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Tumor heterogeneity and cancer cell plasticity

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Preface

Phenotypic and functional heterogeneity arise among cancer cells within the same tumor as a consequence of genetic change, environmental differences, and reversible changes in cellular properties. Some cancers also contain a hierarchy in which tumorigenic cancer stem cells differentiate into non-tumorigenic progeny. However, it remains unclear what fraction of cancers follow the stem cell model and what clinical behaviors the model explains. In this review we will evaluate the implications of recent lineage tracing and deep-sequencing studies for the cancer stem cell model and the extent to which it accounts for therapy resistance and disease progression.

Introduction

The cancer stem cell model provides one explanation for the phenotypic and functional heterogeneity among cancer cells in some tumors[1–5]. The model posits that some cancers are hierarchically organized into subpopulations of tumorigenic cancer stem cells and their non-tumorigenic progeny. In these cases, cancer stem cells are thought to drive tumor growth and disease progression, perhaps including therapy resistance[6–8] and metastasis[9,10]. However, difficulty replicating solid cancer stem cell markers, variability from patient to patient, and variation in results from different xenograft models have meant that it remains unclear what fraction of cancers follow this model – most, or only a minority[11]?

Even in cancers that clearly contain a hierarchy of tumorigenic and non-tumorigenic cells, this hierarchy must co-exist with other sources of heterogeneity including clonal evolution[12], heterogeneity in the microenvironment [13,14], and reversible changes in cancer cell properties that can occur independently of hierarchical organization[15–18]. Under these circumstances it is not necessarily clear which phenotypic and functional differences among cells arise from which sources of heterogeneity. To what extent do metastasis, therapy resistance, and disease progression reflect intrinsic properties of cancer stem cells as opposed to genetic evolution or other sources of heterogeneity? Integration of results from multiple experimental approaches will be necessary to distinguish the relative contributions of these sources of heterogeneity to disease progression.

New experimental approaches have provided perspective and insight into these questions. Genetic approaches to fate-map the contributions of cancer cells to tumor growth in mice

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have provided evidence in support of the cancer stem cell model in some contexts and evidence against the model in other contexts[19–23]. Since transplantation assays evaluate the potential of cancer cells to form tumors, rather than their actual fate in the native tumor, fate-mapping complements what we have learned from transplantation assays (Figure 1). High-coverage sequencing of human tumors has also provided new insights into genetic heterogeneity within tumors and the cells responsible for relapse after therapy[24–28]. In this review, we will evaluate the implications of these new data for the cancer stem cell model and the extent to which this model accounts for clinically important forms of heterogeneity in cancer.

Testing tumorigenic potential

The central idea in the cancer stem cell model is that tumor growth and disease progression are driven by minority populations of tumorigenic cells, and that many other cancer cells have little or no capacity to contribute to tumor growth. This means that therapeutic strategies should particularly focus on killing the tumorigenic cells. Experimentally, the cancer stem cell model has primarily been tested using transplantation assays, which test the potential of a cancer cell to form a tumor. These assays have demonstrated the existence of phenotypically distinct subpopulations of tumorigenic/leukemogenic and non-tumorigenic/non-leukemogenic cells in a number of human cancers including acute myeloid leukemia (AML)[29,30], chronic myeloid leukemia (CML)[31], breast cancer[32], glioblastoma [6,33], colorectal cancer [34–36], pancreatic cancer [37], and ovarian cancer [38–40]. Operationally, the cells that formed tumors in these studies were rare. Nonetheless, in principle a cancer could have common tumorigenic cells and still be hierarchically organized consistent with the cancer stem cell model[41].

Tumorigenic potential can only be tested in a permissive environment. A persistent concern is that there may be cancer cells that have the potential to contribute to tumor growth and disease progression in patients but that do not have the opportunity to exhibit this potential after transplantation. There are many reasons why a transplantation assay might underestimate the frequency of cancer cells with tumorigenic potential. Human cells must be transplanted into highly immunocompromised mice to escape the powerful xenogeneic immune response that kills human cells in mice. Although a succession of increasingly immunocompromised mice has been used to assay the tumorigenic potential of human cancer cells, from nude, to severe combined immunodeficient (SCID), to non-obese diabetic SCID (NOD/SCID), to NOD/SCID IL2Rγ^{null} mice (NSG), all of these mice retain some xenogeneic immune barrier [42]. There is no opportunity to test whether human cells have tumorigenic potential if they are killed by a xenogeneic immune response. The frequencies of human AML cells[30,43], acute lymphoblastic leukemia (ALL) cells[44,45], melanoma cells[46], and lung cancer cells[47] with leukemogenic or tumorigenic activity are much higher in more highly immunocompromised mice.

Other mechanisms also contribute to an underestimation of tumorigenic potential. Many mouse malignant peripheral nerve sheath tumor (MPNST) cells have tumorigenic potential, but cells of one genotype depend upon exogenous laminin to form tumors while cells of another genotype express laminin cell-autonomously[48]. This raises the arresting possibility

that tumors from different patients may require different assay conditions to read out their full spectrum of tumorigenic cells. There are other cases in which key adhesion molecules or growth factors are required for cells to exhibit clonogenic activity but are not available to human cells in mouse tissues because of the inability of mouse ligands to bind human receptors[49]. Xenotransplanted tumors can also lack the architecture and stroma of tumors growing in native sites[50]. There may be many variables that influence the permissiveness of the environment for tumorigenesis, including uncharacterized factors that have not yet been taken into account in assays.

Given these concerns about xenotransplantation it is reassuring that syngeneic transplantation of mouse cancers has also supported the cancer stem cell model. Studies of cells from mouse germ lineage cancers [51], AML[52,53], chronic myelomonocytic leukemia (CMML)[8], CML[54], breast cancer[55,56], and medulloblastoma[57,58] have all been consistent with the cancer stem cell model. Many of these studies demonstrated that some fractions of cancer cells are enriched for tumorigenic activity even when there is no xenogeneic barrier to engraftment. The cancer stem cell model cannot, therefore, be entirely an artifact of xenotransplantation.

Improved transplantation assay conditions have revealed that some cancers have common leukemogenic/tumorigenic cells (some AMLs[59], many melanomas[15,46,60], some ALLs[59,61,62], and mouse MPNSTs[48]), while other cancers continue to have only rare leukemogenic/tumorigenic cells no matter what assay conditions are used[31,41,47]. More work will be required to determine what fraction of cancers falls in each category.

For all of the reasons described above, it will be critical to continue to optimize transplantation assays to most accurately estimate the spectrum of cancer cells that retain the potential to contribute to tumor growth. This will require systematically testing variations in xenotransplantation assay conditions, including the addition of human cytokines [63] or other variables that might influence human cell engraftment[64]. Many human cancers continue to be transplanted into heterotopic sites in mice without a careful comparison of the consequences of heterotopic versus orthotopic engraftment. It will not be surprising if we are continuing to vastly underestimate the frequency of cells that can contribute to disease progression in some human cancers despite the advances made in xenotransplantation assays over the past several years.

