Glioblastoma: A Pathogenic Crosstalk between Tumor Cells and Pericytes

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Abstract

Cancers likely originate in progenitor zones containing stem cells and perivascular stromal cells. Much evidence suggests stromal cells play a central role in tumor initiation and progression. Brain perivascular cells (pericytes) are contractile and function normally to regulate vessel tone and morphology, have stem cell properties, are interconvertible with macrophages and are involved in new vessel formation during angiogenesis. Nevertheless, how pericytes contribute to brain tumor infiltration is not known. In this study we have investigated the underlying mechanism by which the most lethal brain cancer, Glioblastoma Multiforme (GBM) interacts with pre-existing blood vessels (co-option) to promote tumor initiation and progression. Here, using mouse xenografts and laminin-coated silicone substrates, we show that GBM malignancy proceeds via specific and previously unknown interactions of tumor cells with brain pericytes. Two-photon and confocal live imaging revealed that GBM cells employ novel, Cdc42-dependent and actin-based cytoplasmic extensions, that we call flectopodia, to modify the normal contractile activity of pericytes. This results in the co-option of modified pre-existing blood vessels that support the expansion of the tumor margin. Furthermore, our data provide evidence for GBM cell-pericyte fusion-hybrids, some of which are located on abnormally constricted vessels ahead of the tumor and linked to tumor-promoting hypoxia. Remarkably, inhibiting Cdc42 function impairs vessel co-option and converts pericytes to a phagocytic/macrophage-like phenotype, thus favoring an innate immune response against the tumor. Our work, therefore, identifies for the first time a key GBM contact-dependent interaction that switches pericyte function from tumor-suppressor to tumor-promoter, indicating that GBM may harbor the seeds of its own destruction. These data support the development of therapeutic strategies directed against co-option (preventing incorporation and modification of pre-existing blood vessels), possibly in combination with anti-angiogenesis (blocking new vessel formation), which could lead to improved vascular targeting not only in Glioblastoma but also for other cancers.

Introduction

Glioblastoma Multiforme (GBM) is a highly invasive brain cancer, with prominent vascular involvement, characterized by twisted blood vessel [1] and infiltration along external vessel walls [2], which makes it resistant to treatment. Evidence from a rat GBM model has shown that early tumor vasculature forms by co-option of pre-existing brain blood vessels and precedes new vessel formation (angiogenesis) [3]. Vessel co-option also occurs during metastasis of other tumors, as recently demonstrated for the spread of breast cancer into the brain [4]. Furthermore, co-option is also responsible for tumor recurrence and metastasis following anti-angiogenic therapies, both in GBM and in other types of cancer [5]-[8]. Therefore, vessel co-option is likely to be a principle cause of malignancy, which occurs during tumor initiation/progression, metastasis and re-initiation after treatment. However, in contrast to angiogenesis that is well understood, the cellular and molecular bases of vessel co-option in tumors are currently unknown. The normal brain microvasculature is made up of narrow tubes (capillaries), consisting of endothelial cells surrounded by contractile pericytes, which function normally to regulate vessel tone and morphology [9], [10]. Because pericytes are located on the abluminal wall of blood vessels, they are good candidates for a role in mediating vessel co-option by tumor cells. Brain pericytes are pluripotent cells with stem cell properties [11]-[13], similar if not identical to the mesenchymal stem cells that occupy an equivalent perivascular location in bone marrow. There is a growing realization that, in addition to their critical role in maintaining blood vessel integrity and controlling blood flow, pericytes are also key players in other aspects of brain homeostasis and disease. For example, evidence suggests that they are regulators of innate immunity and, depending on the context, can mediate not only pro-inflammatory functions associated with host defense [14], but also the anti-inflammatory response to malignant tumors such as human GBM, which includes the inhibition of T cell function and local immunosuppression [15]. Consistent with a role in normal cerebral immunity, purified brain pericytes have been shown to be interchangeable with macro-
organization is linked to the appearance of tumor cell protrusions, that we called ‘flectopodia’ (luctere, to bend, and podos, foot), characterized by an unusual, discontinuous (moniliform) organization of actin, with highly dynamic beads (0.6–3.0 μm in diameter), moving bidirectionally. Flectopodia are 7–30 μm long, present in 9% of GFP-actin+ cells on vessels (n = 266), with the ability to elongate for up to 20 μm or more, at a rate of 14–99 μm/hour, and to form a bent vessel segment in 30 minutes. A gap of approximately 10 μm separates the GFP-actin labeling from the vessel lumen, suggesting that flectopodia are in close contact with perivascular cells. Flectopodia-associated bending often involves a pair of GFP-actin labeled cells, cooperating via a cytoplasmic bridge. During the process, while the leading cell performs the re-arrangement, the lagging cell translocates the bent segment ahead of the first cell (Figure 1D–F; Movie S1). This suggests that flectopodia mediate the ongoing ratchet-like recruitment of host vessels at the co-option front of the tumor.

Next, to validate and extend our ex-vivo observations, we established a xenograft model that recapitulates human GBM in mice (Figure 1G) and used it to confirm the presence of flectopodia and vessel co-option in situ. High resolution-microscopy of sections from GFP-actin-labeled xenografts identified flectopodia-like extensions as early as 2 days (Figure 1H). In situ hybridization at 7 days for the activated mouse (m)-pericyte marker Rgs5, a gene controlling tumor vasculature remodeling [27], [28], showed the presence of m-Rgs5+ cells surrounding abnormally dilated vessels throughout the graft (Figure 1l–K). m-Rgs5+ pericytes are also detected in the infiltrating margin of 1-month xenografts (Figure 1L–N). Taken together these data strongly suggest that host brain perivascular cells are a target cell type for GBM vessel co-option and modification throughout tumor progression.

To confirm that GBM cells interact with pericytes, GFP-actin labeled U373 and U87 cells were co-cultured with brain slices pre-labeled in situ for pericytes, using either a DiRed-transgene reporter for the pericyte marker NG2 [9] or a fluorescent Dextran tracer (Methods). Our results show that tumor cells on vessels interact with Dextran (Dextran Labeled Pericytes, DLPs) or NG2-DsRed labeled perivascular cells (Figure 2A, B; Figure S2 A–G). Unexpectedly, we also found tumor-derived cytoplasm within the cortex of the target pericyte (Figure 2B and Figure S2 G), implying a role for cytoplasmic transfer in the co-option process [see also Movie S2].

Our identification of pericytes as a specific GBM cell target raised the possibility that the altered blood vessel morphology in tumors could be caused by deregulated pericyte contraction. To test this, we established an in vitro system using isolated mouse brain pericytes (Methods) cultured on deformable silicone substrates [29], with the novel coating of human-laminin to reproduce the blood vessel basal lamina that houses perivascular cells in vivo (Figure 2C). Pericytes in vitro express NG2 and

**Results**

First, we challenged human GBM cells (U87) with mouse brain slices where blood vessels were pre-labeled with black ink (Figure 1A, B). This assay revealed a remarkable ability of GBM cells to pull pre-existing blood vessels into the graft. Vessel co-option happens quickly (within 15 hours) with conversion of normal capillaries into highly twisted structures, demonstrating a capacity for rapid vascular network acquisition, even in the absence of new vessel formation. Next, we used GFP-actin labeling and 2-photon live imaging to identify the intrinsic GBM cellular mechanisms involved in vessel co-option (Figure S1; Figure 1C–F).

We found that, 6 hours after seeding, tumor cells in contact with blood vessels can convert straight segments (up to ~60–70 μm long) into hairpin bends (Figure 1C and Movie S1). Vessel re-option is linked to the appearance of tumor cell protrusions, that we called ‘flectopodia’ (luctere, to bend, and podos, foot), characterized by an unusual, discontinuous (moniliform) organization of actin, with highly dynamic beads (0.6–3.0 μm in diameter), moving bidirectionally. Flectopodia are 7–30 μm long, present in 9% of GFP-actin+ cells on vessels (n = 266), with the ability to elongate for up to 20 μm or more, at a rate of 14–99 μm/hour, and to form a bent vessel segment in 30 minutes. A gap of approximately 10 μm separates the GFP-actin labeling from the vessel lumen, suggesting that flectopodia are in close contact with perivascular cells. Flectopodia-associated bending often involves a pair of GFP-actin labeled cells, cooperating via a cytoplasmic bridge. During the process, while the leading cell performs the re-arrangement, the lagging cell translocates the bent segment ahead of the first cell (Figure 1D–F; Movie S1). This suggests that flectopodia mediate the ongoing ratchet-like recruitment of host vessels at the co-option front of the tumor.

