CHAPTER TEN

Matrix Regulation of Tumor-Initiating Cells

Sophie Y. Wong, Sanjay Kumar
Department of Bioengineering, University of California, Berkeley, Berkeley, California, USA

Contents

Abstract

The recognition that the progression of many tumors may be driven by specific subpopulations of cells with stem/progenitor-like properties (tumor-initiating cells or TICs, a.k.a. cancer stem cells) represents an important recent paradigm shift in cancer biology and therapeutics. TICs in solid tissues are expected to interface with the extracellular matrix (ECM), which can strongly influence cell behavior through a variety of biochemical and biophysical mechanisms. Understanding ECM regulation of TIC behavior is important for developing strategies to isolate, expand, and characterize TICs in a laboratory setting and for understanding the roles ECM-based inputs may play in disease progression and therapy. In this chapter, we discuss how the ECM regulates TICs, starting with a brief overview of TIC biology, isolation, and characterization, molecular mechanisms through which TICs may be regulated by ECM-based signals, and the potential importance of these signals to TIC-driven tumor progression and metastasis.

Progress in Molecular Biology and Translational Science, Volume 126
ISSN 1877-1173
http://dx.doi.org/10.1016/B978-0-12-394624-9.00010-5

1. INTRODUCTION

1.1. What are tumor-initiating cells?

It is an unfortunate reality in cancer that a single treatment strategy is rarely if ever effective for all patients at all times. For example, a chemotherapeutic regimen that initially produces tumor regression may fail later as a resistant population of tumor cells emerges. Moreover, two patients with clinically and histologically similar tumors may exhibit dramatically different responses to a given chemotherapeutic regimen depending on the genomic and proteomic profile of the patient and tumor. This immense heterogeneity between and within tumors frustrates efforts to identify reliable molecular and cellular targets and thus represents a key therapeutic barrier to treatment.[1–3] While the nature and implications of this heterogeneity remain incompletely understood, one important and recently appreciated manifestation of this heterogeneity is the variable ability of cells within a given tumor to propagate the tumor and seed new tumors. In particular, it is becoming clear that for many tumors, a privileged and comparatively rare subpopulation of cells is uniquely able to seed new tumors, whereas the vast majority of tumor cells, presumably this rare subpopulation's more differentiated progeny, contribute to the tumor "bulk" (Fig. 10.1). In this sense, this tumor-initiating subpopulation shares conceptual similarities with stem or

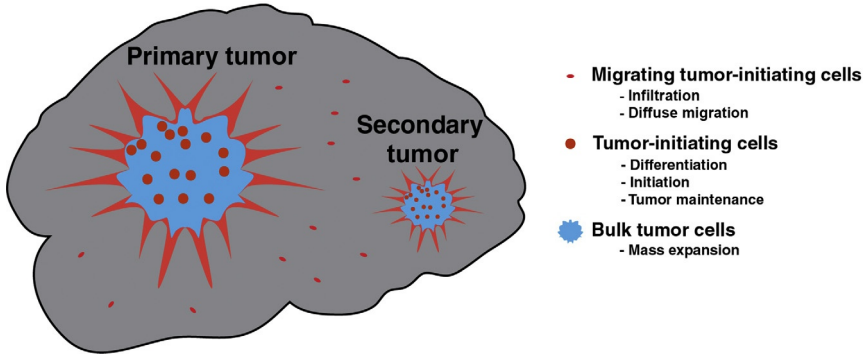

Figure 10.1 Tumor-initiating cells drive secondary tumor formation. Tumors are recognized to consist of a highly heterogeneous population of cells, only some of which can propagate and seed new tumors. This population of tumor-initiating cells (TICs) can diffusely infiltrate tissue (in this example, brain parenchyma), leading to secondary tumor formation. TICs also give rise to more differentiated progeny, which can both add to the tumor "bulk" as well as contribute to more specialized stromal functions such as angiogenesis.

progenitor cells, in that these cells can both self-renew and differentiate to yield more specialized progeny.

Some of the first evidence that a population of cancer cells may have stem-like capabilities emerged in 1994 when Lapidot and colleagues found that only the $CD34^{+}/CD38^{-}$ subpopulation of leukemia cells generated new tumors in mice.[4] The authors also found that the frequency of immature tumor cells was 1000 times less than mature tumor cells, suggesting that this tumor-initiating population could be rare. These findings led to what the field now recognizes as the cancer stem cell (CSC) hypothesis,[5–7] which states that a subpopulation of tumor-initiating cells (TICs) is more tumorigenic than the rest of the tumor cell population and is capable of recapitulating all components of the tumor (e.g. those involved in growth, invasion, and metastasis) and may be highly resistant to therapeutics. TICs have been identified in several cancers including breast cancer,[8,9] prostate cancer,[10,11] lung cancer,[12] and brain cancer.[13,14] These cells now go by many names, including TICs, CSCs,[6,15,16] and tumor-propagating cells. Whatever the terminology, the common concept is that these cells share key functional properties of stem cells and can initiate new tumors when introduced in small numbers to tumor-free tissue. Most importantly, TICs can initiate and propagate tumors that are histologically equivalent to their tumors of origin when orthotopically implanted into immunocompromised animals.

