Evolution of the Cancer Stem Cell Model

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Genetic analyses have shaped much of our understanding of cancer. However, it is becoming increasingly clear that cancer cells display features of normal tissue organization, where cancer stem cells (CSCs) can drive tumor growth. Although often considered as mutually exclusive models to describe tumor heterogeneity, we propose that the genetic and CSC models of cancer can be harmonized by considering the role of genetic diversity and nongenetic influences in contributing to tumor heterogeneity. We offer an approach to integrating CSCs and cancer genetic data that will guide the field in interpreting past observations and designing future studies.

Introduction
Despite advances in cancer treatment, many patients still fail therapy, resulting in disease progression, recurrence, and reduced overall survival. Historically, much focus has been on the genetic and biochemical mechanisms that cause drug resistance. However, cancer is widely understood to be a heterogeneous disease and there is increasing awareness that intratumoral heterogeneity contributes to therapy failure and disease progression (Hanahan and Weinberg, 2011). A tumor is not simply a “bag” of homogenous malignant cells. Rather, a tumor is a complex ecosystem containing tumor cells, as well as various infiltrating endothelial, hematopoietic, stromal, and other cell types that can influence the function of the tumor as a whole. These extraneous cell types can influence tumor cells directly and can create metabolic changes such as a hypoxic environment and nutrient fluctuations, which contribute to heterogeneity in the function of malignant cells. By functioning as a complex ecosystem, overall tumor fitness may be enhanced, ultimately impacting therapy failure (Junttila and de Sauvage, 2013). Aside from these non-cell-autonomous effects, even individual malignant cells within a tumor can possess variation in growth, apoptosis, metabolism, and other “hallmarks of cancer.” However, the mechanisms driving intratumoral variation in cellular function have, until recently, been uncertain.

Three avenues of cancer research are coming together to provide increasing clarity to the underlying mechanisms of tumor heterogeneity and uncovering how these are linked to therapy resistance, tumor progression, and recurrence. Advanced genome sequencing has demonstrated that cancer within a single patient is a heterogeneous mixture of genetically distinct subclones that arise through branching evolution (Burrell et al., 2013; Greaves and Malley, 2012). The unique driver mutations within each subclone can impact the cancer hallmarks differently, thereby contributing to functional heterogeneity. In parallel, strong evidence is emerging that nongenetic determinants, largely related to developmental pathways and epigenetic modifications (DNA methylation, histone modification, chromatin openness, microRNA [miRNA], and other noncoding RNA) contribute to functional heterogeneity (Dick, 2008; Meacham and Morrison, 2013; Nguyen et al., 2012). These determinants are generally ascribed to the maintenance of normal tissue stem cell hierarchies. Similarly, nongenetic determinants create hierarchically organized tumor tissues where a subpopulation of self-renewing cancer stem cells (CSCs) sustains the long-term clonal maintenance of the neoplasm. Although considerable controversy remains as to which tumor types are hierarchically organized and how best to define CSCs, this developmental and/or hierarchical model has generated considerable interest because CSCs appear to possess properties that make them clinically relevant. Evidence from both experimental models and clinical studies indicate that CSCs survive many commonly employed cancer therapeutics. Moreover, the properties and transcriptional signatures specific to CSC are highly predictive of overall patient survival pointing to their clinical relevance. Although this area will not be discussed in our review, an additional promising avenue is the recognition that there are many nontumor cell elements associated with tumors, referred to collectively as the tumor microenvironment (TME) (Hanahan and Coussens, 2012). The juxtaposition of a tumor cell with the TME influences the function of that cell, resulting in significant variation in cellular function. The complexity imposed by the TME is amplified due to crosstalk between tumor cells and the TME. The TME plays a role in adaptive drug resistance, as cells of the same genetic make-up can be sensitive or resistant to drugs depending on the context they are in. Recent studies also point to the potential for the TME to initiate stem cell-like programs in cancer cells (Charles et al., 2010; Vermeulen et al., 2010). Collectively, all three mechanisms are strongly linked to therapy failure and tumor recurrence and all are important determinants of tumor fitness (Figure 1).

We will focus our Review on the genetic and developmental mechanisms that generate tumor heterogeneity, and we will emphasize human studies. Although often considered as mutually exclusive models to describe tumor heterogeneity, we propose that the genetic and developmental and/or hierarchical models of cancer can be harmonized. Indeed, recent findings in leukemia and solid tumors indicate that gene-expression signatures specific to CSC and normal stem cells are highly prognostic for outcome across a wide spectrum of patients with diverse driver mutations (Bartholdy et al., 2014; Eppert et al., 2011; Gentles et al., 2010; Merlos-Suárez et al., 2011), suggesting that stemness is a central biological property or process...
Figure 1. Stemness as a Guiding Principle that Governs Therapeutic Response
Three fields in biology—cancer genetics, epigenetics, and microenvironment—are coming together to provide increasing clarity to the processes that determine stemness and in turn influence clinical outcome. These three factors can influence stemness simultaneously, but they can also act independently over time. Through evolutionary time, different forces can impact a cell’s stemness properties and thereby shape tumor progression and therapeutic response.

Defining Stemness
The modern era of stem cell research began in 1961 with the pioneering studies of Till and McCulloch who developed a clonal in vivo repopulation assay and used it to show that a single hematopoietic cell had multilineage differentiation potential while still retaining the property of self-renewal. Although multilineage differentiation potential is often considered a stem cell property, studies in the hematopoietic system have clearly identified distinct cells capable of both repopulation and multilineage potential but lacking self-renewal potential. Thus, the cardinal property of a stem cell is self-renewal, whether normal or malignant. Self-renewal is the key biological process where, upon cell division, a stem cell produces one (asymmetric division) or two (symmetric division) daughters that retain the capacity for self-renewal, ensuring that the stem cell population is maintained or expanded for long-term clonal growth. Operationally, the gold standard measure of a stem cell is maintenance of long-term clonal growth in functional repopulation assays, involving either transplantation into serial recipients or in situ tracking. Indeed the lack of adherence to this principle has generated much confusion in the CSC field. Many studies employ surrogate in vitro assays such as serial replating of tumorspheres or report on serial passage of bulk tumor cells. However, only clonal serial in vivo repopulation assays can formally test self-renewal of stem cells. The molecular programs that underlie the stem cell state are only just emerging as studies are defining critical epigenetic states and the transcription factors and epigenetic modifiers (e.g., Polycomb complexes and miRNA) that are responsible for endowing self-renewal to a cell. The term “stemness” is increasingly being used in the literature to refer collectively to the integrated functioning of molecular programs that govern and maintain the stem cell state. We will adopt the term “stemness” throughout this review to denote this meaning.

