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Systems Biology of Tumor Dormancy

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Foreword

Numerous organisms in nature have evolved defense behaviors to preserve themselves against predators. Ironically, many of these behaviors are seemingly at odds with the ultimate goal of survival. One such behavior, thanatosis (of Greek origin, meaning "putting to death"), is a defense mechanism in nature whereby an animal feigns death in order to avoid detection and possible death by a predator. This behavior is most commonly associated with the Virginia Opossum, where when threatened, it can switch on a near death appearance by "playing possum" and fooling would-be predators. This evolutionary trait holds insight for cancer research, because similar behaviors may be invoked by the cancer cells within the animal providing a defense for tumor cells.

Cancer is a complex disease and, by reputation and outcome, also an aggressive disease that can quickly overtake and kill its host. However, recent scientific advancements have shown that cancer is capable of a variety of growth patterns, from rapid replication and spreading to a more controlled dormant phenotype evading detection. Unfortunately, a dormant phenotype is by its very nature more difficult to detect and treat.

Despite amazing biomedical advances and billions of research dollars, cancer remains one of the most destructive and elusive diseases known to humankind. Statistically, cancer will be the cause of death for 25 % of the US population, and according to the World Health Organization, it will be the number one global killer this year. Ironically, part of the challenge is due to the success we have had in preventing and treating cancer and other acute diseases which increases survival, and subsequently, the at-risk population. Part of this can be explained by the increase in lifespan throughout the world and the knowledge that cancer is primarily a disease of the aged. In addition, the diversity of the disease and patient population suggests a multitude of etiologies and subsequent treatment strategies. The Cancer Genome Atlas (TCGA) has highlighted the complexity of cancer at the molecular level. Human behavior also plays a significant role especially in the prevention of cancer. Smoking and diet are the most common behaviors that continue to have an important impact on cancer incidence but remain difficult to alter.

With the knowledge that cancer incidence is increasing throughout the world, we must continue to advance detection and treatment of the disease. With encouraging and important exceptions, treatment remains somewhat generic and unchanged over the past decades. Surgery, which was pioneered over a century ago, remains the most recommended and successful treatment for solid tumors. Most chemotherapeutic strategies reflect more broad-based agents targeting fundamental cellular processes such as DNA replication. We currently know more about the physiology and biology of cancer than ever before, and we are beginning to use this knowledge for a more specialized approach to the prevention, detection, and treatment of cancer. Most indicative of this has been recent success of targeted therapies such as Herceptin or Gleevec, which are used to treat aggressive forms of breast cancer and leukemia, respectively. Unfortunately, these treatments, while promising, have pitfalls of patient selection and resistance development. Since cancer is most successfully treated at early or less aggressive stages of the disease, research into the growth kinetics of cancer continues to hold a great deal of promise for future advances against the disease.

Tumor dormancy is a critical stage in cancer development where cancer cells can remain occult and asymptomatic. Dormancy can occur at various stages of the cancer's progression from early stage development, as micro-metastasis, or as a residual disease following "successful" treatment. This last niche as residual disease is critical in long-term survival of the patient. While many questions remain unknown about tumor dormancy, we do know that the process, like so many in biology, involves multiple components and physical scales. At the cellular level, the cell cycle, senescence, and apoptosis are critical, while at the micro-environmental and organismal level, angiogenesis and the immune system are major players. The role that all of these components play in the initiation and cessation of dormancy remains a central question in cancer biology. Other questions exist as to the molecular and cellular markers of dormancy and how this phenotype is manifested in diverse tumor types under various conditions including current therapies. Obviously, answers to these questions will require a systems biology approach that can consider the variety of molecular and cellular components at work.

The National Cancer Institute (NCI) established the Integrative Cancer Biology Program in 2004 to study cancer biology from a systems biology perspective. The Center of Cancer Systems Biology at Steward St. Elizabeth's Medical Center is part of this effort and sponsored the first Annual Workshop on Systems Biology of Tumor Dormancy. The organizing committee included: Nava Almog, Heiko Enderling, Cassedra Enayo, Lynn Hlatky, Clare Lamont, and Melissa Klumpar. This international workshop brought together clinicians, biologists, mathematicians, and computer scientists to discuss the critical issues of tumor dormancy with emphasis on angiogenesis, the immune system, and cancer stem cells. The workshop included presentations by mathematicians Heiko Enderling, Kathleen Wilkie, and Philip Hahnfeldt, and biologists Nava Almog, Stefano Indraccolo, Tobias Schatton, Julio Aguirre-Ghiso, Bruno Quesnel, and Dean Felsher. Working groups held during the meeting allowed workshop participants to discuss current problems related to tumor dormancy and develop novel mathematical/computational models. Mathematicians,

biologists, and clinicians in each working group engaged in interdisciplinary dialogues and model development. During the three-and-a-half day workshop, the modeling groups developed exciting new projects and laid the foundation for collaborations and joint manuscript submissions. The proceedings in this book reflect those presentations and discussions and in collection, represent an important reference for the state of science and hope in the field of tumor dormancy.

Tumor dormancy remains one of the least understood aspects of cancer biology. While its obvious phenotype represents a challenge in detection, elimination, and long-term survival, it also gives new hope in cancer treatment. If we can understand the mechanism of control of dormancy or gain new insights into the molecular and cellular controls of cancer growth or dormancy, then we have the potential to manipulate those processes for better therapies and outcomes. Knowledge gained from publications such as this will bring the field closer to practical approaches, knowing that while the tumor "can run, it can't hide." In the end, even the tricks of the opossum can be detected by the knowledgeable predator.

