Spheres without Influence: Dissociating In Vitro Self-Renewal from Tumorigenic Potential in Glioma

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The capacity for self-renewal is thought to be a critical property of tumor-initiating cells. This capacity is often associated with the ability to generate spheres in vitro. In this issue of *Cancer Cell*, Barrett et al. show that cells lacking sphere-forming ability can still be very efficient at propagating tumors.

One of the great frustrations of current cancer therapy is that the disease often returns even after aggressive surgery, radiation, and chemotherapy. Consequently, the discovery of unique populations of tumor cells that may be responsible for recurrence—and that could be targeted to prevent it–has garnered a great deal of interest. These cells, termed tumor-initiating cells (TICs) or cancer stem cells, were originally defined based on their ability to reinitiate tumors following transplantation. However, studies from a variety of systems have shown that TICs frequently share characteristics with normal stem cells, including marker expression, ability to self-renew, and ability to give rise to progeny of multiple lineages. Because FACS analysis and in vitro assays of self-renewal are much simpler than in vivo studies of tumorigenicity, the former are sometimes used as a surrogate for the latter.

One system in which TICs have been studied extensively is glioma. Numerous reports have suggested that glioma intiating cells share markers with neural stem cells (NSCs), and that when cultured at low density in the presence of growth factors, they can give rise to clonally-derived "tumorspheres," analogous to the neurospheres generated when NSCs are cultured under similar conditions. In fact, the ability of glioma cells to self-renew under neurosphere conditions has prompted many investigators to propagate patient samples as spheres rather than as traditional adherent cell lines (Galli et al., 2004; Lee et al., 2006). However, the assumption that all gliomas can be propagated by stem-like sphere-forming cells has not been carefully tested.

In this issue of *Cancer Cell*, Barrett et al. (Barrett et al., 2012) show that in a subset of gliomas, cells that lack stem cell markers and are unable to form spheres are more tumorigenic than cells that have these properties.

The investigators used a model of glioma in which mice lacking the *Arf* tumor suppressor gene were injected with viruses encoding platelet-derived growth factor (PDGF) or its downstream signaling mediator KRAS (Fomchenko et al., 2011). Because the PDGF pathway is aberrantly activated in the "proneural" form of human glioma (Verhaak et al., 2010), this model has been used to study that subtype of the disease. To investigate the relationship between self-renewal and tumor initiation, they crossed their animals with *Id1*^{VenusYFP} reporter mice (Nam and Benezra, 2009). This allowed them to separate cells based on expression of Id1, a transcriptional regulator that has been shown to control self-renewal in NSCs. Consistent with the role of Id1 in NSCs, Id1^{high} cells (which represented <1% of tumor cells) were enriched in expression of stem cell markers (e.g., Prominin-1/CD133), and were very efficient at sphere formation. In contrast, Id1^{low} cells expressed progenitor markers (e.g., Olig2, Mash1, NG2), and showed minimal self-renewal capacity in the sphere assay (Figure 1).

The investigators then sorted Id1^{high} and Id1^{low} tumor cells and tested their ability to give rise to tumors following transplantation. Surprisingly, the ability to form spheres in vitro did not correlate with in vivo tumorigenic potential. Although Id1^{high} and Id1^{low} cells could both generate tumors, Id1^{low} cells did so much more quickly and with higher penetrance. For example, in the KRAS-driven model of glioma, Id1^{low} cells generated tumors in 52% of mice, with a latency of 37 days, whereas Id1^{high} cells gave rise to tumors in only 5% of mice, with a latency of 119 days. These studies suggested that sphere-forming cells are not necessarily more tumorigenic.

To determine if Id1 function is required for tumor growth, Barrett et al. (2012) crossed their animals to *Id1* knockout mice or to mice expressing a conditional allele of *Id1* that could be deleted using Cre recombinase. In both cases, they found that loss of Id1 dramatically impaired sphere formation but did not affect tumorigenicity. In contrast, knockdown of Olig2 (a progenitor marker that is enriched in Id1^{low} cells and has been shown to be required for glioma formation [Ligon et al., 2007]) significantly impaired tumor formation. These studies reinforce the notion that in vitro self-renewal may not be linked to tumorigenic potential.

