

PATHOBIOLOGY IN FOCUS

Patient-derived xenografts, the cancer stem cell paradigm, and cancer pathobiology in the 21st century

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Cancer is a heterogeneous disease manifest in many forms. Tumor histopathology can differ significantly among patients and cellular heterogeneity within tumors is common. A primary goal of cancer biologists is to better understand tumorigenesis and cancer progression; however, the complex nature of tumors has posed a substantial challenge to unlocking cancer's secrets. The cancer stem cell (CSC) paradigm for the pathobiology of solid tumors appropriately acknowledges phenotypic and functional tumor cell heterogeneity observed in solid tumors and accounts for the disconnect between drug approval based on response and the general inability of approved therapies to meaningfully impact survival due to their failure to eradicate these most important of cellular targets. First proposed to exist decades ago, CSC have only recently begun to be precisely identified due to technical advancements that facilitate identification, isolation, and interrogation of distinct tumor cell subpopulations with differing ability to form and perpetuate tumors. Precise identification of CSC populations and the complete hierarchy of cells within solid tumors will facilitate more accurate characterization of patient subtypes and ultimately contribute to more personalized and effective therapies. Rapid advancement in the understanding of tumor biology as it exists in patients requires cooperation among institutions, surgeons, pathologists, cancer biologists and patients alike, primarily because this translational research is best done with patient-derived tissue grown in the xenograft setting as patient-derived xenografts. This review calls for a broader change in the approaches taken to study cancer pathobiology, highlights what implications the CSC paradigm has for pathologists and cancer biologists alike, and calls for greater collaboration between institutions, physicians and scientists in order to more rapidly advance our collective understanding of cancer.

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INTRODUCTION

Despite significant time, money, and effort expended on cancer research over the last half century, the contribution of newly developed therapeutics to improved patient survival has been limited (Table 1).^{1–3} Treatment options for patients with many solid tumors remains unchanged vs therapies offered decades ago. Furthermore, improvements to overall survival have resulted from successes associated with early detection and cancer prevention initiatives.^{4,5} Technological innovations such as genetic testing (eg, characterizing BRCA1 and BRCA2 mutations), biomarker detection (eg, PSA), and tissue monitoring (eg, colonoscopy) have produced significant improvements in the prevention and early detection of breast, prostate, and colorectal cancers, respectively, often permitting preventative or curative localized resection at early stages of disease. The reduced incidence of smoking has also significantly contributed to the reduced prevalence

of lung cancer, and consequently decreased the number of deaths in the United States as a result of this and other smoking-related malignancies (though the death rate for women with lung cancer rose 6% between 1990–2008 and 2012).^{3,6} Notwithstanding some successes, overall cancer mortality continues to rise and is estimated to nearly double by 2030 if better early detection technologies and therapies that significantly impact survival are not discovered and developed.³

The vast majority of cancer patients continue to be treated with untargeted chemotherapeutic agents and/or radiation following surgical resection as primary, and often secondary, courses of action. For many indications, these standard of care chemotherapy and/or radiation treatments have changed little over the past several decades.^{1,2,7–9} Although these interventions often result in substantial tumor regression, chemo- and radiotherapy regimens are poorly tolerated and

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Table 1 Five-year survival statistics

	% 5-Year survival			% 5-Year survival with distant disease			US deaths 2012
	'75-'77	'95-'00	'01-'07	'75-'77	'95-'00	'01-'07	
Lung	12	15	16	ND	2	4	161 000
Colorectal	51	63	65	ND	10	12	52 000
Breast	75	88	90	ND	26	23	39 000
Pancreatic	2	4	6	ND	2	2	38 000
Prostate	68	99	100	ND	33	29	28 000
Liver	3	8	15	ND	3	4	21 000
Ovarian	26	44	44	ND	29	27	16 000

Abbreviation: ND, not determined.

generally result in the evolution of a more aggressive and refractory disease that emerges to cause death. As a result, cancer mortality rates remain at levels similar to what was observed three decades ago (Table 1; <http://seer.cancer.gov/data/citation.html>). The discovery and development of successful targeted therapies has proceeded at an agonizingly slow pace, possibly because cancer biologists have traditionally not had the correct tools, ample access to patient tumor specimens that provide insight into the full heterogeneity and diversity of human malignancies, nor employed appropriate experimental design paradigms to address tumor biology as it exists in patients. Technical and conceptual advancements for how cancer biologists study and think about cancer calls for a change that should result in greater success in the next several decades than what has been achieved over the last century.

ADDRESSING TUMOR HETEROGENEITY

Each cancer patient's tumor is heterogeneous and unique. Diagnosing patients based on histomorphological features and an abbreviated panel of immunohistochemistry markers provides little information that can be considered distinctive to any given patient. Although these tools have been successful at segregating tumors into broad subclasses, they have largely failed to capture or elucidate the enormous variation of disease within indications. For many malignancies, the existence of additional subtypes has been implied by the differential responses of patients to therapeutic regimens. For others, molecular markers, gene expression profiling, and the more recent implementation of next-generation DNA-sequencing technologies have helped reveal a broader spectrum of heterogeneity among patient tumors.¹⁰⁻¹⁴ In an age where next-generation whole-genome and transcriptome sequencing are not only possible, but increasingly feasible and rapid, diagnoses and treatment options will increasingly be tailored to each patient's tumor using information from these and other novel platforms.¹⁵⁻¹⁷ For example, genomic DNA sequencing efforts have already begun to identify

existing approved or experimental drugs likely to be efficacious in particular patient subgroups.^{15,18,19} Despite these instances, population studies have revealed that most tumors do not have an isolated driver mutation that can be targeted with an existing therapeutic agent.^{11,20,21} Consequently, few patients currently benefit from data obtained using novel platform technologies such as next-generation sequencing, and thus diagnostic and treatment option considerations are not widely influenced by this information (reviewed in previous studies^{18,22-24}). This should change as more knowledge is accumulated around how particular genomic mutations impact and alter specific signaling pathways that can be targeted by small molecule or biological inhibitors, or combinations thereof. Inherent levels of complexity need to be better understood for these next-generation platforms to have their utmost utility in realizing the promise of personalized medicine, and development of unique treatment regimens for individual patients may not be practical for some time. Nevertheless, most would agree that patient diagnosis and treatment considerations should take into account both an individual tumor's genetics and biology, determined as close to the initial time of diagnosis as possible.