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Figure 2. GBM cells target pericytes and modify their contractility. GFP-actin-GBM cells (green) contacting an NG2-DsRed⁺-pericyte (A, red iso-surface in magnified box) and a DLP (B, blue; confocal section) through flectopodia (arrowheads indicate moniliform actin). Note the presence of GFP-actin within the DLP (merged channels, inset in B). v, vessels (DiD-blue in A; Ink-filled-grey in B). C, Scheme showing pericyte (colored cells) in-vivo (top; BV, blood vessel), in-vitro (middle) and on silicone-substrate (bottom). Wrinkling is associated with high αSMA-expression (red-color). D and E (boxed area in G), DIC-optic video-frames of the same field before (D) and after (E) GBM cell addition to pre-plated pericytes. Pericytes alone
produce stable drifting wrinkles (red arrows) that are de-stabilized by GBM cells. White and yellow arrowheads indicate the appearance and disappearance of wrinkles, respectively. Dashed line marks the upper-limit of GBM cell population, transposed from F and G, which show the low magnification of FR Dextran-labeled GBM cells (white false-color in F and magenta in G), plated on cultured pericytes. Time in minutes. H, Traces of two wrinkles, produced before (i) and after (ii) U87-GBM cell-addition, revealing the spatial evolution and colored to indicate lengthening (violet to green) or shortening (green to red) for each time-frame (numbers). I, 3D-plot summarizing the wrinkling behavior of pericytes, either alone (red points, n = 40) or with U87-GBM cells (green points, n = 23). Note the lack of green points in clusters 1 and 2. E1, E2 and C: track-straightness of the ends (E) and center (C) of each wrinkle. Scale bars: 10 μm (A, B), 30 μm (D), 100 μm (G). doi:10.1371/journal.pone.0101402.g002

display attributes consistent with stem (CD14; Vimentin; Nestin), contractile (α-smooth muscle actin, αSMA) and macrophage (CD68 and phagocytosis) potential (Figure S2 H). Two days after plating, cells generate compression forces (indicative of vasoconstriction activity [29]) visible, using Differential Interference Contrast (DIC) imaging, as wrinkles in the silicone sheet (Figure 2D). These are organized around local nodes of higher contractility (Movie S3), correlated with the expression of αSMA protein, a key determinant of pericyte contraction (Figure S2 I–K). Remarkably, αSMA is also enriched in DLPs in vivo, suggesting that they may represent strategic nodes for the regulation of brain vessel tone (Figure S2 L–N'). Time-lapse confocal analysis showed that individual wrinkles change in position and length over time (between 20 and 200 μm) and cycle with a period of approximately 25/40 minutes (Movie S3; Figure S2 O–O' and data not shown).

We then investigated if pericyte wrinkling-activity was affected by GBM cell addition. Although neither U373 nor U87 cells alone deform the substrate, in co-cultures they induce nearby pericytes to generate both new wrinkles and destabilize pre-existing ones (Figure 2E–G; Movie S4; Figure S2 P–P'). Quantification of the contractile activity with and without GBM cells, by tracking the behavior of identified wrinkles (Figure S2 O–O', P–P'), revealed a difference in the way the wrinkles move in space and time (E2–E1, P = 0.02, where E1 and E2 are defined as track-straightness of each wrinkle end, Methods). Moreover and interestingly, including the track-straightness of the wrinkle center (C) and plotting all the wrinkle data in a 3D-scatter graph (Figure 2I), showed that GBM cells abolish the formation of wrinkles that drift laterally or drift laterally and pivot, typical of pericytes in nodes and inter-nodes (point clusters 1 and 2, respectively), leaving only the less organized activity characteristic of anti-nodes (point cluster 3; Movie S3). In conclusion, therefore, our data provide strong evidence that tumor cells can corrupt the intrinsic contractility of brain pericytes.

Next, we investigated the cellular mechanisms employed by GBM cells to induce pericyte dysfunction. Live imaging of U373 and U87 GBM cell/pericyte co-cultures on silicone-laminin substrates identified long extensions (maximum length 81 ± 32 μm) (Figure 3A; Figure S2 P, P'), characterized by a discontinuous distribution of both cytoplasmic varicosities and GFP-actin (Figure 3B, C). Moreover, co-transfection assays in vitro showed that the small GTPase Cdc42, a principle regulator of cell polarity and actin cytoskeletal organization [30], is co-localized with the actin beads within cytoplasmic varicosities (Figure S3 A) and the native protein is visible within the GBM cell extensions on silicone substrate (Figure 3D). Importantly, analysis of human CD44, a cancer-associated cell surface adhesion molecule [31], revealed that the edges and tips of GBM cell elongations contact the target pericyte (Figure 3E, F). Remarkably, tumor cell projections predict the locations where the wrinkling pattern is changing (Figure S4 A–C’; Movie S5 and Figure 3C). Taken together, these converging lines of evidence strongly suggest that the cellular extensions seen on silicone substrates are similar, if not identical, to the flectopodia described above (Figure 1, 2).
supporting our cell-hybrid data in mouse xenografts (Figure S5).
Thus, these results identify pericytes, for the first time, as a specific
GBM cell-target for the production of fusion-like hybrids, with the
potential to generate novel malignant cell variants, such as the
hyper-contractile GDH cells, strategically located to maintain a
hypoxic penumbra at the invasive edge of the tumor.

Next, considering that flectopodia are actin-based extensions
from highly polarized cells, we reasoned that inhibition of the actin
GTPase Cdc42 might block vessel co-option. Immunohistochem-
istry on GBM cells seeded onto brain slices showed that Cdc42 is
enriched in flectopodia (Figure 4A). Reducing Cdc42 in tumor
cells, using either siRNA (iCdc42) or the specific Cdc42-inhibitor
Secramine-A [33], results in shortened extensions to vessels and in
reduced angle of vessel bending in brain slices (Figure 4B-E).
Secramine-A also decreases the likelihood of a bend occurring
where a tumor cell is attached to a blood vessel (Figure S8 A, B).
To test Cdc42-function at the tumor/host margin, GBM cell-
pericytes, with and without inhibition for Cdc42, were grafted either
separately or adjacent to each other into brain slices (Figure 4F).
Importantly, while wild type-cells pull some vessels into the graft
and use others for radial migration, iCdc42-treated cells show little
affinity for blood vessel and no co-option (Figure 4F–H; Figure S8
C, C’). Co-opted vessels present abnormal constrictions, dilations and
localized hairpin bends, while vessels adjacent to iCdc42-
grafts maintain a straight morphology (Figure 4F1 and F2, respectively).

We then showed that CD44, a GBM marker with fusogenic
properties [34], is enriched at vessel contact sites and cooperates
with Cdc42 in vessel co-option/modification in brain slices. Our
data demonstrated that knocking down CD44 (by shDNA) in
combination with Cdc42 increases the inhibitory effect of iCdc42
alone on flectopodia-length and the angle of vessel bending, with a
reduction in the number of glomeruloid-like structures by 70%
(Figure S8 D–H). Additionally, iCdc42 shifts tumor cell-phenotype
from ensheathing/re-arranging vessels, to a loosely associated
state, a tendency amplified when CD44 is also reduced (Figure S8
I, J). Taken together, these data suggest that Cdc42 and CD44 act
eysnergistically during flectopodia-induced vessel co-option/modi-
fication.

Subsequently, we tested the effect of iCdc42-GBM cells on
pericyte behavior on silicone/laminin substrates. In addition to the
initial pericyte activation induced by wild-type GBM cells (Figure
S9 A–B’, E–F’, M), confocal live imaging showed a further
pericyte-transformation into hyper-activated macrophage/dend-
ritic-like cell phenotype, capable of killing and engulfing
iCdc42-treated tumor cells, with concomitant overall reduction in
wrinkling activity (Figure 4I; Movies S8, S9; Figure S9 C–D’,
G–H’, I–L). In summary, this study uncovers a switching role for
Cdc42 not only in flectopodia-mediated vessel co-option, but also in
suppressing the activation of pericytes into cytotoxic, macro-
phage-like phenotypes.

We next investigated whether iCdc42-treatment could block
vessel co-option and promote an immune response in vivo. Seven-
day xenografts of wild type-GBM cells show high levels of h-CDC44
and h-Nestin, with m-Rgs5−/−-cells around diluted, co-opted vessels
(Figure 4J). In contrast, iCdc42 tumors appear to be compromised.
Three days after grafting, implants present only a thin h-CDC44−/−
shell and an intense m-Rgs5−/−-core, with no evidence for h-Nestin−/-cells or vessel co-option (Figure 4K; Figure S9 P). By 7-days, the h-
CDC44−/−-shell and the m-Rgs5−/−-core are reduced or even absent
(Figure 4L), with an accumulation of vimentin−/−-microglia at the
implantation site, demonstrating an increased host phagocytic
response (Figure S9 Q). Taken together, our data indicate that
Cdc42 activity in GBM cells favors tumor-establishment over
clearance.

