Cancer Cell

A Decade of Discovery

Cancer Stem Cells:
Impact, Heterogeneity, and Uncertainty

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The differentiation of tumorigenic cancer stem cells into nontumorigenic cancer cells confers heterogeneity to some cancers beyond that explained by clonal evolution or environmental differences. In such cancers, functional differences between tumorigenic and nontumorigenic cells influence response to therapy and prognosis. However, it remains uncertain whether the model applies to many, or few, cancers due to questions about the robustness of cancer stem cell markers and the extent to which existing assays underestimate the frequency of tumorigenic cells. In cancers with rapid genetic change, reversible changes in cell states, or biological variability among patients, the stem cell model may not be readily testable.

Sources of Heterogeneity within Cancer

Many tumors contain phenotypically and functionally heterogeneous cancer cells (Fidler and Hart, 1982; Fidler and Kripke, 1977; Heppner, 1984; Nowell, 1986). This heterogeneity among cancer cells in the same patient can arise in multiple ways. The most well-established mechanism involves intrinsic differences among cancer cells caused by stochastic genetic (Nowell, 1976) or epigenetic (Baylin and Jones, 2011) changes (clonal evolution; Figure 1A). Differences can also arise among cancer cells through extrinsic mechanisms in which different microenvironments within a tumor confer phenotypic and functional differences upon cancer cells in different locations (Figure 1B) (Polyak et al., 2009; Bissell and Hines, 2011). Finally, some cancers follow a stem cell model in which tumorigenic cancer stem cells "differentiate" into nontumorigenic cancer cells, creating a hierarchical organization (Figure 1C; Table 1) (Dick, 2008; Reya et al., 2001; Shackleton et al., 2009). The differentiation of cancer stem cells provides a mechanism for generating phenotypic and functional heterogeneity beyond the heterogeneity that can be attributed to clonal evolution or environmental differences (Figure 1D). However, the fact that heterogeneity can arise through multiple mechanisms means that heterogeneity alone does not imply the existence of a cancer stem cell hierarchy.

The Cancer Stem Cell Model

The cancer stem cell model is not a new idea (Hamburger and Salmon, 1977). It has been clear for decades that some cancers, including some germ lineage cancers (Kleinsmith and Pierce, 1964), some neuroblastomas (Shimada et al., 1984), and some myeloid leukemias (Fearon et al., 1986; Ogawa et al., 1970), can differentiate into progeny that have limited proliferative potential despite retaining the oncogenic mutations of their malignant progenitors.

Some germ lineage cancers contain rapidly dividing cells that differentiate into postmitotic derivatives (mature teratoma elements) in a process that resembles aberrant embryogenesis (Chaganti and Houldsworth, 2000). The presence of only mature differentiated cells in residual tumor masses after chemotherapy is a favorable prognostic factor, while the presence of residual undifferentiated cells predicts disease recurrence (Stenning et al., 1998). These and other data suggest that undifferentiated cells are primarily responsible for tumor growth and disease progression, consistent with the cancer stem cell model.

Neuroblastomas also exhibit variable degrees of differentiation (Ambros et al., 2002; Shimada et al., 1999a, 1999b, 1984). Neuroblastomas with widespread differentiation have a better prognosis than those with limited differentiation (Shimada et al., 1999b). Highly differentiated neuroblastic tumors are typically focal and can often be cured with surgery (Nitschke et al., 1988). Conversely, poorly differentiated neuroblastomas are often widely disseminated and are usually fatal despite aggressive treatment (Matthay et al., 2009, 1999; Shimada et al., 1999b). Therapies that promote differentiation significantly improve survival (Matthay et al., 2009, 1999). In some infants, disseminated tumors undergo spontaneous differentiation, leading to a favorable outcome even without therapy (Baker et al., 2010). While staging of neuroblastoma is complex and involves a number of variables other than differentiation status, these clinical observations are consistent with the cancer stem cell model in suggesting that undifferentiated neuroblastoma cells sometimes drive disease progression.

