Constitutive Activation of Myosin-Dependent Contractility Sensitizes Glioma Tumor-Initiating Cells to Mechanical Inputs and Reduces Tissue Invasion

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Abstract

Tumor-initiating cells (TIC) perpetuate tumor growth, enable therapeutic resistance, and drive initiation of successive tumors. Virtually nothing is known about the role of mechanotransductive signaling in controlling TIC tumorigenesis, despite the recognized importance of altered mechanics in tissue dysplasia and the common observation that extracellular matrix (ECM) stiffness strongly regulates cell behavior. To address this open question, we cultured primary human glioblastoma (GBM) TICs on laminin-functionalized ECMS spanning a range of stiffnesses. Surprisingly, we found that these cells were largely insensitive to ECM stiffness cues, evading the inhibition of spreading, migration, and proliferation typically imposed by compliant ECMS. We hypothesized that this insensitivity may result from insufficient generation of myosin-dependent contractile force. Indeed, we found that both pharmacologic and genetic activation of cell contractility through RhoA GTPase, Rho-associated kinase, or myosin light chain kinase restored stiffness-dependent spreading and motility, with TICs adopting the expected rounded and nonmotile phenotype on soft ECMS. Moreover, constitutive activation of RhoA restricted three-dimensional invasion in both spheroid implantation and Transwell paradigms. Orthotopic xenotransplantation studies revealed that control TICs formed tumors with classical GBM histopathology including diffuse infiltration and secondary foci, whereas TICs expressing a constitutively active mutant of RhoA produced circumscribed masses and yielded a 30% enhancement in mean survival time. This is the first direct evidence that manipulation of mechanotransductive signaling can alter the tumor-initiating capacity of GBM TICs, supporting further exploration of these signals as potential therapeutic targets and predictors of tumor-initiating capacity within heterogeneous tumor cell populations.

Introduction

Glioblastoma (GBM) is the most aggressive primary brain tumor and is characterized by poor survival even in the setting of surgery, radiation, and chemotherapy (1). Diffuse invasion of single cells within the parenchyma and along vascular structures frequently renders complete surgical resection impossible and leads to recurrence and eventual mortality. This in turn has motivated much effort to understand mechanisms of GBM invasion, an important goal of which is to discover potential molecular targets that could be manipulated to slow disease progression (2, 3). Although soluble and cell-bound factors have long been recognized as important regulators of tumor invasion, it has only recently become clear that biochemical and biophysical cues encoded in the extracellular matrix (ECM) can also strongly regulate tumor invasion. For example, in previous work, we showed that ECM rigidity can regulate GBM cell adhesion, motility, and proliferation, which in turn requires the contractile activity of myosin II (4), as well as the cell–ECM adhesion proteins α-actinin (5) and talin-1 (6). More specifically, when GBM tumor cells are cultured on comparatively stiff ECMS, they spread and migrate very readily whereas they adopt a rounded and immotile phenotype on highly compliant (soft) ECMS. This is consistent with observations in other tumor types that ECM stiffening can promote tissue dysplasia and local invasion through integrin-dependent potentiation of cell–ECM adhesion (7–9).

Much of our understanding of the importance of cell–ECM mechanotransduction in tumor progression is based on the use of continuous cell lines or, less commonly, heterogeneous primary tumor samples that are derived from the bulk tumor. Although these studies have provided much valuable mechanistic insight, it has become clear over the past decade that a very specific and comparatively rare subpopulation of cells plays especially key roles in populating the tumor and driving tumor recurrence.
following chemo- and radiotherapy. In the case of GBM, these tumor-initiating cells (TICs) are formally defined by their ability to recapitulate the original tumor when orthotopically xenografted into immunocompromised mice and are characterized by expression of a specific complement of molecular markers (e.g., CD133, nestin) and stem cell-like properties of self-renewal and differentiation into various tissue lineages. In addition to populating tumors, TICs directly participate in the invasion process in vivo. For example, both primary GBM TICs (10) and H-Ras-transduced neural stem cells (11) invade brain tissue before forming the tumor mass. Moreover, hypoxia, which is often associated with the TIC niche (12) and the necrotic tumor core in which GBM TICs may reside, can enhance migration of GBM tumor cells through induction of the family of hypoxia-inducible factors (13, 14).