Targeting the Cdc42/CD44/actin/pericyte/hypoxia axis (dem-
onstrated above), to block vessel co-option in patients, depends
critically on the underlying mechanism being conserved from
mouse to human. Strikingly, we found that 88% (7/8) of an
unbiased sample of 8 primary human GBM tumors show
abnormally elevated levels of CDC42 and CD44, restricted to
perivascular locations on abnormal blood vessels at the boundary
between tumor tissue and normal looking brain (Figure S10 A–C),
a feature reproduced in an independent GBM biopsy (Figure S10
E). Interestingly, these two genes are part of a coordinately
expressed group of genes (synexpression group), that may function
together in tumorigenesis, including Hypoxia induced factor-1
alpha (HIF1A), the actin binding protein Transgelin (TAGLN,
SM22) and Platelet-derived growth factor receptor beta (PDGFBR),
markers of abnormal tumor vessels [35] and pericytes [36],
(Figure S10 A–C). Notably, all the tumors (2/8) that recur 6 months after radio-
chemotherapies correlate to those cases where expression of these genes are greatest. Taken
together, this validates the Cdc42-CD44/actin/pericyte/hypoxia
axis as a desirable target for GBM therapy.

Overall, our results suggest a 2-signal model for GBM
progression that involves two distinct tumor cell-derived signals,
which act on contractile brain pericytes (Figure 5 and Figure S11
A–D). The first signal (signal-1) causes pericyte activation and
conversion to phagocytic, macrophage-like cells. In contrast,
signal-2, which is flectopodia-dependent and requires active
Cdc42 function, promotes vessel co-option by engaging contractile
pericytes. The combination of these events generates fusion-like
hybrids. In the presence of both signals, the tumor expands by
continuous co-option and diversification, while, in the absence
of signal-2, it is cleared by the unrestricted generation of cytotoxic
cells, derived from the activation of contractile pericytes.

Discussion
Recent advances in glioblastoma research have shown that
GBM is propagated by perivascular stem-like cells [37] and that
Figure 4. Cdc42-inhibition in GBM cells blocks flectopodia-mediated co-option and activates innate immunity. A, Cdc42 is present in U373 cell-flectopodia in brain slices (arrows). B, GFP-actin-labeled tumor cell (green; red color indicates transfection of the oligonucleotide siRNA control) co-opting a bent vessel (red arrowheads). C, A non-polarized, iCdc42-treated, GFP-actin+ U373 cell (yellow, arrowhead, indicates double labeling of green [GFP] and red [negative control for transfection]), on a straight vessel (Ink-filled, black-filled-white arrowheads). D–E, Graphs of the...
Our discovery of a role for pericytes in vessel co-option provides a plausible explanation for these salient features of GBM. Recruitment of activated-pericytes may determine the perivascular location of tumor propagating cells, with transfer of stemness resulting from a fusion-like process and/or cytoplasmic inheritance. The requirement for ongoing co-option at the expanding tumor margin also suggests a reason for the failure of anti-angiogenic therapies, which target only new vessel formation [39].

In contrast to current cancer models that emphasize mutation and clonal expansion from a single-cell of origin [40], our work implies that glioblastoma is a dual cell of origin-disease, where tumor diversification is driven by GBM/perivascular cell coupling (Figure S1 E, F; Discussion S1).

A novel finding of this work is the discovery of flectopodia, as an important GBM cell specialization with the ability to alter vessel morphology. Flectopodia show a unique organization of actin in beads, which seems to reflect the ongoing trafficking of tumor cytoplasm into the cortex of the recipient pericyte. We found that vessel co-option and flectopodia formation and function are dependent on the activity of Cdc42 in tumor cells. Although the precise mechanism of Cdc42-action in this process remains to be determined, it likely relates to its well-established role as an evolutionary conserved organizer of the cortical actin cytoskeleton during cell polarization [30]. This suggests a number of possibilities. First, Cdc42 could act to polarize GBM cells towards the target and to initiate pericyte contact. Second, Cdc42 also controls the focal development of matrix-degrading structures called podosomes, found in normal cell types, such as macrophages and smooth muscle cells [41], and of functionally similar structures called invadopodia in several metastatic tumors [42] including glioma [43]. In spite of the dissimilarities in the actin organization in flectopodia and invadopodia (beads, described here, versus short filaments in bundles [44], respectively), it is intriguing to speculate that flectopodia represent the tissue-version of the in-vitro invadopodia. Alternatively, they might be GBM extensions highly specialized for the interaction with vessel pericytes. Finally, and perhaps more interesting, GBM cell-Cdc42 may act directly within the target pericyte to modify its behavior, as suggested by our demonstration that it is one of the proteins transferred (Figures S3 D-G and S7 B). In smooth muscle cells, physiological contraction in response to vasoconstrictor stimuli requires signaling through Rho-GTPase and Rho-kinase (ROCK), which act specifically by remodeling αSMA and myosin containing stress fibers. Smooth muscle cells also contain a second pathway that leads to actin polymerization and contraction, which is localized in the cortical cell cytoplasm and is mediated, through integrin activation by mechanical stress and contraction stimuli, by Cdc42 [45]. Since contractile pericytes behave similarly to smooth muscle cells and since overexpression of activated-Cdc42 is linked to hyper-contractility of the acto-myosin cortical cytoplasm [46], it
is interesting to speculate, therefore, that local transfer of GBM-Cdc42 could cause inappropriate contraction of the cortical cytoskeleton, with consequent reorganization of blood vessel morphology. Our identification of Cdc42 as a molecular switch that drives flectopodia-mediated GBM cell/pericyte interaction reveals a new role for Cdc42 in addition to its previously proposed function in GBM cell migration [47], [48]. Interestingly, Cdc42 is implicated in regulating altered cell morphology downstream of TP53 [49] and PTEN [50], two tumor-suppressor genes mutated or inactivated in glioblastoma [51]. Hence, this suggests the intriguing possibility that Cdc42-mediated flectopodia formation results from inactivation of these two key regulatory genes.

Our findings also support pre-existing evidence indicating a direct molecular interaction of Cdc42 and CD44 in association with tumor cell-actin cytoskeleton [31], by demonstrating that they synergize in altering vessel architecture in brain slices. In particular, our data suggest that the adhesion protein CD44 could be involved in anchoring the cell to the vessel, facilitating the transfer/bending process.

The presence of Nitrotyrosine co-localized either within, or close to, fusion-like derivatives (GDH cells) suggests that these cells could represent a focal source of oxygen and nitrogen free radicals that might cause long lasting alterations in pericyte contraction. This is reminiscent of the Nitrotyrosine-containing pericytes that control the capillary ‘no-tello’ phenomenon in ischemia reperfusion models of stroke [52]. The location of GDH cells on constricted and dilated blood vessels beyond the tumor margin indicates that they may contribute to creating the hypoxic penumbra, which surrounds the tumor core and which may promote tumor progression. It has also been suggested that reactive Nitrogen species and their derivatives, such as Nitrotyrosine, provide a “chemical barrier” that maintains both a tumor-specific immune response and supports the escape phase of cancer [53]. The presence of Nitrotyrosine associated with GDH cells, in our work, therefore implicates these strategically placed tumor cell/pericyte derivatives as part of such an immunological barrier.

Our work provides additional support for the idea that brain pericyte are part of the innate immune system and can behave as macrophage-like cells which possess phagocytic activity [17]. We have shown, here, that isolated brain pericytes in vitro express the macrophage [54] and general phagocytic [55] marker CD68 and are able to engulf fluorescent beads. Furthermore, the cell morphologies of our pericytes co-cultured with GBM cells on laminin-coated silicone substrates present a similarity to M1 and M2 polarized macrophage phenotypes described by others [56]. Specifically, our round and/or spindle-shaped pericytes, induced by wild-type tumor cells, resemble the anti-inflammatory M2, Tumor Associated Macrophages (TAMs) involved in tumor promotion. In contrast, dendritic-like morphologies found in our pericyte co-cultures with iCdc42-GBM cells, which are able to pursue and phagocytose them, are more similar to pro-inflammatory, tumoricidal M1 macrophages. Thus, pericytes are highly plastic cells that can respond to GBM-mediated signals by changing their contractility, detaching from the substrate and acquiring a pro- or anti-tumor activity. Although it remains to be determined how our proposed two signal-model (Figure 5) fits with the well characterized signaling systems involved in tumor immunology, we speculate that it could involve inflammatory cytokine networks and purinergic signaling (Figure S11 G, H).

In conclusion, our findings reveal that brain pericytes not only provide the materia prima for GBM progression, through blood vessel co-option and immune suppression, but remarkably may also represent its ‘Achilles’ heel’. Taken together, Cdc42-inhibitors (possibly in combination with anti-angiogenic drugs) may prove therapeutically beneficial not only against primary and recurrent glioblastoma, but also other tumors that co-opt blood vessels during brain metastasis [57].