While the overt differentiation in some germ lineage cancers and some neuroblastomas provided clinical evidence consistent with the cancer stem cell model, these rare and unusual malignancies are of uncertain relevance to more prevalent adult cancers. Thus, the cancer stem cell model gained increased attention when evidence emerged supporting the model in leukemia and breast cancer. The advent of flow cytometry made it possible to separate phenotypically distinct subpopulations of live cancer cells to compare their tumorigenic potential. Using this approach, some human acute myeloid leukemias (AMLs) (Bonnet and Dick, 1997; Lapidot et al., 1994) and breast cancers (Al-Hajj et al., 2003) were found to follow the cancer stem cell model, suggesting that a broad spectrum of cancers might be hierarchically organized into tumorigenic and nontumorigenic components. In each of these studies, cells capable of forming leukemias/tumors were rare when transplanted into immunocompromised mice but could be enriched by selecting
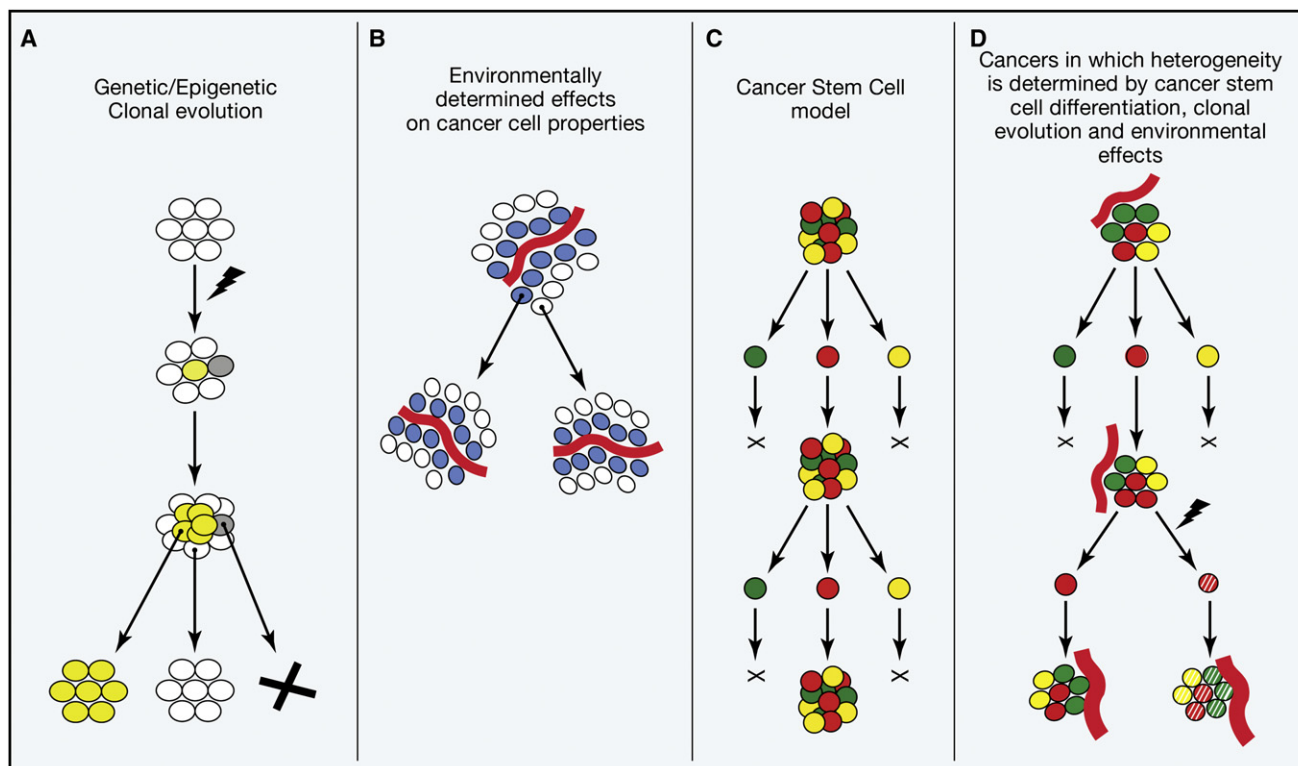
Figure 1. Sources of Heterogeneity within Cancer

(A) Heterogeneity can arise within tumors through stochastic genetic (Nowell, 1976) and epigenetic (Baylin and Jones, 2011) changes that confer heritable phenotypic and functional differences upon cancer cells. This process is known as clonal evolution because the genetic/epigenetic changes are subject to selection within tumors. This process tends to lead to more aggressive cancers over time; however, some cancer cells (gray) would be predicted to lose their tumorigenic capacity as a consequence of disadvantageous genetic changes.

(B) Heterogeneity can arise in response to extrinsic environmental differences within tumors: cancer cells (blue) adjacent to blood vessels (red) are different from cancer cells further from blood vessels (white) (Charles et al., 2010). The differences are shown as being reversible, though environmental differences could also cause irreversible changes in cancer cell properties.

(C) Cancers that follow the stem cell model contain intrinsically different subpopulations of tumorigenic (red) and nontumorigenic cells (yellow and green) organized in a hierarchy in which a minority population of tumorigenic cells gives rise to phenotypically diverse nontumorigenic cells. Nontumorigenic cells are thought to compose the bulk of tumors but have little capacity to contribute to cancer progression (Dick, 2008; Reya et al., 2001; Shackleton et al., 2009). Tumorigenic cells can be serially transplanted, re-establishing phenotypic heterogeneity with each passage.