1.2. Significance of TICs

In addition to lending fundamental new insight into the pathophysiology of tumor progression, a deeper understanding of TIC biology may accelerate the optimization and discovery of treatment regimens. There are at least two ways in which deeper engagement of TICs could aid therapy: first, the development of modalities that directly and specifically target TICs would theoretically represent the most effective way to contain or eradicate a tumor. For example, even after surgical resection of a primary tumor and aggressive follow-up chemo- and/or radiation therapy, the tumor would be expected to recur if a small number of TICs are left behind that could seed new tumors, perpetuate angiogenesis, and invade surrounding tissue. In principle, precise neutralization of TICs would effectively stop tumor initiation[17]. Second, TICs offer a route to personalized medicine, in that they may be specifically isolated from a given patient's tumor and used for patient-specific molecular profiling, drug screening, and disease modeling. Molecular sequencing technologies can be combined with tumor sampling techniques to quantify tumor heterogeneity, trace cell population ancestry,

and measure tumor-specific characteristics. For example, Sottoriva and colleagues[18] developed a framework that could generate patient-specific profiles days after tissue collection and did not require xenotransplantation. TIC characteristics considered in this model included a variety of factors such as: fraction of TICs in the tumor, TIC symmetric division rate, methylation/demethylation rate per cell division, relative tumor age from malignant transformation, and rate of apoptosis. The modeling results matched those reported from xenotransplantation assays, supporting the clinical relevance of this model and its potential for designing patient-specific treatments.

The role of TICs in driving glioblastoma (GBM) has been an especially active area of study. GBM is the most aggressive primary brain tumor and has a median survival time of about 15 months, even with surgery and aggressive chemo- and radiotherapy.[19] A variety of laboratories have isolated subpopulations of TICs from GBM tumors that can recapitulate characteristics of the original tumor when transplanted into immunocompromised mice, such as migratory and infiltrative capabilities, nest-like formations, vascular proliferation, nuclear pleomorphism with mitotic figures, and areas of pseudo-palisading necrosis.[5,6,20–24] The continuous cell lines that have been extensively used as culture models of GBM (e.g. U87-MG) typically grow *in vivo* by direct expansion and do not recapitulate the infiltrative character and other key histologic features of the original tumor when transplanted into mice.[22,25] Thus, while these lines may adequately capture more differentiated elements of the tumor that primarily participate in tissue infiltration, TICs may serve as a more clinically relevant model for investigating cellular aspects of the initiation and maintenance of GBM.

A number of studies have supported a role for TICs in tumor propagation[26] and correlated TIC presence with clinical outcome.[27,28] One study used matched TIC and nonstem tumor cells and followed the single cells from injection to tumor growth to show that TICs are more tumorigenic than more differentiated tumor cells.[26] The TICs proliferated faster than the nonstem tumor cells and more fully recapitulated tumor heterogeneity. Furthermore, analysis of secondary tumors contained a high population of TICs and their progeny. A clinical study that compared expression of the TIC marker CD133 (see below) and patient outcome using a panel of 95 gliomas found that high-grade glioma is strongly associated with high CD133 expression. This study also found that high frequency and clusters of CD133 positive cells—independent of tumor grade, extent of resection, and patient age—could be prognostic factors for gliomas.[27] Finally, another study found

that low-grade gliomas have low expression of the neural stem cell marker nestin, whereas more aggressive, high-grade gliomas have higher nestin expression and produce shorter survival times. Xenotransplantation of tumor-derived spheroids in mice gave rise to tumors in which nestin-positive cells localized to the invasive front.[28]