Testing hierarchical organization

Beyond showing a cancer has tumorigenic and non-tumorigenic cells, the other criterion that must be satisfied according to the cancer stem cell model is that the tumorigenic cells give rise to the non-tumorigenic progeny. Without demonstrating a lineage relationship, the functional differences between cells may reflect genetic differences. Tumorigenic human cancer cells from AML [29,30], CML [31], breast cancer [32], glioblastoma [6,33], colorectal cancer [34–36], pancreatic cancer [37], and ovarian cancer [38–40] formed more tumorigenic cells as well as phenotypically distinct non-tumorigenic cells in immunocompromised mice. For example, $CD34^+CD38^-$ AML cells from many patients are not only enriched for leukemogenic activity but also form $CD34^-$ and $CD38^+$ non-leukemogenic cells upon

transplantation[65]. Similar observations were made in mouse models of germ lineage cancers[51], AML[52,53], CMML[8], CML[54], breast cancer[55,56], and medulloblastoma[57]. This is the basis for the idea that cancer stem cells form heterogeneous tumors by undergoing epigenetic changes akin to the differentiation of normal stem cells.

There are also cancers for which there is compelling evidence against the stem cell model. Tumorigenic cells are common and phenotypically diverse in stage III and IV human melanomas[15,46]. Nonetheless, Boiko et al.[66] suggested that only $CD271^+$ melanoma cells can form tumors in immunocompromised mice. Civenni et al.[67] reported that CD271 negative melanoma cells can form tumors in NSG mice but that they cannot form $CD271^+$ progeny or tumors in NOD/SCID mice. In our hands, CD271 negative melanoma cells readily form tumors in both NOD/SCID and NSG mice and all of the tumors are heterogeneous for CD271 expression[15,46]. In an attempt to resolve the inconsistencies we have compared the tumorigenic capacity of $CD271^+$ and CD271 negative melanoma cells isolated from multiple patients using our dissociation protocol[15,46] as well as the dissociation protocols described by Civenni et al.[67] and Boiko et al.[66]. Irrespective of which dissociation protocol we used, whether or not we injected the cells with Matrigel, or whether we transplanted into NOD/SCID or NSG mice, both $CD271^+$ and CD271 negative melanoma cells readily formed tumors and all the tumors were heterogeneous for CD271 (unpublished data). We have now tested over 20 heterogeneously expressed markers in tumors obtained from many patients but have not been able to detect any fraction of melanoma cells that lacks the potential to form a tumor[15,46].

Difficulty reproducing cancer stem cell markers has been a common problem in solid cancer studies. For example, CD133 appeared to robustly distinguish tumorigenic from non-tumorigenic brain tumor cells in early studies[6,33] but a series of subsequent studies found tumorigenic cells in both $CD133^+$ and CD133 negative fractions[68–71]. Since the existence of markers that can distinguish tumorigenic from non-tumorigenic cancer cells is the experimental basis for the conclusion that these cancers follow the stem cell model, the inability to widely confirm markers undermines the evidence supporting the model.

A related problem is that cancer stem cell markers originally characterized in a limited number of tumors have often been assumed to be generalizable. Such markers have often been used in other tumors, or even in cell lines, without independently confirming that the markers were informative in these other contexts. For example, it was clear from the initial study of breast cancer stem cells that the $CD44^+CD24^-$ surface marker phenotype enriched tumorigenic cells in some, but not all, breast cancers[32]. Yet many studies subsequently characterized $CD44^+CD24^-$ breast cancer stem cells in other tumors or cell lines without confirming that these markers distinguished tumorigenic from non-tumorigenic cells in those contexts. Consequently, many studies of cancer stem cell properties are based upon markers of uncertain validity.

Variability among patients

One of the reasons for variability among studies is that markers expressed by tumorigenic cells differ among patients. Early studies in AML indicated that leukemogenic cells were

highly enriched among $CD34^+CD38^-$ cells but not $CD34^+CD38^+$ AML cells [29,30]. More recent studies with larger numbers of samples showed that while the $CD34^+CD38^-$ fraction consistently contains leukemogenic cells, leukemogenic activity is also commonly found in the $CD34^+CD38^+$ and $CD34^-$ fractions[43,65,72]. Approximately half of AMLs have most leukemogenic cells in the $CD34^+CD38^-$ fraction while the other half have most leukemogenic cells in the $CD34^+CD38^+$ fraction[43,65]. Many human AMLs with nucleophosmin mutations have leukemogenic activity exclusively in the $CD34^-$ fraction, while some have leukemogenic activity in both $CD34^-$ and $CD34^+$ fractions[73]. This indicates that differences in mutations can cause differences in the phenotype of leukemogenic cells among patients. The same is true in solid cancers, as $Sca1^+$ cells are enriched for tumorigenic activity in mouse lung adenocarcinomas with *Kras* and *p53* mutations but not in tumors with only *Kras* mutations[74].

Differences among patients may also reflect differences in the cell-of-origin. For example, some medulloblastomas arise in the cerebellum from sonic hedgehog pathway activation in granule neuron precursors and frequently have a poor prognosis[75]. Other medulloblastomas arise in the dorsal brainstem and are highly curable[75]. Similarly, neural progenitors from different regions of the central nervous system form different subtypes of ependymomas with different properties[76]. Both hematopoietic stem cells and restricted myeloid progenitors can serve as the cell-of-origin for AML [53,77–79] but the leukemogenic cells have somewhat different properties in each case [80]. The distinct developmental origins of tumors, both with respect to regional identity and position in the normal tissue hierarchy, contribute to differences among patients in tumorigenic cell properties.

Tumorigenic cell phenotype can also change over time. In some ovarian cancers, only $CD133^+$ cells contain tumorigenic activity whereas in other ovarian cancers tumorigenic cells are in the $CD133^+$ and CD133 negative fractions [40]. Ovarian cancers with only $CD133^+$ tumorigenic cells sometimes give rise to CD133 negative tumorigenic cells upon passaging[40]. If tumorigenic cell phenotypes commonly change upon passaging of tumors, this could explain some of the inconsistencies observed among studies that use small numbers of tumors.

The frequency of tumorigenic cells in some cancers also varies widely among patients. Side-by-side studies of AMLs from different patients revealed frequencies of leukemogenic cells in the $CD34^+CD38^-$ cell fraction that varied 1000-fold [65]. B-ALLs from different patients had frequencies of leukemogenic cells that varied 100-fold [44]. Ovarian cancers from different patients had tumorigenic cell frequencies that varied almost 1000-fold [40]. It remains uncertain to what extent this reflects biological variability in the frequency of cells that can contribute to tumor growth within patients as opposed to variability in the extent to which transplantation assays are permissive for tumorigenesis by cells of different genotypes. The variability in the frequency and identity of tumorigenic cells between patients shows that markers identified in one tumor cannot be assumed to distinguish cancer stem cells in other tumors or in other contexts.