The disconnect between sphere formation and tumorgenicity has a number of important implications. First, it highlights the fact that not all tumor-propagating cells resemble stem cells. Whereas normal NSCs expand when cultured under neurosphere conditions, normal progenitors typically do not. Thus, tumors that are propagated by progenitor-like TICs would not be expected to grow under these conditions. This is supported by studies of tumors in *Patched* mutant mice, a model for Sonic hedgehog-driven medulloblastoma; these tumors are propagated by progenitor-like CD15^{+} cells that

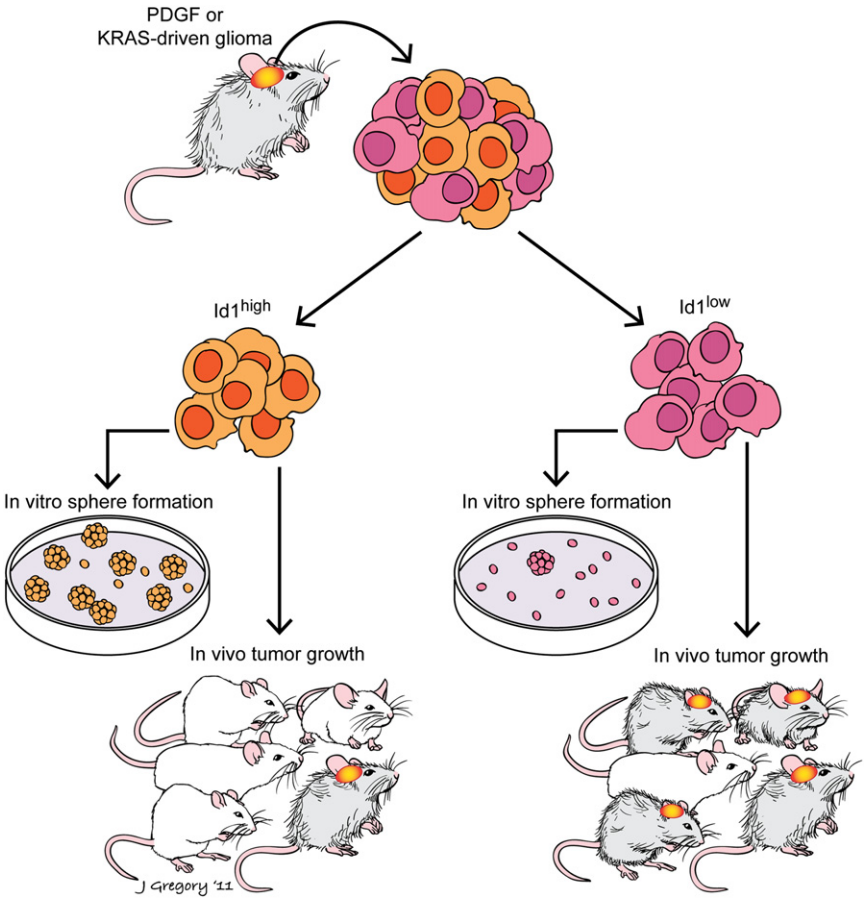

Figure 1. Distinct Populations of Cells Mediate Sphere Formation and Tumor Initiation
PDGF- and KRAS-driven gliomas contain mixtures of cells expressing high and low levels of the transcription factor Id1. Id1^{high} cells (orange) are much more efficient than Id1^{low} cells (pink) at forming self-renewing spheres in vitro. In contrast, Id1^{low} cells are much more efficient at forming tumors following transplantation into mice. (Illustration by Jill Gregory.)

do not grow, or even survive, under neurosphere conditions (Read et al., 2009). Importantly, if not all TICs can form spheres, using this approach to expand patient tumor samples might result in a significant selection bias; only tumors that can form spheres would be available for study. Indeed, one recent study noted that only half of primary gliomas were able to give rise to tumorsphere lines (Chen et al., 2010).

Even when tumors can be grown under sphere-forming conditions, the cells that grow out may not be representative of the original tumor. As Barrett et al. (2012) show, these conditions may select for subpopulations of cells (i.e., Id^{high} cells) that do not represent the full tumorigenic potential of the original tumor. In addition to selection, culturing cells at low density in the presence of growth factors may change their behavior. This was shown many years ago for NSCs, which can undergo marked changes in cell fate potential when cultured under neurosphere conditions (Gabay et al., 2003). Barrett et al. (2012) provide further evidence for this, by showing that glioma cells cultured as spheres undergo changes in marker expression (including Id1 and Olig2) and lose the ability to generate tumors upon transplantation. The fact that culture conditions can dramatically alter tumorigenic potential raises cautions about using tumorspheres to screen for drugs that might be effective at killing tumors in patients.

One possible interpretation of the disconnect between sphere-forming capacity and tumorigenic potential is that tumor initiation does not depend on self-renewal. However, the fact that Id1^{low} cells cannot form spheres in vitro does not necessarily mean that they cannot self-renew. It is possible that under the appropriate culture conditions, these cells might show extensive self-renewal. More importantly, the fact that Id1^{low} cells can form tumors following transplantation indicates that they are capable of significant expansion in vivo. The degree to which this expansion involves self-renewal (e.g., by asymmetric division) versus differentiation remains to be determined. Moreover, the ability of Id1^{low} tumor cells to serially transplant tumors without exhausting—another measure of in vivo self-renewal–has not been tested. Further studies may shed light on the capacity of Id1^{low} tumor cells to self-renew in vivo.