Cancer Stem Cells and Tumor-Initiating Cells
By definition, both CSCs and normal tissue stem cells possess self-renewal capacity; however, self-renewal is typically deregulated in CSCs. For many cancers, CSCs represent a distinct population that can be prospectively isolated from the remainder of the tumor cells and can be shown to have clonal long-term repopulation and self-renewal capacity—the defining features of a CSC (Clarke et al., 2006; Nguyen et al., 2012). However, in some cancer types it has not been possible to distinguish CSCs from non-CSCs because most cells have CSC function. Such tumors seem to be homogeneous or possess a very shallow hierarchy. As well, some evidence is emerging that certain cancer cells exhibit plasticity by reversibly transitioning between a stem and non-stem-cell state (although this is a controversial and intensely debated topic). Thus, even though some tumors may not be organized into a rigid hierarchy, the stemness state contributes a variety of functions that enable cells to survive therapy. A key proposition of our review is that the determinants of stemness are the core contributors that affect therapy failure, regardless of whether these determinants are present within a transitory state or in well-defined CSC populations. Like CSCs, transitory cells also possess clonal tumor-initiation capacity; however, prospective isolation is difficult. Thus, in terms of nomenclature they pose a problem and formally they should not be termed CSCs, a term restricted to cases where self-renewing CSC can be prospectively purified. We and others refer to such cells on the basis of the functional tumor-initiating cell (T-IC) or leukemia-initiating cell (L-IC) assays that identify them. T-IC or L-IC are defined by their ability to: (1) generate a xenograft that is representative of the parent tumor, (2) self-renew as demonstrated by serial passage in a xenograft assay at clonal cell doses, and (3) give rise to daughter cells that may possess proliferative capacity but are unable to establish or maintain the tumor clone upon serial passage (Clarke et al., 2006). The T-IC/L-IC terms can also be applied in situations where a bona-fide CSC exists, but the proper combination of cell surface markers required for their prospective isolation has not been found. For ease of reading, we have adopted the term T-IC/L-IC throughout our review to refer to all cells with clonal long-term tumor initiating function and not just to those where prospective isolation has been possible.

Historical Perspectives on Tumor Heterogeneity
Heterogeneity in the cellular morphology of tumors was noted by the great experimental pathologists of the 1800s. Aside from cellular morphology and tumor histology (Heppner, 1984), improved technology has uncovered additional features of heterogeneity between tumors, including variation in cell surface markers (Dexter et al., 1978; Pertzchuk et al., 1978; Poste et al., 1980; Raz et al., 1980), genetic abnormalities (Mitelman
tions differed with respect to their sensitivity to chemotherapy. A primary cell through limiting dilution plating. Cloned subpopulations were used as a marker for clonal subpopulations derived from the parental tumor cells enabling only some clones to metastasize (Fidler and Kripke, 1977). Important evidence for diversification of tumor cell characteristics came from studies in malignant glioma (Shapiro et al., 1981), where primary human tumor cells were isolated and mitoses analyzed by karyotyping. The established karyotypic heterogeneity in the primary tumor was used as a marker for clonal subpopulations derived from primary cells through limiting dilution plating. Cloned subpopulations differed with respect to their sensitivity to chemotherapeutics (Yung et al., 1982) and genetic stability (Shapiro et al., 1981). Evidence that functional tumor cell heterogeneity exists in vivo came directly from human acute myeloid and lymphoblastic leukemia patients, where in vivo 3H-TdR radioactivity showed marked differences in the proliferation kinetics of individual leukemic cells that could be distinguished on the basis of morphology (Clarkson et al., 1970; Gavosto et al., 1967; Killmann et al., 1963). Thus, this era yielded many observations describing variation in functional parameters and established that growth properties of individual cells within a tumor were far from homogeneous.

Of particular importance from this earlier era of cancer research was quantitative evidence from syngeneic mouse tumor grafting experiments showing that the capacity to initiate a new tumor and sustain disease was variable, with not every cell able to function as a T-IC (Bruce and Van Der Gaag, 1963; Hewitt, 1958). The same observations were made in studies that were carried out in human patients, where tumors were autotransplanted subcutaneously into the same patient (Southam et al., 1962). These studies not only illustrated that tumor reinitiation was variable, but that even in syngeneic recipients T-IC were rare. Collectively, these clonal studies established that tumors are not a collection of homogeneous cells with equal capacity for proliferation. Instead, analogous to an intricate ecosystem, tumors are complex networks where individual cells display a diverse set of characteristics and function together to support the growth and maintenance of the tumor as a whole.

Since the original conception of evolutionary reasoning (Darwin, 1859), it has become evident that genetic diversity within a species’ gene pool enhances its ability to survive and adapt to changing environments over time. Likewise, the stability and robustness of ecosystems depends on the degree of biodiversity (Loreau et al., 2001; Tilman et al., 2006). In developmental biology, different specialized cell types need to exist for the effective functioning of organs. For example, for the proper functioning of the blood system, hematopoietic stem cells (HSCs) need to produce a heterogeneous pool of specialized cell types that differ in structure and function. Heterogeneity even within the HSC pool has been described (Cheung et al., 2013; Yamamoto et al., 2013). Without this diversity, the function of the blood system would be compromised. While the evolution of species and biodiversity in ecology are consequences of changes at the genetic level, diversity in cell function and tissue development within an organism are the result of nongenetic developmental programs.

**Genetic Mechanisms as the Source of Tumor Heterogeneity**

A guiding principle in cancer research is that tumor initiation and progression result from the sequential acquisition of genetic mutations that contribute to subsequent clonal expansions (Nowell, 1976). This view is strongly supported by early studies where genetic mutations were analyzed across different stages of colorectal cancer (Vogelstein et al., 1988). These studies established that genetic changes cause phenotypic manifestations, a finding that added significant weight to the idea that cancer development follows the rules of Darwinian evolution (Cairns, 1975; Nowell, 1976). The basic premise of this long-standing idea is that a cell that is endowed with an advantageous heritable mutation generates progeny that has a survival advantage over other cells that lack this mutation. Consequently, the progeny of the cell with increased fitness will flourish and produce a clonal population that dominates the site where it originated. Over time, additional advantageous mutations can arise, endowing a further growth advantage to another cell within the clone. As unique subclones arise, different outcomes are possible: less fit subclones can be completely lost with the most fit subclone dominating, or many minor subclones persist alongside the dominant clone, forming reservoirs from which evolution can continue. Overall, models where subclones persist and/or contribute to independent phylogenetic lineage trees within single tumors are highly reminiscent of the branching evolution that Darwin described as leading to increased fitness and overall robustness of a species (Figure 2).

Technological advances have made high-throughput sequencing of tumor genomes possible. The last 6 years have seen a flood of whole exome sequencing (WES) and whole genome sequencing (WGS) of thousands of tumors, enabling complex analyses of the mutations that are present within a single tumor and across multiple tumors (Garraway and Lander, 2013). Several principles are emerging from this work, including that fact that the mutational burden is highly variable across tumor types (Lawrence et al., 2013). For example, leukemias tend to have the lowest number of mutations per tumor compared to adult solid tumors. Even within the same tumor type, there is considerable variation in driver mutations and the same driver mutations can occur in different tumor types, suggesting that the same pathways can be active in different tumors (Alexandrov et al., 2013; Kandoth et al., 2013). The high intersub- and intratumor heterogeneity makes it difficult to establish without functional testing whether a particular somatic polymorphism is a driver mutation or a passenger variant. WGS shows that tumors contain thousands of variants, making resolution of the passenger and driver issue a substantial challenge. The origin of passenger mutations was recently elegantly documented in acute myeloid leukemia (AML) genetic studies. Of the many hundreds of mutations that are found in AML blasts,
many were actually present in the founder HSC that was the cell of origin for the AML subclone; these mutations arose every time the normal HSC divided and remained functionally neutral and thus can be considered passengers (Jan et al., 2012; Welch et al., 2012). The acquisition of the oncogenic driver within one such HSC “trapped” the preexisting spectrum of mutations within the AML subclone that expanded and progressed from this initiating cell. After development of leukemia, very few additional mutations are needed to drive the last population expansion, although hits continue to be acquired as the disease progresses (Jan et al., 2012; Welch et al., 2012). Thus, the driver and cooperating mutations that the AML subclone acquires during leukemic progression need to be filtered out from the large spectrum of passengers that preexisted and will continue to arise as AML cells proliferate. Similar approaches have been taken in breast cancer and other solid tumors to distinguish drivers from passengers (Nik-Zainal et al., 2012; Stephens et al., 2012). While these approaches clearly document how the mutational landscape of individual patients can influence the heterogeneous properties between patients, more sophisticated approaches are needed to determine how genetic mechanisms contribute to heterogeneity within tumors.