Invasive motility through tissue is a physically integrated process that requires tumor cells to sense ECM-based mechanical inputs, dramatically change their shape, and exert propulsive forces against the microenvironment (2, 15). Interestingly, many TIC markers such as Oct 3/4 (16) and SOX2 (17, 18) have been shown to regulate cell behaviors that require cell–ECM mechanotransduction, such as motility and invasion. Conversely, several proteins known for their adhesive or cytoskeletal function have more recently been discovered to be highly enriched and functionally important in TICs, such as the actin-binding protein girdin (19), the α6 integrin subunit (20, 21), and the hyaluronan receptor CD44 (22) and its effector moesin (23).

These and other studies led us to speculate that cell–ECM mechanotransductive signaling systems play key roles in the ability of GBM TICs to interact with brain ECM and infiltrate tissue, and that targeting these systems may limit tumor growth and progression in vivo. Here, we explore this important open question by combining materials fabrication, single-cell biophysical tools, in vitro characterization of primary GBM TICs, and mouse xenograft models. We show that GBM TICs are capable of evading restrictions on spreading, motility, and self-renewal normally imposed by ECMs with compliance comparable with brain tissue, and that this mechanosensitivity may be restored by activation of myosin-dependent contractility. This contractile activation has the additional effect of limiting tumor invasion in a mouse orthotopic xenograft model and dramatically enhancing survival. Our work establishes the importance of cell–ECM mechanotransductive signaling in the initiation of GBM tumors and suggests a new set of molecular targets that may be manipulated to limit tumor infiltration.

Materials and Methods

Tumor sample and primary cell culture

The two patient-specific human brain tumor samples used in this study, L0 and L2, were collected in a previous study (10) after informed consent from male patients who underwent surgical treatment and Institutional Review Board approval. Briefly, the extracted tissue was placed in an enzymatic cocktail containing trypsin/ethylenediaminetetraacetic acid (0.05%) for 10 minutes at 37°C and filtered through a 40 μm filter. Cells were then propagated in neurosphere assay growth conditions (24) with serum-free media (Neurocult NS-A Proliferation kit, Stem Cell Technologies) that contained EGF (20 ng/mL, R&D), basic fibroblast growth factor (bFGF, 10 ng/mL, R&D), and heparin (0.2% diluted in PBS, Sigma). The tumor cells form gliomaspheres in suspension under these culture conditions and were serially passaged every 5 to 7 days when spheres reached a diameter of about 150 μm according to a previously established protocol (10). Gliomaspheres were dissociated with trypsin/ethylenediaminetetraacetic acid (0.05%) for 2 minutes and then replated in fresh media with addition of EGF, bFGF, and heparin. Both lines used were only passaged less than 20 times. These cells have been transcriptionally characterized and classified as the Classical subtype of GBM (25). Short tandem repeat (STR) analysis (University of Arizona Genetics Core; Tucson, AZ) confirmed that these cells had not been contaminated by any known cell lines.

Continuous cell line culture

U373-MG human GBM cells were obtained from the University of California, Berkeley (Berkeley, CA) Tissue Culture Facility in 2007, which obtained its cultures directly from the ATCC in 1995. Frozen stocks were made immediately upon receipt, and cultured for less than 6 months for experiments. We note that STR analysis has recently revealed that ATCC U373-MG cells share a common origin with the U251-MG glioma cell line (26), although these lines may have subsequently diverged to exhibit differential drug sensitivities (27). The tumor cells were cultured adherently in DMEM (Life Technologies) supplemented with 10% calf serum (I.R. Scientific), 1% penicillin/streptomycin, MEM nonessential amino acids, and sodium pyruvate (Life Technologies).

Differentiation of brain tumor-initiating cells

A previously established protocol for differentiating TICs was followed (10). Briefly, 5% calf serum was added to basal culture media that lacked growth factors. After 7 to 10 days, expression of lineage markers was assayed using immunofluorescence. For BMP-4–induced differentiation (Supplementary Fig. S2), TICs were incubated with BMP-4 (100 ng/mL; R&D Systems) in the absence of growth factors (bFGF, EGF, heparin) in adherent culture for 48 hours (28).