A key question raised by the differences among patients is whether tumors of the same type differ in the extent to which they are hierarchically organized. For example, one possibility

is that all breast cancers follow a stem cell model even though existing markers do not distinguish tumorigenic from non-tumorigenic cells in some tumors. Another possibility is that only a subset of breast cancers follows the stem cell model. Or perhaps the hierarchy is steep in one subset of each cancer, with rare tumorigenic cells that give rise to abundant non-tumorigenic cells, and shallow in another subset of each cancer, with common tumorigenic cells that form small numbers of non-tumorigenic cells (Figure 2). Until enough tumors are carefully studied to observe consistent patterns among patients with particular subtypes of disease, tumors will have to be tested individually to determine whether tumorigenic cells are common or rare and whether markers can distinguish tumorigenic from non-tumorigenic cells.

Fate versus potential in tumors in vivo

Which cells actually contribute to the growth and progression of tumors in vivo? Most cancer stem cell studies are designed to assess the identity of cancer cells with the potential to contribute to tumor growth. A separate question relates to the identity of cells fated to contribute to the growth and progression of specific tumors (Figure 1). The question of fate addresses what cells actually do in a specific circumstance while the question of potential addresses what cells can do under permissive conditions.

Some cells that have the potential to drive tumor growth do not actually do so in the native tumor because they are not in a permissive environment, they are killed by immune cells, or by therapy. In the native tumor slowly proliferating clones may be at a competitive disadvantage to rapidly proliferating clones and therefore may not contribute much to tumor growth. However, these slowly proliferating clones may form tumors after transplantation. Environmental cues from stromal cells can restrict the growth of cancer cells in the native tumor environment [81], but the absence of these cues may permit the same cells to form tumors after transplantation. In some circumstances, a high percentage of cells with tumorigenic potential may contribute to tumor growth (Figure 1). In other circumstances, there may only be a small percentage of cells with tumorigenic potential that actually contribute to tumor growth.

One context in which the fate of tumorigenic cells can be tracked is after xenotransplantion of human cancer cells. Primary human colorectal cancer cells were marked by lentiviral infection and the relative abundance of distinct clones was tracked as tumor cells were serially transplanted [82]. Individual clones differentially contributed to tumor growth over time: some were always abundant, some were abundant then became rare, and some were rare then became abundant. When human B-ALL cells from a single patient were transplanted at limiting and non-limiting cell doses, different dominant clones emerged in each recipient mouse[44]. Leukemogenic clones thus do not contribute equally over time after transplantation.

Lineage tracing experiments have recently been performed in mouse models of benign tumors to test whether many, or few, cells contribute to their growth [20,21]. Mice bearing benign papillomas were treated with low doses of tamoxifen, allowing Keratin14-CreER to recombine a conditional reporter in a small percentage of papilloma cells[20]. These rare

marked clones were tracked over time to assess their contribution to tumor growth. The frequency of clones declined over time, with only 20% persisting after seven weeks. Non-persisting clones appeared to be lost through terminal differentiation. The average size of persisting clones increased over time: by seven weeks the clones ranged from hundreds to thousands of cells. These observations demonstrate that only a minor subpopulation of tumor cells drives papilloma growth, though the rate at which these cells divide is increased relative to what is observed in the normal epidermis [83]. Benign papillomas are therefore hierarchically organized, consistent with the stem cell model, though this is perhaps not surprising for a benign tumor.

The same study also fate mapped cells in tumors after they progressed to squamous cell carcinomas[20]. Cells in these tumors were more highly proliferative, more undifferentiated, and formed larger clones as compared to cells in benign papillomas. A much greater percentage of cells sustainably contributed to the growth of squamous cell carcinomas. The squamous cell carcinomas in this study, therefore, have only a shallow hierarchy, with few non-tumorigenic cells (Figure 2). Additional studies are required to determine whether there is any hierarchical organization among the persisting clones - perhaps some have more proliferative potential than others. Overall, the data suggest that as benign adenomas progress to carcinomas, the hierarchy becomes more shallow and more cells can contribute to tumor growth.

Schepers et al. used a multicolor conditional reporter to track the contribution of *Lgr5*$^{+}$ cells to the growth of pre-malignant intestinal adenomas[21]. *Lgr5* expression marks normal stem cells in the intestinal crypt [84]. Administration of tamoxifen activated Lgr5-CreER recombinase. This deleted the *APC* tumor suppressor in *Lgr5*$^{+}$ cells, forming adenomas as well as activating the expression of a multicolor reporter in *Lgr5*$^{+}$ cells and their progeny [21]. Administration of a second pulse of tamoxifen reactivated Cre recombinase, recombining the multicolor reporter again, leading to a color switch within some *Lgr5*$^{+}$ cells in the adenomas. *Lgr5*$^{+}$ cells in the normal epithelium are thus capable of giving rise to adenomas and the *Lgr5*$^{+}$ cells within the adenomas can contribute extensively to tumor growth. Since most of the progeny were *Lgr5* negative cells it was speculated that *Lgr5*$^{+}$ adenoma stem cells give rise to *Lgr5* negative cells with little proliferative capacity; however, the *Lgr5* negative cells have not yet been fate mapped and it is unknown whether they have less ability to contribute to tumor growth.

A recent study shows that *Lgr5* negative cells can also act as the cell-of-origin for intestinal adenomas in the context of Wnt pathway activation and inflammation. Adenomas that arise in this context do not, based on currently available data, appear to follow the cancer stem cell model[22]. The *Lgr5* negative cells in these tumors give rise to *Lgr5*$^{+}$ cells, but both *Lgr5* negative cells and *Lgr5*$^{+}$ cells can form spheroids in culture and tumors in vivo with similar efficiency. This argues that at least some intestinal adenomas are not hierarchically organized into *Lgr5*$^{+}$ tumorigenic cells and *Lgr5* negative non-tumorigenic cells. Additional studies of the *Lgr5*$^{+}$ and *Lgr5* negative cell fractions from adenomas in more genetic backgrounds will be required to assess what fraction of adenomas exhibit hierarchical organization. It will also be important to test whether adenomas that do exhibit hierarchical

organization continue to exhibit hierarchical organization after they progress to malignancies.

These lineage-tracing experiments thus provide limited support for the cancer stem cell model. Although the growth of benign skin papillomas was driven by a minor subpopulation of cells, a much larger fraction of cells contributed to the growth of squamous cell carcinomas[20]. A key question now is whether some of the persistent clones that exhibit ongoing contribution to tumor growth might nonetheless have limited tumorigenic potential in transplantation assays. The data on intestinal adenomas also offer limited support for the cancer stem cell model as both *Lgr5*$^{+}$ and *Lgr5* negative cells have the ability to serve as the cell-of-origin and to propagate tumors upon transplantation, at least in certain genetic backgrounds. Ultimately, it will be necessary to integrate the data from both transplantation studies and fate mapping studies in significant numbers of human and mouse tumors to understand the biological diversity.