Intratumoral Genetic Diversity
A key proposition of the multistep tumorigenesis model put forth by Cairns and Nowell is that there are sequential sweeps of clonal dominance that are variably detected depending on when a tumor is sampled. However, they also conceived that the tumor might contain multiple branches or subclones that are evolving independently (Figure 2). Indeed, with deeper sequencing and improved bioinformatic methods, it is becoming clear that tumors are often composed of a dominant genetic clone plus one or more genetically distinct subclones. For example, topological sampling of tumors has shown that different regions possess distinct mutations that are reflective of genetic subclones seedling different parts of a single tumor (Gerlinger et al., 2012). In the case of metastatic renal cancer, 70% of somatic variants were not found in all biopsies of the same tumor; only a VHL mutation and loss of a region on 3p were ubiquitous. Even gene-expression signatures of good and poor prognosis were detected in different regions of the same tumor. Intratumoral diversity with respect to metastatic progression was shown in pancreatic cancer. By sequencing the genomes of metastases and different regions of matched primary tumors obtained through rapid autopsies from seven individuals with end stage pancreatic cancer, the primary tumor was found to harbor geographically and genetically distinct subclones that gave rise to lung, liver, or peritoneal metastases within the same patient (Yachida et al., 2010). Importantly, despite the presence of founder mutations within the parental clones, the cells giving rise to metastatic lesions had a large number of additional mutations, indicating that further clonal evolution had taken place during metastasis. Others have also reported genetic heterogeneity between metastasis-initiating cells in pancreatic cancer (Campbell et al., 2010). These studies highlight the complexity in predicting which subclones will progress to metastasis, even after the genomic architecture of the primary tumor is established.

With the ability to detect genetic subclones within tumors, it is now possible to create lineage maps that provide insight into the subclonal evolution. Such advancements have made it possible to reconstruct the life histories of breast cancers (Nik-Zainal et al., 2012). By sequencing 20 breast cancers to an average 30–40× coverage and one cancer to 188× depth and applying a new bioinformatics algorithm (Greenman et al., 2012) to reconstruct the genomic history, the authors showed that breast cancer evolves through acquisition of driver mutations that produce clonal expansions. Interestingly, the driver mutations occur infrequently in long-lived lineages that passively accumulate mutations without expansions. The most recent common ancestor appeared surprisingly early, indicating that much of the time is spent driving subclonal diversification and evolution among the nascent cancer cells (Nik-Zainal et al., 2012). These studies do not just give a snapshot of the tumor, but narrate the steps it has taken before it was diagnosed, providing promising avenues for earlier screening. One caveat of these studies is that interpatient tumor genetic variability is likely extensive and it may thus be challenging to delineate a common set of steps that are characteristic of different breast cancer subtypes. The key question that the discovery of subclonal diversity raises is which clones will survive therapy and progress to cause recurrence and/or metastasis.

Studies in leukemia have been particularly instructive in revealing the presence of subclones and their role in tumor progression. Analysis of chromosomal translocation breakpoints and DNA copy-number alteration (CNA) profiling in twins with ETV6–RUNX1 positive acute lymphoblastic leukemia (ALL) showed that a preleukemic clone is initiated in utero that expands, seeds both twins, but then evolves with different kinetics and CNA acquisition in each twin (Bateman et al., 2010; Hong...
et al., 2008; Li et al., 2003; Zuna et al., 2004). Genome-wide CNA profiling of paired diagnostic and relapse samples of ALL has been informative (Iminba et al., 2013; Muligahen et al., 2008). In approximately 40% of cases, the leukemic subclone present at relapse was identical to the subclone present at diagnosis or it was a direct evolutionary product. However in 50% of cases, the relapse subclone shared only limited genetic identity with the diagnostic subclone and did not evolve from it. Similar findings came from WGS studies of paired diagnosis and relapse AML samples: the major population at relapse shared only limited genetic identity with the major population at diagnosis and did not evolve from it. These findings from B-ALL and AML predicted the presence of genetically distinct subclones at diagnosis and the existence of ancestral, prediagnostic subclones. These results indicate that tumor evolution may occur through a more complex branching model that gives rise to genetically distinct subclones at diagnosis that vary in aggressiveness and response to therapy (Greaves, 2009, 2010). Moreover, these data establish that there is subclonal variation in both the response to therapy and the probability that a subclone will survive and regenerate a new tumor.

However, these are still in silico depictions of intratumoral diversity and they are inferred from bulk tumor tissue. This limitation makes it difficult to determine how and when population expansions occurred to generate subclones and there are questions of whether the sequencing was deep enough to reveal the entire population substructure. Like population studies of human evolution or in ecology, lineage trees that describe evolutionary history are best undertaken with single cells. If large numbers of single cells are analyzed, their relationship to one another can be mapped and phylogenetic lineage trees can be created (Melchor et al., 2014; Navin et al., 2011; Potter et al., 2013; Shlush et al., 2012). Early studies of this type have now been reported for some leukemias and lymphomas, and they reveal a high degree of complexity within single tumors. Shlush et al. tracked polymorphic somatic mutations in large numbers of single leukemia cells taken at diagnosis and relapse and reconstructed cell lineage trees based on their divisional history. The reconstructed lineage trees from cells at relapse were shallow (indicating that they divide rarely) compared to cells at diagnosis, which showed many more subpopulations. Interestingly, relapse cells were closely related to the L-IC enriched subpopulation from the diagnostic sample, which is known to be relatively quiescent. This result implies that in these instances, relapse might have originated from rarely dividing L-IC. Given the importance of L-ICs to tumor growth, it will be important to broaden this type of single cell analysis to more samples and to determine the extent to which L-IC are involved in establishing a genetically diverse relapse.

Collectively, the identification of genetically diverse subclones within single tumors provides strong evidence that intratumoral heterogeneity can be driven by the unique mutation spectrum present within each subclone (Figure 1). However, key questions remain that sequencing studies alone cannot resolve. One major challenge is determining which mutations are able to drive tumor growth and how to link these drivers to the clonal propagation potential of subclones. Does genetic diversity exist in tumor cells that are responsible for long-term tumor propagation? Which subclones will evolve further? Will all cells within a subclone be equally sensitive to therapy? Which clones will recur or metastasize? Studies to answer these questions require functional assays.

Nongenetic Mechanisms as the Source of Heterogeneity—the Cancer Stem Cell Model

Although the idea that cancer retains features of embryological development has a long history (Cohnheim, 1875), the modern idea that developmental programs underlying normal tissue organization may still function to some extent in cancer began with seminal studies of teratocarcinoma (Pierce et al., 1960), small cell lung carcinoma (Baylin et al., 1978), and mammary carcinoma (Bennett et al., 1978; Hager et al., 1981). They suggested that many tumor cells were differentiated and that those “differentiated” cells were generated by tumor “stem” cells, similar to normal tissue stem cells producing normally differentiated tissues. Thus, tumors can be considered as caricatures of embryogenesis or normal tissue renewal (Pierce and Cox, 1978; Pierce and Speers, 1988). Early studies in the hematopoietic system were also instructive. There was clear evidence from cytokinetic labeling studies that the majority of leukemia blasts were postmitotic and needed to be replenished from a small population of highly proliferative cells (Clarkson et al., 1967; Clarkson et al., 1965; Clarkson, 1969). Presciently, these studies also predicted the existence of a rare leukemic population that cycled very slowly and showed resistance to antiproliferative therapies and therefore was thought be the source of recurrence. Since similar cytokinetics were observed for normal hematopoietic stem cells, it was proposed that the slow-cycling leukemia cells were responsible for the continued generation of the proliferative fraction, representing a leukemic “stem cell” population (Clarkson, 1974). These early studies, together with efforts to identify clonogenic AML progenitors (Buick et al., 1977; McCulloch, 1983; Metcalf et al., 1969; Moore et al., 1973), sparked an interest in thinking about leukemia in terms of hierarchical organization, as was being established for normal hematopoiesis at that time.