As pathologists are all too well aware, malignant tumors consist of heterogeneous cellular compositions: an interconnected mass of dividing, differentiating and dying cells, supporting stromal cells, infiltrating vasculature and ensemble of hematopoietic cells. Since Rudolf Virchow first proposed that cancer was a cell-based disease in the mid-1850's,²⁵ the cellular complexity comprising neoplastic malignancies has been widely recognized and studied. Underlying complexities are now coming into focus. Specifically, three interrelated and increasingly important concepts in cancer biology are revolutionizing our understanding of tumor pathobiology and should lead to improved therapeutic options for cancer patients. First, patient-derived xenograft (PDX) tumor models are increasingly feasible and available.²⁶⁻²⁹ Second, evidence is amassing that supports the cancer stem cell (CSC) paradigm: a theoretical model for cancer that accounts for the importance of specific tumor cell subpopulations to a tumor's growth and potential for driving tumor recurrence (reviewed in previous studies³⁰⁻³²). Finally, next-generation DNA and RNA sequencing efforts (eg, The Cancer Genome Atlas) have demonstrated that very few common mutations unite particular solid tumor types; rather, patient tumors have a spectrum of mutations that collectively drive tumor growth.^{11,33,34}

In the face of this rapidly emerging and previously unappreciated complexity, it is not surprising that therapeutics developed using overly simplistic paradigms and models have been only modestly successful at impacting survival. The development of personalized medicine and its efficient implementation will likely leverage PDX models, the CSC paradigm and next-generation DNA and RNA sequencing to improve our collective understanding of tumor pathobiology,

beginning with the more precise identification of patient subtypes and the identity of CSC in each of these respective tumor subtypes. Critical to these analyses is the availability of tumor models that more accurately reflect patient tumor biology—and not just several crude models, but many that provide the resolution necessary to represent the diversity of cancer patients encountered in the clinic. As the field of cancer biology evolves to recognize and embrace the functional heterogeneity within tumors, so too must experimental paradigms evolve, and access to patient tumor specimens and interdisciplinary collaboration increase, so that meaningful progress can be made towards significantly impacting survival of cancer patients in the 21st Century.

TRADITIONAL MODELS HAVE NOT PREDICTED CLINICAL SUCCESS

More than 68 drugs with varying efficacy have been developed and approved for oncology over the last several decades,³⁵ yet the unfortunate paucity of success stories serves as a reminder that the current arsenal of therapies fail far too many patients. It is tempting to speculate that failure of current therapies stems from the limitations of the tissue culture model systems in which they were discovered, validated and/or evaluated for potency during preclinical development. In the late 1970's, colony-forming soft agar assays emerged as a means by which scientists attempted to study the nature and potential of human tumor stem cells.^{36,37} These *in vitro* colony-forming assays have facilitated insight into the differentiation potential and relationship of hematopoietic stem and progenitor cell populations in normal hematopoiesis and hematopoietic malignancies;³⁸ however, little practical insight has been garnered in solid tissues and tumors.

Traditional cell lines originally derived from patient tumors and adapted to proliferate in *in vitro* culture conditions have been widely established and studied for more than a half century.³⁹ These lines have served as a foundation for cancer research due to their ability to be easily propagated and studied under defined conditions.³⁹ Unfortunately, continuous passage and culture of cells *in vitro* tends to select for cells adapted to thrive in plastic dishes and eliminates variables introduced by tumor resident cell populations such as supporting non-tumor stroma, hematopoietic cells and other tumor microenvironmental factors such as extracellular matrix proteins. In conjunction with the harsh enzymatic manipulations and centrifugation conditions employed during the passage of traditional cell lines, *in vitro* culture conditions have selected for cellular subsets that flourish in their newfound laboratory setting, and have generally selected for populations that are phenotypically uniform—a gross departure from the natural tumor state.

Traditional cell lines are maintained in culture conditions that depart markedly from the natural setting of human tumors. Specifically, cell lines are cultured in nutrient-rich

media in high oxygen tension as plastic-adherent monolayers or in suspension with a lack of any attachment substrate. Not only are these cell lines typically clonal, but they are generally inefficient at initiating tumors when transplanted into compatible, immunocompromised hosts such as nude, SCID, NOD/SCID, or NOD/SCID/ γ^c -null (ie, NSG) mice.^{29,40} When tumors do arise, these masses are generally homogeneous in nature and do not reflect tumor biology as it exists in patients (eg, Figures 1a and b). The expansion of cells in aphysiologic oxygen concentrations (ie, 21% O₂: ambient oxygen levels) has contributed to the perpetuation and study of cells that have accrued dozens, if not hundreds, of mutations and chromosomal abnormalities over the course of their many passages *in vitro*.⁴¹ Profound karyotypic dysmorphisms, including hypotriploid genotypes, are surprisingly common within the cell lines that have come to define the standard models studied by many cancer biologists, such as the NCI60 cell lines (also see <http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi>). Recent studies have demonstrated that brief periods of *in vitro* culture irreversibly change gene expression, indicating that even low-passage cell lines may be compromised.^{40,42} Collectively, these observations call into question the physiological relevance of traditional cell lines as sufficient, let alone relevant, models for studying tumor biology as it exists in cancer patients.

Despite the increasingly apparent limitations of traditional cell lines, they have made significant contributions to our understanding of tumor biology. The uniformity and control over experimental conditions afforded by *in vitro* cell culture has assisted the development and execution of highly reproducible studies under defined conditions that enable insight into drug sensitivity, basic cell biology, and the elucidation of signaling pathways. For example, *in vitro* cell culture has facilitated the development of high-throughput approaches leading to the discovery of second and third generation anti-neoplastic agents (see DeVita and Chu⁴³ for review). The flexibility and accessibility of traditional cell lines perpetuated *in vitro* has also enabled key mechanistic studies to shed light on the contribution of specific genes and mutations to cell survival, proliferation, and migration, contributing to the development of, for example, kinase-specific inhibitors such as erlotinib, vemurafenib, and crizotinib targeting EGFR, BRAF, and ALK/c-MET, respectively (reviewed by Sawyers⁴⁴). Although initially promising, these tailored kinase inhibitors have largely failed to be curative and typically extend life only 3–6 months.^{15,44} Although chemotherapeutic compounds have also proven highly effective *in vitro*, in traditional *in vivo* tumor models and often in the clinic, the untargeted nature of these drugs results in significant toxicity, and consequently, a narrow therapeutic window in cancer patients. Moreover, residual tumors generally recur as more aggressive, refractory, and lethal; likely due to the inability of chemotherapy to eliminate CSC, and resulting in additional mutations accumulated in these cells during exposure to genotoxic drug regimens.⁴⁵