(D) Cancers that follow the stem cell model are also subject to clonal evolution as well as heterogeneity from environmental differences within tumors. Thus, these sources of heterogeneity are not mutually exclusive and may each apply to variable extents depending on the cancer.

cells that expressed specific combinations of surface markers: leukemia-initiating cells were CD34$^+$CD38$^-$ (Bonnet and Dick, 1997; Lapidot et al., 1994) while breast cancer-initiating cells were CD44$^+$CD24$^{-/low}$ (Al-Hajj et al., 2003). This suggested that in some cancers only a small minority of cells can proliferate extensively and that some therapies that shrink tumors might not be curative because they fail to eliminate cancer stem cells.

Since these studies were published, other studies have taken similar approaches to provide evidence that other human cancers also follow the cancer stem cell model, including colon cancer (Dalerba et al., 2007; O'Brien et al., 2007; Ricci-Vitiani et al., 2007), pancreatic cancer (Li et al., 2007), brain tumors (Bao et al., 2006; Piccirillo et al., 2006; Singh et al., 2004) and ovarian cancer (Alvero et al., 2009; Curley et al., 2009; Stewart et al., 2011; Zhang et al., 2008b). In each case, the capacity to propagate the malignancy appeared to be restricted to a small, phenotypically distinct subpopulation of cancer cells. In tumorigenesis assays, many unfractionated cells had to be transplanted in order to transfer disease, suggesting that tumorigenic

cells were rare. These studies suggested that many cancers follow the stem cell model and might be more effectively treated by targeting cancer stem cells.

Sources of Heterogeneity among Cancers

Despite these studies, the generalizability of the model remained uncertain. Does the model apply to all cancers, or only some? Do all AMLs and breast cancers follow the cancer stem cell model, or only in certain patients? Differences among cancers in driver mutations and in the cell-of-origin create great diversity in cancer biology. Sometimes these differences are reflected in the histopathology of the cancer, but in other cases they may influence the underlying biology without recognized effects on histopathology. Nonetheless, these sources of heterogeneity complicate the testing of the cancer stem cell model and mean that observations in a cancer from one patient may be true of cancers in certain other patients, but not all patients.

Some cancers, including hierarchically organized cancers that follow the stem cell model, can arise from normal stem cells

Table 1. Testing the Cancer Stem Cell Model

Properties of Cancers That Follow the Stem Cell Model

	Experimental Evidence
Phenotypic and functional heterogeneity	Flow cytometry distinguishes phenotypically distinct subpopulations of cancer cells that are transplanted to test whether some are tumorigenic while others are nontumorigenic.
Hierarchical organization	Cancer stem cells are tumorigenic cells that give rise to a hierarchy of tumorigenic and nontumorigenic progeny. Upon transplantation, tumorigenic cells should give rise to more tumorigenic cells as well as phenotypically distinct nontumorigenic cells.

Properties Sometimes Ascribed to Cancer Stem Cells but Not Required by the Model

	Experimental Evidence
Therapy resistance	Are tumorigenic cells more likely to survive therapy than nontumorigenic cells? Are tumorigenic cells enriched by therapy?
Rarity	Tumorigenic cells have been rare in many studies that supported the cancer stem cell model but, in principle, such cells do not have to be rare in a hierarchically organized cancer.
Quiescence	While cancer stem cells are sometimes claimed to be quiescent, little data support this assertion and the cell cycle distribution of most cancer stem cells is unknown.
Asymmetric division	Cancer stem cells are sometimes claimed to divide asymmetrically, but this has never been demonstrated in vivo and cannot be an obligate property because it would prevent the numerical expansion of cancer stem cells.
Derive from normal stem cells	Experimentally, cancer stem cells can arise from either normal stem cells or from restricted progenitors/differentiated cells. In practice, the cell-of-origin of most cancers is unknown.