In order to demonstrate that a tumor is organized in a hierarchical manner, it is crucial to establish that it consists of functionally distinct cell types that can be prospectively purified and assayed. With the development of fluorescence-activated cell sorting techniques (Bonner et al., 1972), coupled with refinements in xenografting techniques in immune-deficient mice, it was possible to engraft normal human hematopoietic cells (Kamel-Reid and Dick, 1988; Lapidot et al., 1992) and leukemic cells in mice (Dick et al., 1991; Kamel-Reid et al., 1991; Kamel-Reid et al., 1989). These tools, along with quantitative assays, set the stage for the first purification of T-IC, the operational term for human CSCs (Clarke et al., 2006).

Flow sorting using cell surface markers CD34 and CD38 was used to prospectively isolate human T-IC in AML, termed leukemia-initiating cells (L-IC) (Lapidot et al., 1994). The leukemia initiation potential was in the CD34+CD38- fraction and no engraftment was detected from the CD34+CD38+ or CD34- fractions. By injecting different numbers of cells per mouse and establishing a linear correlation with engraftment, it was calculated that 1 in 2.5 × 10^5 cells could initiate a leukemic graft (Lapidot et al., 1994). Analysis of additional AML samples in a more sensitive immune-deficient mouse model (using non-obese diabetic/severe combined immunodeficiency [NOD/SCID] mice) is required to determine the exact initiating potential.
followed this initial study (Bonnet and Dick, 1997), further establishing that AML is organized as a hierarchy with CD34+CD38+ L-IC at the apex. These studies provided proof for the hypotheses from the 1960s and established that not every AML cell was equal and only rare cells were L-IC.

The initial studies in AML laid the foundation for the generation of CSC studies in solid tumors that followed. The first identification of CSCs in a solid tumor was achieved over ten years ago in human breast cancer (Al-Hajj et al., 2003). A subset of breast cancer cells (CD44+/CD24-`) was prospectively isolated and shown to be solely responsible for sustaining the disease in immune-deficient mice. The CSC subset could be serially passaged and the xenografts generated were histologically heterogeneous, resembling the parent tumor from which they were derived. These results demonstrated that the same CSC principles that had previously been shown to apply in an AML model could also be translated to a solid tumor. Since the initial publication in breast cancer, a plethora of papers have been published identifying CSCs in numerous cancers including brain (Singh et al., 2004), head and neck (Prince et al., 2007), pancreas (Hermann et al., 2007; Li et al., 2007), lung (Eramo et al., 2008), prostate (Collins et al., 2005; Patrawala et al., 2006), colon (O’Brien et al., 2007; Ricci-Vitiani et al., 2007), and sarcoma (Wu et al., 2007). In all cases, non-T-ICs were generated in the xenografts providing evidence for hierarchical organization. However, in most cases where patient-derived cancer samples were used, no genetic analysis was undertaken to compare the xenografts to the primary tumor to determine whether there was selective outgrowth of one or more subclones. Nonetheless, CSCs appeared to be a common feature across different cancer subtypes and tumors from different tissues. Collectively, the studies illustrated that the ability to initiate and propagate tumor growth varies between different cells within a cancer and that this variation is due to a hierarchical relationship between tumorigenic and nontumorigenic cells. This relationship is comparable to developmental hierarchies seen in normal tissues where stem cells reside at the apex and are responsible for generating progeny that in turn exhibit increasing commitment and lineage restriction.

**Xenografting and CSC Detection**

Because tumor initiation is one of the defining features of CSCs, xenografting is central to the CSC model. A limitation of xenograft studies is that even orthotopic transplantation may not faithfully reproduce the TME or the growth factor milieu found within a patient’s tumor. Some murine growth factors are not available (as for many solid tumor types) or where they are uninformative (Amendola et al., 2013). In addition to miRNA, the use of reporter assays that measure cellular signaling pathway activity, such as the Wnt reporter (Vermeulen et al., 2010), can be used as alternative means of measuring distinct cellular fractions that may segregate T-IC and non-T-IC. The tools for analyses of other important pathways in human cells is only beginning to emerge and it will be interesting to see how intracellular signaling markers will impact the identification of T-ICs. At least in AML, the strongest independent piece of evidence supporting the utility of the xenograft assay to detect bona fide L-IC is that only gene signatures from functionally validated L-IC populations are strongly prognostic for patient survival (Eppert et al., 2011). This and other evidence is presented in the Linking Stemness, Prognosis, and Therapy section.
In the context of solid tumors, there have been many more discordant findings regarding the phenotype and properties of T-IC depending on experimental conditions and the type of xenograft assays employed. Through the use of recipients with increased immune-deficiency, the assayed frequency of T-IC in melanoma changed by many orders of magnitude such that the most permissive recipient read out a T-IC frequency of virtually 1 in 1 (Quintana et al., 2010). As most tumor cells were T-IC, such tumors appear to be homogeneous and not following a hierarchical model of tumor organization, although some have argued that there may be methodological explanations (Boiko et al., 2010). Nevertheless, use of more immune-deficient mouse models does not necessarily change the evidence documenting the existence of CSC and for many tumors a hierarchy containing T-IC and non-T-IC was seen (Ishizawa et al., 2010; O’Brien et al., 2012). In others cases such as breast cancer, the implantation of human stromal elements appears to mitigate many problems and enables reliable detection of T-IC (Kuperwasser et al., 2004). Overall, future use of recipients that express components of the human immune system, as well as cross-species reactive growth factors, should be valuable modifications to the xenograft assay and enable more reliable evaluation of hierarchical organization in tumors.

Given the central importance of the xenograft assay to measure functional cancer cell properties and its heavy use in CSC research and experimental drug studies, it is important to fully describe the characteristics, including the genetic make-up, of cancers that grow as xenografts. A major limitation of CSC studies to date is that there has been a lack of integration of genomic and functional properties of T-ICs as we describe later in this Review. Notably, it remains to be determined which genetic clones can generate grafts in mice and how this influences the corresponding CSC measurements.

Clonal Dynamics, Dormancy, and Therapy Failure

Although the studies described above indicate that not all tumor cells possess T-IC function, another major question is whether all T-IC are equal in their tumor propagation ability. If there is variation, it will be important to establish whether the variation exists within cells of a single genetic clone. The answer to this question holds major importance to the design of future cancer therapies. Addressing this question requires genetic analyses combined with functional assays that measure tumor propagation at the resolution of individual clones. We have recently characterized colon cancer xenografts and shown that single genetic subclones from the patient tumor can be separated and stably propagated over multiple passages in mice. Being able to propagate a genetic clone allowed us to track the behavior of cells within unigenetic lineages. By using lentivirus-mediated cell marking, we mapped the growth dynamics of 150 marked cells from ten primary human colon cancer samples in serial transplants that spanned 387 days of tumor growth on average (Kreso et al., 2013). In every genetic clone that we analyzed, we detected significant variation of cellular behaviors: some marked cells were proliferative and persisted at every transplant, whereas others were less robust and could not be detected at later points during transplantation. Thus, these results directly identify functional diversity among cells that are part of a single genetic clone in a solid tumor.