Materials and Methods

Human tumor cells and in vivo labeling

Human glioblastoma cell lines U87-MG and U373-MG were purchased from American Type Culture Collection (ATCC) and European Cell Culture Collection (ECACC), respectively, and grown in α-MEM medium with 10% fetal bovine-serum (Invitrogen). Cells for imaging were transfected with: GFP-actin plasmid (pEGFP-actin, Clontech), encoding a fusion of EGFP and human cytoplasmic beta actin; EGFP-plasmid alone (pCAGGS-GFP); Cherry-Cdc42 plasmid, encoding an N-terminal fusion of mCherry and human Cdc42 (GeneCopoeia); GFP-RR (for GFP targeting to the outer mitochondrial membrane, kind gift from Dr. Nica Borgese, CNR, University of Milano, Italy) and GFP-beta3 integrin (kind gift from Dr. Victor Small, Institute of Molecular Biotechnology, Vienna, Austria). For chemical transfection, we used Superfect reagent (Qiagen) according to manufacturer’s instructions. Dextran-labeled U87 and U373 cell pellets (biotinylated mini-Ruby, MiRU, Fluoro-Emerald, FlEm, or Alexa-Flour 647: D3312, D7178 and D22914, respectively, Invitrogen/Molecular Probes) were prepared in 20 μl hanging drops [58], containing 45,000 cells, 17.5 μl growth medium and 2.5 μl of Dextran stock (0.5 mg in 40 μl H2O). Cultures were incubated for 48 hours and cell pellets were washed prior to use, either as grafts into mouse brain slices or as implants into mouse brains. In some experiments, single cell suspensions of Dextran-labeled cells were generated by trypsinization and trituration of labeled cell pellets. GBM cells for 2-day mouse xenografts were transfected with h-Cherry-Cdc42 and used to prepare hanging drops. In some cases GFP-actin transfected cells were co-labeled with CMITM (5-6-4-Chloromethyl-Benzoyl-Amino-Tetramethylrhodamine).

Pericyte isolation and in vitro co-cultures

Mouse brain pericytes for co-culture experiments were isolated according to the method of Oishi et al. [59]. Pericytes and GBM cells were labeled separately with different color Dextrans, as hanging drops. Prior to co-culture, cell aggregates were dispersed to a single cell suspension with trypsin, mixed at a ratio of 1:1 with GBM cells and plated on glass coverslips for 15 hours. To identify hybrid cells, co-cultures were trypsinized, replated and checked for double-labeling 12 and 48 hours later.

Inhibition of Cdc42 and CD44 function

For Cdc42-inhibition, U373 and U87 cells were transfected using Hyperfect reagent (Qiagen) with a mixture of 3 siRNA (small interfering RNA) oligonucleotides against human-Cdc42 sequences (100 nM/each) (these were from 5'-3': Cdc42-10505, GGCU- GUCAGAUGUGGGAGGtt; Cdc42-10429, GGAUUAUGACAGAUAGGTt; Cdc42-10324, GGCGAGAGAGGUUGAAGAt; Ambion) or with an Alexa Fluor red-conjugated siRNA, as a transfection control. Inhibited cells were used after 72 hours for seeding on brain slices or after 48 hours for making hanging drops. Scramble-A (Cdc42-inhibitor; kind gift of Dr. T. Kirchhausen, Harvard Medical School, Boston [60]) was added in serum-free medium, at 25 nM, for in vitro experiments, and at 15–50 nM in 2% FBS-containing medium for brain slices. To inhibit CD44, we
used 1.5 μg/3.5 cm dish of a mixture containing equal amounts of 5 different shRNA lentiviral-plasmids (cDNA-inserts were: CD44-57563, CCGGGGCCCTATGGTTCCCAATCGAGTT-TTGAAAATCCTAATAGGCTTTGTT; CD44-57564, CCGGGGCCCTATGGTTCCCAATCGAGTT-TTGAAAATCCTAATAGGCTTTGTT; CD44-57566, CCGGGGCCCTATGGTTCCCAATCGAGTT-TTGAAAATCCTAATAGGCTTTGTT; CD44-57567, CCGGGCCCTATGGTTCCCAATCGAGTT-TTGAAAATCCTAATAGGCTTTGTT; all purchased from Sigma), which were co-transfected with GFP-actin. CD44-inhibited cells (with a reduction of CD44 protein level by 27%, which reflects the transfection efficiency of GFP-actin of approximately 30%) were used after 5 days (for slices) and 60 hours (for hanging drops).

**In vivo vessel visualization**

To label cerebral blood vessels with DiI or DiD (Invitrogen/Molecular Probes), mice (Harlan Laboratories) were anaesthetized with Ketamine (100 mg/kg) and Xylacine (10 mg/kg) and 0.8 ml of a 0.05 mg/ml solution of DiI or DiD (1:10 dilution of 0.5 mg/ml-DiI/ethanol in 30% sucrose) was injected into the left atrium of the heart, followed 2 minutes later by transcardial perfusion with PBS. To label with ink, anaesthetized mice were transcardially perfused with 15 ml of a 20% solution of black drawing Ink mixture of 20% Ink and 4% PFA. Brains were either embedded in paraffin-wax (standard procedures) and cut at 7 μm or in cryoprotectant and cut at approximately 30 μm. This produced grafts that integrated into the striatum, the cortex or the hippocampus.

Mice, 2-days to 3-months post grafting, were perfused using a mixture of 20% Ink and 4% PFA. Brains were either embedded in agarose and cut at 120 μm using a vibratome, or embedded in paraffin-wax (standard procedures) and cut at 7 μm. For thick slice-grafts, cell pellets were manipulated using tungsten needles and pushed inside the region of the striatum. For grafting into NG2DsRedBAC brain slices, hanging drop cell pellets were injected through the edge of a 1 mm-coronal slice using a hand pulled Pasteur pipette to leave a tumor cell implant extending through the striatum. Slices were fixed between 12 and 72 hours after grafting and cut at 30 μm.

**Western blot, immunocytochemistry, immunohistochemistry and in-situ hybridization**

For western blots, U373 cell lysates were separated on 10% SDS-polyacrylamide gels, and probed for: human Cdc42 (using mouse monoclonal antibody, BD Bioscience, 1:350) plus anti-mouse horseradish peroxidase-labeled horse secondary; human CD44 (polyclonal rabbit antibody, Abcam, 1:4,000) plus anti-rabbit horseradish peroxidase-labeled goat secondary. Rabbit polyclonal (GeneTex) and mouse monoclonal (Sigma) antibodies were used to detect human β-actin (1:6,000), for loading control. Secondary antibodies were visualized by chemiluminescence (ECL, Amersham) and quantified using Quantity One software (BioRad).

For immunocytochemistry and immunohistochemistry, we used mouse monoclonal antibodies against human-CD44 (1:100, BD Pharmingen), mouse/human-Cdc42 (1:50, BD-Transduction Lab), human-Nestin (1:200, Chemicon) and human-alpha3beta1 Integrin (1:50, Chemicon); chicken polyclonal against mouse/human-Cdc42 (1:50, GeneTex); mouse monoclonal against mouse/human-Vimentin (1:60; clone V-9, Sigma) and alpha-
Smooth Muscle Actin (αSMA) (1:70, in vitro, and 1:50 in thick vibratome slices, Abcam); rabbit polyclonal against Laminin (1:100; Chemicon), NG2 chondroitin sulphate proteoglycan (1:60, Chemicon), Nitrotyrosine (1:50, Millipore) and RFP (1:400 and 1:250, in vitro and thick vibratome slices, respectively; MBL); rat anti mouse-CD44 (1:30, BD Pharmingen), mouse-CD68 (1:60, Abd-Serotec) and clone rat-401 against mouse-Nestin (1:150, Millipore). Actin cytoskeleton was visualized by Phalloidin, conjugated with Alexa-488 or 594 (Molecular Probes). Single and double labeling patterns were revealed by fluorescence microscopy, using goat anti-mouse secondary antibodies coupled to Alexa 488 and 594 or to Cy3 (Invitrogen/Molecular Probes). In some cases, fluorescence samples were counterstained with Hoechst (0.001%, Sigma) to label the nuclei and reveal the cytoarchitecture, prior to mounting in Moviol. Alternatively, labeling patterns were revealed by bright field microscopy, using HRP-conjugated anti-mouse secondary antibodies followed by ABC reagents (VectorLabs), plus either Diaminobenzidine (DAB, brown) or 3-aminon-9-ethylcarbazole (AEC, red; Sigma). ABC reagents were also used to directly reveal biotin in MiRu-labeled cells, after boiling in citrate-buffer (pH 6). Cytoarchitecture in bright field sections was visualized using Cresyl Violet staining (0.25%). Finally, human nuclei were detected by hybridization with a FTTC-labeled human specific pan centromeric probe (1695-F-01 Star*FISH, Cambio, Cambridge UK) and visualized using an alkaline phosphatase coupled anti fluorescein antibody and BM-purple substrate (Boehringer).

In situ hybridization for Rgs5 was performed according to standard methods using a full-length mouse cDNA (purchased from imaGenes, Berlin) as a template for the probe.

Gene expression analysis on Human Glioblastoma Tumors. Resected Human GBM tumors were analyzed for tumor associated gene expression markers, by mining the Allen Brain Atlas database of in situ hybridization data on Glioblastoma tumor sections. An additional Human GBM biopsy was used for immunohistochemistry (provided by Dr. J. Sola, Hospital ‘Virgen de la Arrixaca’ Murcia, University of Murcia, Spain).

Ethics Statements. All experiments with mice were in accordance with the Spanish law 32/2007 of November 7th, for the care, use, transport, experimentation and sacrifice of animals. The use of human GBM biopsies was approved by the Ethical Committee of Clinical Investigation.