2. IDENTIFICATION AND ISOLATION OF TICs

Manipulation of TICs in culture presupposes an ability to reliably identify and isolate these cells. As a result, much effort has been devoted to the search for sensitive and specific TIC markers that may be exploited in flow cytometry, fluorescence-activated cell sorting, immunofluorescence, and other applications. For identification and isolation of GBM TICs, many studies have used neural stem cell surface markers such as CD133,[29] CD15,[30] and A2B5.[31] Similarly, the integrin subunit α6 was shown to be expressed at high levels in GBM TICs and to play a functional role in GBM TIC maintenance and tumor formation capacity.[32] Although several markers have been identified, use of any one marker alone has proven to be somewhat unreliable. For example, both $CD133^+$ and $CD133^-$ glioma cells can display stem-like properties and can generate secondary tumors in orthotopic mouse models.[33,34] As a result, while the field continues to search for sensitive and specific molecular markers, the gold standard for verification of GBM TICs remains a functional one—i.e., GBM TICs are defined by their ability to recapitulate the tumor of origin when orthotopically implanted into immunocompromised mice. A variety of *in vitro* functional screens have been developed to streamline and augment *in vivo* implantation studies. For example, cell survival in neural stem cell medium over several passages has been successfully used to select GBM TICs from bulk tumor tissue.[20] Another study exploited the inverse correlation between proliferation rate (cell cycling speed) and tumorigenicity to select for GBM TICs.[5,35] Improved characterization of TIC properties and the development of new screening/isolation methodologies are critical for further studies that seek to better understand tumor pathogenesis.

3. ROLE OF EXTRACELLULAR MATRIX AND MECHANICAL SIGNALS IN REGULATING TIC FUNCTION

3.1. Extracellular matrix

Having described the identification and isolation of GBM TICs, we now turn to a more detailed discussion of how the extracellular matrix (ECM)

may regulate TIC behavior, with a special focus on GBM. In GBM, it is now evident that TICs invade along perivascular spaces, which have a high concentration of ECM proteins such as collagen, fibronectin, and laminin.[36] TICs sense and process these matrix-bound factors through adhesion receptors such as integrins[32,37] and CD44.[38] For example, Lathia and colleagues discovered that integrin α6, a laminin receptor, is necessary for TIC survival and proliferation and directly correlates with TIC stem cell marker expression.[32] Another recent study showed that integrin α3, which adheres to laminin and fibronectin, is overexpressed in CD133-positive TICs. Suppression of α3 slowed random migration and reduced transwell invasion in glioma cell lines, which in turn depended on ERK1/2 phosphorylation.[37] In addition to integrins, the adhesion receptor CD44 has been widely studied and characterized in multiple cancers.[39] High expression of CD44 in GBM TICs correlates with poor clinical prognosis and has been shown to regulate TIC growth through Akt and other signals.[38,40] These adhesion receptor studies support an important role of ECM in TIC function and tumorigenesis.

While these and other studies clearly demonstrate that ECM ligation can trigger signals that modulate TIC behavior, it has also become clear over the past two decades that mechanical cues encoded within the ECM can also direct tumor invasion and growth. Features within the ECM such as matrix geometry, density, and rigidity have been shown to regulate fundamental cellular functions such as motility, proliferation, and gene expression.[41–45] For example, endothelial cells and fibroblasts have higher cell spreading area and motility on stiff matrices when compared to soft matrices.[44,46] It has also been shown that continuous GBM culture models have increased motility, spreading area, and proliferation on stiff matrices.[41] Interestingly, differences in ECM rigidity can also direct the differentiation of adult stem cells, including mesenchymal and neural stem cells.[42,47] In the first and perhaps best-known such study, Engler and colleagues showed that mesenchymal stem cells preferentially undergo neurogenesis on soft ECMs ranging in stiffness from 0.1 to 1 kPa, myogenesis on ECMs ranging from 8 to 17 kPa, and osteogenesis on stiff ECMs ranging from 25 to 40 kPa.[42] They also found that inhibition of nonmuscle myosin II blocked differentiation, thus implicating myosin-based contractile signaling in stiffness-dependent differentiation. Later, Keung and colleagues showed that soft matrices (0.1–0.7 kPa) directed neural differentiation of adult neural stem cells, whereas stiff matrices (1.5–75 kPa) produced relative enrichment of astrocytic differentiation.[48] Mechanistic studies then revealed that the GTPases RhoA and

Cdc42 were key to these effects, with suppression of these proteins rescuing neuronal differentiation on stiff ECMs.

3.2. Propagation of TICs in ECM-adherent cultures

Since the ECM can instruct or select for specific cellular behaviors, it is important to consider the role the ECM may play in culturing TICs in the laboratory setting.[49] For example, GBM TICs can be grown in adherent cultures[21] or as neurospheres in suspension[50,51] (Fig. 10.2). While TICs were long thought to retain their tumor-initiating capacity only when propagated long term as neurospheres, more recent studies reveal that TICs may be propagated as adherent cultures without loss of marker expression or tumor-initiating capacity.[21,22] Specifically, the authors of this study verified the tumorigenicity of each adherent TIC line by injecting 100,000 TICs intracranially into immunocompromised mice. After the mice were sacrificed, the resulting tumors had infiltrated brain tissue and expressed characteristic molecular markers (e.g. nestin) and displayed histopathological hallmarks of GBM. Remarkably, limiting dilution studies revealed that some TIC lines could form aggressive tumors upon transplantation of as few as 100 cells. In addition, adherent cells could be differentiated in culture into marker-positive neuronal, oligodendrocytic, and astrocytic lineages. Some important practical advantages of adherent culture over neurosphere culture include more straightforward quantification of cell proliferation, improved cellular homogeneity, and fewer gradients in oxygen, nutrients, and other