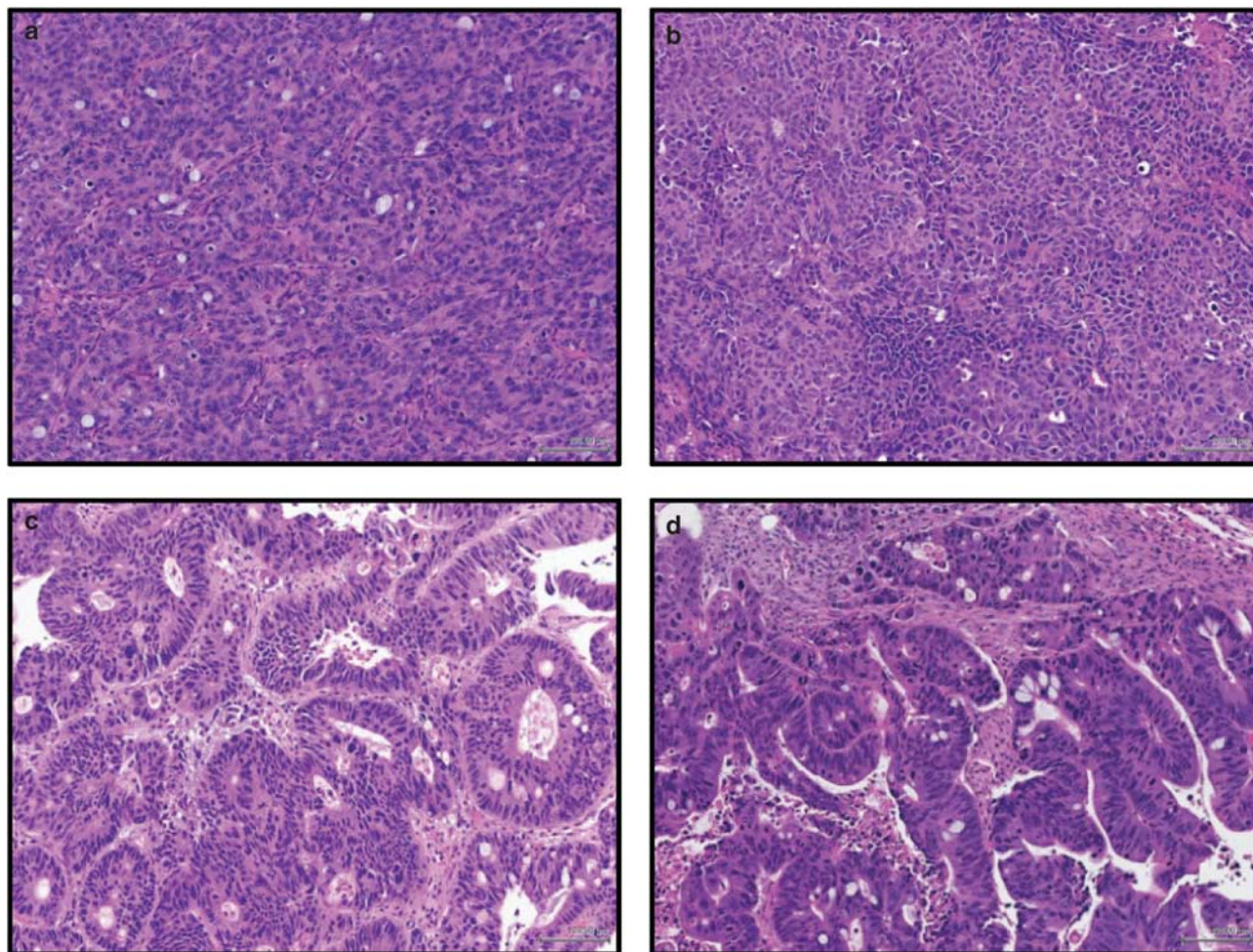


Figure 1 Patient-derived xenograft (PDX) models of colorectal cancer recapitulate primary tumor heterogeneity. Hematoxylin and eosin (H&E) stained FFPE slides of xenografts generated by traditional HT-29 (a) or SW480 (b) colorectal cancer cell lines, vs a primary colorectal tumor, SCRXPDX-CR101-p0 (c), and the same patient's PDX tumor following passing through NOD/SCID mice, SCRXPDX-CR101-p1 (d). Note the relative uniformity of the HT-29 and SW480 tumors relative to the primary or PDX tumor following minimal passing in immunocompromised mice.

Genetically engineered mouse models (GEMMs) are also popular models through which tumor biology is studied. Unfortunately, these tumor models also have their intrinsic shortcomings. It is becoming increasingly clear that more than one driver mutation is needed to initiate tumorigenesis—both in mice and in men.^{20,34} Efficient tumorigenesis in GEMMs must often be driven by the introduction of at least two defined oncogenes and/or mutated tumor suppressors, depending on the mouse model and/or models needed to be crossed to drive tumorigenesis.^{46,47} Generating GEMMs with more than one driver mutation is extremely time-consuming and difficult. Another weakness of these models is that transgene expression (eg, mutated KRAS) is commonly driven to superphysiological levels, such that protein expression often exceeds that ever encountered in patients.⁴⁶ Moreover, when tumors do arise in these models, they are sporadic in their growth such that it is difficult to design studies powered

by a significant number of animals, while also being difficult to monitor without labor-intensive *in vivo* imaging technologies. As previously discussed, the Cancer Genome Atlas project has demonstrated that the spectrum of driver mutations differs significantly among patients, thus the relevance of particular GEMMs to patients in the clinic is increasingly questionable.^{33,34} Notwithstanding the above criticisms, GEMMs may have advantages over xenograft models in their cellular composition and/or sensitivity to therapeutic agents.⁴⁸ Specifically, GEMM tumors contain stromal and hematopoietic components not possible in a human tumor xenograft setting, and thus these tumors may respond more appropriately to small molecules, immunomodulatory, and/or biological agents that cross-react with mouse antigens. Nevertheless, for the various reasons outlined above, GEMMs will likely provide limited insight into oncogenesis and patient tumor heterogeneity.