In addition, approximately 20% of marked cells were initially undetectable, but following serial transplantation such cells became activated and continued to function (Kreso et al., 2013). This provided formal evidence for the existence of dormant cell populations that drive tumor growth in primary human colon cancer. Moreover, by treating xenografts with conventional chemotherapy, we discovered that while some long-term persisting cells were eradicated, the dormant cells survived treatment and contributed to tumor regrowth. These changes were not accompanied by selection of distinct genetic subclones, as the control and treated tumors displayed close genetic identity. Tumor cell dormancy has been observed in other systems, including breast cancer, melanoma, and leukemia (Pece et al., 2010; Roesch et al., 2010; Saito et al., 2010). Because most conventional chemotherapies are largely cytotoxic to dividing cells, dormancy may provide cells with a means of escape or survival (Figure 3), although other mechanisms, such as acquisition of new mutations or selection of cells with preexisting genetic mutations, could also be at work to ensure survival following therapy. Collectively, these studies provide evidence that even within a single genetic clone, cancer cells are heterogeneous in their ability to survive chemotherapeutic insults. This added layer of functional diversity adds a new tier of complexity within tumors.

Plasticity and CSC Detection

In vitro studies have often been used as surrogate means of studying T-IC. A number of reports using cell lines that have been cultured in vitro have shown that the T-IC state is not static. Sorted T-IC enriched populations generated non-T-IC, but some studies found that sorted non-T-IC populations could generate T-IC (Gupta et al., 2011; Magee et al., 2012; Sharma et al., 2010). Studies of JARID1B, a histone demethylase, have been informative and highlight the complexity of the T-IC state in melanomas. JARID1B was shown to mark slowly cycling melanoma cells that are essential for continuous tumor growth of established melanomas and metastatic progression, but are not required for tumor initiation (Roesch et al., 2010). JARID1B expression was limited to a small subpopulation of melanoma cells, but the maintenance of this subpopulation was dynamic: while purified JARID1B-positive cells generated JARID1B-negative cells, as expected by the CSC model, single JARID1B-negative cells also gave rise to heterogeneous progeny, including JARID1B-positive cells. This study indicates that some cells that are essential for tumor maintenance may not be static entities, but rather can acquire tumor maintenance capabilities depending on the context.

The finding that normal stem cells can reenter the stem cell state (Mani et al., 2008) gave way to the idea that it may be possible to generate T-IC from non-T-IC under some conditions. Indeed, EMT factors have been used to generate T-IC from non-T-IC in breast cancer (Chaffer et al., 2013). The environment in which tumor cells reside can also induce stem-like states in cancer cells. For example, myofibroblast-secreted factors, including hepatocyte growth factor, can induce Wnt signaling in colon cancer cells and consequently induce a T-IC-like state in more differentiated tumor cells in vivo (Vermeulen et al., 2010). In mouse models of intestinal tumor initiation, epithelial nonstem cells can reexpress stem cell markers upon Wnt activation and...
can “dedifferentiate” to T-ICs (Schwitalla et al., 2013). Likewise, perivascular nitric oxide that is released by endothelial cells can activate Notch signaling and induce a stem-like state in PDGF-induced gliomas (Charles et al., 2010). These studies highlight the dynamic nature of cancer cells and show the importance of the stem cell state in tumor generation.

Given the importance of these concepts, it will be important to show whether other cancers possess such properties. As well, it will be critical to determine to what extent plasticity exists in primary tumor tissue, as opposed to cell lines, and whether it is induced in vivo. Although provocative, some studies reporting plasticity were not done clonally and this is essential to understand the homogeneity of cells in each state and the frequency of cells that are able to change states. Is every non-T-IC able to generate a new T-IC, or are only some non-T-ICs responsible for the generation of new T-ICs? If only some, does this reflect heterogeneity of the non-T-IC population? Clearly tumors with a high probability of interconversion between T-IC and non-T-IC states render hierarchical cellular organization less meaningful than if such interconversions are rare. Normal tissue stem cells can also “dedifferentiate” into a more primitive state when normal tissue homeostasis is perturbed, for example during transplantation procedures or following stem cell ablation (Rinkevich et al., 2011; Tata et al., 2013; Van Keymeulen et al., 2011). Thus, it will be important to determine the probability of being in one state versus another and the factors that influence such interconversions (Gupta et al., 2009). However, even in tumors where the interconversion rate is high, the available data indicates that when a cancer cell possesses stemness properties it is more likely to progress, metastasize, resist therapy, and self-renew, compared to when it is in the opposite state. Thus, even for tumors that do not strictly follow the CSC model, the concept that stemness is an important aspect of the biology of that cell remains strong. As such, novel approaches will be needed to eradicate cells that display determinants of stemness.

Epigenetics and Stemness

The primary, nonmutational mechanism that governs developmental hierarchies is epigenetic regulation of the genome. Epigenetic modifications of DNA, histones, and nucleosomes as well as noncoding RNAs, including miRNA, allow for modification of gene expression (Baylin and Jones, 2011; Iorio and Croce, 2012). Alterations in the epigenome dictate cell fate specification and have been used as means of reprogramming noncancerous cells. Although epigenetic modifications are not as stable as mutational changes and can be reversed, some types of modification are a stable, heritable means by which distinct cellular states and functions can be generated. The importance of epigenetic regulation in generating diversity apart from genetic mutation has been shown in several systems. For example, a small proportion of slowly cycling melanoma cells that are essential for tumor growth can be purified based on the expression of JARID1B, a member of the jumonji/ARID1 histone 3 K4 demethylases (Roesch et al., 2010). Other epigenetic factors including members of the Polycomb group of transcriptional repressors (BMI-1 and EZH2) that are linked to normal stem cell self-renewal have been shown to exhibit variation in expression levels within tumors and play a role in tumor progression (Sparmann and van Lohuizen, 2006). Further support for the role of stemness in cancer biology is emerging from cancer genome-sequencing efforts showing that genetic disruption of epigenetic regulators of normal stem cell function is critical for cancer pathogenesis. Mutation in DNMT3A, which is highly recurrent in AML, causes major dysregulation of gene expression leading upregulation of stemness genes and increased repopulation and self-renewal of normal HSC (Ley et al., 2010; Shah and Licht, 2011). Other highly recurrent mutations in genes such as IDH1/IDH2 and TET2 affect epigenetic programs that underlie stemness for many cancers, including AML (Abdel-Wahab and Levine, 2012). Alterations in the epigenome dictate cell fate specification and have been used as means of reprogramming noncancerous cells. Although epigenetic modifications are not as stable as mutational changes and can be reversed, some types of modification are a stable, heritable means by which distinct cellular states and functions can be generated. The importance of epigenetic regulation in generating diversity apart from genetic mutation has been shown in several systems. For example, a small proportion of slowly cycling melanoma cells that are essential for tumor growth can be purified based on the expression of JARID1B, a member of the jumonji/ARID1 histone 3 K4 demethylases (Roesch et al., 2010). Other epigenetic factors including members of the Polycomb group of transcriptional repressors (BMI-1 and EZH2) that are linked to normal stem cell self-renewal have been shown to exhibit variation in expression levels within tumors and play a role in tumor progression (Sparmann and van Lohuizen, 2006). Further support for the role of stemness in cancer biology is emerging from cancer genome-sequencing efforts showing that genetic disruption of epigenetic regulators of normal stem cell function is critical for cancer pathogenesis. Mutation in DNMT3A, which is highly recurrent in AML, causes major dysregulation of gene expression leading upregulation of stemness genes and increased repopulation and self-renewal of normal HSC (Ley et al., 2010; Shah and Licht, 2011). Other highly recurrent mutations in genes such as IDH1/IDH2 and TET2 affect epigenetic programs that underlie stemness for many cancers, including AML (Abdel-Wahab and Levine, 2012).

