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The dormancy dilemma: Quiescence versus balanced proliferation

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Abstract

Metastatic dissemination with subsequent clinical outgrowth leads to the greatest part of morbidity and mortality from most solid tumors. Even more daunting is that many of these metastatic deposits silently lie undetected, recurring years to decades after primary tumor extirpation by surgery or radiation (termed metastatic dormancy). As primary tumors are frequently curable, a critical focus now turns to preventing the lethal emergence from metastatic dormancy. Current carcinoma treatments include adjuvant therapy intended to kill the cryptic metastatic tumor cells. Because such standard therapies mainly kill cycling cells, this approach carries an implicit assumption that metastatic cells are in the mitogenic cycle. Thus, the pivotal question arises as to whether clinically occult micrometastases survive in a state of balanced proliferation and death, or whether these cells undergo at least long periods of quiescence marked by cell cycle arrest. The treatment implications are thus obvious – if the carcinoma cells are cycling then therapies should target cycling cells, whereas if cells are quiescent then therapies should either maintain dormancy or be toxic to dormant cells. Because this distinction is paramount to rational therapeutic development and administration, we investigated whether quiescence or balanced proliferation is the most likely etiology underlying metastatic dormancy. We recently published a computer simulation study that determined that balanced proliferation is not the likely driving force and that quiescence most likely participates in metastatic dormancy. As such, a greater emphasis on developing diagnostics and therapeutics for quiescent carcinomas is needed.

Introduction to metastatic dormancy

Advances in cancer treatment, underpinned by a growing understanding of tumor biology, have rendered the majority of localized solid tumors either curable or controllable. Surgical and radiological interventions have improved to the point that the initial primary tumor can be extirpated. However, if the primary tumor gives rise to clinically-detectable metastatic lesions, current therapies usually only delay mortality and are not curative. Most daunting to patients faced with treatment decisions for disease that is “non-metastatic” at the time of primary tumor diagnosis is that metastatic dissemination may have already occurred despite the primary lesion “being cured” by removal. Many of these metastatic tumors will appear years or more than a decade later, thus termed metastatic dormancy. Although this phenomenon afflicts nearly half of carcinoma patients that develop metastases (1), the cell biology of this remains unknown. Proposed dormancy mechanisms range from cell cycle arrest, immune function dysregulation, angiogenic insufficiency, stress related kinase and

Conflict of Interest Statement

Two of the authors (A.W. and L.G.G.) have intellectual property positions and patent(s) on the bioreactor that have been assigned to their respective Institutions (Univ Pittsburgh and MIT).

urokinase receptor imbalance, to tumor/stroma biomechanics (2–5). Fundamentally these vary between the orthogonally opposed dormancy mechanisms of cellular quiescence or balanced proliferation (mitogenesis equally offset by apoptosis). Spotlighting this difference is critical because therapeutics in the clinic and under development largely target cycling, not quiescent carcinoma cells.

This metastatic dormancy, while present in most all cancers, is particularly insidious for carcinomas of the mammary gland. For breast cancer, up to one third of small (1 cm or less), non-invasive (or micro-invasive) carcinomas with no evidence of metastasis will become evident at distant sites within the decade following diagnosis of the primary tumor (1). For this reason, adjuvant chemotherapy, aimed at killing cycling tumor cells, is an option after successful lumpectomy. This adjuvant therapy has been met with limited success for most incarnations of breast carcinoma, as adjuvants reduce metastatic recurrences by only a third (on average) at ten years (6). The reasons for this therapeutic failure in breast and other epithelial carcinomas can be summarized by two possibilities – the tumors are inherently resistant to the agents used, or the disseminated tumor cells are not cycling (1, 7).