Xenotransplantation of human primary brain tumor-initiating cells into mice

Female 8-week-old nonobese diabetic/severe combined immunodeficient γ (NSG) mice (NOD.Cg-Prkdc(scid)Il2rgtm1Wjl/J; Sj) were implanted intracranially with 200,000 L0 TICs (CA Rhoa or Control) following institutional and national regulations, and according to a previously established protocol (10). The mice were treated daily with doxycycline (625 mg/kg) beginning at 4 days after implant until endpoint. Five mice were used for each cohort and followed until death.

Statistical analysis

Data are reported as mean ± SE. All pairwise statistical comparisons were performed with the Student unpaired t test, except as noted. Details of comparisons and replicates are provided in the appropriate figure legend.

Results

Tumor-initiating population is maintained on soft and stiff ECMs

Before asking whether ECM-encoded stiffness cues play instructive roles in regulating GBM TIC behavior, we first assessed the degree to which ECM stiffness might exert selective pressures that could compromise GBM TIC tumor-initiating or differentiation properties. To do this, we isolated two GBM TIC lines, L0 and L2,
from human primary GBM tumors as previously described; unlike continuous culture models of GBM, these TICs generate tumors with pathologic hallmarks and invasion patterns of GBM when orthotopically xenografted in immunocompromised mice, even after multiple passages in culture (10). We then cultured these GBM TICs in neurobasal growth medium on laminin-coated polyacrylamide gels of rigidity varying from 0.08 kPa to 119 kPa (4). In a previous study, it was shown that the top 5% most slowly cycling GBM TIC fraction contained the most tumorigenic cells (10). To rule out the possibility that ECM rigidity might be selecting for or against this key subpopulation, we first applied a serial dye dilution assay to measure the distribution of cell-cycling time (Fig. 1A). Flow cytometry of carboxyfluorescein-treated cells revealed fluorescence distributions similar to previously reported results and highly overlapping across all ECM rigidities. This result indicates that a slow cycling, tumor-initiating subpopulation is preserved across all ECM rigidities (Fig. 1A, arrow), prompting us to use the overall unsorted population for all subsequent studies. As additional evidence for an absence of selection, 100% of GBM TICs were found to express the self-renewal markers nestin and SOX2 (Fig. 1B) and could be differentiated by serum into glial and neuronal marker-positive cells across all ECM rigidities (Fig. 1C). Both cell lines demonstrated qualitatively similar behavior. Thus, alterations in ECM rigidity do not preferentially select for or against slow-cycling highly tumorigenic cells, alter stem cell marker expression, or compromise differentiation.

Tumor-initiating cells can spread, migrate, and proliferate on soft and stiff ECMs

In previous studies with continuous human GBM cell lines, we demonstrated that soft ECMs comparable with brain tissue (0.08–0.8 kPa) can strongly limit cell spreading, motility, and proliferation (4). We therefore asked whether similar regulatory effects would be observed for GBM TICs. To our surprise, L2 TICs adopted the same elongated morphology on soft ECMs as on stiff ECMs (Fig. 2A), with no significant differences in projected spread area (Fig. 2B). Moreover, GBM TICs did not exhibit prominent stress fibers or mature focal adhesions (Fig. 2C), structures strongly promoted by stiff ECMs in many other cell types (4, 8, 29). Qualitatively similar results were obtained for the L0 TIC line (data not shown). For both L0 and L2 TICs, random cell migration speed was only weakly sensitive to ECM rigidity (Fig. 2D), whereas proliferation was completely insensitive to ECM rigidity, with GBM TICs robustly migrating and proliferating even on the most compliant matrices (Fig. 2E). It occurred to us that the reduced stiffness sensitivity of GBM TICs relative to continuous GBM cell lines might simply be a consequence of the fact that GBM TICs are cultured in a neurobasal, serum-free medium, whereas continuous lines are typically cultured in a DMEM-based medium supplemented with 10% serum, which contains both ECM components and activators of adhesion and contractility (4, 10). To rule out this possibility, we cultured the continuous human GBM cell line U373-MG in both neurobasal and DMEM-based medium with or without 10% serum supplementation and measured average cell speed across a range of ECM stiffnesses (Supplementary Fig. S1). To assess correlations between stiffness-sensitivity and tumor-initiating capacity, we repeated the spreading and motility experiments with TICs after a brief (48 hours) treatment with bone morphogenetic protein 4 (BMP4), which has previously been shown to strongly inhibit tumorigenic potential (Supplementary Fig. S2; ref. 28). Notably, BMP4 treatment restored ECM mechanosensitivity with respect to cell spread area but not motility, with the exception of L2 on the stiffest ECM.