through mutations that overactivate self-renewal mechanisms (Barker et al., 2009; Merlos-Suárez et al., 2011; Yang et al., 2008). Other cancers, including hierarchically organized cancers, can arise from restricted progenitors or differentiated cells as a result of mutations that ectopically activate self-renewal mechanisms in these cells (Figure 2A) (Cozzio et al., 2003; Huntly et al., 2004; Krivtsov et al., 2006; Schüller et al., 2008; Yang et al., 2008; Zhao et al., 2010). Thus, hierarchical organization in a cancer does not imply that it originated from normal stem cells, and the cancer stem cell model does not address the cell-of-origin (Wang and Dick, 2005). However, the cell-of-origin can influence the hierarchical organization of cancers as it influences the mutations that are competent to transform (Wang et al., 2010). The interaction of driver mutations with cellular context influences the frequency of leukemogenic cells and perhaps the degree of hierarchical organization (Heuser et al., 2009; Somervaille et al., 2009). Nonetheless, these inferences are all based upon experimentally induced cancers. The cell-of-origin for most cancers that spontaneously arise in patients has not been identified, at least not with precision, making it difficult to assess which biological differences among human cancers reflect differences in cell-of-origin.
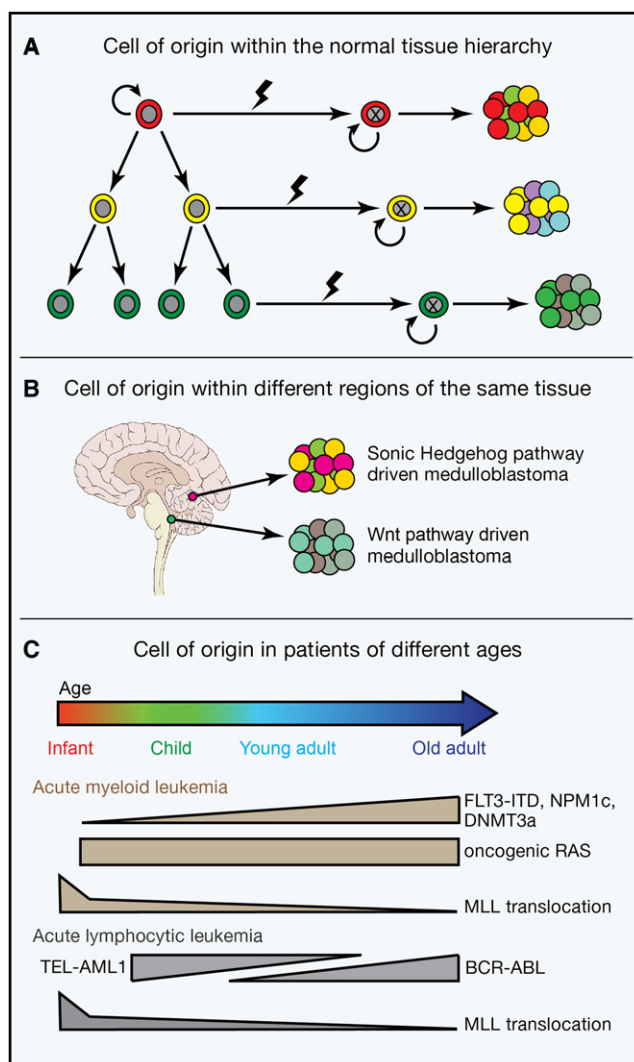
Spatial differences in the cell-of-origin can also influence cancer properties. In some tissues, regional differences in cellular properties influence the driver mutations that are competent to transform. Medulloblastomas can arise either from Sonic Hedgehog pathway activation in granule neuron precursors of the cerebellum or from Wnt pathway activation in dorsal brainstem progenitors (Gibson et al., 2010; Schüller et al., 2008; Yang et al., 2008) (Figure 2B). Medulloblastomas are hierarchically organized, consistent with the cancer stem cell model (Read et al., 2009; Singh et al., 2004; Ward et al., 2009), so it is likely that the regional identity of the cell-of-origin influences cancer stem cell properties, though this has not yet been tested. Ependymomas that arise from different regions of the central nervous system have different mutations, different patterns of gene expression, and different prognoses (Johnson et al., 2010; Taylor et al., 2005). Because ependymomas appear to arise from radial glia and are hierarchically organized into tumorigenic and nontumorigenic components (Taylor et al., 2005), these results suggest that regional differences in radial glia lead to regional differences in driver mutations and cancer stem cell properties.

Temporal differences in the cell-of-origin also affect the properties of leukemogenic cells. Stem cell properties and self-renewal mechanisms change with age (He et al., 2009; Levi and Morrison, 2008). This likely alters the types of mutations that are competent to initiate cancers. Consistent with this, the mutation spectrum changes with age in human leukemias. Some driver mutations are found primarily in older patients (e.g., in FLT3, Nucleophosmin1, and Dnmt3a), whereas other mutations occur throughout life (e.g., in Ras) or more commonly in young patients (e.g., translocations involving AML1, MLL, and NUP98) (Figure 2C) (Armstrong and Look, 2005; Berman et al., 2011; Brown et al., 2007; Downing and Shannon, 2002; Falini et al., 2005; Ho et al., 2011; Kiyoi et al., 1999; Kottaridis et al., 2001; Ley et al., 2010; Meshinchi et al., 2001; Stirewalt et al., 2001; Thiede et al., 2006; Vogelstein et al., 1990; Zwaan et al., 2003). Because many AMLs are hierarchically organized (Lapidot et al., 1994; Yilmaz et al., 2006) and driver mutations influence the frequency of leukemogenic cells (Heuser et al., 2009; Somervaille et al., 2009), temporal changes in the cell-of-origin likely influence cancer stem cell properties.