Prognostic assays for metastatic dormancy

This is the key unknown in metastatic dormancy, whether the clinically silent micrometastases are in a state of balanced proliferation and death or if they exist as mitogenically quiescent (8). The phenotype and genomic fingerprints of the primary tumor often suggests quiescence. Two distinct molecular algorithms are used clinically to predict breast cancer recurrence (http://www.agendia.com/pages/mammaprint/21.php and www.oncotypedx.com), and while they do not utilize the same genes, there is a preponderance of mitosis-related genes that correlate with recurrence consistent with most proposed signatures (9). Further, triple negative (ER-, PR-, HER-2-) mammary tumors are generally aggressive, highly proliferative, highly recurrent tumors that are treated with adjuvant chemotherapy, but can be considered eliminated (or senescent) if there is no metastatic recurrence after about three years. These clinical correlates suggest that the latent tumor cells at the metastatic sites may also have low rates of cycling.

The drawback to these and other current prognostic assays is that the cells examined are from the primary tumor site, where the preponderance of cells likely lacks the necessary traits for successfully seeding, surviving or expanding in the metastatic microenvironment. While each step in the metastatic cascade (separation from the primary tumor, intravasation into a conduit, survival in that conduit, extravasation, and finally successful seeding of an ectopic organ) is inefficient, ectopic survival appears to be the most rate-limiting step. Careful enumeration of these stages in animal models shows that it is the establishment of a small number of surviving cells in the metastatic target organ that is the least efficient (10, 11). Interestingly, the few cells found weeks after seeding in these animal models were not highly proliferative (12). Still, these are among the few studies that have examined the early stages of tumor metastasis. As true human metastatic dormancy (as noted after years to decades) cannot be approached in rodent models (though initial steps of attaining dormancy may be noted in these short-lived animals), it is critical to consider the quiescent behavior of these tumor cells in the human context to direct therapeutic and diagnostic approaches.

Computer simulation revealing quiescence as integral to metastatic dormancy

We recently implemented an *in silico* model to determine the survival probability range that carcinoma micrometastases in a state of balanced proliferation would yield the dormant phenotype (13). Although many other computer simulations have been developed to model

metastasis (14–16), none of these approaches have explored the survival probability requirements of the metastatic niche necessary to confer a dormant phenotype. We employed a Markov chain Monte Carlo approach that sampled from a probability distribution to assign either a divide or die condition to each cell in each simulated metastasis. Survival probabilities were assigned ranging from 30 to 70 percent with an additional stochastic 10 percentage point probability adjustment at each sampling. So for example, at 40 percent survival probability metastatic cells would survive between 30 and 50 percent of the time, thus providing an additional alignment to the fluxing survival conditions of the metastatic niche. We assumed that any given patient could have 1000 independent metastatic deposits at the time of primary tumor removal. By defining active cycling as a 3 – 5 day mitogenic window, 1218 cycles equated to a 5 – 10 year metastatic dormancy before the metastases reached the clinically detectable level of one million cells or more. By traversing the survival probability from 30 to 70 percent during each cycle, we identified the boundary conditions at which any micrometastases might remain in a dormant phenotype over the entire time period (without any metastases in that ‘peron’ clinically emerging). Surprisingly, this survival probability window was narrow, being only about one percentage point wide, from a probability of 49.7 to 50.8 percent (Figure 1). Even more interesting, the width of this survival probability was independent of the assumed starting size of the micrometastases (1 – 8 starting cells). While it is generally accepted that solitary, extravasated cells initiate metastases and may become quiescent, we also simulated a burst of proliferation prior to establishment of a micrometastasis by modeling metastases starting at 2000 cells. While these larger starting metastases maintained the dormant phenotype, the survival probability window remained the same one percentage point. At survival percentages even just slightly lower than 49 percent, all micrometastases rapidly died out, and by 55 percent there was rapid outgrowth of clinical metastases (<100 cycles to exceed one million cells) that were more indicative of progressive metastatic disease. This suggested (at least theoretically) that it would be unlikely during extended human clinical dormancy that the tumor cells would exclusively exist in a state of continuous cycling with matched cell death (balanced proliferation).