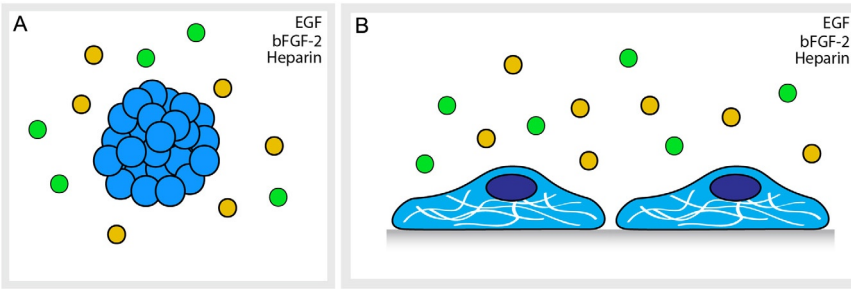

Figure 10.2 Comparison of adherent and neurosphere-based TIC cultures. Both neurosphere and ECM-adherent cultures are widely used to propagate TICs. (A) An acknowledged limitation of neurosphere culture is the possibility of gradients across the sphere in oxygen, nutrients in the culture, and cell-secreted factors, as well as uneven access to extracellular matrix. (B) Adherent culture has recently emerged as a complementary paradigm to neurosphere culture, with the prospect of minimizing these gradients while also offering greater scalability.

soluble factors. Perhaps most importantly, the adherent culture paradigm facilitates high-throughput screening; to illustrate this, the authors screened their TIC lines with 450 drugs from the NIH Clinical Collection and found that 23 of these drugs killed all TIC lines tested, including, unexpectedly, seven agents that target monoamine signaling (e.g. serotonin-specific reuptake inhibitors).

This is not to say that TICs in a small neurosphere (e.g. 150–200 μm) cannot maintain stem-like properties; however as the neurosphere grows larger, the percentage of stem-like cells rapidly decreases,[49] which has been ascribed to the increasingly uneven access to growth factors and oxygen as the neurosphere grows and may be further complicated by increases in juxtacrine and paracrine signaling. In adherent culture, all cells have effectively equal access to soluble factors in the medium, and cells may be plated at sufficiently low density as to minimize cell–cell contacts. In addition, the increased exposure to laminin in the matrix can promote maintenance of stem-like properties for adherent cells, which has been found to be an important factor in identifying TICs.[32] Although debate continues about which culture method is best for a given application, both are used to successfully propagate TICs *in vitro*.

3.3. Mechanisms of mechanotransduction

The finding that TIC behavior is regulated by ECM engagement and biophysical properties raises the question of whether the molecules that mediate these effects may bear value as drug targets. For example, Cilengitide, an αv integrin antagonist, inhibits GBM growth in preclinical models and is currently being evaluated in clinical trials.[52] Recent studies with breast and prostate cancer have used integrins to select for a tumor-initiating subpopulation from the bulk tumor.[8,11] As described earlier, the laminin receptor integrin α6 is highly expressed in GBM TICs and is necessary for BTIC self-renewal, proliferation, and tumor formation capacity.[32] Since laminin is abundant in the BTIC perivascular niche, this result is significant because it suggests a mechanism through which this ECM protein can contribute to maintenance of stemness.

Several actin binding proteins[53,54] and transcription factors relevant to integrin signaling and mechanotransduction[55,56,57,58] have been identified to have the capability of regulating GBM initiation, invasion, and chemosensitivity. For example, the transcription factor ZEB1 is highly expressed in GBM TICs and is known to be correlated with shorter survival

and poor response to Temozolomide[58(p1),59]. ZEB1 is regulated by tyrosine receptor type A, and increased expression leads to increased binding to E-box regions of E-cadherin, resulting in highly motile cells and increased tumor invasion. Downstream targets of ZEB1 have subsequently been shown to include ROBO1, OLIG2, CD133, and MGMT. Knockdown of ZEB1 sensitizes cells to temozolomide and decreases expression of stem cell markers SOX2, OLIG2, and CD133.[58(p1)]

Another example is the influence of the actin binding protein, Girdin, which is activated by the PI-3-Kinase/Akt pathway. Activation of the Akt pathway can induce conversion from low-grade to high-grade glioma and regulates angiogenesis, apoptosis, and invasion.[60,61] Girdin is known to regulate cell migration, cell polarity, and epithelial–mesenchymal transition, and in GBM TICs contributes to self-renewal and tumorigenicity.[54] Tumor grade is positively correlated with Girdin expression, and knockdown of Girdin decreases motility and invasion, neurosphere formation, tumorigenicity, and expression of nestin and CD133, and induces differentiation. These studies collectively show that tumor initiation, invasion, and chemoresistance are linked by pathways that are activated by biochemical and potentially mechanical factors in the ECM.