Myosin II-dependent contractility restricts rigidity-dependent spreading, motility, and invasion

Previously, we had shown that myosin II-dependent contractility is required for GBM tumor cells to sense ECM rigidity, in as
much as pharmacologic inhibition of myosins rescues spreading and motility on compliant ECMs (4). This observation is consistent with a model in which cells must exert traction forces through myosin motors to deform the ECM and sample local stiffness (30, 31). We therefore hypothesized that the ability of GBM TICs to evade motility limitations imposed by soft ECMs derives from insufficient generation of myosin-based contractile forces. Myosin-dependent cell contractility is activated by RhoA GTPase, which promotes myosin phosphorylation by activating Rho-associated kinase (ROCK; ref. 32). We thus performed gain-of-function studies in which we overexpressed a constitutively active (CA) mutant of RhoA (Fig. 3A) to determine whether increased contractility could restore suppression of motility on soft ECMs. Western blot analyses confirmed elevation of phosphorylated myosin light chain expression in the constitutively active GBM TIC lines (Fig. 3B). To investigate the effect of CA RhoA on cellular mechanical properties, we used atomic force microscopy (AFM) to measure cell elasticity (Fig. 3C). We found that transduction with CA RhoA conserved cell stiffness for L0 while pushing the observed stiffness range to higher values and significantly increased cell stiffness for L2 relative to control GBM TICs transduced with the control empty vector.

Expression of CA RhoA remarkably restored sensitivity to ECM stiffness, with CA RhoA GBM TICs significantly rounding up on soft ECMs and spreading on stiff ECMs (Fig. 3D and E; L2 CA RhoA GBM TICs shown, with L0 CA RhoA GBM TICs yielding similar results). In addition, CA RhoA strongly retarded GBM TIC motility on soft ECMs compared with stiff ECMs (Fig. 3F).

Notably, GBM TIC proliferation (Fig. 3G) and the universally positive expression of nestin and SOX2 (Fig. 3H) remained relatively insensitive to ECM rigidity. In addition, both control and CA RhoA TICs could still form neurospheres (Fig. 3I and J), which is a predictor of glioma tumor progression and is associated with poor clinical outcome (33). Altogether, these data confirm that constitutive activation of RhoA does not compromise GBM TIC self-renewal properties.

To verify that the observed increase in contractility is due to myosin activation per se as opposed to other downstream effects...
Increased Rho GTPase activation leads to stiffness-dependent spreading and motility. A, a genetic strategy for inducible activation of contractility. A constitutively active (CA) mutant of RhoA (or CA ROCK/CA MLCK as noted) was placed under the control of a doxycycline-inducible promoter, placed into a lentiviral vector, and used to transduce GBM TICs to yield stable cell lines. B, RhoA induction increases myosin light chain phosphorylation. Western blot analysis confirms that induction of CA RhoA increases myosin light chain phosphorylation relative to control cells transduced with an empty vector. Data shown are for the L0 TIC line. n = 3 independent experiments. C, CA RhoA preserves or enhances cell stiffness. AFM measurements reveal that overexpression of CA RhoA preserves mean cell stiffness in L0 cells (while pushing the range of observed stiffness values to higher values) and increases mean cell stiffness in L2 cells, consistent with an increase in cell contractility. n = 10 to 20 cells pooled from at least three biologic and technical replicates. D, CA RhoA restores ECM stiffness-dependent spreading. Phase contrast imaging reveals that overexpression of CA RhoA produces cell rounding on soft ECMs, in contrast with control-transduced cells, which spread on ECMs spanning the entire range of stiffness. E, quantification of projected cell area shows that CA RhoA rescues stiffness-dependent cell spreading, with significantly decreased spread area for CA RhoA TIC on soft ECMs. * P < 0.05 and ** P < 0.001 relative to control. n = 30 cells (pooled from at least three technical and three biologic experiments) for all conditions. F, CA RhoA rescues stiffness-dependent cell motility. Time-lapse phase contrast imaging of random cell motility over 8 to 12 hours shows significantly decreased motility of these cells on soft ECMs relative to stiff ECMs. * P < 0.05 and ** P < 0.001 relative to glass. n = 30 cells for all conditions. G, CA RhoA does not alter cell proliferation. Cells were incubated with bromodeoxyuridine (BrdUrd) for 45 minutes and stained with 7AAD before analysis by flow cytometry. The data reveal that overexpression of CA RhoA neither produces statistically significant changes in proliferation for any given ECM stiffness nor does it render proliferation sensitive to ECM stiffness. n = 3 independent experiments. H, CA RhoA TICs express the stem cell markers nestin (top row; 100% positive) and SOX2 (bottom row; 100% positive) on all ECM stiffnesses. I, CA RhoA does not compromise the ability of TICs to form neurospheres. * P < 0.001 relative to control, n = 384 TICs. J, quantification of neurosphere size for L0 and L2 cells as a function of RhoA expression. * P < 0.001 and ** P < 0.05 relative to control, n = ~ 1500 spheres for all conditions.