This analysis assumed a homogenous population behavior (divide or die at each cycle) even though the survival probabilities were stochastic at any given cycle for each metastatic cell. The remarkably small probability window for dormancy in a balanced proliferation/death model holds from at least 1 to 2000 starting cells. Obviously, if we significantly extended the cycle time beyond 72 hours, or introduced intermissions into cycling, the probability window would broaden slightly; however this would merely reinforce the finding that balanced proliferation and death is not likely the sole mechanism for metastatic dormancy. It should be noted that the model does not account for the processes leading to micrometastatic establishment (escape, transit, extravasation, etc), nor does it address the starting the point for quiescence, nor determine that the quiescent state is uninterrupted; these issues are to be addressed in further modeling studies. Although this model has shortcomings as noted here and in the original article (including that simulated metastases do not spawn secondary metastases), that such a narrow survival probability was defined is highly suggestive of a critical role for quiescence. Although this model was not designed to statistically reject a null hypothesis that balanced proliferation plays a dominant role in metastatic dormancy, that only a single, narrow, and contiguous survival probability led to metastatic dormancy does not reasonably align with our biological understanding of the metastable nature of the metastatic niche (unless the cells undergo periods of quiescence). A conceptually different invocation of additional events for metastatic emergence, such as angiogenesis or failure of suppressive events to allow for further metastatic growth (4, 17, 18), only invoke the trigger for quiescence but do not alter the idea that for extended tumor dormancy, the micrometastatic cells likely undergo mitogenic quiescence for at least extended periods.

In vivo investigations into metastatic dormancy

The question arises as to whether dormant metastases that undergo cellular quiescence align with clinical and experimental findings. Studies that directly examine dormant micrometastases are few and hampered by the lack of clinical samples or limitations of experimental systems (4, 7). From the experimental side, there have been a number of interventions to regulate cellular quiescence and determine the effects on tumor growth and persistence in host animals (19–21). The outcomes and correlation of low proliferative rate with metastatic dormancy are consistent with a cellular quiescence hypothesis rather than balanced proliferation. Further, the conversion of micrometastatic cells from a mesenchymal phenotype to a more epithelial one (22–24), also is supportive as these epithelioid tumor cells tend to have a reduced mitogenic rate. Additional support come from recent *in vivo* work that demonstrated that TGF-β receptor blockade prevents dormancy by microenvironmental BMP (25). In fact, the authors identified a genetic signature that is predictive of breast cancer organotropism to lung versus other common metastatic sites such as liver and brain.

Another open question concerns that state at which disseminated tumor cells enter dormancy. While the angiogenic switch model implies a later event after the micrometastasis goes through numerous cell cycles, others suggest a very early event possibly at the single cell level (25). Work by the Welch group has investigated single cell metastatic dormancy *in vivo* and provides insights into mechanisms such as breast cancer metastasis suppressor 1 (BRMS1) and kisspeptin (KISS1) (26–28). These provide mechanistic bases to the earlier work which counted solitary cells surviving in ectopic sites (10). However, in these experiments it is not clear whether these persisting disseminated cancer cells give rise to the later outgrowth. Regardless, the model tested demonstrated the same narrow probability range whether the initially established micrometastatic nodule contained 1 or up to 2000 cells; only the number of dormant metastases varied based on cell number (13).

The human observations are also consistent with the cellular quiescence model. Disseminated tumor cells isolated from bone marrow even after removal of the primary lesion have been found to have low proliferation rates by mitogenic markers, and low to undetectable levels of activated AKT as a key signaling nexus in tumor cell mitogenesis (29, 30). Still these singular cells in bone marrow are not the micrometastatic foci one might consider as potentially emergent. Unfortunately, such early small lesions are not often observed and studied in human patients. However, where data have been gathered, it has been found that such tumor foci are often highly epithelial, and morphologically quiescent rather than the mesenchymal-like phenotype displayed by their paired primary lesions (31–33).