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Introduction

Awareness of the existence and importance of tumor dormancy has come from a number of disparate clinical and translational directions, attesting to the broad applicability of this phenomenon. To do the topic justice, it is necessary to briefly recount the settings in which dormancy has been encountered, and most importantly, to assess what has been learned and what stands yet to be learned from those encounters, both in the clinic and in the laboratory.

Curiously, attention has turned to the subject of tumor dormancy amidst an intense clinical focus on the opposite phenomenon—advanced, "out-of-control" cancers. It is not lost on anyone in the field that, while very important therapeutic strides have been made against particular cancers, including blood-borne, germ-cell, and childhood cancers since the declared War on Cancer in 1971, the situation for adults manifesting most advanced epithelial cancers remains problematic. These high-profile refractory cancers, including those of the lung, breast, brain, pancreas, colon, and ovary, carry fearsome statistics and metastatic disease often foreshadows an inevitable course. Our mainstay strategies of direct tumor attack, employing a growing repertoire of chemotherapeutic and radiation protocols, often provide impressive initial responses, but over the long run frequently prove inadequate.

It therefore stands to reason attention is feverishly focused on finding new methods to detect cancer earlier while the condition remains treatable. Indeed, the search for cancer in asymptomatic people has taken on a life of its own, placing as much emphasis on discovering it in the seemingly healthy as treating it in the obviously sick. The battle has even pitted alternative methods of detection against one another. The National Lung Cancer Screening Trial (NLST) was conducted to resolve the issue of whether people at risk for lung cancer would benefit more from screening with the powerful low-dose spiral computed tomography (CT) than conventional chest X-ray. The trend was sufficient to end the trial early—a sizeable 20 % improvement in survival was noted when CT was employed. But there were some tradeoffs. With CT, only 3.6 % of lesions requiring clinical follow-up proved to be positive for cancer, while for X-rays, the rate was 52 % higher at 5.5 %.

It may at first seem paradoxical that a clear improvement in detection technology for cancer should also be yielding higher rates of false detection. One might argue

that, as our ability to resolve increases, so should the accuracy of the claims surrounding what we are examining. But this would not take into account that with the power to resolve comes not only a better resolution of what was visible before, but also the ability to view what were previously undetected lesions, some of which may pose a distinctly different level of threat. This possibility was brought to the fore in a seminal study (Black and Welch, *NEJM*, 1993), which reported on histological findings from autopsies of adults dying of non-cancer causes. Similar to the NLST study, the limits of diagnostic capabilities using refined methods for gross visualization were tested. Surprisingly, for a range of cancer types, it was determined that the prevalence of microscopic detectable cancer far outweighs the actual macroscopic disease incidence—that virtually all of us by adulthood are cancer carriers, whether we manifest symptoms or not. Thus, by looking more closely for cancer disease in our quest to avoid its advanced refractory state, one is discovering that cancers commonly exhibit growth dynamics not characteristic of symptomatic disease. The picture emerging is that overtly transformed cancer cells commonly face cancer-host interaction bottlenecks that limit tumor growth before becoming overt disease.

One major realization of this altered dynamic is the state of tumor dormancy. Once thought an exceptional occurrence, dormancy is now appreciated to be a common stage in the course of many cancer types. The implications of this realization are nothing short of dramatic—extending in three major directions. The first is the epidemiologic notion of cancer risk, which if properly defined as the eventual experience of symptomatic disease, must now be conceptually disconnected from its current interpretation as the risk of creation of the first cancer cell. Secondly, we must reconsider the practical implications for whether to treat the ever-smaller tumors detectable by our improving technologies that may be dormant and therefore pose a much-reduced threat. Lastly, and perhaps most importantly, we must understand how intrinsic dormancy bottlenecks can effectively control cancer in ways we have not been able to match with our therapeutic anti-cancer armamentarium.

A proper accounting of dormancy in cancer progression would clearly improve risk estimation for symptomatic cancer presentation. Heretofore, classic thinking has maintained that stochastic DNA damaging events and gene mutations lead to eventual cell transformation and the first cancer cell, from which symptomatic cancer inevitably arises. The prevalence of tumor dormancy has removed the word "inevitably" from this statement, radically altering the classic risk models. Understanding the ramifications stands to better inform policy-making decisions, e.g., limits for exposures to carcinogens in the workplace and the environment. As cases in point, the Biological and Environmental Research Division of the Department of Energy (DOE) is charged with researching the cancer risk associated with nuclear waste cleanup, and more generally the hotly debated question of whether there exist low-dose limits to exposure below which there is no lasting damage. In addition, the Space Radiation Program Element of the Human Research Program at the National Aeronautics and Space Administration (NASA) is committed to estimating the excess radiogenic cancer risk for astronauts embarking on extended space missions. The matter of tumor dormancy is proving pivotal to both objectives.

The therapeutic question of treatment of slow-growing tumors, although not new, has also attracted attention in light of the question of "over-diagnosis" of cancers that would not progress. In contrast to "false positives," these represent the detection of histologically confirmed cancers, but cancers that are destined not to present as symptomatic disease over the person's lifetime. Contributing to this class are dormant and sufficiently slow-growing tumors—ironically the very types of tumors our early-detection technologies are best designed to detect. One recently published study of the subject involved 39,888 Norwegian women with diagnosed invasive breast cancer who had either participated in their new breast cancer mammography screening initiative, or not. What the investigators found was that, when tracking the number of detected cancers in the unscreened group, they never quite added up to the number detected by screening—the difference representing "pseudodisease," i.e., dormant or near-dormant tumors detected by screening that never would have advanced to routine clinical presentation over the patient's lifetime. They estimated that for every 2,500 people screened, one cancer death would be avoided, but six to ten individuals would undergo unnecessary treatment for a disease they were never destined to experience. More generally, the problem of overdiagnosis tends to exaggerate the success statistics for any screening study, as every treatment for screen-detected pseudodisease contributes a guaranteed "cure."