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Figure 3.

of RhoA activation, we treated CA RhoA GBM TICs with the myosin II ATPase inhibitor blebbistatin, which rescued spreading of CA RhoA TICs on the softest ECM (Fig. 4A). Consistent with this finding, treatment with blebbistatin did not yield systematic dependences of spreading area (Fig. 4B) or migration speed (Fig. 4C) as a function of matrix stiffness for either control or
Nonmuscle myosin II regulates TIC mechanosensitivity. A, CA RhoA-mediated suppression of cell spreading can be rescued with pharmacologic inhibition of myosin-II. Addition of 25 µmol/L of blebbistatin results in spreading of CA RhoA GBM TICs on soft ECMs, similar to the control GBM TICs. B, quantification of projected cell area shows that addition of blebbistatin reverses CA RhoA effects and caused CA RhoA TICs to spread on soft ECMs. *, P < 0.05 relative to glass; n = 30 cells pooled from at least three technical and three biologic experiments for all conditions. C, quantification of blebbistatin effects on cell migration speed. Time-lapse phase contrast imaging of random cell motility was conducted over 8 to 12 hours.

Figure 4. RhoA TICs. To investigate potential effects of blebbistatin on growth, we measured TIC proliferation (Fig. 4D) and viability (Fig. 4E) as a function of ECM stiffness and CA RhoA expression, which did not reveal systematic effects of the drug. Given the demonstrated importance of the myosin II isoforms A, B, and C to glioma invasion (2, 34), we also measured isoform expression as a function of ECM stiffness and CA RhoA status. Western blot analyses (Fig. 4F and G) revealed that TICs express all three isoforms under all stiffnesses and in the presence or absence of C. A, B, and C show that control and CA RhoA TICs express all three isoforms on all ECM stiffnesses (L2 shown; L0 had similar results). G, quantification of Western blot data; n > 2 independent experiments. H, relative levels of myosin II heavy chain isoforms measured by LC/MS-MS. n = 2 experiments per condition.

Thus, stiffness- and RhoA-mediated effects in this system do not appear to be a consequence of changes in the myosin II isoform ratio.

To test the generality of the effect of increased myosin activity on cell migration, we developed additional GBM TIC lines overexpressing CA mutants of the contractile activators ROCK1 (Fig. 5A) and myosin light chain kinase (MLCK; Fig. 5B), the latter of which activates myosin through an orthogonal, RhoA-independent mechanism. Indeed, overexpression of either molecule produced highly rigidity-dependent motility, with soft ECMs strongly suppressing motility. Thus, suppression of motility on these highly compliant ECMs is consistently associated with generation of high contractile forces against the ECM.
To determine whether activation of contractile signaling could also limit motility in three-dimensional ECMs that better capture steric and architectural features of tissue ECM, we performed spheroid invasion assays in soft collagen I gels (Fig. 6A). Consistent with our 2-D migration studies, RhoA activation almost completely abrogated invasion relative to control cells transduced with an empty vector. RhoA activation also produced a 39% reduction in migration through laminin-coated 3-µm pore size Transwell inserts (Fig. 6B). Notably, treatment of TICs with BMP-4 also significantly reduced invasion in both cell lines (Supplementary Fig. S2C).