Work on defining metastatic dormancy signatures provide fodder for both cellular quiescence and externally constrained growth (balanced proliferation). For instance, an angiogenic signature predicted tumor outgrowth in a mouse model (34), while G0-like quiescence was noted in a squamous carcinoma xenograft model (35). Combining such mouse model and *in vitro* networks derived from arrested cells, a 49 gene signature emerged that correlated with clinical dormancy in a retrospective analysis of human mammary carcinomas (36). These genetic signatures might be predictive of metastatic dormancy in the human condition and serve as a foundation to explore mechanisms to either maintain metastatic dormancy or induce emergence.

Another data set from which we may draw conclusions relates to the seeming chemoresistance of micrometastases. This can be due to cellular quiescence, inherent

chemoresistance, resistance due to the microenvironment (signals or privileged site), or a combination of the preceding; or even simply an artifact of observation. Artifactual observations can arise from the inherent nature of logarithmic doubling in which an impressive 99% reduction in cell number would translate to only a two-week delay during the emergent phase of tumor cell outgrowth. However, studies have shown that metastatic cells are relatively more chemoresistant than paired primaries when challenged with a range of therapies due to the combination of microenvironmental signals and inherent cell properties (37–40). It also must be noted that noncycling or quiescent cells are generally more resistant to killing by a variety of insults including chemotherapies, and while this is not likely the sole reason for the resistance of metastases, mitogenic quiescence would be contributory. Still, the clinical experience suggests that in most carcinomas, chemotherapy rarely cures clinically-evident metastases but rather reduces the tumor burden and prolongs life. However, the integration of the experimental and observational findings to-date suggest that the cellular quiescence model is consistent with clinical dormancy, but these suggestions fall short of being convincing.

What is needed is a comprehensive approach to metastatic dormancy in which the cellular processes can be evaluated longitudinally, and in real time. True appreciation of the full span of metastatic dormancy requires human patients due to the time-length of the phenomenon and the spontaneous nature of such metastases that confound the existing experimental models (8). However, attempts have been made to use the human patient as an experimental system by examining disseminated tumor cells found in bone marrow and circulation (4). Given the relative abundance of such cells coupled with the rarity of actual emergent metastases, it is quite possible that the vast majority of these cells are pre-senescent or pre-apoptotic and thus not representative of dormant micrometastases. Rather, one would desire intravital imaging of human micrometastases, a capability for which advances in PET and other imaging modalities are striving. However, this is currently not available, and one could envision that the putative cellular quiescence would defeat efficient labeling and detection of such micrometastases.

Microphysiological 3D bioreactors to study metastatic dormancy

We are taking a different approach to examining the early stages of metastatic seeding and dormancy in an all human microphysiological bioreactor (23) (Figure 2). This *ex vivo* system allows for metastatic seeding and entry into dormancy to be examined at a cellular level for up to several weeks. This exceeds the hours-long window possible in animal models (11, 41, 42) and is beneficial over the random terminal endpoints of long-term metastasis studies in animals and humans. Even with the limitations of having incomplete vascular and immune systems and lacking neural innervation, this system provides for the complex multicellular and matrix interactions within the metastatic niche comprising human cells. In this manner, one can determine whether tumor cells can enter cellular quiescence. Initial work suggests that cellular quiescence or entry into dormancy is finely regulated, and dependent on numerous factors including tissue rheology, fluid/blood flow, oxygen tension and nutrient and hormone levels. For instance, a stiff supporting matrix, possibly representative of fibrosis, drives carcinoma cells towards progressive metastatic growth; this may underlie the puzzling phenomenon of cells that attain at least short term dormancy *in vivo* growing in 3D cultures *in vitro*. While there are other systems with which to probe dormancy, we are providing this on a template for discussion.