Hypoxia detection. To detect hypoxia in mouse brains, mice were injected intra-peritoneally with Pimonidazole-HCL (Hypoxyprobe, Inc) (60 mg/kg body weight) and fixed 30 and 80 minutes later by transcardial perfusion with PFA/Ink. Bound-Pimo was detected in 7µm sections. GBM cells were injected onto the brain surface and the window was then closed with a glass coverslip and held in place with silicone grease. Inverted and upright multiphoton microscopes were connected to either a Millenia-Tsumani or a Mai Tai HP iSapphire picosecond laser (SpectraPhysics, Mountain View, CA, USA). All images were collected using internal spectral detectors and LCS Lite Software (Leica). The objective lenses we used were: 20x/0.50 and 63x/0.9 N.A. (both in water) HCX Apo U-V-I; 63x/1.20 N.A. (water) HCX Plan Apo CS; 63x/30 N.A. (glycerol) HCX Plan Apo CS. For 2-photon and confocal microscopy we used glass bottom-dishes (MatTek Corporation) and imaged between 2 and 14 hours. Image and video processing (all acquired at 1024×1024 pixel resolution) were performed using LCS-Lite (Leica), Image J-1.41 (NIH Image package) and Imaris Software (bitplane). 3D video deconvolution was performed using Blind Deconvolution (algorithm developed by Autoquant, Media Cybernetics, and implemented by Leica). For multiphoton imaging, GFP and DiI fluorophores were excited at 900 nm laser wavelength and the emission filters were used were 500/524 and 549/582 nm, respectively.

Statistical analyses

For statistical analysis, we used Microsoft Excel (2003) and MATLAB Software. Tests were aimed to compare either features and behavior between control and iCdc42 and/or shCD44-treated GBM cells in brain slices or pericyte wrinkling behavior, with and without GBM cells, on deformable substrates. For CD44-interference, we co-transfected shCD44-plasmid with GFP-actin and included only green cells in the analysis. Normal or non-normal distributions were evaluated using Kolmogorov Smirnov (K-S) test for non-parametric data (one-sample). K-S test for non normal data sets was used for comparing wrinkling behavior of the same region, before and after adding GBM cells, and length of protrusions of GBM cells interacting with blood vessels in brain slices. Length of protrusions (µm) has been grouped as follows: 0(0), 1(0–20), 2(20–40), 3(40–60), 4(60–80), 5(80–100), 6(100–120), 7(120–140), 8(140–160). For statistical analysis on the angle of vessel bending: controls: n = 13, median = 10, range: 0–40°; iCdc42: n = 15, median = 0, range: 0–65°, fraction (0 µm) = 73%; Secramine: n = 22, median: 0, range: 0–21 µm, fraction (0 µm) = 0%; iCdc42 + Secramine: n = 15, median = 0, range: 0–63 µm, fraction (0 µm) = 73%; Secramine: n = 22, median: 0, range: 0–21 µm, fraction (0 µm) = 0%; iCdc42 + Secramine: n = 15, median = 0, range: 0–43 µm, fraction (0 µm) = 87%. K-S was also used for the estimation of the angle of vessel bending in brain slices (angle of bending, in degrees, has been grouped as follows: 0(0), 1(0–20), 2(20–40), 3(40–60), 4(60–80), 5(80–100), 6(100–120), 7(120–140), 8(140–160). For statistical analysis on the angle of vessel bending: controls: n = 13, median = 106, range = 45–125°, fraction bending-angle
Pericytes Control Glioblastoma Malignancy

(0°) = 0%; iCdc42: n = 13, median = 0, range: 0–160°, fraction (0°) = 54%; iCdc42 shCD44: n = 13, median = 0, range: 0–160°; fraction bending-angle (0°) = 62%. For evaluation of bending events: controls: n = 29, total fraction of cell-associated bent vessels = 83%; Secramine A-treated: n = 29, total fraction = 36%. Wilcoxon Mann Whitney test for non-parametric data was used for: vessel co-option in thick slices (n = number of grafts in a total of 14 thick slices). Chi Square (χ²) test for descriptive statistic was used to compare differences in the frequency of vessel bending (bending events) and differences in glomeruloid-like structure formation in brain slices (controls: n = 36, total fraction of cell-associated glomeruloids = 36%; treated: n = 31, total fraction = 6%). Student’s t-test for parametric data was used for the number of infiltrating cells on vessels in thick slices (n = number of grafts in a total of 14 thick slices). K-S and t-tests were two-tailed. Significance value (P < 0.05) was adjusted to avoid inflated type-1 error: α = 0.05/4 (for brain slices); α = 0.05/2 (for thick slices). Protrusion length was counted only for GFP-actin labeled cells contacting vessels, either directly or through extensions, using Leica Application Suite (LAS) AF6000 software. For statistical analyses on protrusion length, a length = 0 μm was assigned to each cell lacking protrusions. To measure the angle of vessel bending, each bent segment was manually traced from the microscope screen onto an acetate sheet and the angle of deviation, from vessel axis, was measured using a protractor. A bending angle degree = 0 was assigned to straight vessels. For tracking selected wrinkles on silicone substrates, xyzt-data from confocal videos were imported into Imaris Software (5.7 version, Bitplane) and used to generate 4D movies. Identified wrinkles were tracked using the Filament Tracking package on the z-series frame offering the sharpest focus. Specifically, we tracked the displacement of the ends of each wrinkle (designated as e1 and e2), for 5 wrinkles across each area of 0.03 millimeters squared. Among the various statistical parameters offered by Imaris Software for the analysis of each track (e1 and e2), we selected the value defined as ‘track straightness’ (E1 and E2, indicating track displacement/track length), which represents a numerical measurement of how the ends of the wrinkle can move in space and time. With pericytes alone, the track straightness is generally higher than in co-culture with tumor cells. This reflects the tendency for wrinkles to either: 1) drift laterally or drift laterally and pivot, which represents the majority of the pericytes located in the regions surrounding the nodes and in the regions connecting them (internodes); 2) increased local movement and/or less lateral displacement, shown by a relatively small percentage of pericytes located in the anti-node regions. In the presence of tumor cells, the reduction in the wrinkle track straightness indicates the second behavior as the main modality of pericyte contraction. Imaris and Leica Application Suite programs were used for cell measurements and quantification in brain explants and on silicone substrates. The 3D-scatter plot used to illustrate wrinkle data distribution was obtained using MATLAB Software.

Supporting Information

Figure S1 Vessel co-option and remodeling by GBM cells in brain slices. GFP-actin transfection of GBM cells allowed to investigate the actin cytoskeleton dynamics during tumor cell/vessel interaction. A, In vitro, phalloidin staining (red) of glioblastoma cells transfected with GFP-actin construct (below) shows complete overlapping (yellow) with the actin cytoskeleton (green), at intracellular level (stress fibers, white arrowhead) and in cellular protrusions, both in ruffles (white arrows) and in filopodia (arrow in inset), while GFP transfection alone shows very little co-localization in ruffles (white arrow and yellow color) and no co-localization either in stress fibers (arrowhead) or filopodia protrusions (arrow in inset). B, 2-photon video frames showing a co-opting glioblastoma cell making initial contact with a vessel (DiI-red) through cell polarization and emission of actin-enriched extensions (arrowheads); the white dotted line indicates the absence of protrusions at t0. Longer extensions with discontinuous actin (arrows) are polarized towards another vessel (red dotted line). C, Cell co-option of mouse brain meningeal vessels, following intracranial injection of GFP-actin labeled-GBM cell suspensions. Intravital imaging of the superficial neocortex confirms that injected U373 tumor cells (also labeled with CMTMR, red), after initial polarization towards blood vessels (v, DiI, red, dashed lines), emit actin-enriched thin cellular extensions (white arrow in i), which contact the vessel abluminal surface (inset: beaded organization of actin in the protrusion, arrows). Although thicker protrusions are detectable (yellow arrows in ii and iii), they always bear thinner terminal elongations that contact the vessel (dotted lines in magnification, iii). D E, frames from two 4D rendered-confocal videos (in E only the vessel is rendered), showing U373 cells modifying blood vessels (Ink-filled, grey) in brain slices. D, an additional example of a flectopodia-linked vessel modification (yellow arrows and lines); white arrows point to moniliform actin-distribution in flectopodia. Another, less elaborate, type of local vessel modification is also observed (E, live, and F, fixed; yellow arrowheads in E F and yellow lines in magnified insets in E), in which a cell envelops and kinks a narrow vessel, as indicated in the scheme (G). This type of local vessel alteration is coupled to the retraction of a long GBM cellular extension (E, white arrows) and formation of subcortical actin fibers (yellow arrow). D and E are taken from sequential videos of the same cell, with an interval of 1 hour (red arrows: vessel previously bent in D). Time in minutes. Scale bars: 6 and 1.5 μm (A and insets), 10 μm (B D), 20 and 11 μm (G-i and G-ii), 9 μm (F).