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Thus, epigenetic factors, classically ascribed to govern normal cell diversification, are becoming increasingly relevant for the maintenance of different cancer cell states.

Epigenetic mechanisms can also be important for the observed variability in response to therapy (Glasspool et al., 2006). A small population of cells that remain drug-tolerant following treatment has been reported across several cell lines, including cells derived from melanoma, lung, gastric, colon, and breast cancers (Sharma et al., 2010). Following treatment of these drug-sensitive cell lines with anticancer agents, the authors observed a small proportion of cells that persist, remaining viable while the majority of cells are killed by the therapy. This drug-tolerant phenotype was related to changes at the level of global chromatin, with high expression of the histone demethylase JARID1A and IGF-1R signaling in drug tolerant cells (Sharma et al., 2010). Importantly, heterogeneity in drug response can be generated even when cultures are initiated from single cancer cells, indicating a nongenetic mechanism. Others have found that escape of cells from anticancer drug treatment involves a survival advantage conferred by cell-to-cell variability in the dynamics of specific proteins (Cohen et al., 2008). Substantial variation between daughter cells in response to antimitotic drugs has also been reported that is not the result of genetic differences, but rather due to competing intracellular networks involving caspase activation and cyclin B1 levels (Gascoigne and Taylor, 2008). Overall, these studies highlight the importance of nongenetic mechanisms governing both cellular fates and drug response. It will be important to discern how these in vitro studies translate to in vivo growth properties of cancer cells following drug administration.

Gene-expression analysis is another important means by which different cellular states can be identified. By using single cell multiplex PCR analysis in combination with fluorescence-activated cell sorting, it has been shown that colon tumors contain subpopulations of cells whose transcriptional states mirror those of the lineages found in the normal colon epithelium (Dalerba et al., 2011). Importantly, these authors show that a tumor derived from a single cell can exhibit the morphological diversity and transcriptional variability reflective of multilineage differentiation seen in normal colon tissue. These gene-expression programs are also important indicators of patient survival (Dalerba et al., 2011), formally proving that epigenetic heterogeneity due to multilineage differentiation processes can establish phenotypic and functional diversity in tumor clones. As such, it is evident that tumor heterogeneity can arise due to transcriptional programs that are reminiscent of normal tissue differentiation, which are independent of genetic diversity.

**Noisy Gene Expression and Heterogeneity**

Studies in lower organisms have found that stochastic nongenetic processes involving protein production or degradation can account for numerous phenotypic effects (Losick and Desplan, 2008; Süel et al., 2007; Wernet et al., 2006). In mammalian cells, survival of apparently homogeneous cells can be dictated by natural differences in protein levels, which regulate receptor-mediated apoptosis between cells and illustrate the dramatic effects that noise in gene expression can have (Spencer et al., 2009). The variability in levels of proteins within cells, albeit transiently heritable, quickly changes in daughter cells due to different growth rates and noise in gene expression. As such, it is inherently different from epigenetic regulation discussed above. Nonetheless, the variability between cells with respect to noise in gene expression and variability in signal transduction components has implications for tumor biology and therapeutics. Traditionally, the failure of a therapy to eradicate all cells has been ascribed to genetic differences, proliferative status, or the microenvironment, but it is possible that the variability of cells to respond can also be governed by natural differences in protein levels.

At the level of cell populations, evidence is emerging to support stochastic processes governing cell state equilibriums. For example, breast cancer cell lines, separated into different phenotypic fractions or “states” based on cell surface marker expression, return to equilibrium proportions over time in vitro. This progression toward equilibrium proportions was the result of interconversion between different phenotypic states, which can be modeled as stochastic processes that occur with each cell division using the Markov process, where interconversion rates depend only on the cell’s current state (Gupta et al., 2011). This study provided a theoretical framework for explaining phenotypic equilibriums in breast cancer cell lines. It will be important to see these principles established in primary cancers where T-IC can be highly resolved by sorting and tested in robust clonal serial T-IC assays.

Collectively, these studies indicate that in apparently homogeneous environments, cells of the same genotype can exist in different states that influence their behavior. The implications of such variability to biology and medicine are important. The implicit assumption most experimental studies take is that a uniform cell population reacts in a uniform manner. However, given the biological noise between individual cells, the effects of treatments on populations are likely underestimated, as averaging data across many cells can have the net effect of masking heterogeneity at the single cell level. With new technological advances, an increasing number of single-cell studies are being reported that demonstrate considerable cell-to-cell variability in apparently homogeneous populations. For example, quantitative PCR gene-expression analysis of 280 genes was undertaken for 1,500 single cells that span a variety of highly purified mouse HSC and progenitor populations. This study uncovered a large degree of heterogeneity within cell types that were classically thought to represent a uniform collection of cells (Guo et al., 2013). It will be important to establish whether such variation is the result of technical variability in the assays used, or whether it represents true differences in biological function. In the context of the CSC model, extensive cellular variability within what is thought to be a uniform CSC population has implications for therapeutic targeting. Therapy directed against a molecular target might not be effective if intrinsic variability in the cellular context renders subsets of cells within the population unequally responsive to drug targeting.

**Limitations of Genetic and Nongenetic Models**

As we have argued throughout this Review, both genetic and nongenetic determinants influence tumor heterogeneity and often these two views have been presented as mutually exclusive models, stimulating intense debate (Clevers, 2011; Marusyk et al., 2010). Thus, epigenetic factors, classically ascribed to govern normal cell diversification, are becoming increasingly relevant for the maintenance of different cancer cell states.
et al., 2012; Shackleton et al., 2009). However, each view in isolation is insufficient to explain fully the diversity seen within cancers. The genetic model focuses on genetic heterogeneity without considering that individual cells within a genetically homogeneous subclone might still exhibit variation in function due to any of the nongenetic determinants described above. Similarly, a major limitation of the CSC model or hierarchical model is that it views the tumor as being genetically homogeneous and static, without accounting for the existence of genetically distinct subclones or tumor evolution. For example, a tumor might contain different subclones, some of which are virtually homogeneous in terms of T-IC activity because they are highly progressed and possess a high mutational burden, whereas other subclones with fewer oncogenic mutations might be almost devoid of T-IC. Such subclones could also possess differences in the cell surface markers used for sorting. Thus fractionation of the bulk tumor into T-IC and non-T-IC populations could simply be the result of segregating subclones with very low T-IC activity from those with high T-IC activity (Figure 4). In this scenario, sorting has simply segregated on the basis of genetic identity rather than providing the essential test of the CSC model, which requires testing the T-IC ability of genetically identical cells within a single subclone (Figure 5), as described recently in our study of the clonal dynamics of T-IC from human colon cancer (Kreso et al., 2013). Clearly the recent findings on subclonal diversity raise an important challenge to the validity of the broad literature on T-IC. Despite these concerns, as we will argue below, we propose that the genetic clonal evolution and CSC models can be unified into a comprehensive view of cancer heterogeneity.

Three independent studies in human B-ALL and T-ALL have provided the essential evidence that subclonal genetic diversity exists within functionally defined L-IC (Anderson et al., 2011; Clappier et al., 2011; Notta et al., 2011). Elegant single cell FISH studies established that the diagnostic tumor contained genetic subclones and provided evidence for the evolutionary relationship between them. In all three studies, diagnostic samples were transplanted into xenograft recipients and since only L-IC are able to initiate leukemic propagation, the genetic makeup of the xenografts reflected the genotype of the L-IC(s) that were transplanted. Individual mice transplanted with cells from the same sample were shown to contain genetically distinct subclones, proving that genetic diversity exists among L-ICs.