Conclusions

The real importance of determining whether the balanced proliferation or cellular quiescence model holds sway is the therapeutic implications of such. Proliferating cells allow for a

distinct set of agents and strategies; mainly the routine chemotherapies could be valuable as they target mainly cycling cells. On the other hand, if the metastatic cells are quiescent (in a G0 or G1 state), these agents would likely not be efficacious. Therefore, strategies should then be aimed not just at extirpating the cells but also could be designed to keep the cells in a state of indolent dormancy. In fact, generalized toxic therapies might actually be detrimental as the collateral damage to the parenchymal or stromal cells may lead to an inflammatory state; there are suggestions that such an inflammatory milieu may 'awaken' the surrounding dormant micrometastases. In such a situation, adjuvant chemotherapy may not only be contra-indicated due to toxicity, but also due to shortening overall patient survival. Thus, the critical question as to whether the micrometastatic cells are in a state of quiescence or balanced proliferation is the key to dealing with tumor dormancy, and should be at the forefront of new approaches to tumor management.

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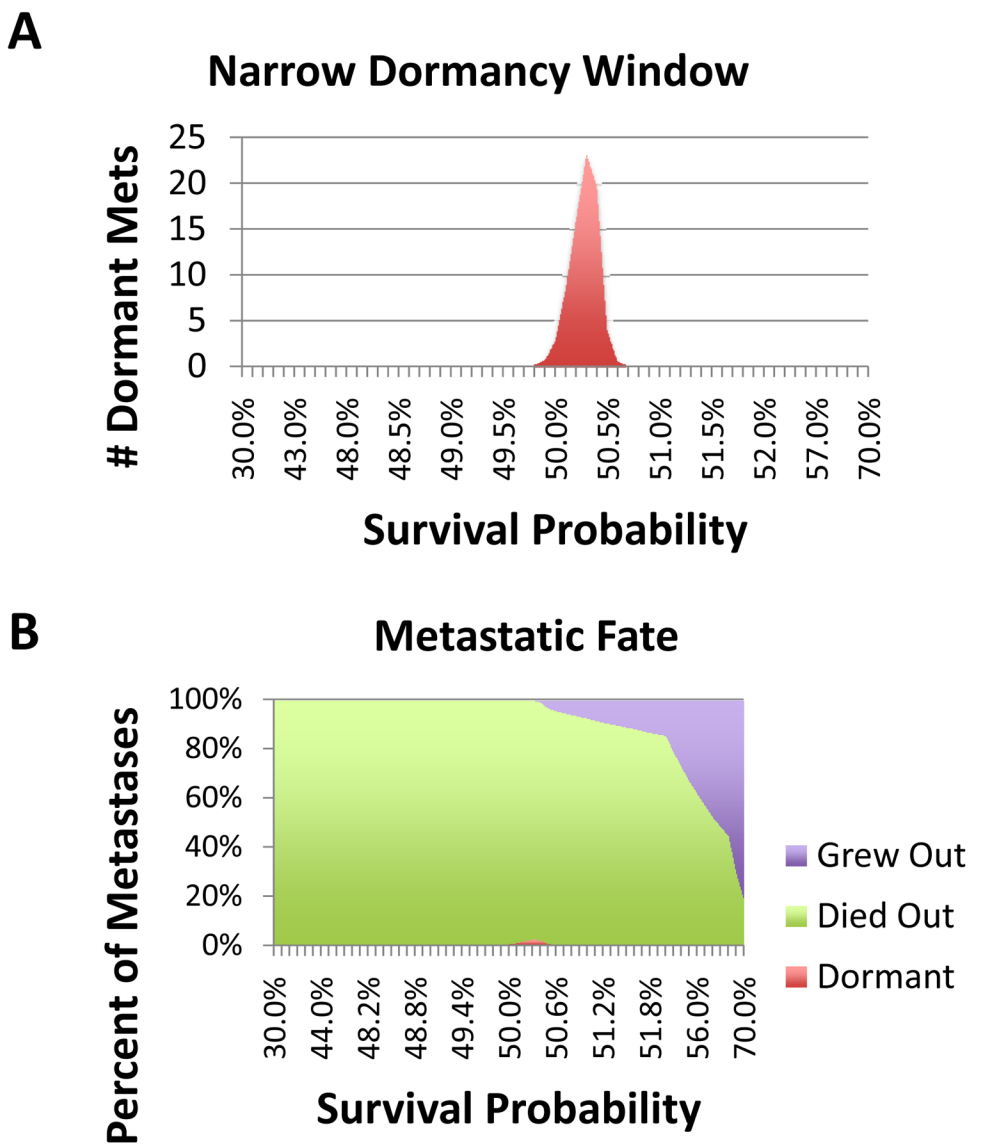

Figure 1.

