic activity.

Our findings may have considerable therapeutic implications. On the one hand, endothelial cells bearing the same genomic alteration as cancer cells may show a different sensitivity to conventional anti-angiogenic treatments, such as VEGF/VEGFR targeting. On the other hand, our data indicate the possibility of targeting the process of GSC differentiation into endothelial cells, thus offering new therapeutic options for cancer treatment.

METHODS SUMMARY

Cell culture. Glioblastoma neurosphere cultures were established from freshly dissociated surgical specimens as described[3,21,22]. Primary cultures of glioblastoma differentiated cells were obtained by plating cells from freshly dissociated samples in DMEM-F12 medium containing 10% FBS. For primary culture of glioblastoma microvascular endothelial cells, $CD31^+$ cells were purified using Miltenyi Microbead Kit (Miltenyi Biotech) according to manufacturer's instructions and grown in endothelial basal medium (EBM Bullet kit; Biowhittaker Cambrex).

Immunohistochemistry, immunofluorescence and flow cytometry. Immunohistochemistry was performed as described[22] on deparaffinized sections of glioblastoma tissue. For immunofluorescence, cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. Cytofluorimetric analysis was performed using a FACS Canto flow cytometer (Becton Dickinson). Cell sorting was performed with a FACS Aria cell sorter (Becton Dickinson).

Interphase FISH and combined immunohistochemistry and FISH (FICTION). Single- and dual-probe interphase FISH was performed as described[3]. Images were captured using a high-resolution black and white CCD microscope camera AxioCam MRm REV 2 (Karl Zeiss) and analysed using AxioVision 4 multichannel fluorescence basic workstation (Karl Zeiss).

Lentiviral infection. Selective targeting of the cells expressing endothelial phenotype was obtained by modifying the pRRLsin.Tie2p.TKiresGFP.spre lentiviral vector provided by L. Naldini[15,16]. Viral particle production and GSC infection were performed as previously described[23].

***In vivo* experiments.** Nude athymic and SCID mice (female, 4–5 weeks of age; Charles River) were used. Partially dissociated glioblastoma neurospheres were used for both orthotopic and subcutaneous injection, typically 10^5 and 5×10^5, respectively. For *in vivo* endothelial targeting, mice were injected with Tie2-*tk* glioblastoma neurospheres into the right flank and control vector glioblastoma neurospheres into the left flank. After having developed bilateral nodules mice received ganciclovir at 50 mg kg^{-1} day^{-1} intraperitoneally for 5 days. Ganciclovir-treated mice were killed at different time points to collect samples for histology and immunofluorescence.

Statistical analysis. Student's *t*-test was used to analyse data using Statistica (version 5.5; Statsoft) or Fig.P (version 2.7; Biosoft) softwares.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions L.R.-V. and R.P. performed most of the experiments and coordinated the project; M.B. performed cell sorting and flow cytometric analysis; M.T. and G.S. detected and characterized human endothelial cells in mouse xenografts; G.I. and E.A.P. developed the functional assays of the endothelial cell cultures; G.M. recruited the patients and performed surgery; T.C. and L.M.L. were involved in pathology assessment and detection of genomic aberration in endothelial cells; R.D.M. conceived the study and wrote the paper.

Author Information Data have been deposited at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MEXP-2891. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to R.D.M. (demaria@iss.it) or R.P. (pallini@rm.unicatt.it).