Figure 4. Failure to Separate Genetic Subclones May Confound Conclusions Regarding Source of Cancer Heterogeneity

Left panel shows that if cancer cells are not separated into distinct genetic subclones before they are tested for the presence of a hierarchical organization, then T-IC measurement may not reflect the complexity of the parental cancer. Right panel shows that cells from the purple clone have a high number of engrafting cells irrespective of a marker that is used to prospectively purify T-ICs. On the contrary, T-ICs can be prospectively purified from the green clone. Of note, the clones depicted in yellow and blue contain T-IC but in this example they are not positive for the marker of choice. This highlights that multiple markers may be required to identify T-ICs from distinct genetic subclones.

Unification of the CSC and Clonal Evolution Models

From a conceptual standpoint it is clear that therapy failure and recurrence are not simply due to the acquisition of new mutations. Rather, the surviving tumor cells must also have regenerative potential in order to regrow the tumor; cells contributing to recurrence must behave like T-IC. Further, tumors are dynamic entities: cells are dying, proliferating, or entering dormancy. Thus, static genetic analysis of bulk tumor tissue or single-cell topological sampling of different tumor sites cannot formally prove that any particular genetically distinct cell or subclone is functionally important. For instance, a laser-captured cell used for genomic analysis might be on a trajectory toward death just before it is sampled and therefore is not relevant for tumor growth. Arguably, the only important cells in a tumor are the ones that are responsible for long-term clonal growth; any other cell ultimately leads to clonal exhaustion. As such, clone-propagating cells represent the unit of selection for the tumor (Greaves, 2013). As we argue above, the hallmark of such a cell is the capacity to self-renew, as without self-renewal clonal exhaustion is inevitable. Thus, a critical question that arises from the many sequencing studies that have described intra-tumoral subclonal diversity is whether diversity exists in long term propagating cancer cells. The best way to test this question is by combining cancer genetic analyses with functional T-IC assays of primary human cancers.
Moreover, the different mutations that distinguished each subclone resulted in variability in functional properties including stemness, L-IC frequency, and aggressiveness of xenograft repopulation. Indeed, some subclones with less aggressive growth properties could only be detected in clonal assays when they were transplanted at limiting dilution, without competition from more aggressive subclones. Characterization of subclonal genetic diversity enabled reconstruction of the evolutionary process reaching back to the ancestral subclone, and demonstrated that functionally distinct subclones were related by branching evolution (Notta et al., 2011). Importantly, these studies showed that the same markers may not identify CSCs in all patient samples of a specific tumor type. Collectively, these data indicate that T-ICs are not static entities but can evolve. When they evolve and acquire additional mutations, the T-IC frequency can increase, indicating that the increasing genetic burden can lead to increased self-renewal as well as interfere with the malignant maturation process. Thus, a dynamic model emerges where early in tumor progression the tumor is a close caricature of the developmental hierarchy of the tissue from which it arose, with a minority T-IC and a high proportion of more differentiated non-T-IC (Figure 2). As T-IC accumulate advantageous mutations, these perturb differentiation processes further and increase self-renewal such that the T-IC expand in the subclone, reading out as having increased frequency in assays. As tumors progress, the mutational burden becomes high resulting in impairment of the remaining maturation programs and even higher capacity for self-renewal, and further expansion of cells possessing T-IC properties. In this model, as cancers progress, tumor hierarchies become shallower within genetic subclones. In some cases, once the clone has progressed to such an advanced state, the frequency of T-IC may be so high that the tumor subclone essentially becomes functionally homogeneous without evidence of a hierarchy. If this model is accurate, it could contribute a plausible explanation for why independent studies of the same tumor type (for example, melanoma) might yield different results between adenocarcinomas driven by K-rasG12D alone, Sca-1 did not significantly enrich for T-IC activity. Furthermore, for adenocarcinomas expressing a mutant human EGFR transgene, only Sca-1-negative cells harbored T-IC activity (Curtis et al., 2010). This study predicts that the same markers may not identify CSCs in all patient samples of a specific tumor type. Importantly, while CSC markers varied between the different genetic tumor models, a hierarchical organization was present within each model, supporting the notion that on top of genetic diversity, nongenetic functional variability governs tumor growth. Thus, these models highlight the influence that the genetic background of a cancer has on CSC properties.

A key finding of these combined genetic and functional studies was that genotype influences L-IC frequency. B-ALL samples with CDKN2A/B mutation had an L-IC frequency that was on average 1,000-fold higher than that of samples without these mutations (Notta et al., 2011). Mouse models with defined genetic lesions also support the conclusion that genotype can influence T-IC properties. For example, three different mouse models of lung cancer showed variability with respect to the phenotype of the T-IC (Curtis et al., 2010). In tumors initiated with activation of oncogenic K-rasG12D and p53 deficiency, cells expressing Sca-1 could be prospectively purified as T-IC. However, in models, a hierarchical organization was present within each model, supporting the notion that on top of genetic diversity, nongenetic functional variability governs tumor growth. Thus, these models highlight the influence that the genetic background of a cancer has on CSC properties.

Collectively, these data indicate that T-ICs are not static entities but can evolve. When they evolve and acquire additional mutations, the T-IC frequency can increase, indicating that the increasing genetic burden can lead to increased self-renewal as well as interfere with the malignant maturation process. Thus, a dynamic model emerges where early in tumor progression the tumor is a close caricature of the developmental hierarchy of the tissue from which it arose, with a minority T-IC and a high proportion of more differentiated non-T-IC (Figure 2). As T-IC accumulate advantageous mutations, these perturb differentiation processes further and increase self-renewal such that the T-IC expand in the subclone, reading out as having increased frequency in assays. As tumors progress, the mutational burden becomes high resulting in impairment of the remaining maturation programs and even higher capacity for self-renewal, and further expansion of cells possessing T-IC properties. In this model, as cancers progress, tumor hierarchies become shallower within genetic subclones. In some cases, once the clone has progressed to such an advanced state, the frequency of T-IC may be so high that the tumor subclone essentially becomes functionally homogeneous without evidence of a hierarchy. If this model is accurate, it could contribute a plausible explanation for why independent studies of the same tumor type (for example, melanoma) might yield different results between...
labs with respect to T-IC phenotype, frequency, etc.; tumors at different stages of progression might not be comparable because their mutational burden differs.

Because mutations arise in both T-IC and non-T-IC populations, and because only T-IC contain long term propagating and self-renewing cells, it is likely that most T-IC arise from the genetic evolution of T-IC and not from the non-T-IC compartment, which lacks self-renewal. However, it is also possible that occasionally mutations will arise in non-T-IC that endow them with self-renewal capacity and convert them into T-IC. Therefore, during progression, the T-IC compartment might be a composite of T-IC generated from evolving T-IC, as well as some newly generated T-IC. Different tumors and mutations might have higher rates of non-T-IC conversion to T-IC than others. Computational simulations have shown that non-T-IC may also be important for the overall robustness of T-ICs, while T-ICs are the units of selection during the evolution of a cancer (Greaves, 2013; Sprouffske et al., 2013). We propose this revised model as a unification of the genetic evolution and developmental/CSC hierarchy models. Indeed, the other nongenetic determinants described above (noise, stochasticity, plasticity, TME) can also be accommodated as mechanisms that can convert non-T-IC into T-IC. Overall, this unified model provides a framework for future studies to determine which tumor types might follow these predictions.