(TIF)
distribution of actin proteins (phalloidin, green, in i and αSMA, red, in f) in pericytes plated on silicone plus human laminin. Wrinkles in magnified box (arrowheads, K) are strongly correlated to αSMA expression (Ref [64] in Methods), as indicated in K, L. Coronal section through the striatum (Str) of a brain pre-labeled for DLPs and perfused with black-Ink shows that DLPs (M, green, asterisks) express αSMA (magenta, arrowhead in magnification in N), which correlates with constricted segments (N′, yellow arrowhead) of a Ink-filled vessel (N, white arrowhead). Nuclei (Hoechst) are in blue. O–P. The dramatic effect of GBM cells (ER dextran, magenta) on pericyte contraction is illustrated by comparing wrinkling patterns from confocal videos of the same field, recorded before (O) or after (P) GBM cell addition (host/tumor border indicated by dashed line in P). Asterisks (red in O) indicate the positions of 3 nodes, two of which (yellow in P) are destroyed. In the presence of GBM cells, destabilization of the wrinkles along the margin (replacement of stable pre-existing wrinkles, red arrows in O′), by unstable wrinkles that come, white arrowheads, and go, yellow arrowheads, in P) correlates with dynamic protrusion and retraction of GBM cell flectopodia-like extensions (indicated by dashed arrows in P′), O″ and P′: show the tracking data illustrated in Figure 2H projected onto the original, initial time point for each trace (t2 and t14, respectively). Time in minutes. Scale bars: 40 μm (A, D), 20 μm (B), 25 μm (C).

(TIF)

Figure S3 Cdc42 protein localizes in flectopodia varicosities and is transferred into pericytes in xenografts. A. Confocal video-frames of a U373 GBM cell in vivo. Co-transfection with GFP-actin (green) and CherryCdc42 (Ch-Cdc42, red) reveals the striking dynamic association between actin beads and local accumulations of Cdc42 (white arrowheads in magnifications) in the cytoplasmic varicosities within the long cellular extensions (white arrows in A). Arrowheads (B) and dashed-arrowed (C) indicate varicosities positive for GFP-tagged outer mitochondrial membrane protein (GFP-RR) and β3-integrin (arrowheads in insets), respectively, in the extensions of GBM cells cultured as 3D aggregates on laminin-coated glass (confocal video frames). Asterisks point to a labeled fragment released from the tip of the extension. Time in minutes. D, 3D confocal reconstruction (100 μm section) of a 2-day Ch-Cdc42-U373 cell xenograft, immunostained for the Cherry-tag (red); dextran-labeled-host cells, cyan, th, thalamus; hip, hippocampus; men, meninges. E, F. Graft-host interaction-zone from an adjacent section to the boxed-area in D, magnified in F; white arrows point to a cell double-labeled for Ch-Cdc42 and host-dextran. G shows localized tumour-Cdc42 transfer into a dextran-labeled, CD68 host cell (white arrows), indicating that cell/cell transfer can occur by a mechanism other than phagocytosis. White arrowheads point to a CD68′-dextran labeled-phagocyte. G is an adjacent section in the region boxed in D. Co-localizations (in F and G) are confirmed in confocal zy-sections (white asterisks). Scale bars: 8 and 2 μm (A and magnification), 25 μm (B), 15 μm (C), 550 μm (D), 50 μm (E), 20 μm (F), 10 μm (G).

(TIF)

Figure S4 GBM cell-flectopodia modify local wrinkling patterns of pericytes plated on flexible substrates. Videos frames (combined DIC and fluorescence confocal microscopy) of GBM cell/pericyte co-cultures on laminin-coated silicone substrates. A. Flectopodia extending from a FarRed-labeled U87 cell (arrows) coincide with the presence of new wrinkles (arrowheads). B. Wrinkles, centered around terminal varicosities (white arrowheads) of the flectopodium (white arrow) extending from a MiR−labeled-GBM cell (red color shown in first and final frames, only), are induced with a period of approximately 40–50 min (red arrowheads; presence, yellow arrowheads; absence). C. Flectopodia of two MiR+−GBM cells (arrowheads in C′′, lower magnification of C–C′), one of which transfected with GFP-actin (green, white arrowheads in C and C′, light blue arrowheads in C′′), show local accumulations of GFP-actin and dextran. Note that the waves of altered contraction of the substrate (red/yellow arrowheads in C) are in phase with periodic enrichment of actin at the tip of the flectopodium (white arrowheads in C′; blue arrowheads in C′′). D, Cytoplasmic particles (1 and 2, indicated by green dots), either pre-existing (magenta arrowheads) or released as varicosities (dark blue arrowheads) from the tips of flectopodia (arrow) translate and divide within the target pericyte. Time in minutes. Scale bars: 40 μm (A, D), 20 μm (B), 25 μm (C).

(TIF)

Figure S5 Tumor cell/host pericyte interaction generates fusion-like hybrids in xenografts and brain slices. A. Co-opted blood vessels (white and black-filled white arrows), in the graft 7-days post-implantation. B, MiR+−perivascular cells (arrowheads) inside the graft (i) are negative for human-centromeric DNA (h-cen DNA) (i′) and positive for mouse Rgs5 (i″), suggesting potential fusogenic zones in which human tumor cytoplasmic determinants are mixed with mouse nuclei. C, Scheme showing two FiEm (green)-injection sites, used to pre-label brain pericytes (DLPs) and the position of the GBM graft (MiR+labeled, red). Dashed line indicates the plane of section in D, E, F and J, D and E. Combined bright field and fluorescent images indicating a 3D confocal reconstruction of 120 μm-vibratome sections through a U373 (D) and a U87 (E)-graft, respectively, showing co-opted blood vessels in the tumor core (ink-filled; white arrowheads). cp, choroid plexus. D, E, I, FiEm and MiR+−GDH cells (arrows in D, E; arrowheads in I, magnification of an adjacent section, at the position indicated in D and E) on a constricted (J, arrow) and dilated (I, thicker arrow) vessel segment (grey color). Cell nuclei in blue (Hoechst). F–G, A combined bright field and fluorescent image showing DLPs from a control brain (arrows in F; arrowheads in G), located on straight vessels (ink-filled, black in F; grey in G). Central panel in G shows lack of MiR (red) signal. H, 3D rendering confirms that DLPs are pericytes, based on their perivascular location and large nucleus (arrow), compared with the smaller size of an endothelial cell nucleus (arrowhead). J, Staining for h-centromeric DNA of a 7-day MiR+−U373-xenograft, grafted into an unlabeled host. Black arrows in low magnification point to cells magnified in K and L, whose nuclei are either strongly positive (L, white arrows, type-1 cells) or negative (K, black arrows, presumptive GDH cell) for h-cen DNA. Another putative GDH cell is coupled to infiltrating type-1 cells (black arrows in L). Equivalent fields are shown as fluorescence (left hand side; MiRu, red, and Hoechst, pale blue) and bright field micrographs (right hand side); vessels v, ink-filled, black. Type-1 cells (N, arrowheads) express h-CD44 (green), which is absent in the presumptive GDH cell (M, white arrowheads); v: ventricle; v: vessel; yellow arrowheads: vessel dilations; white arrow: vessel constriction. O, Table showing the labeling characteristics and phenotypes for cells located in the tumor core (Type-1fusion-like, Type-1) and at the infiltrating margin (Type-1, Type-2 and GDH), for both U373 and U87 GBM xenografts. P−R, Confocal pictures of brain slices, pre-labeled with FiEm-Dextran for pericytes (green) and seeded with MiR+−U873 cells (red). Examples of novel recombinant cell types, that resemble...
those found in mouse xenografts: \( \text{P} \) shows a type-1-like cell located on a blood vessel (yellow arrow) and associated with a FIEm\( ^\text{a} \)-particle (white arrowhead in magnification); type-1-like cells are also present, either closely apposed (arrowheads in \( \text{Q} \) or partially co-labeled (white arrow in the confocal section, and asterisk in inset); \( \text{R} \), Double labeled cells (FIEm\( ^\text{a} \) and MiRu\( ^\text{a} \)), similar to GDH cells, are also visible. Arrows point to the almost complete co-localization of the two Dextras. Vessels: Ink-filled (white) or outlined by dotted lines; nuclei, Hoechst, blue. Scale bars: 100 \( \mu \text{m} \) (\( \text{A}, \text{D}, \text{J} \)), 30 \( \mu \text{m} \) (\( \text{F} \)), 20 \( \mu \text{m} \) (\( \text{G} \)), 8 \( \mu \text{m} \) (\( \text{H} \)), 10 \( \mu \text{m} \) (\( \text{B}, \text{K}--\text{N} \)), 7 \( \mu \text{m} \) (\( \text{P}, \text{R} \)).