Although traditional cell lines have helped gain an understanding of the basic biology underlying specific genes, proteins, and/or signaling pathways, cell lines cultured *in vitro* have not significantly contributed to the discovery of targets for directed therapies that have meaningfully impacted patient survival. Trastuzumab and T-DM1 (anti-Her2 biologics) and Imatinib/Gleevec (a BCR/ABL kinase inhibitor) are prime examples of cancer drugs that have meaningfully impacted patient survival; however, neither was discovered in traditional cell lines or GEMMs.^{49,50} Her2/Neu (ErbB2) was originally identified as a potentially interesting cancer target based on the observation of elevated expression in tumor specimens obtained and preserved by pathologists.^{51,52} Moreover, the p210-BCR/ABL fusion protein was identified and associated with chronic myelogenous leukemia (CML) upon close examination of chromosomal architecture using blood smears from CML patients.^{53,54} Traditional cell lines and GEMMs effectively complemented these discoveries, but were most effectively utilized as platforms to precisely dissect the molecular and cellular biology of ErbB2 and p210-BCR/ABL, respectively. To understand tumorigenesis and patient heterogeneity observed in the clinic, cancer research, drug discovery, and development must increasingly look beyond *in vitro* tissue culture models and GEMMs and towards experimental systems that better replicate human tumor biology and enable the study of many patient tumors as they are likely to exist in cancer patients.

PDXs: MODELS FOR THE 21ST CENTURY

Immunocompromised mouse strains have become more widely available over the last 20 years and permit the engraftment, passage, and study of human tumor cells *in vivo* in a xenograft setting.^{26,27,55–57} As a natural extension of standard tissue culture techniques, some researchers have taken to the xenotransplantation of traditional tumor cell lines that have been passaged extensively *in vitro*. These models have empowered remarkable advances in the understanding of angiogenesis and tumor cell invasion, resulting in the development of therapeutic agents such as bevacizumab and sorafenib;^{58,59} however, promising preclinical data obtained using these traditional cell line-initiated xenografts has not translated into dramatic improvements in overall survival in most cancer patients.⁶⁰

To better preserve the genomic integrity and tumor heterogeneity observed in patients, many researchers are increasingly turning to PDX models generated using freshly resected patient tumors immediately transplanted into immunocompromised murine hosts without an intermediate *in vitro* culture step.^{26,28,42} Serial passage and expansion of tumors through successive generations of murine hosts without intervening cell culture permits ongoing propagation of tumor lines and the study of tumor biology without subjecting PDX tumor cells to the stressful and compromising conditions encountered *in vitro*.⁴² Currently, subcutaneous PDX models, wherein tumor cells are transplanted

underneath the skin of the hindquarters or in the mammary fat pad dominate the field of primary xenografts, succeeding intra-ocular, embryonic, and athymic systems.^{29,61} Many groups also transplant cells under the kidney capsule or orthotopically; the latter of which may better replicate the tumor microenvironment than subcutaneous models, and thus may be most physiologically relevant. Regardless of the transplantation site, the cellular complexity and architecture of PDX tumor models remains remarkably faithful to the tumor in its natural state in most cases—complete with invading vasculature and supporting stromal cells (Figure 1d). Tumor growth in the xenograft setting ensures that tumor cells are exposed to physiologically relevant oxygen, nutrient, and hormone levels (in cases where there is interspecies cross-reactivity), and provides natural physical substrates for tumor cell adhesion. In contrast to *in vitro* propagated cultures, cytogenetic analyses of PDX models reveals strong preservation of the chromosomal architecture observed in patient tumors.^{28,62} PDX models of melanoma, breast, pancreatic, ovarian, lung, colorectal, and brain-derived tumors have been successfully established in many laboratories (reviewed by Tentler *et al*²⁹), and many have proven to exhibit similar chemoresponsiveness to anti-neoplastic agents as observed in the same donating patient in the clinic—underscoring the fidelity of these models to the natural disease state^{30,63} (unpublished results).

PDXs faithfully recapitulate much of a tumor's biology, yet these models also have their shortcomings. The engraftment frequency and growth rate of implanted tumors is highly variable by tumor type and subtype, suggesting that some tumors struggle to engraft for reasons that might include a dependence on hematopoietic cells and/or microenvironmental cues not present in mouse stroma or not compatible with human cells. Other factors that likely contribute to inefficient tumor initiation as xenografts include the length of time that passes between when the tumor is resected and the time of transplantation, the absence of an appropriate support matrix and/or growth factors, or an inhospitable site of implantation. Moreover, human tumor stroma and infiltrating lymphocytes are often lost in the initial passages in mice if xenograft tumors were initiated with tumor fragments containing these human cell populations. The extent to which tumor cells from freshly resected tumors are able to withstand mechanical stress and xenotransplantation barriers is also unclear. For example, breast cancer PDX models appear to be particularly difficult to establish, with a 27% engraftment rate in the most successful laboratories, compared with ovarian (65%), lung (50%), melanoma (59%), and colorectal (68%) cancer.^{51,61} Mounting evidence suggests that the exact mouse strain (eg, NOD/SCID vs NSG) does not significantly impact engraftment efficiency of most solid tumor types, but strain differences can impact the rate at which tumors arise.^{29,64,65}

PDX tumor models can be propagated as either discrete tumor fragments or as single-cell suspensions. Whereas the

former has the advantage of retaining cell–cell interactions and some tissue architecture during transplantation, the latter permits single cell-by-cell assessment of phenotype, the interrogation of differential tumorigenicity by isolated tumor cell subpopulations, and an unbiased sampling of the entire tumor, ensuring that spatially segregated subclones are not inadvertently selected during analysis or passaging. Nevertheless the generation of single-cell suspensions from PDX tumors presents unique challenges. Cells in solid tumors naturally attach to neighboring cells and the extracellular matrix, thus requiring tissue and cell disaggregation to generate single-cell suspensions in order to be analyzed individually. Physical and enzymatic dissociation techniques required to generate single-cell suspensions can be harsh, and cells that survive the process are often sensitized to detachment-induced apoptosis (ie, anoikis).⁶⁶ In addition, some cell surface molecules are sensitive to dissociating enzymes such that post-dissociation antigen-staining profiles can be distorted from their natural state in the context of tumors, thus highlighting the importance of keeping tumor dissociation times short and remaining cognizant of the impact of particular enzymatic cocktails and dissociation conditions on antigen expression. It is also critically important to discriminate the live singlet population from cell aggregates and dead cells when analyzing or isolating cells by flow cytometry and fluorescence-activated cell sorting (FACS) to ensure that analyzed, isolated and/or transplanted tumor cell subpopulations comprise single viable cells.⁶⁷ The requirement for single cells is critically important when testing the tumorigenicity of one tumor cell subpopulation vs another, as it is of utmost importance in protecting against artifacts associated with cell clumps and impurities that can mire the interpretation of experimental results.