The most far-reaching implications of tumor dormancy, however, may well come from translational research. Looking forward, the phenomenon of tumor dormancy, or near dormancy, offers a unique opportunity to understand a natural means of modulating disease progression. Appreciating this, the Workshop on Tumor Dormancy held at the SEMC in Boston this last summer was focused on presenting for interactive discussion the various underpinnings and implications of this simple dynamic state. These settled into four broad contexts—the roles of (1) the immune response, (2) cancer stem cells (CSCs), (3) organ context, and (4) induction of angiogenesis.

The immune system was discussed for its rather complicated inclusion of tumor dormancy, sandwiched as it is as the second "E" (for "Equilibrium") between the earlier tumor attrition ("Elimination") phase and the final tumor release ("Escape") phase, known collectively as the three "E's" of immunoediting. The immune influence was portrayed as a contest of sorts between the tumor cells (prey) and the immune cells (predators), with the outcome being anything but intuitive. In line with recent studies, a biphasic immune response was noted. One surprising observation was that limited immunity may actually hasten escape from the equilibrium phase it helps establish and encourage cross-resistance to agents. In this way, tumor dormancy can actually limit the effectiveness of therapy.

By a quite distinct mechanism, CSCs, along with their non-stem counterparts, were proposed to play an analogous role in producing biphasic dependencies between cell targeting and overall population response. When CSC migration or non-stem cell killing is low, CSCs become encased in their own progeny, thwarting CSC expansion and thus population growth overall. However, when CSCs are able to occupy adjacent open areas of the tumor with the help of either higher cell migration or a higher attrition of the non-stem progeny, the tumor may more efficiently

undergo "self-metastasis" at its periphery, thereby helping to expand the tumor as a whole. Other work demonstrated a more transcendent control, operating through the myc oncogene. The effect of its inactivation is to block self-renewal, tying this process again back to CSCs.

The role of context in controlling growth was seen also to extend beyond the stem and non-stem composition. Evidence exists that organ-specific molecular signaling can determine whether a metastatic lesion will expand or remain dormant. By examining the different signaling profiles at these sites, it has been possible to ascertain what may be dominant controlling factors. Key players prove to be stress-activated kinases, transcription factors, e.g., p53, and cell cycle inhibitors, e.g., p21.

Finally, a fourth major topic discussed was the role of tumor angiogenesis in defining the dormant state and its implications for tumor development. Once again cancer growth dynamics are seen to be controlled by a balance of opposing influences; either through balanced proliferation and cell death in the case of prevascular lesions that are not yet angiogenically competent, or a balance between pro- and anti-angiogenic factors emanating from the post-vascular tumor. Potential mechanisms governing dormancy control in these two cases were discussed; the former showing a novel influence of miRNAs, and the latter showing evidence of tumor exploitation of what are likely normal organogenic growth controls. In work that may be glimpsing a global influence of immunity, stem cells, and context in dormancy, a tumor model focusing on a stem-like ABCB5+ subpopulation of melanoma cells revealed simultaneous immune influence along with angiogenic control.

The take-home message from these seemingly disparate underpinnings of the dormancy state may well be the commonalities revealed. Tumor dormancy may generally be described as a balance between opposing forces, working through molecular, population, and inter-tissue levels. Most of the mechanistic drivers are proving not to be de novo creations, but mechanisms "borrowed" in a distorted way from normal tissue controls. This is providing an impetus for a new frontier in treatment approach—one that could conceivably limit progression of refractory cancers by employing existing natural control processes. "Putting the genie back in the bottle," if you will, a goal that has evaded tumor-directed attacks thus far, may well be achievable through exploitation of tumor dormancy—a dynamic which has already proven it can do just that.

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Preface

The concept of this book arose from the first in a series of annual workshops organized by the Center of Cancer Systems Biology at Steward St. Elizabeth's Medical Center, Tufts University School of Medicine, and supported by the National Cancer Institute's Integrative Cancer Biology Program. This inaugural workshop focused on Systems Biology of Tumor Dormancy and was held in Boston, Massachusetts in late July 2011. The goal of the workshop, and by extension, of this book, was to present research advances in the field of tumor dormancy from diverse experimental and clinical perspectives using biological, mathematical, and computational approaches.

As the editors, we are grateful to the team at the Center of Cancer Systems Biology who organized and hosted the workshop and would like to extend our appreciation to all workshop speakers and contributing authors who diligently worked on their respective chapters. We would also like to thank Melissa Klumpar and Brandy Weidow for their help in editing the chapters, and Melanie Tucker and Connie Walsh from Springer Publishing who guided us through this journey and kept us on course.

We hope that this book stimulates your interest in tumor dormancy, as well as in exploring interdisciplinary research techniques.

Enjoy.