Testing fate through selective cell ablation

The selective ablation of genetically defined subsets of tumor cells is another approach to test which cells are fated to contribute to tumor growth or disease progression in the native tumor environment. A recent study addressed the role of *Nestin* positive cells in the maintenance of a mouse model of glioma by ablating these cells [19]. GFP and the herpes simplex virus thymidine kinase (HSV-TK) were expressed under the control of the *Nestin* promoter such that HSV-TK positive cells could be selectively killed upon administration of ganciclovir. *Nestin*-expressing, GFP^{+} glioma cells were relatively quiescent and represented a minority of cells in the gliomas. Administration of ganciclovir modestly extended the lifespan of mice, indicating that the *Nestin*$^{+}$ cells contribute to tumor growth. Importantly, when tumors were reduced in size by treatment with the chemotherapeutic temozolomide, pulse chase experiments suggested that tumor regrowth originated from the *Nestin*$^{+}$ fraction of tumor cells. Co-administration of temozolomide and ganciclovir significantly slowed tumor re-growth; however, it was impossible to assess the long-term effects of eliminating the *Nestin*$^{+}$ cells because mice independently developed lethal tumors unrelated to the original tumor. Consequently, it remains unclear if the *Nestin*$^{+}$ cells are exclusively responsible for driving tumor growth and recurrence after therapy or if the *Nestin* negative cells also contribute. It would be particularly interesting to selectively ablate the Nestin negative cells to determine whether this also slows tumor growth and extends mouse lifespan.

Reversible plasticity among cancer cells

Some cancer cells reversibly transition among states that differ in their competence to contribute to tumor growth[85]. For example, some cancer cells can reversibly transition between epithelial and mesenchymal states and there is evidence that breast cancer cells in the mesenchymal state are more competent to form tumors[9]. Melanoma cells reversibly turn on and off the JARID1B histone demethylase, and JARID1B-expressing cells are more competent to sustain tumor growth[16]. Many other markers are reversibly turned on and off within lineages of melanoma cells in a manner that does not correlate with the ability to

form a tumor[15,17]. Exposure of glioma cells to perivascular nitric oxide reversibly promotes their ability to form tumors[13]. The evidence that some cancer cells can undergo reversible changes in their competence to form tumors offers an alternative explanation for the increased tumorigenic potential of subsets of cancer cells independent of the differentiation of cancer stem cells.

Drug resistance is also a plastic property of some cancer cells, with rare subpopulations in cancer cell lines that exhibit resistance to a variety of therapeutics, reversibly forming sensitive or resistant progeny depending on whether the cells are passaged with or without the therapeutic[18]. This raises the possibility of intrinsic therapy resistance mechanisms that are not necessarily associated with a static hierarchy or an undifferentiated state.

It is critical to distinguish models in which intrinsic differences in tumorigenic capacity reflect reversible changes in cell state as compared to irreversible differentiation as these models make very different experimental and clinical predictions (Figure 3). If the heterogeneity within tumors reflects cells that reversibly and efficiently transition between tumorigenic and non-tumorigenic states[9,85] it may not be possible to experimentally identify any population that lacks tumorigenic potential (Figure 3f). Furthermore, it would remain necessary to eliminate all cancer cells during therapy, as even the non-tumorigenic cells could drive disease recurrence by giving rise to tumorigenic cells (Figure 3i). In contrast, if heterogeneity reflects hierarchical organization in which cancer stem cells irreversibly differentiate into non-tumorigenic cells, then therapies that eliminate cancer stem cells should be necessary and sufficient to cure disease (Figure 3a, g). This distinction is thus critical to understand the underlying biology and to develop more effective therapies. Importantly, almost all of the existing evidence for reversible transitions between tumorigenic and non-tumorigenic states comes from studies of cells in culture, often cell lines, so it remains uncertain to what extent this is observed within spontaneously arising cancers in vivo.

Genetic heterogeneity within tumors

The conclusion that cancer stem cells can recapitulate the heterogeneity of the tumors from which they derive has consistently been based on analyses of small numbers of surface markers, calling into question the degree to which there is genetic heterogeneity within tumors that is not recapitulated after the transplantation of tumorigenic cells[86]. If genetic heterogeneity within tumors is low, then the differentiation of cancer stem cells into non-tumorigenic progeny could be the major driver of heterogeneity (Figure 4). Conversely, if genetic heterogeneity is extensive, every tumorigenic cell could form a genetically distinct tumor rather than recapitulating the tumor from which it derives. In tumors with extensive genetic heterogeneity, phenotypic and functional differences among cells cannot be assumed to reflect the differentiation of cancer stem cells - they could reflect genetic differences.

With these questions as a backdrop it is interesting to consider the implications of recent deep sequencing studies. Deep sequencing has been used to examine the genetic heterogeneity within tumors, the subclonal composition of tumors, and the evolutionary relationships of mutations during disease progression. Deep sequencing cannot directly test

the cancer stem cell model. However, the frequencies of allelic variants in bulk tumor cells can be used to quantify the relative contribution of different clones to tumors. Even neutral passenger mutations can be informative because by following the contribution of the cells bearing these mutations to tumor growth and disease progression we gain insights into the fates of individual cancer cells and their progeny. Data from AML[25,87], CLL[88], breast cancer [27,89,90], renal cell carcinoma [28,91], and pancreatic cancers [92–94] show surprisingly extensive genetic heterogeneity. Extensive genetic heterogeneity provides many opportunities for genetic changes to confer phenotypic and functional heterogeneity within tumors that is not addressed by the cancer stem cell model (and that may complicate the testing of the model; Figure 3c, f, i).

It has long been understood that cancer cells undergo clonal evolution in which mutations occur stochastically in individual cancer cells and then are subject to positive or negative selection depending on whether they confer a competitive advantage or disadvantage[12,95]. Cancer stem cells are no exception. Leukemogenic ALL cells obtained from the same patient exhibit genetic heterogeneity and undergo genetic changes over time when passaged in mice [24,44]. It is not clear whether these ALLs follow the cancer stem cell model as leukemogenic cells are common in some ALLs and it has proven difficult to identify any clear hierarchical organization [61,96]. Human colorectal tumors have also been serially transplanted in mice and their genetic heterogeneity assessed [82]. Only a small number of de novo genetic variants were detected in serially transplanted tumors as compared to primary patient samples. Since there is compelling evidence that colorectal cancers are hierarchically organized into tumorigenic and non-tumorigenic components [34–36], these results show that genetic changes do occur in colon cancer stem cells, though the paucity of such changes raises the possibility that the rate of mutagenesis may be suppressed in those cells. There is no inherent inconsistency between the cancer stem cell model and the clonal evolution model[4] (Figure 5).

Therapy resistance

Tumorigenic cells in certain cancers have been observed to be intrinsically resistant to certain therapies. For example, tumorigenic glioblastoma [6] and breast cancer [7] cells were enriched after irradiation of xenografts. A similar enrichment of tumorigenic cells was observed in cyclophosphamide-treated colorectal tumors [97]. Activation of ATM-dependent DNA-damage repair in tumorigenic glioblastoma cells [6] and decreased reactive oxygen species in tumorigenic breast cancer cells [7] may explain the therapy resistance of these cells.