It is important to note that these studies focus on a specific subtype of glioma and that other forms of glioma may be propagated by stem-like, sphere-forming cells. At the same time, it is worth considering whether other types of cancer that are propagated as spheres might be subject to the same caveats raised by these studies. As convenient as cultured cell lines can be for studying tumor biology, it is critical to remember that tumors only grow in living animals. Finding ways to make them stop growing often requires moving beyond the in vitro sphere and into the complex in vivo microenvironment.

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Mdm2's Dilemma: To Degrade or To Translate p53?

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In this issue of *Cancer Cell*, Gajjar et al. provide insight into how Mdm2 can both inhibit and enhance p53 activity. In the basal setting, Mdm2 binds p53 and promotes p53 degradation. Under stress conditions, ATM-dependent phosphorylation of Mdm2 results in its recruitment to p53 mRNA, thereby stimulating p53 translation.

The p53 tumor suppressor is a transcription factor that is induced in response to a variety of stress signals (Kruse and Gu, 2009). Under normal conditions, the p53 protein is kept at low levels in cells by ubiquitination-dependent proteosomal degradation mediated by its negative regulator, the E3 ubiquitin ligase Mdm2 (Figure 1A). Mdm2 is also a p53 transcriptional target and thus participates in a negative feedback loop with p53. Stress-mediated upregulation of Mdm2 has been considered a means by which p53 is able to regulate the duration and amplitude of its cellular effects.

In response to activation of specific oncogenic pathways, the ARF tumor suppressor is upregulated. ARF, in turn, interferes with Mdm2-dependent inhibition of p53 (Manfredi, 2010) (Figure 1B). In contrast, stimulation of the p53 pathway by genotoxic stress involves the DNA damage-activated kinase ATM, which has been shown to directly phosphorylate both p53 and Mdm2 (Kruse and Gu, 2009; Manfredi, 2010) (Figure 1C). The significance of ATM-dependent phosphorylation of Mdm2 was confirmed by the observation that phosphorylation of serine 395 on Mdm2 led to impaired p53 degradation (Maya et al., 2001). Biochemical studies have indicated that this is likely due to altered oligomerization, thereby attenuating the processivity of the E3 ligase activity of Mdm2 (Cheng et al., 2009). DNA damage has also been shown to induce the relocalization of Mdm2 to the nucleolus (Bernardi et al., 2004). It has been proposed that a nucleotide-binding motif within the Mdm2 E3 ligase RING domain facilitates nucleolar localization of Mdm2 (Poyurovsky et al., 2003). Candeias et al. (2008) then made the surprising observation that the p53 mRNA itself was able to interact directly with the RING domain of Mdm2. This interaction impaired the E3 ligase activity of Mdm2 and promoted p53 mRNA translation. It was unclear, however, under what biological settings such an interaction would have relevance.

In this issue of *Cancer Cell*, Gajjar et al. (2012) provide important insight by demonstrating that the DNA damage- and ATM-dependent phosphorylation of Mdm2 on serine 395 promotes the interaction of Mdm2 with p53 mRNA. This, in turn, is needed for p53 stabilization and apoptotic activity (Gajjar et al., 2012) (Figure 1D). By means of RNAi and overexpression experiments, these authors show that both ATM and Mdm2 are required to achieve full p53 apoptotic activity after DNA damage. Use of an Mdm2 isoform that does not bind to the p53 protein shows that a protein-protein interaction between Mdm2 and p53 is remarkably dispensable for this. It was further demonstrated that the interaction between p53 mRNA and the Mdm2 RING domain is necessary for p53-dependent apoptosis after genotoxic stress. Studies using a mutated p53 mRNA that no longer binds Mdm2 confirmed findings with a mutant Mdm2 protein that has a reduced affinity for the mRNA. These intriguing results support the notion that ATM-mediated phosphorylation of Mdm2 at serine 395 promotes allosteric changes in the RING domain, which in turn facilitate p53 mRNA binding. Finally, Gajjar et al. (2012) show that after DNA damage, the interaction between Mdm2 and p53 mRNA impairs Mdm2-dependent ubiquitination of p53. Thus, it is argued that the p53 mRNA-MDM2 interaction not only increases p53 translation but also inhibits p53 protein degradation as well.

In sum, this study demonstrates that Mdm2 can act as a positive regulator of p53 activity after genotoxic stress. It further provides an additional novel explanation for why Mdm2 is transcriptionally upregulated by p53 after DNA damage.

The finding that p53 mRNA relocalizes with Mdm2 in the nucleolus after DNA damage is especially interesting since the nucleolus is generally thought of as the site of ribosomal RNA transcription.