PDX tumor models arguably provide the most reproducible approximation of tumors in human cancer patients. Information gathered using these models more accurately reflect human tumor biology than other existing models, and thus time and money spent studying and understanding these, and comparing their pathophysiology to patient tumors should yield greater insight into cancer pathobiology. Nevertheless, these models are not perfect and efforts to address PDX tumor model deficiencies are ongoing. For example, transplantation of human CD34⁺ cord blood cells enriched for human hematopoietic stem and progenitor cells can spur the generation of a human innate and adaptive immune system in mice⁶⁸—offering the promise of performing xenotransplantation experiments in mice with a reconstituted human immune system. The combination of PDX tumor engraftment in these humanized mouse models may facilitate insight into the role of the immune system in tumor biology; however, these models will be difficult to employ broadly owing to the difficulty in efficiently generating these models and need to HLA-match xenografted tumors with the human immune cell component such that graft-vs-tumor, let alone graft-vs-host, responses don't

complicate the interplay between the human immune system, tumor, and mouse microenvironment.

Perhaps the most substantial barrier to PDX tumor model establishment and their widespread utilization for research is time and cost. Unlike traditional tissue culture, wherein experiments can be rapidly executed using relatively inexpensive supplies, PDX models involve propagation in expensive genetically engineered mice requiring equally expensive animal husbandry and facility costs. Experiments must be planned months in advance, as PDX tumors can often take up to 24 weeks to arise after transplantation—presenting long experimental cycles. Altogether, the total cost of a traditional cell culture-based project might represent a fraction of a graduate student's stipend, whereas PDX models can easily rival and exceed the cost of personnel. As a consequence, only a minority of the best-funded academic labs are currently equipped to study PDX tumor models, and only then in moderation. The substantial time and cost burden of PDX tumor models will likely require institutional and broader national support to be more widely embraced on the scale needed to displace traditional cell lines from broader use. The long-term benefit of studying more relevant tumor biology merits these expenditures.

PDX MODELS AND THE RESURRECTION OF TUMOR STEM CELLS

For decades, the field of cancer biology has been dominated by the stochastic model for tumor evolution. This model dictates that all tumor cells have equivalent replicative capacity and that mutations conferring a proliferative or survival advantage, for example, result in that cell and its progeny eventually becoming the dominant clone in the tumor. Although clonal evolution and competition is certainly a feature of many tumors,^{69,70} this model alone fails to adequately acknowledge the cellular diversity comprising many solid tumors. The stochastic paradigm for cancer remained largely unchallenged as technologies required to demonstrate functional tumor cell heterogeneity (ie, fluorescent tag-labeled monoclonal antibodies, commercial availability of FACS, and severely immunocompromised mice) did not become broadly accessible to researchers until the late 1990s. These technologies facilitated work demonstrating the recapitulation of tumor cell heterogeneity in passaged PDX tumor models in a remarkable series of studies demonstrating that functional tumor cell heterogeneity accompanies the phenotypic diversity observed within tumors.^{56,57,71,72} This, in turn, led to reassessment and reconsideration of the stochastic model for cancer biology.

The observation that phenotypically distinct tumor cell subpopulations are uniquely capable of fueling tumor growth in serial transplants, whereas the majority of tumor cells appear to be bystanders in the process, has led to emergence of the CSC paradigm for tumorigenesis both in hematological and solid tumor malignancies.^{30,71–73} The CSC paradigm

holds that tumors are comprised of a cellular hierarchy with some similarities to normal tissue, with a self-renewing, multipotent cell at the apex of the hierarchy that no longer responds appropriately to environmental cues.^{30,73} First demonstrated in AML,^{56,57} data supporting the CSC paradigm have since been extended to a multitude of solid tumors, including breast, glioblastoma, colorectal, ovarian, and pancreatic cancer.^{71,72,74–78} Evidence has emerged that only CSC possess the capacity to generate secondary tumors containing both CSC and non-tumorigenic (NTG) cell populations (ie, phenotypic and functional heterogeneity), supporting a model whereby CSC may be rare, but appear solely able to drive fully heterogeneous tumor growth and recurrence.^{30,73} CSC theoretically retain features of normal stem cells, including their ability to symmetrically or asymmetrically divide, remain relatively quiescent, express elevated levels of multidrug resistance transporters and DNA damage repair enzymes, and better handle oxidative stress. Several of these characteristics are believed to be important in the resistance of CSC to traditional chemotherapy and radiation.^{63,73,79–84} The ability of CSC to leverage these characteristics to better handle environmental stresses and genotoxic damage potentially explains the disconnect between therapeutic response (ie, tumor burden reduction) and overall survival in the clinic. Tumors can all but be eradicated; however, if CSC persist, tumors will inevitably recur and are likely to be more aggressive given the genotoxic insults survived. Diagnostics and/or target discovery approaches that simply evaluate bulk tumor cells (*vs* normal tissue), without taking CSC into consideration are unlikely to provide insight on factors that will impact outcome given the relative infrequency of CSC in most tumors (often enumerated as <1% of the tumor). Such diagnostic and target discovery approaches have been taken for decades in naiveté to the underlying presence and biology of CSC.

Since the first functional *in vivo* demonstration for the existence of a solid tumor-initiating cell in human breast tumors in 2003,⁷¹ the semantics and classification schemes surrounding CSC have remained confusing despite the fact that the CSC paradigm has gained significant traction among cancer biologists.^{30,32,85} The exact relationship between CSC, normal stem cell populations, and the ‘cell of origin’ in cancer remains something of a mystery.^{32,86} To be clear, although a tumor’s initial CSC (ie, cell of origin) likely evolves from normal stem cells as a result of accumulating mutations that confer oncogenic properties over time,³³ the cell of origin in cancer and the predominant CSC present in an evolved tumor may possess differing phenotypes and properties depending on the point in time the tumor is being studied. More specifically, the CSC identity in disease may evolve an identity different from the cell of origin. CML serves as an excellent conceptual example for tumor progression wherein the cell of origin and CSC identities differ as a result of tumor evolution over the course of disease progression.^{87–90}