[EPS]

Figure S6 Markers of altered pericyte contractility in short and long-term GBM mouse xenografts and induced hypoxia. \( \text{A} \) and \( \text{C} \) Show triple-labeled confocal reconstructions of 120 \( \mu \text{m} \)-coronal sections from 7-day MiRu-labeled (red) U373 (\( \text{A} \)) and U87 (\( \text{C} \)) tumors, grafted into mice with pre-labeled DLPS (FIEm, green) and stained for \( \alpha \text{SMA} \) (cyan). Although \( \alpha \text{SMA} \), a marker of contractile pericytes, is generally more prevalent in perivascular cells in U373 than U87 grafts (white arrowheads, \( \text{A}, \text{D} \)), both cell lines (\( \text{E} \) and \( \text{F} \)) boxed areas in \( \text{A} \) and \( \text{C} \) express very high levels of \( \alpha \text{SMA} \) (arrowheads in \( \text{E} \) \& \( \text{F} \)) in GDH fusion-like hybrids (arrow), located outside the graft on highly modified vessels (Ink filled, black). Similarly, Nitrotyrosine, a marker of nitritative stress, is also present in the grafts (arrowheads in \( \text{H} \) \& \( \text{J} \)), but shows its highest level (arrowheads in 1-1 in association with \( \alpha \text{SMA} \)-GDH cells (arrow), accumulating either within the GDH cell itself (\( \text{G} \), \( 3 \)) or in immediately adjacent pericytes (\( \text{I} \), \( 3 \)). This differential localization of Nitrotyrosine between U373 and U87 is also evident in the tumor core, where it is found either in putative fusogenic zones at the tumor/host interface (\( \text{G2} \)) or in contact with MiRu\( ^\text{a} \)-cells in perivascular locations in the graft core (\( \text{T2} \)), respectively. Infiltrating tumor cell-tongues (\( \text{K}, \text{MiRu}, \text{red}, \text{white arrows} \)) and Pimo-stained hypoxic regions (\( \text{L}, \text{brown}, \text{Pimonidazole} \)) in a 7-day U373-xenograft. White and black dotted lines: core of the implant; red dotted line: 100 \( \mu \text{m} \); white arrowheads). Particularly striking is the range of double-labeled cell morphologies seen at 12 h after replating, white arrowheads). Particularly striking is the range of double-labeled cell morphologies seen at 12 h, which includes cells containing both an enormous macronucleus (yellow arrowhead in \( \text{D} \)) and multiple micronuclei (dashed arrow), each surrounded by local accumulations of co-labeled cytoplasm, suggesting both abnormal DNA replication and possible segregation of aneuploid mini-cells. Furthermore, the differential distribution of human stem markers in the fusion-like derivatives suggests that a highly asymmetric process of cytoplasmic segregation occurs rapidly following mixing (\( \text{E} \)). In addition to complete cytoplasmic co-labeling, we also found cell/pairings (arrowheads in \( \text{F} \)) point to double-labeled zones) that appear to involve flectopodia-like extensions (arrows). Asterisk: accumulation of \( \alpha \text{S1} \) integrin at the tip of the flectopodium. Nuclei, Hoechst, blue. Scale bars: 30 \( \mu \text{m} \), 15 \( \mu \text{m} \) \( \text{B}, \text{C}, \text{E} \), 40 \( \mu \text{m} \) \( \text{D} \), 20 \( \mu \text{m} \) \( \text{F} \).

[TIF]

Figure S7 Generation of pericyte/GBM cell fusion-like hybrids \( \text{in vitro} \). Fluorescence micrographs showing the presence of double labeled-progeny (arrowheads in \( \text{A} \) \& \( \text{C} \)), 48 hours after co-culturing FIEm (green)-pericytes with GBM cells, labeled either with MiRu \{\( \text{MR}, \text{red}, \text{A} \)\} or Cherry-tagged Cdc42 protein (red, \( \text{B}--\text{C} \)). Arrow indicates \( \text{A} \) parental-type single labeled-cells and in \( \text{B} \) plurinucleated (black-filled white arrowheads), putative fusogenic zones. Immunocytochemistry on double-labeled derivatives shows that two human GBM markers associated with stenness (\( \text{h-Nestin} \) and \( \text{h-CD44} \), \( \text{D} \) \& \( \text{E} \)) respectively are maintained in early stages (12 h after replating, white arrowheads). Particularly striking is the range of double-labeled cell morphologies seen at 12 h, which includes cells containing both an enormous macronucleus (yellow arrowhead in \( \text{D} \)) and multiple micronuclei (dashed arrow), each surrounded by local accumulations of co-labeled cytoplasm, suggesting both abnormal DNA replication and possible segregation of aneuploid mini-cells. Furthermore, the differential distribution of human stem markers in the fusion-like derivatives suggests that a highly asymmetric process of cytoplasmic segregation occurs rapidly following mixing (\( \text{E} \)). In addition to complete cytoplasmic co-labeling, we also found cell/pairings (arrowheads in \( \text{F} \)) point to double-labeled zones) that appear to involve flectopodia-like extensions (arrows). Asterisk: accumulation of \( \alpha \text{S1} \) integrin at the tip of the flectopodium. Nuclei, Hoechst, blue. Scale bars: 30 \( \mu \text{m} \), 15 \( \mu \text{m} \) \( \text{B}, \text{C}, \text{E} \), 40 \( \mu \text{m} \) \( \text{D} \), 20 \( \mu \text{m} \) \( \text{F} \).

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Figure S8 CD44 synergizes with Cdc42 during GBM cell co-option and modification of blood vessels in brain slices. \( \text{A} \), GFP-actin transfected tumor cells (green) treated with the Cdc42-inhibitor Secramine-A appear morphologically round and not polarized (white arrowhead), on a straight vessel (black-filled white arrowheads). \( \text{B} \), The graph shows the effect on vessel modification due to the inhibition of Cdc42 activity by Secramine-A. Controls, \( n = 29 \); Secramine, \( n = 42 \). \( \text{C} \), Front and side views (\( \text{C} \) \& \( \text{C} \)), respectively) from the 4D rendering of a confocal video, showing a wild type-U373 cell implant (MiRu, red) next to an iCdc42 (FIEm, green)-treated implant. Wild-type cells (arrows) exit the graft on blood vessels (grey), while iCdc42-cells (arrowheads) are found scattered in the parenchyma. \( \text{D} \), High level of CD44 (blue) at contact sites with bent vessel segments (red arrowheads) in GFP-actin-transfected GBM cells (white arrows). Double inhibition for CD44 and Cdc42 leads to a round cell phenotype (GFP-actin\( ^\text{a} \), green in \( \text{E} \), CD44 in blue). Inset shows lack (red dotted-line) of CD44- labelling (grey color), with no evidence for vessel bending (yellow arrowhead). \( \text{F} \), \( \text{H} \), Statistical analysis of the outcome of Cdc42 interference, coupled to down-regulation of CD44, shows stronger inhibitory effect compared to Cdc42 inhibition alone; \( \text{F} \), controls and iCdc42 shCD44, \( n = 15 \); \( \text{G} \), controls and inhibited, \( n = 13 \); \( \text{H} \), controls, \( n = 36 \); treated: \( n = 31 \) (asterisks: see Methods for length/angle grouping). \( \text{I}--\text{J} \), Quantitative effects of Cdc42 and CD44 interference (graph, left hand side) on the frequency of 5 tumor cell morphologies in brain slices (hypothetical temporal sequence, right hand side). Scale bars: 10 \( \mu \text{m} \) (\( \text{A}, \text{C}--\text{D} \)).
Figure S9 Inhibition of Cdc42 in GBM cells converts contractile pericytes into a phagocytic, macrophage-like phenotype on silicone substrates and leads to tumor suppression in vivo. A–H’. To provide a sketch of the global dynamics of GBM cell/pericyte interactions, we created a single 2D image (maximum projection in both z and time axes) for each 4D movie of pericytes alone (A, C, E, and G; DIC optics) and cocultures, using FarRed-labeled (FR)-GBM cells, either U87 (B–B’), iCdc42-U87 (D–D’), U373 (F–F’), and iCdc42-U373 (H–H’). X, X’ and Y’ represent DIC, DIC plus fluorescence and fluorescence alone, respectively. This analysis showed a striking difference between the behavior of the two cell lines. U87 cells occupy a contained region of the field, with a clearly discernable boundary (dotted line in B’), reflecting a random cell movement within a circumscribed area. In contrast, U373 cells produce more or less parallel lines distributed throughout the field (arrows in F’), which represent the trajectories of directed cell migration. Red arrowheads: node regions present in the field, before and after GBM cell addition. Wild-type GBM cells also cause the activation of flat and contractile pericytes (P’ into raised (P’) and spheroidal pericytes (P’, green filled-black arrowheads in B’–B’ and F’–F’), normally characteristic of plastic anti-node regions of pericytes plated alone (tabulated in M). P’ pericytes also include mitotic and pseudo-mitotic cells (those with a transient cleavage furrow). The analysis of 4D-2D projection patterns for GBM cells inhibited for Cdc42 showed that the overall field features for both U373 and U87 cells were completely transformed. First, the normal organization of U87 as a circumscribed block and of U373 in quasi-parallel lines is replaced, in each case, by discrete compact FR-cell patches (D’’ and H’’), respectively, reflecting locally confined, rotating trajectories. Second, we found an overall reduction in wrinkling activity, correlated with a further activation of pericytes into a macrophage-like, phagocytic phenotype (P’/M, blue-filled black arrowheads point to one example), which come to surround the iCdc42-GBM cells (dotted lines in magnifications D’’’ and H’’’). I, J. Two examples of tumor cells (yellow arrows) been killed and engulfed by activated, macrophage-like pericytes (white arrowheads in I). Double arrows indicate end-points in the phagocytic process. Note the morphological transformation of an initially flat pericyte (P’ in J) into a spheroidal, macrophage-like phenotype (P’/M), via a transient raised state (P’). Time in minutes. K, L. Single time point video-frames, indicating that the appearing of round, macrophage-like pericytes in the top-half of the field (blue-filled black arrowheads in L) is coupled to the disappearance (yellow arrowhead) of a node (red arrowhead in K). A second node (red-filled black arrowhead) is established subsequently in the bottom-half of the field, where no GBM cells are visible. M. Tabulation of activated pericyte phenotypes (number of pericytes from a total of 600 cells/millimeter squared). N–O. Quantification of Cdc42 and CD44 knock down efficiency by Western blot and immunohistochemistry. Western blot (N) shows Cdc42 and CD44 levels (reduced to 20% and 73%, respectively), normalized for actin across different experimental groups. O. Histological and immunohistochemical analysis of control or iCdc42-U373 cell pellets pre-implantation, using the markers indicated. P. Cresyl Violet staining of U373 graft/host boundary (7-day xenografts), labeled in vivo for blood vessels (black Ink). Red arrow (P1) indicates abnormally dilated vessels; 1, boxed area in P1, showing the infiltrating margin of a control-graft (red dotted line); red arrows in 2 (boxed area in P1) point to dilations and constrictions of a vessel colonized by tumor cells. Boxed areas in P1 show, respectively, the well-defined margin (dotted line in P1’) and a morphologically normal vessel (red arrows in 2’), from an iCdc42-graft; c, cortex; cc, corpus callosum.