Increased cellular contractility extends survival time in an orthotopic mouse model

Finally, to test whether contractility-mediated restriction of GBM TIC motility could reduce tumor invasion and initiation in vivo, we compared the tumorigenicity of CA RhoA with control GBM TICs in a mouse orthotopic xenograft model. We intracranially injected GBM TICs transduced with either CA RhoA or an empty vector into immunocompromised (NSG) mice and followed each cohort until death (Fig. 7A). Remarkably, RhoA activation prolonged survival time by 30% (Fig. 7B). IHC analysis further revealed that tumors seeded by CA RhoA GBM TICs had reduced invasion capacity and highly circumscribed growth patterns (Fig. 7C), whereas control GBM TICs diffusely spread throughout both hemispheres and generated a secondary tumor in the contralateral hemisphere, consistent with human GBMs (Fig. 7D, i–iii, v–vii). Moreover, although both CA RhoA and control GBM TICs were able to form tumors in vivo, only tumors derived from the control GBM TICs exhibited all of the defining morphological characteristics of GBM: pseudo-palisading necrosis, nest-like formations, angiogenesis, and mitotic figures. Notably, both control and CA RhoA GBM TICs also continued to express nestin (Fig. 7D, iv, viii), illustrating retention of an important TIC marker throughout the growth and invasion process. Quantification of tumor cross-sectional area confirmed that the area occupied by CA RhoA TIC-induced tumors is 3.5-fold lower than control TIC-induced tumors (Fig. 7E). To verify that the difference in tumor occupancy was not a result of cellular proliferation, tissue sections were stained with the antibody Ki-67 (Fig. 7F). We found no difference in proliferation between control and CA RhoA GBM TICs, lending further support to the notion that the reduction in tumor invasion more likely stemmed from limited migration than altered cell division.

Discussion

Mechanical signals encoded in the ECM are increasingly recognized as important regulators of tumor progression (8, 35, 36). It has also been shown that continuous culture models of GBM require myosin II and its upstream regulators to sense and respond to ECM rigidity (4, 5, 37, 38). However, it has remained unclear whether these principles apply to primary TICs, or whether manipulation of the mechanosensing machinery can influence tumor initiation and progression in vivo. Here, we have addressed these questions by elucidating connections between mechanosensitivity in vitro, activation of contractile signals, and tumor progression and survival in vivo. We discovered that primary GBM TICs can evade limits on spreading and motility normally imposed by compliant matrices by generating low contractile signals.
Constitutive activation of RhoA in GBM TICs extends survival time and reduces tumor invasion in an orthotopic xenograft model. A, mice orthotopically implanted with L0 CA RhoA GBM TICs live significantly longer than mice implanted with L0 control TICs. Eight-week-old NSG mice were implanted intracranially with 200,000 cells. Mice were treated daily with doxycycline (625 mg/kg) for 4 days post implant to endpoint. **, P = 0.0018, log-rank test. n = 5 mice per condition. B, CA RhoA results in a 30% increase in overall mouse survival. ***, P = 0.0002, t test. C, tumors formed from L0 CA RhoA GBM TICs do not infiltrate brain tissue as extensively as L0 control TICs. Hematoxylin and eosin stain shows (i) a large area of infiltration and secondary tumor formation (*) in mice implanted with L0 control TICs; and (ii) a defined area of infiltration with clear borders and no secondary tumor formation in mice implanted with L0 CA RhoA GBM TICs. D, CA RhoA reduces diffuse invasion in GBM. Hematoxylin and eosin (i, v) and human nestin stains (iv, viii) reveal a clear border between CA RhoA tumors and the parenchyma whereas control GBM TICs infiltrate readily. Ipsilateral (ii) and contralateral (vi) hemisphere hematoxylin and eosin stains show secondary tumor formation (*) for control cells and not for CA RhoA cells. E, human GBM cells were identified using anti-human Nestin antibody and visualized using the ABC-Elite peroxidase method (Vector Laboratories). Counterstaining of the nuclei was performed using hematoxylin. Analysis of cross-sectional area shows significantly decreased tumor occupancy for mice brains implanted with L0 CA RhoA TICs compared with L0 control TICs. Graph shows average ± SE over 5 to 8 sections per group. ***, P < 0.0001. F, Ki-67 staining and quantification show no difference in cell proliferation across multiple regions in control and CA RhoA GBM TICs.
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