In early chronic phase CML, CSC appear to have a normal hematopoietic stem cell (HSC) phenotype, though the CSC behaves differently from normal HSC by skewing cell division towards the production of more abnormal stem cells (via an increased frequency of symmetric, self-renewing cell divisions) and slightly altering the normal course of hematopoietic differentiation towards certain myeloid lineages.^{90–92} As such, the cell of origin in this disease is thought to be the normal HSC. As the disease progresses, additional mutations accrue that result in, for example, the constitutive nuclear localization of β -catenin, conferring self-renewing properties with phenotypes akin to hematopoietic progenitor cells, thus converting the tumor progenitor cell to a CSC as a result of its newfound ability to self-renew indefinitely.⁸⁹ These abnormal progenitor cells (myeloid blast cells) are unable to differentiate and also proliferate more rapidly than their CSC counterparts. Thus, the severe manifestation of acute phase blast crisis is driven by a more aggressive CSC with a progenitor cell phenotype. CSC in CML may thus have a stem cell or progenitor cell phenotype, depending on the stage of disease (chronic phase *vs* blast crisis). In fact, both likely co-exist in blast crisis CML patients, but the less aggressive chronic phase CSC clone(s) are dramatically outnumbered. Similar observations have recently been made in both human AML and a mouse model of AML, wherein several tumor-initiating cell populations have been demonstrated to co-exist.^{64,93}

Solid tumors likely also follow this progression from normal stem cell to pre-oncogenic stem cell to CSC. Unlike CML, where pre-oncogenic HSCs are not necessarily retained in a specific niche and their progeny may disseminate throughout the bone marrow and circulation, stem cells for most solid tissues are confined to a defined three-dimensional space, or niche, due to their attachment to neighboring cells. As a result of these attachments, expansion of the stem cell compartment and maintenance of the differentiation program might be predicted to culminate in the formation of a mass of more crowded cells with some semblance of normal tissue architecture. In cases where there is space available for tissue expansion, this crowding can cause the formation of involutions, or polyps (Figure 2b; *vs* normal differentiation—Figure 2a). If differentiation is restricted to a particular lineage, the mass of cells might be less organized (Figure 2d). Finally, if all differentiation pathways are blocked, an amorphous mass of cells with no recognizable structure might result (Figures 2e and f). The above description for how solid tumors may manifest and evolve at the histomorphological level as a result of CSC identity and differentiation capacity reflects what is observed in the case of gastrointestinal cancer progression.^{94–97}

Early stages of adenocarcinoma in the intestinal tract, for example, may produce one of several histomorphological outcomes. In normal intestinal epithelium, stem cells in the crypt base divide to produce, through a hierarchical series of fate-restricted progenitor cells (ie, transit amplifying cells),