Boston, MA, USA

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Part I
Angiogenesis

Chapter 1
Genes and Regulatory Pathways Involved in Persistence of Dormant Micro-tumors

Nava Almog

Abstract Micro-tumors can remain dormant for prolonged periods of time before they switch and enter the rapid growth phase. This initial stage in tumor progression is clearly understudied. In spite of high prevalence, significant clinical implications and increased interest by the research community, tumor dormancy is still poorly understood. The topic of tumor dormancy also suffers from a lack of definition and an agreed upon terminology to describe it. Additionally, the number of reproducible experimental models available for studying indolence of human micro-tumors is quite limited. Here, we describe the development of a general class of in vivo models of indolent human tumors and how these models can be used to elucidate molecular and cellular mechanisms involved in the regulation of dormancy. The models consist of human tumor cell lines that form microscopic cancerous lesions in mice. Although these lesions contain viable and fully malignant cancer cells, the tumors do not expand in size but remain occult for prolonged periods until they eventually spontaneously switch and become fast-growing tumors. Consistent with Judah Folkman's vision that tumors will remain occult and microscopic until they acquire the ability to recruit new and functional blood vessels, the dormancy period of the micro-tumors is associated with impaired angiogenic capacity. Such models can be used for dissecting the host and the tumor-derived regulatory mechanisms of tumor dormancy. Understanding the process by which dormant tumors can overcome growth constraints and emerge from dormancy, resuming size expansion, may provide insights into novel strategies to prolong the dormancy state or to block tumor formation in the early stages, before they are physically detected or become symptomatic.

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Introduction

Tumor dormancy is a clinical phenomenon in which tumors do not expand in size over a prolonged period of time [1–6]. It has long been recognized as a significant problem in the management of cancer patients [3, 7–10]. Tumors can enter a latent phase during various stages in tumor progression including post-angiogenic stages of tumor progression [11]. However, in this chapter we only discuss dormancy of *microscopic tumors*, which are usually present with a maximal diameter of 1–2 mm. Here, dormant tumors are defined by their inability to expand beyond a microscopic size (see Fig. 1.1). Importantly, it is demonstrated that such small harmless lesions have the potential to switch to become fast growing, clinically apparent, and lethal. These microscopic cancerous lesions are observed as: one of the earliest stages in tumor development; as micro-metastasis in distant organs; and as minimal residual disease left after surgical removal or treatment of primary tumors [10, 12–15]. Indeed, the mortality of cancer patients is largely determined by the occurrence of metastases, which often are too small to be detected but eventually can lead to relapse [12, 16–19].

Although the concept of dormant tumors is more accepted and better studied when occurring as minimal residual disease, or as micro-metastases which remain

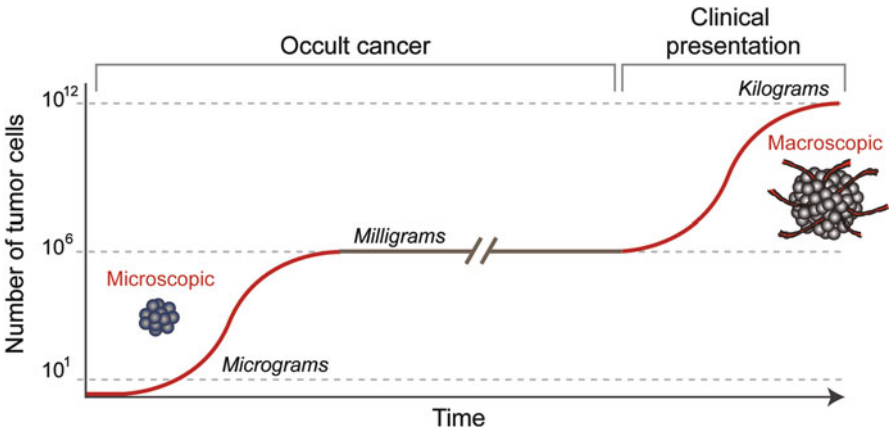

Fig. 1.1 Tumors often remain microscopic and clinically undetectable over long periods of time. This schematic of tumor growth can represent growth of primary or secondary cancers (metastases). Dormant micro-tumors can persist at a small steady size for years, remaining undetectable by commonly used imaging techniques

asymptomatic for decades, tumor dormancy is likely at least as prevalent as a stage of primary tumors. Microscopic and occult cancerous lesions are often found in otherwise healthy people [6, 20]. This implies that many people carry small and occult cancerous lesions without knowing it and that it is more common than frequently recognized.

In recent years, the field of tumor dormancy has been gaining significant attention: A number of reviews and essays on the topic have been published in leading journals such as *Nature* [6], *Nature Reviews Cancer* [2, 3], as well as *Nature Reviews Clinical Oncology* [4] and *Nature Medicine* [15], along with a specially dedicated issue of *APMIS* journal published in 2008. Moreover, dormancy has been the topic of dedicated sessions in several prominent cancer research conferences, and is currently a topic of considerable interest to NCI. Although an improved understanding of the manner by which tumors are induced to remain dormant would have important implications for cancer treatment and screening, and despite increased interest in the research community, to date, the dormant phase of tumor growth remains largely unexplored as a point of therapeutic intervention. The vast majority of cancer research is done on fast-growing and easily detectable tumors, which are more accessible for studies, such as signaling pathways investigations, drug response examinations, and biomarker analyses.