Probability of dormancy from *in silico* simulation. Dormancy is defined by a metastasis achieving 1,218 cycles while having a cell number greater than zero and below 1 million. The simulated balanced proliferation yields a dormant phenotype for patients harboring 1000 cryptic micrometases only between 49.7 – 50.8 percent survival probability regardless of starting cell number in the micrometasasis. Panel A shows the absolute number of dormant metastases at the end of the 1,218 cycles for a starting number of 2 cells per micrometastasis. Panel B depicts the metastatic fate for each of the survival probabilities demonstrating that same dormancy window (in red). The green area demonstrates that the majority of metastases die out even at survival probabilities approaching 60 percent. Metastases that become clinically evident (exceed 1 million cells) are shown in purple. From (13).

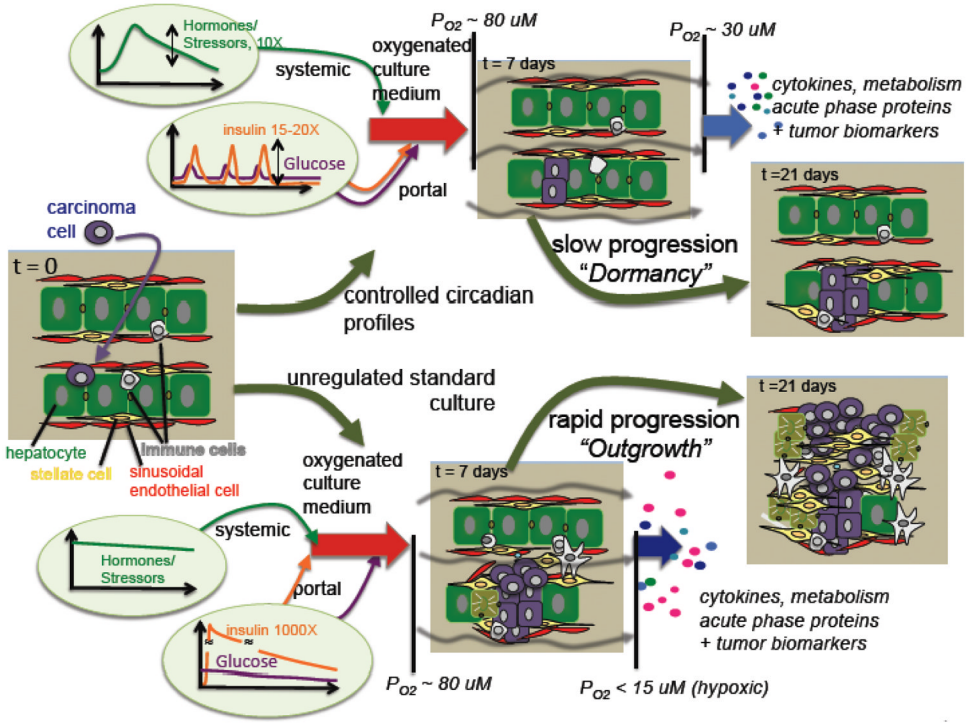

Figure 2.

Schematic of an approach to study metastatic dormancy. The development of microphysiological systems such as organotypic bioreactors allows for the examination of cellular events in metastatic seeding and entry into dormancy or continuous outgrowth for a multiweek period (43). Shown is a cartoon depicting the fate of the micrometastasis following from the host tissue being in a more physiological state (upper pathway) versus a stressed, inflammaned or fibrotic organ (lower pathway). The stressed pathway prohibits micrometastases from entering dormancy while supporting emergence into frank metastases.