Liu and colleagues recently tied together these concepts by showing that the composition and mechanics of the ECM used to culture TICs can exert powerful instructive and/or selective effects that can profoundly influence subsequent tumorigenicity.[62] The authors examined the formation of melanoma TICs in 3D fibrin gels of varying stiffnesses and found that the softest gel (0.09 kPa) generated the most and largest spheroids over a 5-day period when compared to the stiff gel (1.05 kPa). Subcutaneous transplantation of TICs propagated in the soft gel resulted in greater primary tumor formation and lung metastasis compared to TICs propagated on hard plastic. Furthermore, spheroids grown in the soft gel exhibited increased expression of stem cell markers CD133, nestin, and Bmi-1.

4. CONCLUSION

While it has long been recognized that tumors are highly heterogeneous, only recently has it been appreciated that this heterogeneity may reflect a hierarchy of cellular entities in which a comparatively rare subpopulation of TICs are capable of initiating and propagating the tumor. Over the past decade, significant effort has been devoted to identifying and clarifying the function of these TICs, which has allowed investigators to dissect

specific contributions of individual factors to tumor progression. As described in this review, the field is only beginning to understand the importance of the ECM and other solid-state components of the microenvironment in regulating TIC behavior, and there is every reason to expect that mechanical inputs will prove to be an important dimension of this regulation. As our understanding of the role of ECM and mechanical signals to TIC biology advances, there are several open questions to address, each of which presents important opportunities to innovate. First, what are the defining characteristics of the TIC physical microenvironment *in vivo*, how is this different from the normal tissue microenvironment, and which characteristics are most important to tumor initiation and propagation? Second, if mechanical inputs are important to TIC function, how do the signaling systems that process these inputs interface with more canonical oncogenic signaling systems? Specifically, can aberrant mechanotransductive signaling "tip the balance" between TIC quiescence and tumorigenesis, and could this be leveraged in some way to identify new druggable targets? Third, combining these concepts, is it possible to develop advanced *in vitro* culture systems that enable one to investigate ECM and mechanobiological regulation of TICs in a systematic, high-throughput, and physiologically mimetic fashion? One envisions that advances in this last area could dramatically accelerate both fundamental discovery and therapeutic design, with standardized culture platforms serving as key enabling technologies for personalized molecular and chemotherapeutic screening. Realizing this vision will require a highly multidisciplinary effort, including input from biomaterials scientists, micro- and nanotechnologists, cell and ECM biologists, and of course cancer biologists. The coming years and decades are likely to be extremely exciting ones for this field, with advances in basic science directly informing technology and therapeutics and therapeutic advances opening new avenues for scientific inquiry.

REFERENCES

1. Rouzier R, Perou CM, Symmans WF, et al. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res*. 2005;11(16):5678–5685. http://dx.doi.org/10.1158/1078-0432.CCR-04-2421.
2. Magee JA, Piskounova E, Morrison SJ. Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer Cell*. 2012;21(3):283–296. http://dx.doi.org/10.1016/j.ccr.2012.03.003.
3. Banerjee S, Reis-Filho JS, Ashley S, et al. Basal-like breast carcinomas: clinical outcome and response to chemotherapy. *J Clin Pathol*. 2006;59(7):729–735. http://dx.doi.org/10.1136/jcp.2005.033043.
4. Lapidot T, Sirard C, Vormoor J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*. 1994;367(6464):645–648. http://dx.doi.org/10.1038/367645a0.