It is now clear that a number of biological processes can contribute to tumor dormancy, and they all support the role of the microenvironment, tumor stroma, and host response. These include tumor cell senescence, immune response of the host, hormonal control or block or insufficiency of tumor angiogenesis potential [1, 2, 5, 8, 17, 21–26]. Indeed, only in the last few years it has been fully appreciated that the tumor constitutes a highly integrated ecosystem in which different cellular populations depend upon each other. Clearly, dormancy of cancerous lesions depends on crucial signals from the microenvironment and the tumor stroma [27–29]. It is still to be determined, however, whether tumors attain dormancy through mechanisms involving extrinsic interactions (e.g., with the microenvironment) or from intrinsic properties of the cells.

Dormant Primary Tumors

The phenomenon of tumor dormancy can well explain the clinical phenomenon of minimal residual disease left after an apparent successful treatment of cancer and the very late relapses often seen in cancer. For example, it is well known that it can take years to decades following breast cancer treatment before local or distant recurrence becomes clinically detectable. The frequent recurrence of breast cancer strongly suggests that cancer seeds are left at the site of primary tumor growth or shed and seed in distant sites as dormant lesions. These lesions could eventually emerge from dormancy erupting into fast-growing tumors [17]. Moreover, patients can present with a metastasis, yet have “unknown primary tumors” which cannot be located [30].

Studying dormancy of *primary* tumors in clinical settings is extremely challenging. Evidence for the existence of primary micro-tumors in clinically healthy individuals comes primarily from histological studies that report a high prevalence of micro-tumors, even in young children (for a review, see [20]). However, even when such micro-tumors are detected in retrospective autopsy studies, it cannot be determined how long the lesions were present and whether they would remain occult or continue growing.

A feasible way to prove the existence of and to study the prevalence of primary dormant *microscopic* tumors is by comparing the frequency of micro-tumors found at autopsies with the frequency of detectable *macroscopic* tumors. When analyzing such data it is important to make sure that early precancerous lesions are excluded. It is also crucial to determine the prevalence of proliferating cells in the tumor, and exclude cases of very slow growing micro-tumors which have no or very few proliferating cells [31, 32]. While such studies are rare, many reports document the high prevalence of micro-tumors in retrospective autopsy studies.

One of the most striking observations of a high incidence of occult tumors was found in a systemic autopsy study of carcinoma of the thyroid. Although the frequency of detected occult papillary carcinoma in this study was 35.6% (at least one papillary carcinoma was found in 36% of the thyroids examined), the *estimated* frequency of such tumors, based on size and the sampling methodology, was over 100% (suggesting there could be more than one carcinoma in each thyroid examined) [33, 34]. Interestingly, such micro-tumors were found not only in older adults, but also in individuals younger than 40 years old [35]. Since clinically apparent thyroid cancer is found in less than 1% of the population [36], it can be concluded that the vast majority of micro papillary thyroid cancers remain occult. Similarly, it is estimated that over 33% of women aged 40–50 years old have clinically undetectable breast cancer [34], although only 1% or less of the population develops clinically detectable breast cancer [36, 37]. It is statistically evident therefore, that a large proportion of such micro-tumors *never* develop into clinical disease. In fact, tumor dormancy can last for a lifetime [5, 6].

Experimental In Vivo Models of Human Tumor Dormancy: How Can You Measure what You can't See?

Although tumor dormancy has been recognized as a clinical problem for many years, very few examples of spontaneous tumor dormancy have been documented in experimental animal models [20]. However, tumor dormancy in experimental animal models may be a frequent occurrence that goes unrecognized. The fact that it is not commonly seen in the laboratory is most probably due to the fact that the majority of researchers select for rapid and consistent tumor growth.

To better understand the pathogenesis and underlying regulatory mechanisms of dormancy in human tumors, we previously established in vivo xenograft models of human tumor dormancy that include breast cancer, glioblastoma, osteosarcoma, and

liposarcoma. In these models, human tumor cell lines are injected into immunocompromised (SCID) mice and form microscopic dormant tumors. We showed that in these models, tumor dormancy is associated with impaired angiogenic potential. To date, these are the only available in vivo models we are aware of, in which human tumor cell lines derived from malignant cancers form dormant and occult tumors when injected into mice and then spontaneously emerge from dormancy into rapid growth. All of these models were generated from commercially available human tumor cell lines and did not include any artificial genetic modification. These models were generated using two discrete approaches, developed in the Folkman laboratory. Both approaches are based on recognizing the heterogeneity of tumor cell populations in fully malignant tumors.

Achilles et al. described the first approach in 2001 [38]. The angiogenic heterogeneity in a human liposarcoma was studied by the generation of single-cell derived clones from a liposarcoma cell line (Fig. 1.2). While this parental liposarcoma cell line, as well as a majority of the single-cell derived clones, generated fast-growing and highly angiogenic tumors when injected into mice, other clones generated non-angiogenic microscopic tumors that did not grow and instead remained occult over 100 days after cell inoculation. This was the first direct proof that an angiogenic tumor can contain subpopulations of tumor cells with little or no angiogenic activity. These cells, when expanded in culture and injected into mice, will form small, avascular tumors at the site of injection. The angiogenic capacity of tumor cells can be therefore, correlated with the growth rate of the tumors they can generate.

This work implies that non-angiogenic tumor cells can "hitchhike" in tumors that contain angiogenic cells and suggests that the growth rate of a tumor will rely on the total angiogenic output of all the tumor cell subpopulations. This is consistent with the "hot spots" often observed in histological analysis of tumor vascular density [39].

Two of the single-cell derived clones that were generated in the Achilles studies were used for our studies: Clone 9 which generates fast-growing liposarcomas and Clone 4 which generates dormant, non-angiogenic liposarcomas (Fig. 1.3). Although we have used only two of these clones, this method of isolating cells that form dormant tumors is applicable to other tumor types and tumor cell lines (data not shown). However, biochemical markers of tumor dormancy could make this approach much easier and cost-effective.