Q. Vimentin labeling (green) of the tumor mass in wild type-grafts (arrow in Q and magnified box-1) and of the host microglia (arrowheads in Q and Q’). iCdc42-grafts appear almost negative for vimentin (arrow in Q’), while there is an increase in host microglia response (arrowheads in Q’ and in magnification of box-2), which spread through the corpus callosum (small red arrow). Scale bars: 100 µm (A–H’, K, O–Q and magnification), 20 µm (I, J).

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Figure S10 A synexpression group of perivascular contractile/hypoxia cell markers in human glioma. A–C. Three independent glioblastoma tumors, taken from the Allen Brain Atlas database (www.brain-map.org). Note co-expression of the markers indicated (arrows and arrowheads in magnifications) in abnormal tumor vasculature (small arrows in Haematoxylin and Eosin stainings, HE), located at the border between the least (strongly stained) and more (weakly stained) differentiated tumor tissue (shown as dotted lines in A). The comparison of a presumptive fusion zone in the core of a 7-day U373-xenograft (MiRü), D, with an anomalous vascular structure in a human GBM biopsy E, shows the similarity of marker expression around highly modified vessels, raising the interesting possibility that the hypertrophic Cdc42+, laminin-rich-perivascular regions are the sites of ongoing GBM cell/pericyte amalgamation (white arrows in D: in E white arrows point to endothelium-free, hypertrophic perivascular layer). Dotted lines indicate the lumen of abnormal vessels; endothelial cell nuclei (E–iv, small white arrows) are visible only in that part of the vessel-wall lacking Cdc42 (white arrowheads). Nuclei in blue, Hoechst. Scale bars: 17 µm (D), 40 µm (E).

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Figure S11 Co-option signals 1 and 2 at the tumor/host margin and their hypothetical mediators. A. Interaction at the host/tumor margin. In the presence of functional Cdc42 (B), both signal-1 and 2 are active. Signal-1 activates pericytes to trigger the innate immune system and phagocytic response. Signal-2, by co-oping naïve, contractile pericytes, leads to vessel co-option and remodeling, and acts as an immunosuppressor by limiting the number of naïve pericytes available to be converted into macrophage-like cells. Signal-1 plus signal-2 also leads to the production of fusion-like hybrids. In the absence of Cdc42 (C), the constraint on pericyte activation is relieved, leading to rapid tumor clearance. E, Hypothetical scheme showing putative recombinant cell types generated by flectopodia-mediated transfer into naïve pericytes (TP1, TP2, left hand side), or by resolution of the tumor cell/pericyte-fusion hybrid1 (Hy1–Hy4, right hand side). The phenotype of the resulting cells is hypothesized to be specified by the independent segregation of cytoplasmic and nuclear determinants, where the behavioral phenotype depends primarily on the cytoplasmic factor. D and F Key legends. G. Signal 1 could involve pro-inflammatory molecules (red) released by GBM cells (green), which activate contractile pericytes to a macrophage-like, oncolytic phenotype (M1-type, white). During this acute inflammatory process, extracellular ATP (e-ATP), for instance, could promote the M1-type polarization and assembly of the P2X7R/NLRP3 inflammasome [64]. Signal 2, which opposes signal 1 and requires GBM cell/pericyte contact through flectopodia, results in the altered contractility of the pericyte and the localized clustering of F-actin in the pericyte cytoplasm (Figure 3F and Figure S11 H). This clustering could be due to the flectopodia-dependent transfer of F-actin beads into the target pericyte. Alternatively, or in addition, it may involve the production of PPI, from extracellular ATP [64], [65]. ATP hydrolysis involves the activity of the
and 18 min; rate: 4 fps (frames per second). 

**Movie S5 Actin-enriched flectopodia are associated with modifications in pericyte contractility.** Confocal video showing GFP-actin-U87 cells (green) interacting with pericytes plated on a laminin-coated silicone substrate. The contact of actin-enriched extensions of a GBM cell with the substrate (white arrows) is linked to new wrinkle-formation (red arrows), indicative of altered pericyte contractility. Yellow arrows point to substrate-relaxation following the retraction of the cell extension. Cyan arrows point to a GFPActin+cytoplasmic fragment (which varies between 3.6 and 4 μm in diameter, lines indicate the trajectory), moving onto the substrate in tight contact with pericytes. Δtime: 7 minutes; time: 2 hour 30 min; rate: 2 fps. (AVI)

**Movie S6 The contact with pericytes on silicone substrates is mediated by GBM cellular extensions, which can release cytoplasmic fragments.** Magnification of a detail from Video 4. While moving on the top of contractile pericytes spread on laminin-coated silicone substrates, a FR−U87 cell contacts the underlying pericytes through highly polarized protrusions (red arrows), and releases cytoplasmic fragments (asterisks) which are visible as varicosities (white arrowheads) at the tip of the retracting extensions (white arrows). Δtime: 10 minutes; time: 1 hour 10 min; rate: 1 fps. (AVI)

**Movie S7 GBM cells merge with brain pericytes on laminin-coated silicone substrates.** Magnification of a detail from Video 4, showing the step-by-step sequence of a merging between a tumor cell and a pericyte. The FR dextran−cytoplasm from a U87 cell (white arrowheads) is progressively transferred (yellow arrowheads) into an adjacent unlabeled pericyte (white arrows). Asterisks indicate the merging cell product, which then migrates away. Δtime: 10 minutes; time: 1 hour 40 min; rate: 1 fps. (AVI)

**Movie S8 GBM cells are destroyed by activated phagocytic pericytes.** An iCdc42-U87 GBM cell (yellow arrows) is pursued and caught by a non-contractile, macrophage-like, activated pericyte (white arrowheads), which appears to contain cytoplasm from a previous engulfed FR+GBM cell (yellow arrowheads). Blue arrowheads indicate the sequence of enveloping and destruction of the tumor cell, with the resulting uptake of the residual GBM cell into the phagocytic cell-cytosol (blue arrowheads). A second activated pericyte (white arrows) participates in the immune reaction. Δtime: 10 minutes; time: 8 hours; rate: 3 fps. (AVI)

**Movie S9 Inhibition of Cdc42 activity (iCdc42) in GBM cells induces pericyte immune response on laminin-coated silicone substrates.** An iCdc42-U373 GBM cell (yellow arrows) is chased by an activated host pericyte (white arrowheads), which finally engulfs it (blue arrowheads). The result of another phagocytic event is shown in the upper part of the video (second blue arrowheads). Note that the entire field is characterized by dendritic-like cells connected in a network by long thin extensions (red arrowheads). Δtime: 10 minutes; time: 8 hour; rate: 4 fps. Time between frames: 10 min; total time: 8 h. (AVI)

**Discussion S1 Tumor cell/pericyte fusion.**

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Author Contributions

Conceived and designed the experiments: EMC PHC SM. Performed the experiments: EMC PHC CRG. Analyzed the data: EMC PHC. Contributed reagents/materials/analysis tools: SM. Wrote the paper: EMC PHC.

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