5. Deleyrolle LP, Harding A, Cato K, et al. Evidence for label-retaining tumour-initiating cells in human glioblastoma. *Brain*. 2011;134(5):1331–1343. http://dx.doi.org/10.1093/brain/awr081.
6. Dirks PB. Brain tumor stem cells: the cancer stem cell hypothesis writ large. *Mol Oncol*. 2010;4(5):420–430. http://dx.doi.org/10.1016/j.molonc.2010.08.001.
7. Vermeulen L, de Sousa e Melo F, Richel DJ, Medema JP. The developing cancer stem-cell model: clinical challenges and opportunities. *Lancet Oncol*. 2012;13(2):e83–e89.
8. Vaillant F, Asselin-Labat M-L, Shackleton M, Forrest NC, Lindeman GJ, Visvader JE. The mammary progenitor marker CD61/β3 integrin identifies cancer stem cells in mouse models of mammary tumorigenesis. *Cancer Res*. 2008;68(19):7711–7717. http://dx.doi.org/10.1158/0008-5472.CAN-08-1949.
9. Ponti D, Costa A, Zaffaroni N, et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res*. 2005;65(13):5506–5511. http://dx.doi.org/10.1158/0008-5472.CAN-05-0626.
10. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res*. 2005;65(23):10946–10951. http://dx.doi.org/10.1158/0008-5472.CAN-05-2018.
11. Patrawala L, Calhoun-Davis T, Schneider-Broussard R, Tang DG. Hierarchical organization of prostate cancer cells in xenograft tumors: the CD44+α2β1+ cell population is enriched in tumor-initiating cells. *Cancer Res*. 2007;67(14):6796–6805. http://dx.doi.org/10.1158/0008-5472.CAN-07-0490.
12. Eramo A, Lotti F, Sette G, et al. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ*. 2007;15(3):504–514. http://dx.doi.org/10.1038/sj.cdd.4402283.
13. Beier F, Beier CP, Aschenbrenner I, Hildebrandt GC, Brümmendorf TH, Beier D. Identification of CD133−/telomerase low progenitor cells in glioblastoma-derived cancer stem cell lines. *Cell Mol Neurobiol*. 2010;31(3):337–343. http://dx.doi.org/10.1007/s10571-010-9627-4.
14. Huang Q, Zhang Q-B, Dong J, et al. Glioma stem cells are more aggressive in recurrent tumors with malignant progression than in the primary tumor, and both can be maintained long-term in vitro. *BMC Cancer*. 2008;8:304. http://dx.doi.org/10.1186/1471-2407-8-304.
15. Beck B, Blanpain C. Unravelling cancer stem cell potential. *Nat Rev Cancer*. 2013;13(10):727–738. http://dx.doi.org/10.1038/nrc3597.
16. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature*. 2001;414(6859):105–111. http://dx.doi.org/10.1038/35102167.
17. Jordan CT, Guzman ML, Noble M. Cancer stem cells. *N Engl J Med*. 2006;355(12):1253–1261. http://dx.doi.org/10.1056/NEJMra061808.
18. Sottoriva A, Spiteri I, Shibata D, Curtis C, Tavaré S. Single-molecule genomic data delineate patient-specific tumor profiles and cancer stem cell organization. *Cancer Res*. 2013;73(1):41–49. http://dx.doi.org/10.1158/0008-5472.CAN-12-2273.
19. Nakada M, Nakada S, Demuth T, Tran NL, Hoelzinger DB, Berens ME. Molecular targets of glioma invasion. *Cell Mol Life Sci*. 2007;64(4):458–478. http://dx.doi.org/10.1007/s00018-007-6342-5.
20. Galli R, Binda E, Orfanelli U, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res*. 2004;64(19):7011–7021. http://dx.doi.org/10.1158/0008-5472.CAN-04-1364.
21. Pollard SM, Yoshikawa K, Clarke ID, et al. Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. *Cell Stem Cell*. 2009;4(6):568–580. http://dx.doi.org/10.1016/j.stem.2009.03.014.
22. Lee J, Kotliarova S, Kotliarov Y, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary

tumors than do serum-cultured cell lines. *Cancer Cell*. 2006;9(5):391–403. http://dx.doi.org/10.1016/j.ccr.2006.03.030.
23. Kleihues P, Sobin LH. World Health Organization classification of tumors. *Cancer*. 2000;88(12):2887. http://dx.doi.org/10.1002/1097-0142(20000615)88:12<2887::AID-CNCR32>3.0.CO;2-F.
24. Lathia JD, Heddleston JM, Venere M, Rich JN. Deadly teamwork: neural cancer stem cells and the tumor microenvironment. *Cell Stem Cell*. 2011;8(5):482–485. http://dx.doi.org/10.1016/j.stem.2011.04.013.
25. Niclou SP, Danzeisen C, Eikesdal HP, et al. A novel eGFP-expressing immunodeficient mouse model to study tumor-host interactions. *FASEB J*. 2008;22(9):3120–3128. http://dx.doi.org/10.1096/fj.08-109611.
26. Lathia JD, Gallagher J, Myers JT, et al. Direct in vivo evidence for tumor propagation by glioblastoma cancer stem cells. *PLoS One*. 2011;6(9):e24807. http://dx.doi.org/10.1371/journal.pone.0024807.
27. Zeppernick F, Ahmadi R, Campos B, et al. Stem cell marker CD133 affects clinical outcome in glioma patients. *Clin Cancer Res*. 2008;14(1):123–129. http://dx.doi.org/10.1158/1078-0432.CCR-07-0932.
28. Strojnik T, Røsland GV, Sakariassen PO, Kavalar R, Lah T. Neural stem cell markers, nestin and musashi proteins, in the progression of human glioma: correlation of nestin with prognosis of patient survival. *Surg Neurol*. 2007;68(2):133–143. http://dx.doi.org/10.1016/j.surneu.2006.10.050.
29. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature*. 2004;432(7015):396–401. http://dx.doi.org/10.1038/nature03128.
30. Son MJ, Woolard K, Nam D-H, Lee J, Fine HA. SSEA-1 is an enrichment marker for tumor-initiating cells in human glioblastoma. *Cell Stem Cell*. 2009;4(5):440–452. http://dx.doi.org/10.1016/j.stem.2009.03.003.
31. Ogden ATMD, Waziri AEMD, Lochhead RA, et al. Identification of A2B5+CD133− tumor-initiating cells in adult human gliomas. *Neurosurgery*. 2008;62(2):505–515. http://dx.doi.org/10.1227/01.neu.0000316019.28421.95 [Miscellaneous Article].
32. Lathia JD, Gallagher J, Heddleston JM, et al. Integrin alpha 6 regulates glioblastoma stem cells. *Cell Stem Cell*. 2010;6(5):421–432. http://dx.doi.org/10.1016/j.stem.2010.02.018.
33. Clément V, Dutoit V, Marino D, Dietrich P-Y, Radovanovic I. Limits of CD133 as a marker of glioma self-renewing cells. *Int J Cancer*. 2009;125(1):244–248. http://dx.doi.org/10.1002/ijc.24352.
34. Hermansen SK, Christensen KG, Jensen SS, Kristensen BW. Inconsistent immunohistochemical expression patterns of four different CD133 antibody clones in glioblastoma. *J Histochem Cytochem*. 2011;59(4):391–407. http://dx.doi.org/10.1369/0022155411400867.
35. Lyons AB. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. *J Immunol Methods*. 2000;243(1–2):147–154. http://dx.doi.org/10.1016/S0022-1759(00)00231-3.
36. Venere M, Fine HA, Dirks PB, Rich JN. Cancer stem cells in gliomas: identifying and understanding the apex cell in cancer's hierarchy. *Glia*. 2011;59(8):1148–1154. http://dx.doi.org/10.1002/glia.21185.
37. Nakada M, Nambu E, Furuyama N, et al. Integrin α3 is overexpressed in glioma stem-like cells and promotes invasion. *Br J Cancer*. 2013;108(12):2516–2524. http://dx.doi.org/10.1038/bjc.2013.218.
38. Zhu X, Morales FC, Agarwal NK, Dogruluk T, Gagea M, Georgescu M-M. Moesin is a glioma progression marker that induces proliferation and Wnt/β-catenin pathway activation via interaction with CD44. *Cancer Res*. 2013;73(3):1142–1155. http://dx.doi.org/10.1158/0008-5472.CAN-12-1040.
39. Marhaba R, Zöller M. CD44 in cancer progression: adhesion, migration and growth regulation. *J Mol Histol*. 2004;35(3):211–231. http://dx.doi.org/10.1023/B:HIJO.0000032354.94213.69.