The second model approach was developed from the observation that many human tumor cell lines do not "take," or do not form aggressive tumors, when injected into immune-deficient mice, coupled with Dr. Folkman's hypothesis that such cell lines might actually "take" and generate dormant tumors that remain microscopic and occult for long periods of time. Indeed, a number of such cell lines were shown to form microscopic and avascular tumors at site of injection [30]. Some of the dormant tumors generated by this means eventually switch and "escape" from dormancy to form aggressive tumors (Fig. 1.2b).

For our studies, three human cell lines from different tumor types were chosen based on their "no-take phenotype." These include breast carcinoma, glioblastoma, and osteosarcoma. Similar to the dormant clones of the liposarcoma, when injected into SCID mice, these cell lines generated microscopic tumors that remained occult

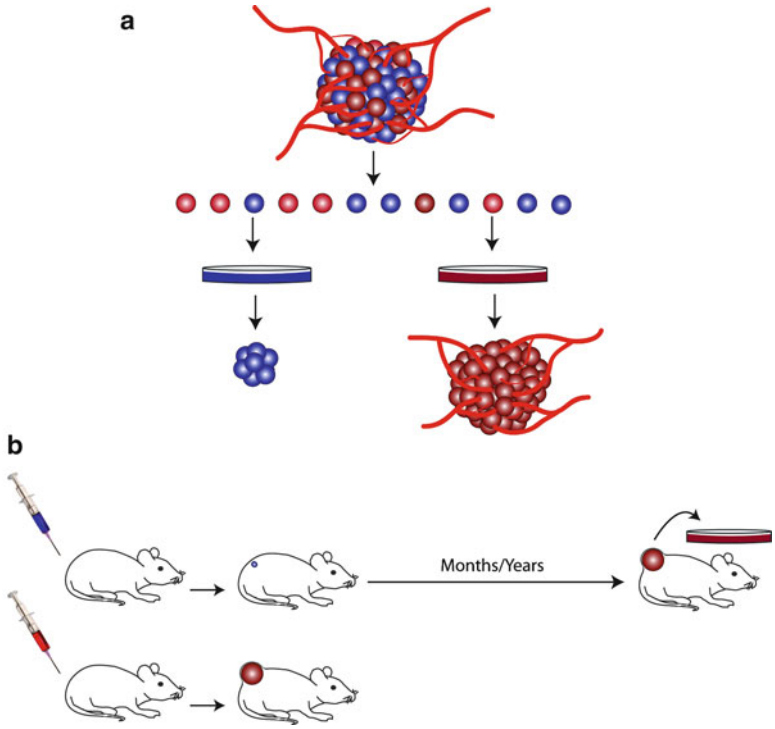

Fig. 1.2 Summary of the two approaches to generate pairs of cell lines that form either dormant or fast-growing tumors. (**a**) Isolation of cellular subpopulations that form dormant or fast-growing tumors. Single-cell derived clones are prepared from a heterogeneous cancer cell population (such as a human tumor cell line) that forms fast-growing tumors (shown in figure as a heterogeneous tumor cell population of *red* and *blue cells*). Tumors generated from these different clones have a spectrum of growth rates. While majority of the single-cell derived clones will generate fast-growing tumors (*red* tumor cells in figure), a percentage of such clones will generate dormant, microscopic tumors (*blue* tumor cells in figure). Screening for clones that form either dormant or fast-growing tumors requires in vivo tumor growth monitoring. (**b**) Isolation of cells from tumors that had spontaneously escaped from dormancy. Human tumor cell lines that are known to have a "no take" phenotype are injected into mice (*blue* tumor cell suspension in figure). These cells form microscopic tumors that remain occult for long periods of time until some of them spontaneously switch to rapid growth (*red* tumor in figure). These tumors that escaped dormancy are used to generate new tumor cell lines (*red* tumor cell in culture dish in figure). Cell lines from tumors that switched (*red* tumor cell suspension in figure) form fast-growing tumors immediately after injection into new mice

for over 100 days [40]. Following prolonged periods of dormancy, however, some of the tumors spontaneously emerged from dormancy and formed fast-growing and aggressive tumors at the site of injection. Cells from these "switched" tumors were cultured, confirmed to be of a human origin and maintained as clones in tissue culture conditions. When these cells, cultured from dormant tumors that had switched to fast growing were re-injected into mice, fast-growing tumors were observed soon after tumor cell injection. This implies that cells from tumors that emerged from