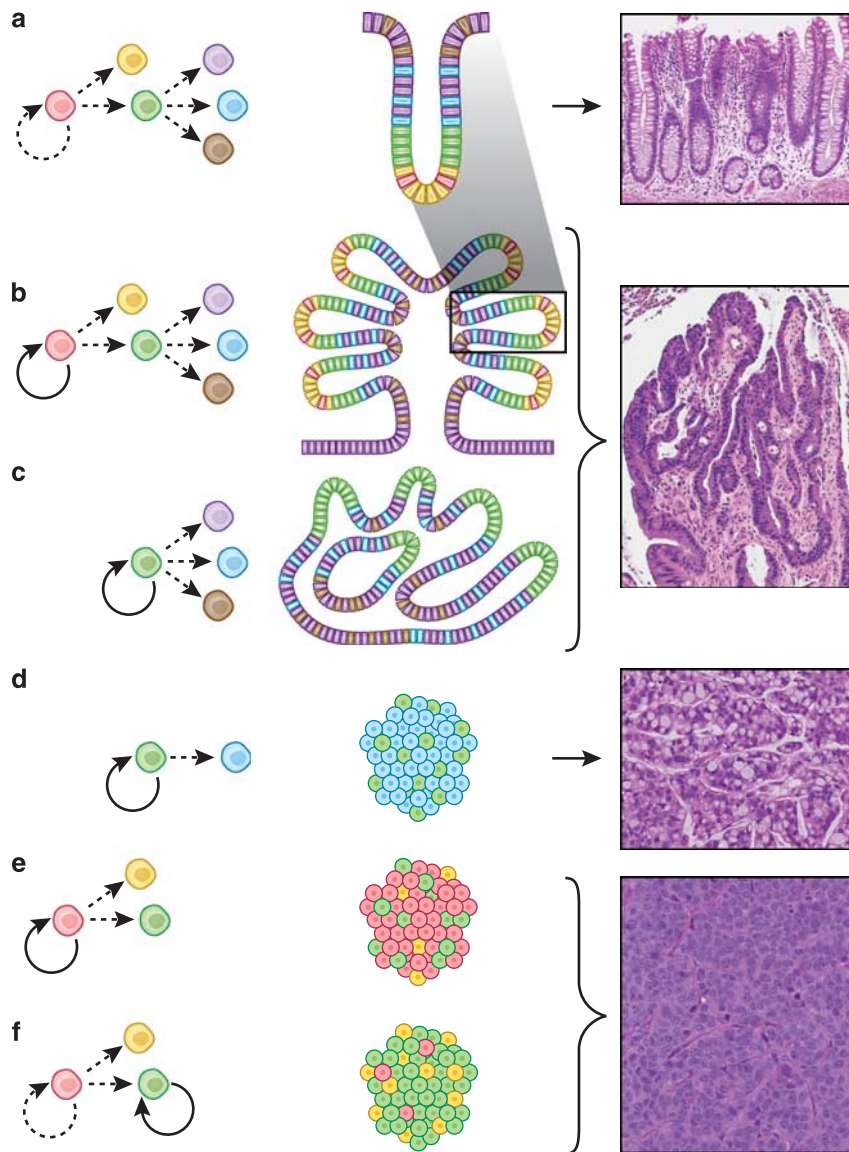


Figure 2 The cancer stem cell (CSC)/tumor perpetuating cell (TPC) identity and differentiation capacity likely influence tumor histomorphology: a gastrointestinal model. **(a)** Normal intestinal differentiation, where progeny of stem cell divisions are appropriately balanced to produce additional stem cells (red), paneth cells (yellow), or progenitor cells (green). Normal differentiation of progenitor cells results in progeny with one of three cell fates: secretory goblet (blue), enteroendocrine cells (brown), or absorptive enterocytes (purple). **(b)** Oncogenic events arising in stem cells may result in an amplified pool of CSC, wherein the CSC have a normal stem cell phenotype and the entire tree of differentiated progeny is also amplified. In the context of solid tissues, the resultant overcrowding causes aberrant higher-order crypt structures (eg, polyps). **(c)** CSC with a progenitor cell identity, wherein normal differentiation is maintained, may result in tumors largely comprised of terminally differentiated cells with involuting structures, but largely devoid of cells with a normal stem cell phenotype. **(d)** CSC with a progenitor cell identity, wherein normal differentiation is skewed towards one particular cell fate may result in tumors containing one predominant cell type (ie, goblet cells) and devoid of both other mature cell lineages and cells with a normal stem cell phenotype. **(e)** Tumors where CSC have a normal stem cell phenotype, but where differentiation programs are impaired may result in tumors comprised of only cells with stem and progenitor cell phenotypes and no structure normally associated with differentiated progeny. **(f)** The CSC with a progenitor cell identity, wherein normal differentiation programs are blocked may result in tumors predominantly comprised of cells with a progenitor cell phenotype and no structure normally associated with differentiated progeny.

the terminally differentiated cell types comprising the intestinal epithelium: enterocytes, goblet cells, and enteroendocrine cells (and in the small intestine, paneth cells). Under homeostatic conditions, these cell fate decisions are regulated to maintain proper numbers and proportions of each cell

type to ensure proper structure and function of the intestinal epithelium (Figure 2a). Oncogenic mutations that arise in the normal intestinal stem cell that leave normal differentiation pathways intact lead to cell divisions skewed to favor stem cell self-renewal (ie, symmetric self-renewing cell divisions

where both daughter cells are stem cells), which results in a tumor containing the full complement of intestinal cell types and largely retaining normal, albeit more crowded, tissue architecture (Figure 2b). Moreover, CSC predominantly driving these well differentiated tumors may have a normal stem cell phenotype (Figure 2b) or a normal progenitor cell phenotype (Figure 2c). These tumors, where differentiation pathways are intact, tend to be classified by pathologists as well differentiated.

In contrast, CSC with mutations that result in the blockade of particular differentiation programs, preventing the generation of certain types of transitional progenitor cells and/or their more differentiated progeny, result in tumors with significantly less resemblance to normal tissue, although they may contain particular recognizable differentiated cell types, such as Goblet cells of the intestine (Figure 2d). Impaired differentiation potential occurring at the level of the normal progenitor cell might manifest as a tumor predominantly consisting of progenitors and no differentiated cell types (Figure 2e). The same histomorphological appearance might be visible in a tumor where the CSC lie at the progenitor cell level and all terminal differentiation pathways were blocked (Figure 2f). These latter examples are classified as poorly differentiated tumors by pathologists. One can also envision the existence of several clones within a tumor resulting in areas of well, moderately and poorly differentiated tissue, depending on the number of competing CSC clones and point in time the tumor is observed. In summary, CSC identity and differentiation potential are likely tightly wed to tumor biology and the histopathological features observed. Research to determine CSC identity and differentiation potential among tumor subtypes will result in the identification of characteristics that facilitate more accurate classification of tumors.

Achieving efficacy and safety in cancer patients dictates that therapeutic targets be differentially expressed on tumor cells vs normal tissue. Moreover, if overall survival is to be impacted, CSC must be successfully targeted and eliminated—highlighting the importance of fully understanding the biology and cellular composition of individual tumors and their relationship vs normal tissue. Successful therapeutic exploitation of CSC will demand a better understanding of CSC identities in different patient populations within any given indication, and possibly at different stages of disease. For example, a patient with a moderately differentiated colorectal tumor fueled by a CSC with a normal stem cell identity (see Figure 2b) might be completely cured by a therapeutic targeting that particular CSC while avoiding normal stem cells. Another patient, also with a moderately differentiated intestinal tumor, may show no response because the therapeutic agent fails to impact that patient's CSC, which has a progenitor cell phenotype (see Figure 2c). To be clear, cells specifically eradicated in the former tumor may not exist in the latter, as tumors in these two patients are fueled by CSC with different identities despite having largely

similar histomorphology at the macro scale. Once CSCs are identified and patient subtypes elucidated, associated antigens, signaling pathways, and/or other unique weaknesses need to be discovered (vs their normal stem and progenitor cell counterparts) and exploited for diagnostic and/or therapeutic benefit.

CANCER STEM CELLS ARE TUMOR PERPETUATING CELLS

To avoid semantic confusion surrounding CSC, a more appropriate term for these cells is tumor perpetuating cells (TPCs), as these cells are defined by their ability to fully recapitulate tumors and do so over an extended period of time as demonstrated by serial transplantation of small numbers of tumor cells with defined phenotypes.^{30,32} Although the current gold-standard for defining a CSC is demonstrating a cell's ability to reconstitute tumors *in vivo*, it is becoming increasingly clear that cells exist within tumors that themselves are devoid of self-renewing capacity but do have significant proliferative capacity supporting their ability to generate tumors in mice (unpublished results).^{59,85} These latter 'tumor progenitor cells' (TProg) would appear to have sufficient proliferative capacity to generate a tumor in a primary transplant, thereby fulfilling the definition of a tumor-initiating cell, but lack the ability to reconstitute the entire cellular heterogeneity of the parental tumor, let alone serially reconstitute tumors. By comparison, normal hematopoietic progenitor cells can reconstitute all lineages of hematopoietic cells in mice for up to 16 weeks (reviewed by Schroeder⁹⁸), thus if normal progenitor cells have significant capacity to recapitulate tissue *in vivo* for a significant period of time, it wouldn't be surprising to find TProg cells within tumors that can similarly reconstitute a tumor that has no TPC and thus cannot be perpetuated in serial transplantation experiments. Of significant importance, however, is the fact that the existence of distinct TPC and TProg populations would complicate the current widely accepted technical definition of CSC. As both TPC and TProg are able to generate tumors in mice in an initial transplant, simple tumorigenesis is not a defining characteristic of CSC, nor can a single transplant be used to distinguish between the two tumor cell subpopulations. Resulting tumors must be phenotyped to look for loss in tumor heterogeneity (eg, loss of TPC) and serial transplants must be done to determine whether self-renewal capacity is inherent or lost.