Heterogeneity among Patients in Cancer Stem Cell Phenotype

Differences in driver mutations and cell-of-origin among patients raise the question of whether similar hierarchies of tumorigenic and nontumorigenic cells, with similar markers, are conserved among patients with similar cancers. Initial studies suggested that AMLs in many patients adopted a similar hierarchical organization in which rare leukemogenic cells were distinguished

Figure 2. Sources of Heterogeneity among Cancers

Differences in the cell-of-origin can directly and indirectly influence the phenotype of tumorigenic cells and, perhaps, whether or not the cancer is hierarchically organized.

(A) Different cell types in a stem/progenitor cell hierarchy within a normal tissue may be transformed into cancer cells. The properties of the cell-of-origin influence the types of mutations that are competent to transform and the properties of the resulting cancer (Huntly et al., 2004; Wang et al., 2010).

(B) Spatial differences in the identity of the cell-of-origin within tissues influence the types of mutations that are competent to transform and the properties of the resulting cancer (Gibson et al., 2010; Johnson et al., 2010).

(C) Temporal differences in the cell-of-origin also influence the types of mutations that are competent to transform and the properties of the resulting cancer (J.A.M. and S.J.M., unpublished data), consistent with the observation that the driver mutation spectrum changes with age in patients (Downing and Shannon, 2002) (see text for references regarding age-related changes in the incidence of specific mutations).

from nonleukemogenic progeny by having a CD34$^+$CD38$^-$ surface marker phenotype, largely irrespective of AML subtype or blast cell maturation state (Bonnet and Dick, 1997; Lapidot et al., 1994). Many studies from other laboratories went on to characterize "leukemic stem cell" properties, such as gene expression signatures, by isolating CD34$^+$CD38$^-$ cells without

verifying that these markers distinguished leukemogenic from nonleukemogenic cells in the patients they studied.

It was subsequently determined that there are leukemia-initiating cells among CD34$^-$ and CD38$^+$ cells in some AMLs (Sarry et al., 2011; Taussig et al., 2008, 2010). Dick and colleagues systematically addressed this issue by comparing the leukemogenic capacity of CD34$^+$CD38$^-$, CD34$^+$CD38$^+$, CD34$^-$CD38$^+$, and CD34$^-$CD38$^-$ AML cells from 16 patients (Eppert et al., 2011). In the 13 AMLs that engrafted, there was leukemogenic activity in the CD34$^+$CD38$^-$ fraction; however, leukemogenic cells were also detected in at least one other fraction in most patients. Most leukemogenic cells were contained in the CD34$^+$CD38$^-$ fraction in half of the patients and in the CD34$^+$CD38$^+$ fraction in the other half of patients. In one case, there were similar frequencies of leukemogenic cells in all fractions, and a second case had leukemogenic cells in 3 of 4 fractions. Leukemogenic activity is therefore not usually restricted to the CD34$^+$CD38$^-$ fraction and there is heterogeneity among patients in leukemogenic cell phenotype (Figure 3). Some AMLs might not follow the cancer stem cell model at all.

The frequency and phenotype of leukemogenic cells is also highly variable in mouse AMLs. Deletion of *Pten* from adult mouse hematopoietic cells leads to the development of AML upon transplantation into wild-type mice (Yilmaz et al., 2006). In these AMLs, leukemogenic activity is most highly enriched among rare cells with a surface marker phenotype similar to normal HSCs, but cells that express mature myeloid markers possess lower levels of leukemogenic activity. Mouse AMLs induced by MLL-AF9 expression appear to have much higher frequencies of leukemogenic cells than observed after *Pten* deletion (Krivtsov et al., 2006; Somervaille and Cleary, 2006; Somervaille et al., 2009). In these leukemias, cells bearing GMP-like surface markers have the highest frequency of leukemogenic cells (Krivtsov et al., 2006); however, leukemogenic activity is also found in other cell fractions (Somervaille and Cleary, 2006; Somervaille et al., 2009). Differences in oncogenic mutations can thus have profound effects on the frequency and phenotype of leukemia-initiating cells.