Although it is sometimes suggested that cancer stem cells can be defined by therapy resistance, this is not true in any general sense. Differentiation therapies specifically target cancer stem cells by exploiting their capacity to differentiate. Acute promyelocytic leukaemia (APL) is treated with arsenic trioxide and trans-retinoic acid to induce terminal differentiation, growth arrest, and apoptosis by clonogenic APL cells [98]. Mouse glioblastoma stem cells can be induced to differentiate into glia by treating with bone morphogenetic protein 4 (BMP4), reducing proliferation, tumor growth, and tumorigenic cell frequency [99]. BMP4 also promotes glial differentiation by normal CNS stem cells [100] suggesting that tumorigenic cancer cells sometimes inherit differentiation pathways from

normal stem cells in the same tissue. Cis-retinoic acid improves survival in high-risk neuroblastoma patients [101,102] by inducing the differentiation of undifferentiated neuroblastoma cells. Thus, tumorigenic cells are more sensitive to some therapies and less sensitive to other therapies as compared to non-tumorigenic cells.

Genetic changes clearly confer therapy resistance in some circumstances[103]. Sequential genetic analysis of cancers before therapy and after relapse have been consistent with this. In ALL [24,26], AML [25], and CLL [88,104], minor subclones prior to therapy often become dominant after therapy. If the inherent therapy resistance of cancer stem cells were the major determinant of survival during therapy then dominant clones prior to therapy would be likely to remain dominant after therapy. The observation that therapy selects for minor subclones suggests that either survival is stochastic (many cells have a similar low probability of surviving), non-cell-autonomously determined, or determined by genetic differences among subclones.

When the dominant pre-therapy clone remains dominant after relapse, the dominant clone often gains de novo mutations [25,26,88]. Relapse-specific mutations may confer therapy resistance. For example, recurrent relapse-specific mutations were identified in the gene NTC52 in 10–20% of T-ALLs[105,106]. NT5C2 is a 5′ nucleotidase that can inactivate nucleoside analogs, such as the chemotherapy drugs 6-mercaptopurine and 6-thioguanine used to treat ALL. The relapse-specific *NTC52* mutations are predicted to be gain-of-function mutations that enhance enzymatic activity[105,106]. Certain genetic changes are likely sufficient for therapy resistance.

The intrinsic resistance of certain cancer stem cells to certain therapies likely collaborates with genetic change to enable disease progression (Figure 6). CML follows a cancer stem cell model as CML stem cells form terminally differentiated myeloid cells during chronic phase [107]. CML stem cells are inherently resistant to imatinib [54,108–110]. Cells with features of CML stem cells persist after therapy, even in patients who achieve a complete cytogenetic remission [111]. Imatinib is thought to restore the health of CML patients primarily by eliminating differentiated CML cells. This profoundly reduces leukemia burden but leaves CML stem cells lurking in the patients, ready to re-establish the disease upon discontinuation of imatinib[112,113]. As long as patients are maintained on imatinib they remain healthy, until a mutation arises in *BCR-ABL* that confers imatinib resistance [114,115]. In this way, the inherent imatinib-resistance of CML stem cells allows the disease to smolder along in remission, but a genetic change is required for more robust imatinib resistance and disease progression in the face of therapy.

Perspective

Cancer stem cell properties have been suggested to explain diverse unsolved clinical problems yet these predictions have often not been carefully tested. The roles played by other sources of heterogeneity (such as genetic) in disease progression have often not been factored into such claims. Moreover, difficulties confirming solid cancer stem cell markers have undermined efforts to confirm their existence in some cancers and to study their biology. It remains unclear to what extent these difficulties reflect variation in marker

expression by cancer stem cells from different patients versus misguided efforts to apply the stem cell model to cancers that lack hierarchical organization. The tendency not to publish data that are difficult to interpret or inconsistent with the model exaggerates the extent to which cancer stem cell markers are conserved among patients. It is time to critically test the model and its predictions, to acknowledge when the data don't fit the model, and to integrate the data with other sources of heterogeneity when they do.

Tumorigenic cells are rare and phenotypically distinct in some cancers. In other cancers, tumorigenic cells are common and phenotypically diverse with no clear hierarchical organization. We do not yet know what fraction of cancers follow the stem cell model.

As we develop a more complete understanding of genetic heterogeneity within tumors there may be some cancers in which genetic heterogeneity is the major driver of phenotypic and functional heterogeneity. In some cancers with pervasive genetic heterogeneity it may not be possible to rigorously test the cancer stem cell model as genetic differences within and between tumors may make it impossible to identify any reproducible hierarchical organization, even if there is differentiation to post-mitotic progeny. Some cancers may have epigenetic heterogeneity that is not well described by the cancer stem cell model. Indeed, a general question concerns the extent to which the phenotypic and functional properties of cancer cells undergo reversible changes. New models of cancer heterogeneity/plasticity may emerge.

In cancers that do not follow the stem cell model it will be important to demonstrate this, to avoid fruitlessly focusing on small subpopulations of cancer cells that have no more capacity to drive disease progression or therapy resistance than other cancer cells. In cancers that do follow the stem cell model, it will be important to clarify the markers that can be used to identify these cells and the contexts in which they work. It will also be critical to integrate our understanding of the biology of these cells with our understanding of other sources of heterogeneity to develop a realistic view of how each contributes to disease progression. For example, it is possible in some cancers that clones with a hierarchy of tumorigenic and non-tumorigenic cells may co-exist in the same tumors with clones that have lost their hierarchical organization as a consequence of additional mutations. This would profoundly complicate the testing of the model and undermine the extent to which it can explain clinical behavior.

- Cancer stem cell markers will have to be tested in larger numbers of patients[15,40,65] to account for heterogeneity among patients and to determine whether certain markers are more reliable in certain subsets of patients. This will provide insight into whether only certain subtypes or stages of disease follow the cancer stem cell model. Studies should not assume that markers that distinguish tumorigenic from non-tumorigenic cells in one tumor will do so in other tumors.
- To date, there is no evidence that any combination of cancer stem cell markers isolates any cancer stem cell population to a high degree of purity. The inability to purify any cancer stem cell is a profound impediment to characterizing the biology of these cells with precision. Until a high degree of purity can be demonstrated,

claims related to the cell cycle distribution and gene expression profiles of “cancer stem cells” will be of uncertain validity.

- It will be informative to perform lineage tracing[20,21] and selective cell ablation experiments[19] in more cancers and at more stages of disease to assess whether many, or few, cells are fated to contribute to tumor growth and disease progression.
- Studies of genetic heterogeneity should be integrated with studies of tumorigenic potential to develop a more realistic understanding of the extent to which individual tumorigenic cells recapitulate the heterogeneity of the tumor from which they derive. In many cancers that follow the stem cell model, tumorigenic cells may reproduce the cellular hierarchy but not the genotype of the tumor-of-origin.