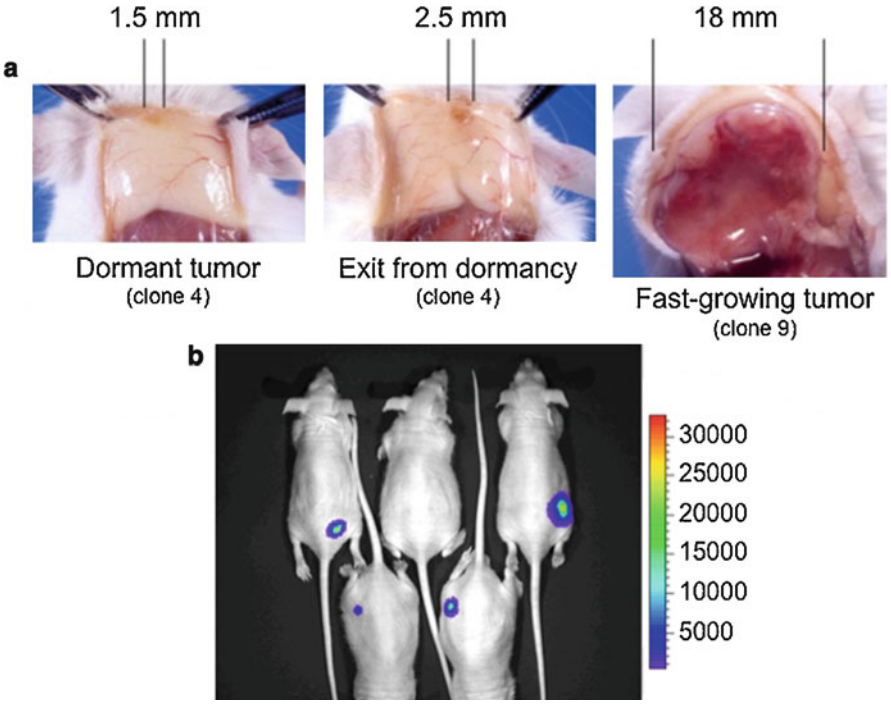

Fig. 1.3 Representative images of human liposarcoma grown in mice. (**a**) 37 days after subcutaneous injection of human SW872 liposarcoma Clone 4 cells into SCID mice, small tumors can be detected only after flipping the skin. Rarely, a more vascularized tumor with a diameter over 2 mm can be observed. Such tumors might be during the initiation of the "switch" from dormancy. In sharp contrast, at that same time point, tumors generated from Clone 9 of the human SW872 liposarcoma are considerably larger, easily detected by gross examination, and highly vascularized. (**b**) Persistence of dormant tumors generated from Clone 4 of the human SW872 liposarcoma can be detected by bioluminescence. Luminescence from tumor cells (that were labeled with luciferase before injection) indicates the presence of viable and metabolically active cells at the site of injection. 80 days after subcutaneous injection, the tumors can be detected by bioluminescence although they are not detected by gross examination

dormancy had acquired *stable intrinsic changes* that confer the tumor growth ability beyond the limiting diameter of a few millimeters.

For each tumor type (glioblastoma, osteosarcoma, and breast carcinoma), we currently have a pair of clones: One clone that generates dormant tumors (the parental cell line) and one clone that generates fast-growing tumors (established from the tumors that escaped from dormancy). Together with the pair of dormant and fast-growing liposarcoma (described above), we have a panel of pairs of cell lines from four different tumor types that each share a common genetic background but differ in their in vivo tumor growth patterns. In all these tumor models, the dormant tumors remain occult at the site of injection for prolonged periods of time until they eventually switch to rapid growth. Once these tumors pass the dormancy threshold, they

grow at kinetics similar to the fast-growing and angiogenic tumors. On the other hand, the fast-growing tumors initiate rapid mass expansion soon after the tumor cell injection and grow exponentially. The same pattern of tumor dormancy or fast tumor growth is seen both in subcutaneous and orthotopic injection sites of our breast cancer, liposarcoma, and glioblastoma models.

Importantly, the observation that tumors remain microscopic until they switch and then grow at a pace similar to fast-growing tumors strongly supports the assumption that the growth of the microscopic tumors is restricted by thresholds or bottlenecks. Only when tumors are able to surpass these, can they expand in size. This is in sharp contrast to tumors that simply have a very slow pace of growth.

Moreover, our experimental models allow us to address a fundamental question in tumor dormancy: Do the elements necessary for the induction of dormancy originate within the host (e.g., tumor microenvironment) or within the tumor cells? Both the dormant and fast-growing tumors are injected at the same sites and are grown in identical "stromal" conditions, yet the dormant tumors will remain microscopic, while the fast-growing ones quickly become macroscopic. This strongly suggests that intrinsic changes in the *tumor cells* are the basis for the differential growth patterns of the tumors. It is also clear that intense intercellular communication with the tumor stroma plays a critical role in dormancy regulation. However, it seems that the signals dictating stromal behavior originate in the tumor cells. Importantly, the selection for cells that "switched" from the dormancy period is evident only in vivo, since prolonged growth of the tumor cells in culture does not affect the growth kinetics of the tumors generated from them (Almog, unpublished). Clearly, the selection for cells that can generate fast-growing tumors is derived from microenvironment pressure and signaling communication with the host.

The unique advantage of the experimental system we developed is the unlimited source of cells that will form dormant tumors (which are otherwise rarely obtained from in vivo tumors), together with counterpart tumor cells that will form fast-growing tumors, both derived from the same parental tumor cell population. This enables detailed and extensive molecular and cellular analyses. Indeed, we are currently using these models to study common pathways that are uniquely expressed in dormant tumors of various tumor types. Understanding the underlying mechanisms of tumor dormancy could have significant implications in the prevention and treatment of cancer: The human tumor cell lines that form dormant tumors in mice can be used not only as models for dormant primary tumors, but also as possible models for the clinical observations of very late cancer recurrences.

Angiogenesis Regulation of Tumor Dormancy

Dr. Judah Folkman was the first to suggest the fundamental relationship between angiogenic potential and the ability of a tumor to grow malignantly, and that dormancy can be associated with lack of angiogenesis [22, 41]. By now, it is well established that tumor growth beyond the size of 1–2 mm is angiogenesis-dependent

[42–45], and several experimental models of angiogenesis-related dormancy have been reported. Evidence of this was first observed using tumor implants in rabbit eyes [22] in which the same tumor remained dormant and avascular when implanted in the anterior chamber, but grew progressively when implanted in the iris. Holmgren et al. described another example of spontaneous dormancy. They observed micrometastases that remained occult as a result of systemic inhibition of angiogenesis mediated by the primary tumor [23].