The same is true in solid cancers. Tumorigenic cells are enriched within the CD44$^+$CD24$^{-/low}$ population of some breast cancers (Al-Hajj et al., 2003). However, other breast cancers studied by Al-Hajj et al. had more phenotypically diverse breast cancer-initiating cells, demonstrating that the CD44$^+$CD24$^{-/low}$ surface marker phenotype does not universally distinguish tumorigenic from nontumorigenic breast cancer cells (Al-Hajj et al., 2003). In mouse models of mammary cancer, different driver mutations give rise to cancers that differ in the extent to which they follow the stem cell model (Cho et al., 2008; Vaillant et al., 2008; Zhang et al., 2008a); for example, CD61 enriched for cancer stem cell activity in mice with Wnt1 driven breast cancers but not in mice with Neu/ErbB2 driver breast cancers (Vaillant et al., 2008). Mouse models of lung cancer with different transforming mutations have tumorigenic cells with different surface marker phenotypes (Curtis et al., 2010). Thus, different oncogenic mutations give rise to cancers that differ in the extent to which they follow the cancer stem cell model and in tumorigenic cell phenotype.

These results demonstrate the importance of testing cancer stem cell markers in significant numbers of patients to
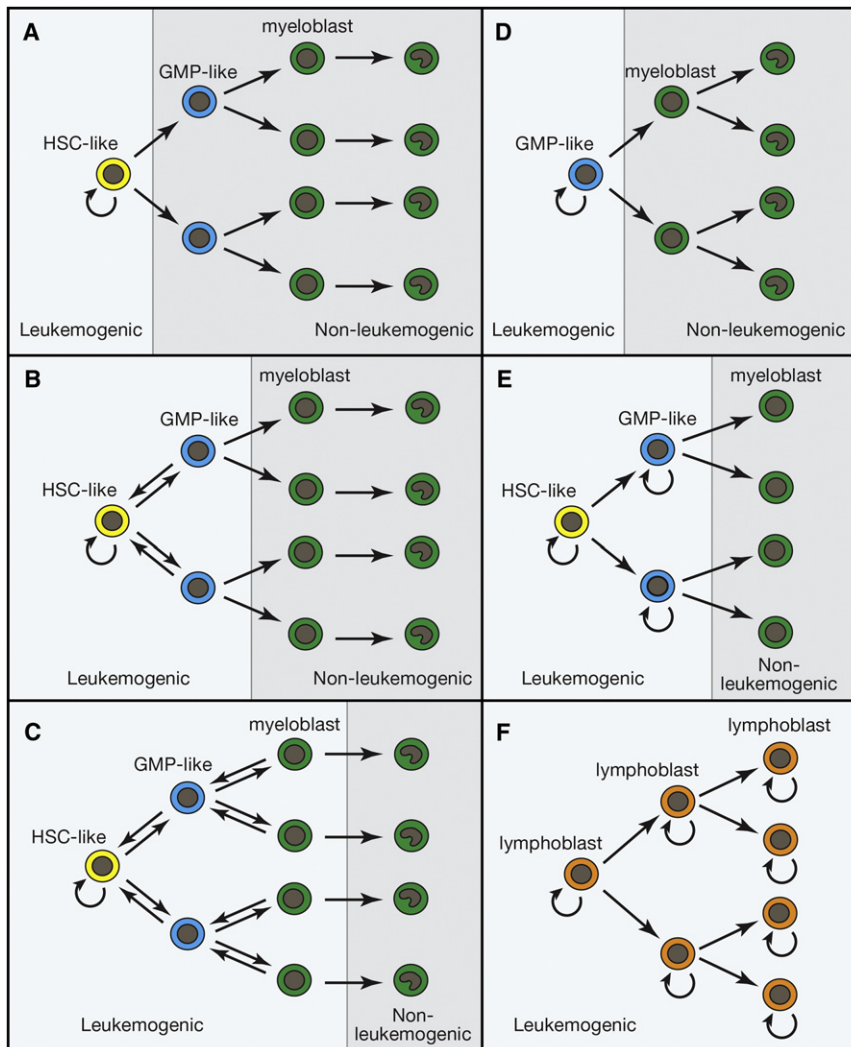
Figure 3. Variation among Leukemias in the Degree and Nature of Hierarchical Organization

Although most AMLs follow a cancer stem cell model, the surface marker phenotypes of the leukemogenic cells vary from patient to patient.

(A–C) Different oncogenic mutations can transform cells at different levels within the hematopoiesis hierarchy (Wang et al., 2010), potentially influencing the frequency, spectrum, and phenotype of cells with leukemogenic potential. Interconversion between leukemogenic cell populations would allow any population to recapitulate the heterogeneity of the leukemogenic cell pool (Eppert et al., 2011; Sarry et al., 2011).