It is clear from the prevailing CSC literature that significant heterogeneity remains among purported CSC populations, as serial transplants done in limiting dilution commonly quantify tumor-initiating cell frequency to be between 1:75–1:1000 for most solid tumors.^{63,74–76} Melanoma appears to be an exception, wherein one in four cells is able to perpetuate tumors without the use of enriching markers.⁶⁵ Accordingly, melanoma has served as the flag bearer for prospective inadequacies of the CSC paradigm due to the relatively high frequency of tumor-initiating cells in this indication. The concept that CSC must be infrequent

may result from the observation that normal stem cells generally represent a tiny fraction of normal tissues. The relative infrequency of CSC in the pioneering studies of AML also set a precedent for this assumption.⁵⁶ Nevertheless, the melanoma example suggests that CSC can also be relatively frequent, depending on the indication, patient and/or stage of disease.

Critics of the CSC paradigm have been vocal in their appraisal of the shortcomings of xenograft tumor models, bolstered by conflicting tumorigenicity results published by various groups when using common markers thought to demarcate CSC (eg, CD44, CD133, and ALDH1A1 activity). These critics tend to overlook substantial functional evidence for tumor cell heterogeneity and rare tumor-initiating cell frequency, which can be traced back 80 years.^{99–103} Specifically, the potency and rarity of tumor-initiating cells in mouse and rat tumors has been demonstrated using autologous transplants since the 1930's.^{99–102} Moreover, autologous human tumor cell transplants, wherein primary tumor cells were excised from a patient and transplanted into the same patient's thigh in limiting dilutions (notably considered as unethical by today's standards), demonstrated that tumor-initiating cell frequencies were remarkably rare in well-differentiated tumors, often requiring inoculums in excess of 1 million cells.¹⁰³ These examples of autologous tumor cell transplantation strongly refute the argument that the CSC phenomenon is an artifact of xenotransplantation. Additional studies demonstrating relationships between tumor differentiation status and patient prognosis have further underscored the ties between cellular differentiation within tumors and the hypothesis that CSC are relatively infrequent in less aggressive tumors, whereas CSC might be more frequent in later stage and/or aggressive tumors.^{104,105}

BRAVE NEW WORLD OF CANCER PATHOBIOLOGY

It is increasingly clear that CSC underlie tumor growth, recurrence, and resistance to current therapeutics. Accordingly, it stands to reason that cancer will be best diagnosed and treated if physicians are educated by knowledge of events occurring at the CSC-level. As such, improved marker panels are needed such that CSC can be identified, isolated, and characterized with much greater accuracy than what is presently possible. If overall survival is to be improved, cancer patients must also be better subtyped based not only on tumor histomorphology, but on genetic composition, mRNA expression patterns, and frequency and subtype of CSC. CSC might be enumerated and characterized in FFPE tissue sections, characterized among biopsy material and/or circulating tumor cells, and targeted with therapeutics that actively kill CSC to leave less opportunity for escape. To achieve these goals, the CSC paradigm for cancer biology needs to be fully validated and embraced despite the implications for cancer biologists accustomed to studying biology using traditional cell lines.

Widespread use of traditional oncology models has contributed to countless manuscripts and thousands of promising

discoveries over the years, yet few have been successfully translated into consistently efficacious therapies that significantly impact overall survival. PDX tumor models currently offer the best path forward, preserving physiological oxygen tension, three-dimensional growth and metabolic conditions allowing for more faithful recapitulation of tumors. This approach is not without fault considering the imperfect cross-talk between murine and human cells, and lack of an intact human immune component in severely immunocompromised mice. Traditional cultured cell lines will continue to be important complements to PDX tumor models, but their existing primacy to understanding basic tumor biology must be challenged. If they are to produce the desired results, cancer research, drug discovery and development must be conducted with patient-derived tissue grown and passaged exclusively in a physiological environment where gross genomic abnormalities do not accrue at unnatural rates and where cellular compositions more accurately represent patient tumor biology.

The diversity of subtypes among cancer patients makes it difficult to systematically and rigorously test hypotheses. There can exist several subtypes of cancer within any particular indication, such as lung cancer, where adenocarcinoma, large cell, small cell, and squamous cell carcinoma represent four different diseases.¹⁰⁶ Patterns of target expression and/or diagnostic/prognostic biomarkers only become apparent with the study of many tumors within any indication and subtype, combined with detailed annotation of a tumor characteristics and patient outcomes. Consequently, the number of PDX models needed to achieve significant insight into any given tumor type might be substantial. As such, each established PDX tumor line marks an important incremental contribution to the understanding of cancer pathobiology. Among the greatest limitations in expanding the number of these models is the availability of primary tumor tissue—an unfortunate irony given the omnipresence of this disease. In this respect, institutions, surgeons, and pathologists must increasingly collaborate with those at the bench to provide the material needed to better study and understand tumor biology. Patients are most certainly supportive of such collaborations and it is our experience that institutional policies or expectations form the most significant barrier to advancing the generation of PDX models and expanded collaboration between physicians and bench scientists.

The establishment and use of PDX tumor models for cancer research is neither inexpensive nor easy, acting as a barrier to this work being done solely at academic institutions without increasing support. Nevertheless, an increasing number of investigators at academic medical centers have found the resources or formed collaborations to build or access PDX tumor libraries. Large pharmaceutical and biotechnology companies are increasingly turning to PDX models to evaluate preclinical drug efficacy and better understand patient heterogeneity. As a result, significant opportunities exist for collaboration between those that have

access to fresh patient tissue and those who have the resources to generate and thoroughly characterize PDX lines. Established and well characterized low-passage PDX tumor lines will serve as the foundation for improved identification and characterization of tumor cell subpopulations (eg, CSC vs NTG cells) in various tumor subtypes, and as a result enhance our understanding of cancer biology.

Work with traditional cell lines has borne tens of thousands of manuscripts at the cost of billions of dollars since Richard Nixon signed the National Cancer Act in 1971. It is time that cancer biologists abandon what is easy and relatively inexpensive, increasingly ask pertinent questions with relevant tumor models, and progressively increase collaboration with oncologists, pathologists, and other clinicians regularly seeing patients and/or their tumors in the clinical setting. Embracing the CSC paradigm, working with PDX tumor models and maintaining constant awareness of clinical relevance are initial and critical steps towards making significant advances in the understanding of cancer biology in the next several decades. Such fundamental pursuits as these should more efficiently yield the discovery and development of novel diagnostics and therapeutics that significantly impact overall survival of cancer patients.

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DISCLOSURE/CONFLICT OF INTEREST

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