In a spontaneous tumor model (RIP1-Tag2) in transgenic mice, tumors arise in the pancreatic islets as a result of the expression of the simian virus 40T antigen (Tag) oncogene. After 13 weeks, only 4% of tumors are angiogenic and contain evidence of neovascularization, whereas the remaining 96% stay microscopic and nonangiogenic. The spontaneous progression of nonangiogenic lesions to the angiogenic phenotype in this model was termed the *angiogenic switch* [46]. Although this name implies a short-acting "on-off" switch, the transition of a non-angiogenic avascular cancerous lesion to a highly angiogenic and fast-growing tumor encompasses a series of steps [47]. The successful culmination of this continuously productive process is the development of fully functional (although possibly abnormal) vessels capable of sustaining sufficient blood flow to support tumor mass expansion.

In our experimental models, tumor dormancy is clearly associated with impaired angiogenic potential. While no major cellular differences can be observed in vitro between cell lines that form dormant or fast-growing tumors, including morphology, proliferation, migration, and colony formation in soft agar, the tumor growth patterns in vivo are strikingly different [40, 49]. Similar to dormant tumors generated from other cell lines [30], dormant tumors generated from all of our models (SW872 liposarcoma, MDA-MB-436 breast carcinoma, T98G glioblastoma, and KHOS-24 osteosarcoma) have a high prevalence of proliferating cells. Tumor mass does not expand due to the high rate of apoptosis of tumor cells, which balances their proliferative capacity.

Noticeably, in contrast to fast-growing tumors that are highly vascularized, dormant tumors are mostly avascular. In most cases, vasculature can be observed only on the periphery of dormant tumors. In immunohistochemistry analysis and staining of endothelial cells, large vessels with open lumens are frequently seen in fast-growing tumors, while in dormant tumors, rare aggregates of endothelial cells are observed [40, 49]. In a detailed examination of tumor vasculature in liposarcomas by confocal analysis, a typical tumor vasculature comprised of interconnected and tortuous vessels is observed in the fast-growing tumors, whereas the vessels observed on the periphery of dormant tumors appear as nonfunctional tubes with aberrant morphology and many blunt ends [1].

Furthermore, when the relative area of endothelial cells in dormant tumors was followed over time, a decrease in microvessel density (MVD) was observed between days 14 and 60 after cell injection. A sharp increase in MVD was associated with the transition of tumors from dormancy to rapid growth and mass expansion [49]. This suggests that tumor dormancy is associated not just with impaired angiogenic capacity, but also with *inhibition* of angiogenesis. The inhibition is terminated following the induction of the angiogenic switch.

A significant and consistent difference between cells that form dormant tumors and those that form fast-growing tumors in our models is the secretion of the angiogenic inhibitor, thrombospondin-1 (TSP-1) [40, 49]. When in vitro secretion of pro- and anti-angiogenic factors from cells that form dormant tumors was compared with those that form fast-growing tumors, the dormant tumor-forming cells, regardless of tumor type, secreted relatively high levels of TSP-1. Other angiognesis inhibitors might also play a role in dormancy regulation, but these have yet to be determined.

It should be noted that once these dormant tumors undergo the angiogenic switch and initiate growth and expansion of mass, the tumor growth kinetics are similar to those of their paired rapidly growing angiogenic tumors. This further supports the concept that the fundamental mechanism underlying tumor dormancy in these models is impaired angiogenic capacity, rather than a decreased proliferation rate.

In summary, in our experimental models, blockage of tumor progression and persistence of micro-tumors is associated with the inability of the tumor cells to sustain the induction of functional new capillary blood vessels. This implies that not only the onset, but also the extent of angiogenesis is a critical determinant of tumor progression and growth.

Molecular Signature of Tumor Dormancy

The fact that tumor cells undergo genetic alterations during the switch from dormancy to rapid growth prompted us to identify the genetic profiles of indolent tumors. For this purpose, we utilized our experimental model of paired dormant and fast-growing tumors originating from the same parental cell lines. We ran genome-wide expression profiling assays to determine the consensus signature across our human tumor dormancy models.

We identified several genes that were differentially expressed between our dormant and fast-growing tumors, regardless of tumor type, and characterized common tumor dormancy-associated genes [50]. Around 700 genes were significantly differentially regulated in the same pattern (either induced or suppressed) in all four dormant and fast-growing tumor models examined. A number of these dormancy-associated genes had previously been shown to be involved, or associated with, tumor angiogenesis and tumor progression.

The molecular process most differentially expressed between dormant and fast-growing tumors was the *regulation of angiogenesis*. *Thrombospondin*, a known angiogenesis inhibitor [43], *angiomotin*, a mediator of the angiogenesis inhibitor angiostatin [51], and *tropomyosin*, a suggested mediator of the anti-angiogenic activity of endostatin [52], were shown to be upregulated in all of the dormant tumor cells examined. Dormant tumors also expressed TGFbeta2, which was previously shown to inhibit FGF-2-induced corneal endothelial cell proliferation [53] and to modulate extracellular matrix component expression [54]. In addition, dormant tumors induced the expression of proline-4-hydroxylase, which was previously shown to upregulate levels of several angiogenesis inhibitors [55]. Interestingly,