(D) Some mutations, such as MLL-AF9 translocations, can confer leukemogenic activity upon restricted progenitors (Cozzio et al., 2003; Huntly et al., 2004; Krivtsov et al., 2006; Zhao et al., 2010).

(E) If multiple populations have leukemogenic capacity but do not interconvert, then only the most immature cells can recapitulate the full heterogeneity of the parent leukemia, but multiple levels of the hierarchy may be able to drive disease progression (Goardon et al., 2011).

(F) In some ALLs, many cells have leukemogenic activity despite heterogeneity in marker expression (le Viseur et al., 2008; Williams et al., 2007).

These clinical observations appear to be explained by the persistence of rare imatinib-resistant CML stem cells (Chu et al., 2011; Graham et al., 2002; Holtz et al., 2002). A mouse model of CML-like disease driven by HIP1-PDGFR and AML1-ETO fusion proteins had leukemogenic and nonleukemogenic subpopulations of cancer cells distinguished by differences in surface marker expression (Oravecz-Wilson et al., 2009). The leukemogenic cells were rare and much more resistant to imatinib than their nonleukemogenic progeny. Imatinib treatment dramatically enriched leukemogenic cells despite reducing overall leukemia burden. Evidence has also been presented for radiation resistance in brain tumor-initiating cells (Bao et al., 2006) and breast cancer-initiating cells (Diehn et al., 2009).

While it has become fashionable to consider therapy resistance a defining feature of cancer stem cells, the sensitivity of tumorigenic and nontumorigenic cells to therapy depends upon the cancer and the therapy. Some therapies actually exploit the capacity of tumorigenic cells to differentiate into nontumorigenic cells by inducing differentiation. Acute promyelocytic leukemia is treated with arsenic trioxide and trans-retinoic acid, which induce rapid terminal differentiation, growth arrest, and apoptosis of the cancer cells (de Thé and Chen, 2010). Differentiation therapy has also been exploited experimentally in glioblastoma by treating with bone morphogenetic protein 4 (BMP4) to induce glial differentiation, reducing proliferation, tumor growth, and tumorigenic cell frequency (Piccirillo et al., 2006). BMP4 also promotes glial differentiation by normal CNS stem cells (Gross et al., 1996) suggesting tumorigenic cancer cells sometimes inherit differentiation pathways from normal

appreciate the heterogeneity among patients. Yet studies of cancer stem cell characteristics often assume that markers identified in one study can be applied without validation to other patients, to cell lines, or to cells transformed in culture. The conclusions in such studies may be undermined by heterogeneity among patients or by context- or oncogene-dependent effects on tumorigenic cell phenotype.

The Implications of the Cancer Stem Cell Model for Therapy

In cancers that follow the stem cell model, the functional differences between tumorigenic and nontumorigenic cells can have important implications for therapy. For example, the BCR-ABL inhibitor imatinib has been incredibly effective at restoring the health and prolonging the lives of patients with chronic myeloid leukemia (CML) (Druker et al., 2006); however, these patients must remain on imatinib indefinitely because many patients fail to completely eliminate BCR-ABL-expressing cells from their bone marrow (Bhatia et al., 2003; Chu et al., 2011) and even patients that achieve a complete molecular remission often relapse upon withdrawal of therapy (Rousselot et al., 2007).

stem cells in the same tissue. Cis-retinoic acid also induces glial differentiation and improves survival in high-risk neuroblastoma patients (Matthay et al., 1999; Thiele et al., 1985). Thus, tumorigenic cells are specifically targeted by some therapies.

Therapy resistance can also arise through genetic mechanisms that are not necessarily related to cancer stem cells. While the intrinsic resistance of CML-initiating cells to imatinib allows these cells to persist in treated patients who are in remission, the ongoing administration of imatinib generally prevents relapse until the CML-initiating cells acquire an amplification of BCR-ABL or point mutations that confer imatinib resistance (Gorre et al., 2001; Roumiantsev et al., 2002). Melanomas carrying V600E BRAF mutations become resistant to the BRAF inhibitor vemurafenib through a variety of genetic mechanisms (Johannessen et al., 2010; Nazarian et al., 2010; Poulikakos et al., 2011; Poulikakos and Rosen, 2011; Villanueva et al., 2010), and we are unable to find any evidence that melanoma follows a cancer stem cell model (Quintana et al., 2010; Quintana et al., 2008). Therefore, cancer progression and therapy resistance may be influenced by the properties of cancers stem cells in cancers that follow the model, but therapy resistance and disease progression can also arise through genetic changes unrelated to the question of whether a cancer follows the stem cell model.