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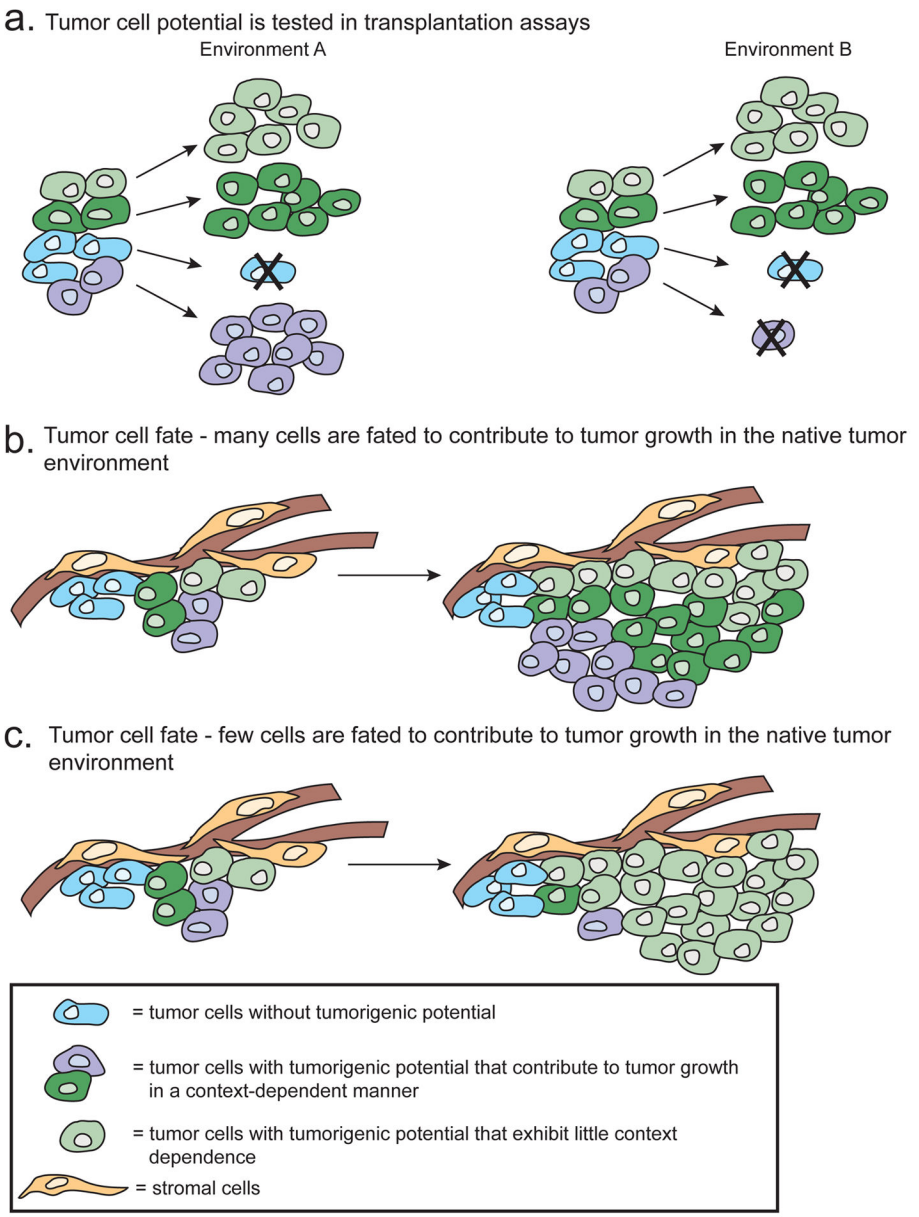

Figure 1. Cancer cell fate versus potential

a, Transplantation assays assess the potential of cancer cells to form tumors. The ability of a cell to form a tumor is context dependent: cells that can form a tumor under one set of conditions may not form a tumor in other conditions. For this reason, tumorigenesis assays must be conducted under the most permissive possible conditions so as not to underestimate the spectrum of cells with tumorigenic potential. Factors such as the site of injection, the genetic background of recipient mice, and co-injection of extracellular matrix all influence the ability of cells to form tumors. Optimization of these and other parameters can substantially increase the frequency of tumorigenic cells detected in various cancers[30,43,44,46,47]. **b** and **c,** Lineage tracing or fate-mapping assays assess the actual fate of tumor cells in a particular context, often the native tumor environment. Thus, while potential

measures what a cell can do under permissive conditions, fate measures what a cell actually does in a particular context. Some cells with tumorigenic potential do not actually contribute to tumor growth – for example because they are in a non-permissive environment or because they are eliminated by immune effector cells. An important question is whether many (**b**) or few (**c**) cells with tumorigenic potential actually contribute to tumor growth. It will be important to integrate transplantation studies of tumorigenic potential with studies of cell fate in the native tumor environment to assess the extent to which the cancer stem cell model describes the growth and progression of individual cancers.

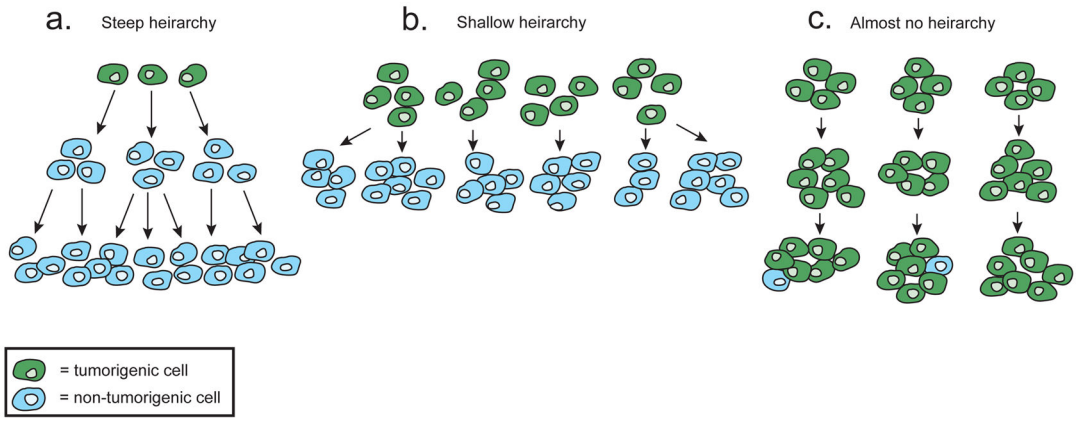

Figure 2. Various cancers may be hierarchically organized into subpopulations of tumorigenic and non-tumorigenic cells but some hierarchies may be steep (a), with only rare tumorigenic cells, while other hierarchies may be shallow, with common tumorigenic cells (b) or even rare non-tumorigenic cells (c)

As hierarchies become increasingly shallow, the value of distinguishing between tumorigenic and non-tumorigenic cells to understand cancer biology and improve therapy declines.