**Challenge with Studying Solid Tumors**

Solid tumor studies pose a particular challenge to capture the subclonal diversity present within the parent tumor. Several studies have shown that genetic subclones are topographically separate (Gerlinger et al., 2012; Yachida et al., 2010). As such, biopsy specimens that are used for research may not be representative of the entire parent tumor. For solid tumors, sampling multiple, different geographical regions from the primary tumor will be important for capturing distinct genetic subclones. This may not be possible for all solid tumors, but it will be instrumental in determining the extent of genetic subclonal variability and it will aid in interpreting concurrent CSC studies. Even if one biopsy is taken, it may represent several genetic subclones that will require separation into individual clones before CSC studies can be carried out on each subclone. While subclonal lineage relationships can be reconstructed using deep sequencing, another method by which subclones can be separated is by transplanting cancer cells at clonal cell doses over multiple recipients (Figure 5). Following engraftment of independent genetic subclones, CSC-related questions can then be addressed in each subclone. Thus, even if the entire tumor specimen is not sampled for genetic analysis, CSC studies will be carried out at least on some of the subclones present in the parent tumor. Studying CSCs in the context of independent subclones is important as all subclones and the corresponding CSCs need to be eradicated for successful therapy.

**Linking Stemness, Prognosis, and Therapy**

If CSCs represent the unit of selection in tumors, as discussed above, then clinically relevant parameters including survival must be more closely related to the properties of CSCs than to non-CSCs. Testing this concept requires understanding the unique molecular circuitry of T-IC as compared to non-T-IC. We have recently reported initial identification of an L-IC-specific transcriptional signature through gene expression analysis of 16 AML samples that were each separated into four fractions and the L-IC activity of each fraction tested in optimized xenograft assays (Eppert et al., 2011). Functional L-IC testing was essential because the cell fraction(s) that contained L-IC were variable for each sample. The L-IC signature was prognostic for overall survival across a wide spectrum of AML patients, providing strong validation of the CSC model and of the clinical relevance of L-IC. In parallel, we carried out analysis of the transcriptional landscape of the entire spectrum of normal human HSC and progenitors. We found that HSC and L-IC gene-expression signatures converged on a shared stemness signature that was also highly prognostic (Eppert et al., 2011). We recently expanded this study and have completed gene-expression profiling of functionally defined L-IC and non-L-IC containing fractions from an additional 84 AML patient samples. Gene-expression analysis of this more representative data set generated a powerful signature that is highly prognostic when tested on approximately 1,000 AML patients in four independent cohorts. The fact that a single signature has such high prognostic power across a diverse spectrum of patients, each with distinct genetic mutations, establishes that stemness is a central biological property or process upon which many driver mutations coalesce. Recently, stemness signatures have been developed from normal stem cells in solid tissues including the intestine and breast (Merlos-Suárez et al., 2011; Pece et al., 2010). Similar to our leukemia results, the stem cell signatures were highly predictive of T-IC content and patient outcome. Overall, these early studies support a link between genetics and stemness and highlight the need to develop more stemness signatures from a wide distribution of tumor types to test the generalizability of this concept.

Strong evidence is emerging to support a link between stemness and therapy resistance in glioblastoma, colon cancer, breast cancer, and numerous other tumors, where studies show that T-IC fractions are more resistant to therapy compared to non-T-IC (Bao et al., 2006; Diehn et al., 2009; Ishikawa et al., 2007; Saito et al., 2010; Viale et al., 2009; Zhang et al., 2010). Indeed, T-IC possess as a number of biological properties that distinguish them from the remainder of tumor cells; not only resistance to treatment (Bao et al., 2006; Li et al., 2008; Tehran-chi et al., 2010) but also evasion of cell death (Majeti et al., 2009; Todaro et al., 2007) and dormancy (Kreso et al., 2013). While many of these papers involve in vitro or xenograft assays, patient data is also accumulating. In patients with 5q- myelodysplastic syndrome (MDS), complete remission can be achieved with lenalidomide treatment but patients invariably relapse. FISH analysis of bone-marrow specimens obtained prior to therapy showed that both progenitor (CD34+CD38+) and L-IC (CD34+ CD38 Thy1+) compartments harbored the 5q– deletion (Tehran-chi et al., 2010). In most patients, the L-IC compartment was resistant to lenalidomide treatment while progenitor cells were eliminated. In one patient with clinically advanced disease, both L-IC and progenitor cell compartments were resistant to treatment, suggesting either that therapy selected for a genetic subclone with an L-IC population that is not marked by the CD34+CD38 Thy1+ surface markers or that with additional mutations non-CSCs gained L-IC properties. Recent studies in
multiple myeloma, a lymphoid malignancy, have also shown that a newly identified L-IC population is resistant to proteasome inhibitor treatment compared to the bulk tumor cells (Leung-Hagensfeijn et al., 2013). Collectively, these studies highlight the interplay between genetics and CSC properties that drive clinical parameters such as therapy response and ultimately survival.

The emerging evidence linking stemness to prognosis and therapy failure suggests that therapeutic targeting of determinants of stemness might be an effective means to eradicate T-IC and prevent recurrence. Although there is still considerable uncertainty as to how stemness is regulated, several regulators including Bmi-1 have been strongly linked to self-renewal and have been implicated in the maintenance of stem cells in several tissues (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003). We have found that human colorectal T-IC function is dependent on BMI-1. Downregulation of BMI-1 inhibits the ability of colorectal T-ICs to self-renew, resulting in abrogation of their tumorigenic potential (Kreso et al., 2014).

Treatment of primary colorectal cancer xenografts with a small molecule BMI-1 inhibitor resulted in colorectal T-IC reduction with long-term and irreversible impairment of tumor growth. These studies point to the need to attempt clinically feasible targeting of this and other predicted components of the self-renewal machinery. Because stemness-associated factors are likely shared between normal stem cells and CSCs, successful eradication of CSCs will require understanding to what extent CSCs differ from normal stem cells to minimize the impact of therapies on normal stem cell function.

The Road Ahead

Over the last several decades, there has been a revolution in our understanding of cancer growth. Advances in sequencing technologies have paved the way to deciphering the tumor genome. It is becoming increasingly clear that a tumor does not have one single tumor genome, but instead comprises multiple genomes that belong to distinct subclones. These subclones may evolve in parallel over the lifetime of a cancer and contribute to intratumoral heterogeneity. However, even within single genetic subclones, not all cells function equally: some cells retain capacity for self-renewal and long-term clonal maintenance, some lay dormant, some fuel tumor growth, and most tumor cells are postmitotic and destined for clearance.

Despite the apparent complexity, there are unifying principles rooted in developmental hierarchies that can guide our approach to targeting cancer. The litmus test for defining a dangerous cancer clone is whether the clone contains cells that exhibit unlimited growth potential. Unlimited growth potential is exhibited by the most primitive cells, which possess stemness properties such as self-renewal. Thus, by understanding stemness properties within tumors, we will be able to gain insight into the most important cells that can drive sequential rounds of tumor growth. Work in several tumor types has shown that cells with stem cell properties are equipped with innate machinery that protects them from radiation and chemotherapy. As well, stem cell gene-expression programs correlate with patient outcome, further supporting the relevance of stemness properties in cancer. By delineating genetic from nongenetic stemness influences, we will be able to tease apart the unique aspects of tumor growth and ultimately gain a unified understanding of how diverse genetic subclones, each with their own superimposed developmental hierarchy, coordinate tumor maintenance.

In nature, evolution creates biodiversity and this in turn makes an entire ecosystem robust. In cancer, diversity within tumor cells at the genetic and functional level together with their coexistence with the microenvironment also increases tumor fitness, allowing tumor cells to offset survival pressures imposed by therapy. More effective therapies will require gaining insight into this diversity.

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