40. Jijiwa M, Demir H, Gupta S, et al. CD44v6 regulates growth of brain tumor stem cells partially through the AKT-mediated pathway. *PLoS One*. 2011;6(9):e24217. http://dx.doi.org/10.1371/journal.pone.0024217.
41. Ulrich TA, de Juan Pardo EM, Kumar S. The mechanical rigidity of the extracellular matrix regulates the structure, motility, and proliferation of glioma cells. *Cancer Res*. 2009;69(10):4167–4174. http://dx.doi.org/10.1158/0008-5472.CAN-08-4859.
42. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell*. 2006;126(4):677–689. http://dx.doi.org/10.1016/j.cell.2006.06.044.
43. Tilghman RW, Cowan CR, Mih JD, et al. Matrix rigidity regulates cancer cell growth and cellular phenotype. *PLoS One*. 2010;5(9):e12905. http://dx.doi.org/10.1371/journal.pone.0012905.
44. Georges PC, Janmey PA. Cell type-specific response to growth on soft materials. *J Appl Physiol*. 2005;98(4):1547–1553. http://dx.doi.org/10.1152/japplphysiol.01121.2004.
45. Cheng C-M, Steward Jr RL, LeDuc PR. Probing cell structure by controlling the mechanical environment with cell–substrate interactions. *J Biomech*. 2009;42(2):187–192. http://dx.doi.org/10.1016/j.jbiomech.2008.10.014.
46. Yeung T, Georges PC, Flanagan LA, et al. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil Cytoskeleton*. 2005;60(1):24–34. http://dx.doi.org/10.1002/cm.20041.
47. Keung AJ, Kumar S, Schaffer DV. Presentation counts: microenvironmental regulation of stem cells by biophysical and material cues. *Annu Rev Cell Dev Biol*. 2010;26(1):533–556. http://dx.doi.org/10.1146/annurev-cellbio-100109-104042.
48. Keung AJ, de Juan-Pardo EM, Schaffer DV, Kumar S. Rho GTPases mediate the mechanosensitive lineage commitment of neural stem cells. *Stem Cells*. 2011;29(11):1886–1897. http://dx.doi.org/10.1002/stem.746.
49. Woolard K, Fine HA. Glioma stem cells: better flat than round. *Cell Stem Cell*. 2009;4(6):466–467. http://dx.doi.org/10.1016/j.stem.2009.05.013.
50. Deleyrolle LP, Reynolds BA. Identifying and enumerating neural stem cells: application to aging and cancer. In: Verhaagen J, Hol EM, Huitenga I, Wijnholds J, Bergen AB, Boer GJ, Swaab DF, eds. *Neurotherapy: progress in restorative neuroscience and neurology*. Vol. 175. New York, NY: Elsevier; 2009:43–51 Available at: http://www.sciencedirect.com/science/article/B7CV6-4WXGYYC-9/2/e6a7879250e9bf6126f9504ae6c027ce. Accessed April 5, 2011.
51. Azari H, Millette S, Ansari S, Rahman M, Deleyrolle LP, Reynolds BA. Isolation and expansion of human glioblastoma multiforme tumor cells using the neurosphere assay. *J Vis Exp*. 2011;56:e3633. http://dx.doi.org/10.3791/3633.
52. Reardon DA, Nabors LB, Stupp R, Mikkelsen T. Cilengitide: an integrin-targeting arginine–glycine–aspartic acid peptide with promising activity for glioblastoma multiforme. *Expert Opin Investig Drugs*. 2008;17(8):1225–1235. http://dx.doi.org/10.1517/13543784.17.8.1225.
53. Terakawa Y, Agnihotri S, Golbourn B, et al. The role of drebrin in glioma migration and invasion. *Exp Cell Res*. 2013;319(4):517–528. http://dx.doi.org/10.1016/j.yexcr.2012.11.008.
54. Natsume A, Kato T, Kinjo S, et al. Girdin maintains the stemness of glioblastoma stem cells. *Oncogene*. 2012;31(22):2715–2724. http://dx.doi.org/10.1038/onc.2011.466.
55. Kobayashi K, Takahashi H, Inoue A, et al. Oct-3/4 promotes migration and invasion of glioblastoma cells. *J Cell Biochem*. 2012;113(2):508–517. http://dx.doi.org/10.1002/jcb.23374.
56. Alonso MM, Diez-Valle R, Manterola L, et al. Genetic and epigenetic modifications of Sox2 contribute to the invasive phenotype of malignant gliomas. *PLoS One*. 2011;6(11):e26740. http://dx.doi.org/10.1371/journal.pone.0026740.
57. Oppel F, Müller N, Schackert G, et al. SOX2-RNAi attenuates S-phase entry and induces RhoA-dependent switch to protease-independent amoeboid migration in

human glioma cells. *Mol Cancer*. 2011;10:137. http://dx.doi.org/10.1186/1476-4598-10-137.
58. Siebzehnrubl FA, Silver DJ, Tugertimur B, et al. The ZEB1 pathway links glioblastoma initiation, invasion and chemoresistance. *EMBO Mol Med*. 2013;5(8):1196–1212. http://dx.doi.org/10.1002/emmm.201302827.
59. Edwards LA, Woolard K, Son MJ, et al. Effect of brain- and tumor-derived connective tissue growth factor on glioma invasion. *J Natl Cancer Inst*. 2011;103(15):1162–1178. http://dx.doi.org/10.1093/jnci/djr224.
60. Enomoto A, Ping J, Takahashi M. Girdin, a novel actin-binding protein, and its family of proteins possess versatile functions in the Akt and Wnt signaling pathways. *Ann NY Acad Sci*. 2006;1086(1):169–184. http://dx.doi.org/10.1196/annals.1377.016.
61. Jiang P, Enomoto A, Jijiwa M, et al. An actin-binding protein girdin regulates the motility of breast cancer cells. *Cancer Res*. 2008;68(5):1310–1318. http://dx.doi.org/10.1158/0008-5472.CAN-07-5111.
62. Liu J, Tan Y, Zhang H, et al. Soft fibrin gels promote selection and growth of tumorigenic cells. *Nat Mater*. 2012;11(8):734–741. http://dx.doi.org/10.1038/nmat3361.