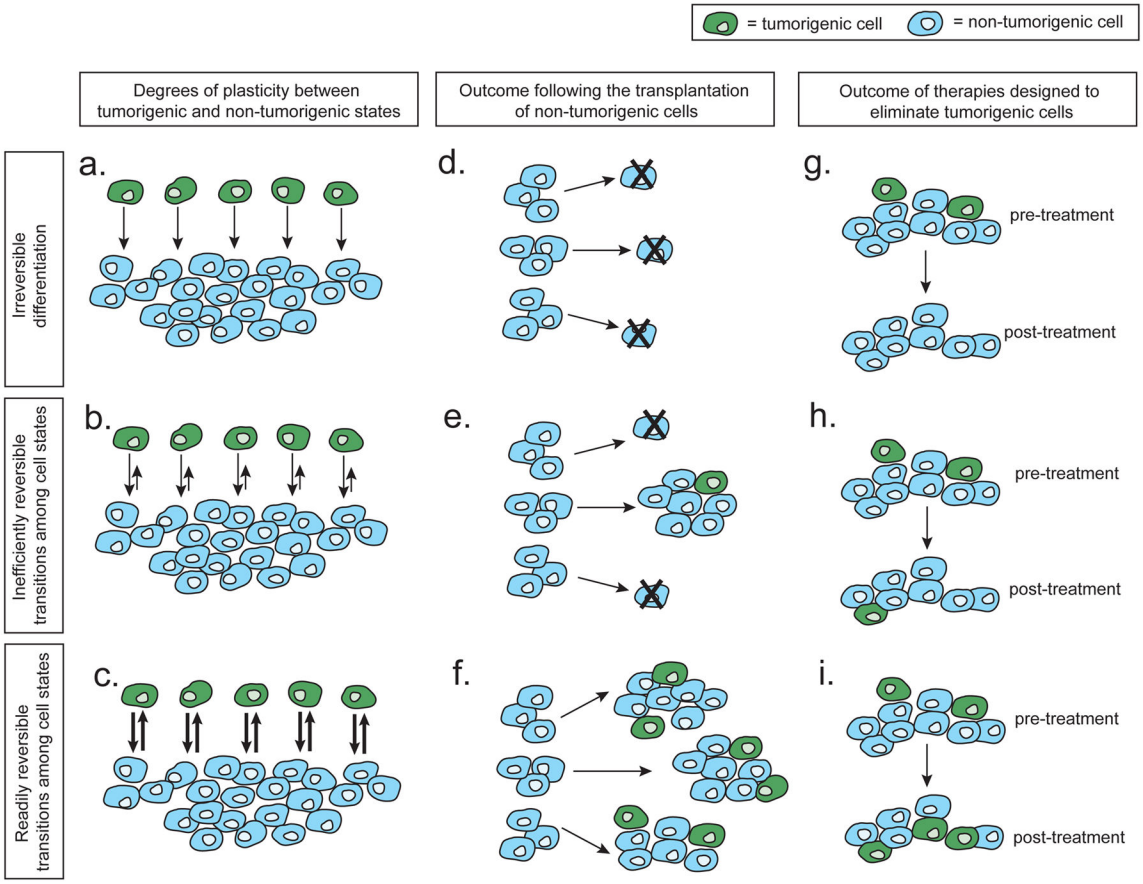

Figure 3. Potential forms of plasticity among tumorigenic and non-tumorigenic cells yield different predictions with respect to transplantability and therapy response

a–c. The differentiation of tumorigenic cells into non-tumorigenic progeny may be irreversible (**a**), inefficiently reversible (**b**), or readily reversible (**c**). **d–f.** This degree of plasticity within cancer cell hierarchies influences the outcome of transplantation assays. If differentiation is irreversible, non-tumorigenic cells should not form tumors after transplantation (**d**). If differentiation is inefficiently reversible, non-tumorigenic cells will inefficiently form tumors after transplantation (**e**). If cells efficiently and reversibly transition between tumorigenic and non-tumorigenic states then cells in the non-tumorigenic state should nonetheless form tumors after transplantation. Under these circumstances, transplantation assays may not be able to distinguish between cells in tumorigenic and non-tumorigenic states and it may not be experimentally possible to distinguish this model from tumors that are composed entirely of tumorigenic cells (**f**). **g–i,** Plasticity within cancer cell hierarchies also influences the predicted outcome of therapies that ablate tumorigenic cells. If differentiation is irreversible, therapy will convert a hierarchically organized malignancy to a benign tumor containing only non-tumorigenic cells (**g**). If differentiation is inefficiently reversible, a single round of therapy will deplete but not eliminate tumorigenic cells (**h**). If cells efficiently transition between tumorigenic and non-tumorigenic states then a single round of therapy will have little effect on tumorigenic cell frequency (**i**).

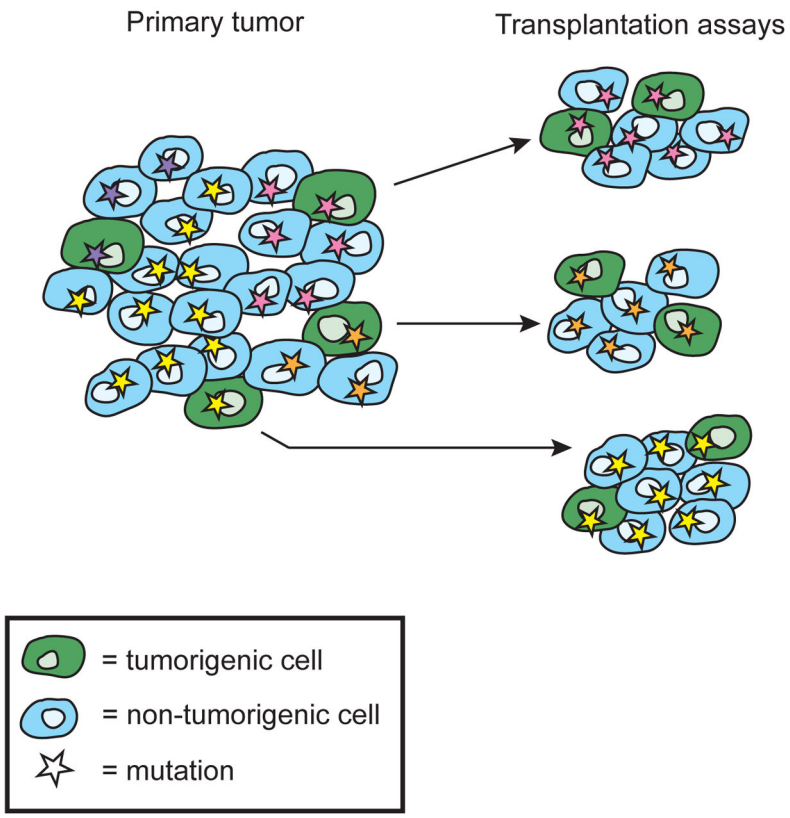

Figure 4. Tumorigenic cells cannot recapitulate the heterogeneity of the tumors from which they derive if those tumors have extensive genetic heterogeneity

If every tumorigenic cell carries a combination of common and unique mutations then none of these cells will recapitulate the genetic heterogeneity of the tumor from which they derive - they will all give rise to genetically distinct tumors upon transplantation. They still may give rise to hierarchically organized tumors with tumorigenic and non-tumorigenic components, as in the tumor of origin. Nonetheless, if the genetic heterogeneity involves mutations that influence cancer cell phenotype or function the genetic heterogeneity will contribute to tumor heterogeneity through mechanisms independent of cancer stem cell differentiation.

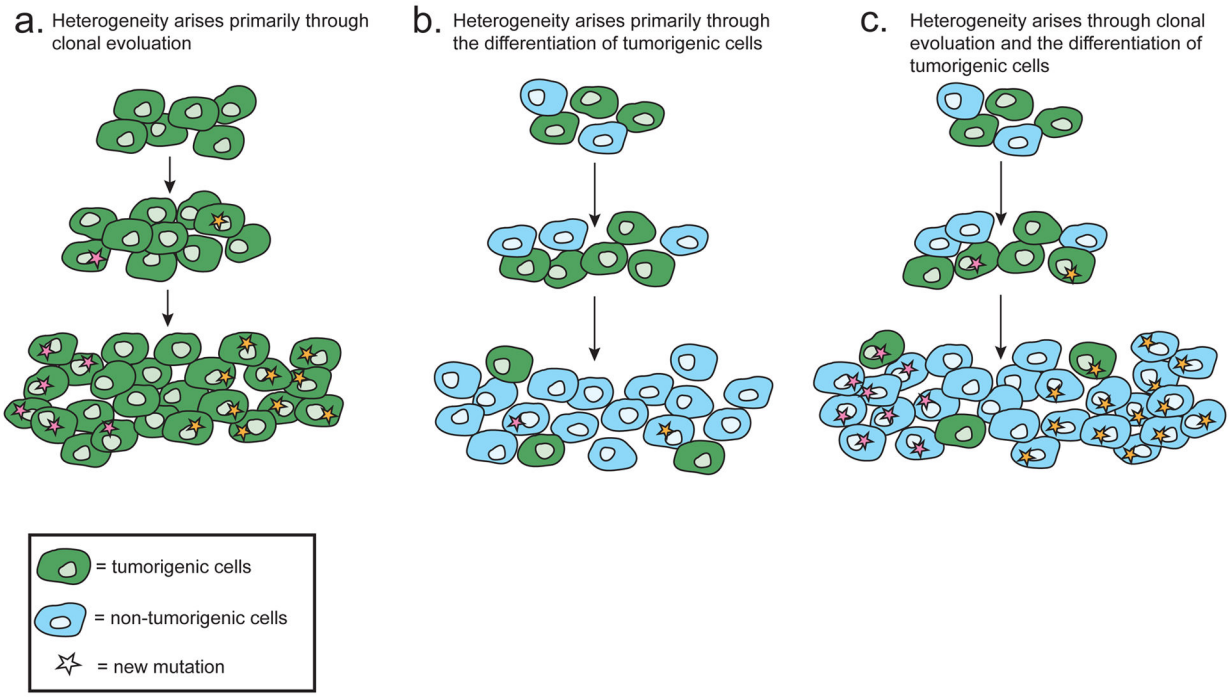

Figure 5. Clonal evolution and the differentiation of tumorigenic cells into non-tumorigenic cells can independently, or jointly, contribute to tumor heterogeneity

a, New mutations can increase the heterogeneity within tumors as long as the mutations influence cell phenotype or function. **b**, The differentiation of tumorigenic cells into non-tumorigenic progeny creates heterogeneity within tumors. New mutations that occur in non-tumorigenic cells would not be propagated (unless they restore tumorigenic potential). **c**, If mutations occur in tumorigenic cells, then both clonal evolution and the differentiation of tumorigenic cells into non-tumorigenic progeny contribute to tumor heterogeneity. This is likely what occurs in cancers that follow the stem cell model. This means that phenotypic and functional differences cannot automatically be ascribed to epigenetic differences among tumorigenic and non-tumorigenic cells as genetic heterogeneity may contribute to some of those differences.

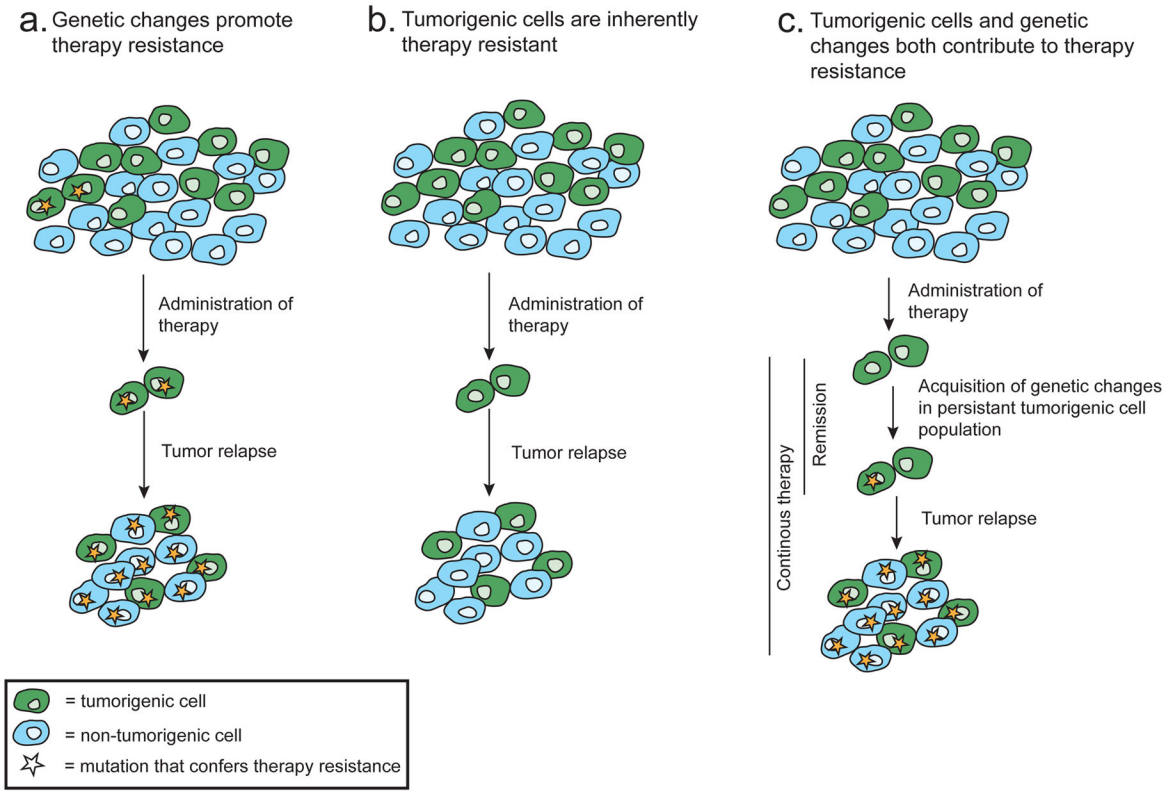

Figure 6. Genetic changes and the inherent properties of tumorigenic cells can independently, or jointly, contribute to therapy resistance

a, Genetic alterations can confer therapy resistance (e.g. ref[105]). **b**, Tumorigenic cells in certain cancers are inherently resistant to certain therapies[6,7,97]. **c**, Tumorigenic cells may persist despite therapy but may not be able to cause disease relapse due to an inability to regenerate significant numbers of non-tumorigenic cells in the presence of therapy. The acquisition of de novo mutations may enhance therapy resistance, enabling relapse and disease progression. CML stem cells are thought to be inherently imatinib resistant, and to persist in the presence of imatinib until they acquire an imatinib resistance mutation and progress to blast cell crisis[112–115].