Tumorigenesis Assays

The xenotransplantation of human cancer cells into mice differs in a number of important respects from the growth of human cancer cells in patients. Mouse tissues differ from human tissues in terms of architecture and stromal cells (Kuperwasser et al., 2004). Mouse growth factors and adhesion molecules sometimes do not bind human receptors (Manz, 2007). Autologous immune cells are an important element of the tumor microenvironment as they can either promote or impair tumor growth (de Visser et al., 2006). Yet there are profound differences in immune regulation between the autologous and xenogeneic settings. Human cells transplanted into mice are subject to powerful xenogeneic immune responses that kill most human cells before they have an opportunity to proliferate (Auchincloss and Sachs, 1989). That is why human cancer cells must be transplanted into highly immunocompromised mice to assay tumorigenic capacity. Even NOD/SCID mice retain an attenuated xenogeneic barrier. Transplantation into more highly immunocompromised mice (such as NOD/SCID IL2Rγ^{null}) can significantly increase the frequency of tumorigenic cells that is detected in some, but not all, human cancers (Ishizawa et al., 2010; Kennedy et al., 2007; Quintana et al., 2008).

Some have speculated that less immunocompromised mice may represent "better models" for studying human cancers because the preservation of some immune activity makes these mice more similar to patients. However, the mechanisms by which mouse immune cells respond to transplanted human cells bear little resemblance to the mechanisms by which human immune cells sometimes respond to autologous cancer cells (Auchincloss and Sachs, 1989). No xenotransplantation assay can model the immune responses that sometimes occur in patients against their own tumors. Normal human hematopoietic stem cells (HSCs) and cancer-initiating cells are therefore more likely to be detected in more highly immunocompromised

mice: NOD/SCID mice treated with anti-CD122 antibody to deplete NK cells or NOD/SCID IL2Rγ^{null} mice (Eppert et al., 2011; McDermott et al., 2010; Quintana et al., 2008).

It is also critical to recognize that transplantation assays, particularly xenotransplantation assays, test the potential of cells to form tumors, not their actual fate in the tumor in which they are born (Figure 4). There are many environmental variables in the tumor environment, such as hypoxia and immune responses, which can prevent cells that have the potential to form a tumor from actually doing so in their normal environment. Consequently, nobody knows whether many, or few, cells with tumorigenic potential actually contribute to disease progression in patients. The question of which cells are actually fated to contribute to disease progression is highly context dependent and is probably not testable in patients because of the experimental manipulations that would be required; however, this is testable by fate mapping of cells in mouse tumors. It will be interesting to compare the results of side-by-side fate-mapping experiments and transplantation assays to assess whether the cells that have the potential to form tumors upon transplantation are the same cells that drive disease progression in situ.

Tumorigenic Cells Are Abundant in Some Cancers

Not all hematopoietic malignancies contain rare cancer-initiating cells. Tumorgenic/leukemogenic cells are common in certain mouse models of B cell lymphoma, T cell lymphoma, and AML in which it is possible to transfer disease to wild-type recipients by transplanting only ten cells (Kelly et al., 2007). In a mouse model of B cell acute lymphoblastic leukemia (ALL), at least 50% of cancer cells are capable of transferring disease into wild-type recipient mice (Williams et al., 2007). In these malignancies, the abundance of tumorgenic/leukemogenic cells suggests that if there is any hierarchy it must be much more shallow than observed in many human AMLs, which so far have consistently appeared to have rare leukemogenic cells (Bonnet and Dick, 1997; Lapidot et al., 1994).

Tumorigenic cells are also common in some human cancers. When human melanomas were first transplanted into NOD/SCID mice, it was estimated that only one in a million melanoma cells were capable of forming tumors, and melanoma was proposed to follow a cancer stem cell model (Schatton et al., 2008). However, simple changes in tumorigenesis assay conditions (including the use of NOD/SCID IL2Rγ^{null} mice) increased the detected frequency of tumorigenic cells to one in four (Quintana et al., 2008). We now routinely transplant single cells directly from patients into NOD/SCID IL2Rγ^{null} mice and on average 30% of single cells form tumors (Quintana et al., 2010). We have quantified the frequency of tumorigenic cells from more than 30 patients with diverse stages and sites of disease, and in every case, the frequency of tumorigenic cells has been high, even in primary cutaneous melanomas obtained directly from patients (Quintana et al., 2010). Similar results have been published with mouse models of melanoma (Held et al., 2010). Thus, xenotransplantation assays sometimes dramatically underestimate the frequency of human cancer cells with tumorigenic potential.

Is melanoma unique among solid cancers in having common tumorigenic cells? We addressed this question in mouse models of malignant peripheral nerve sheath tumors (MPNSTs)
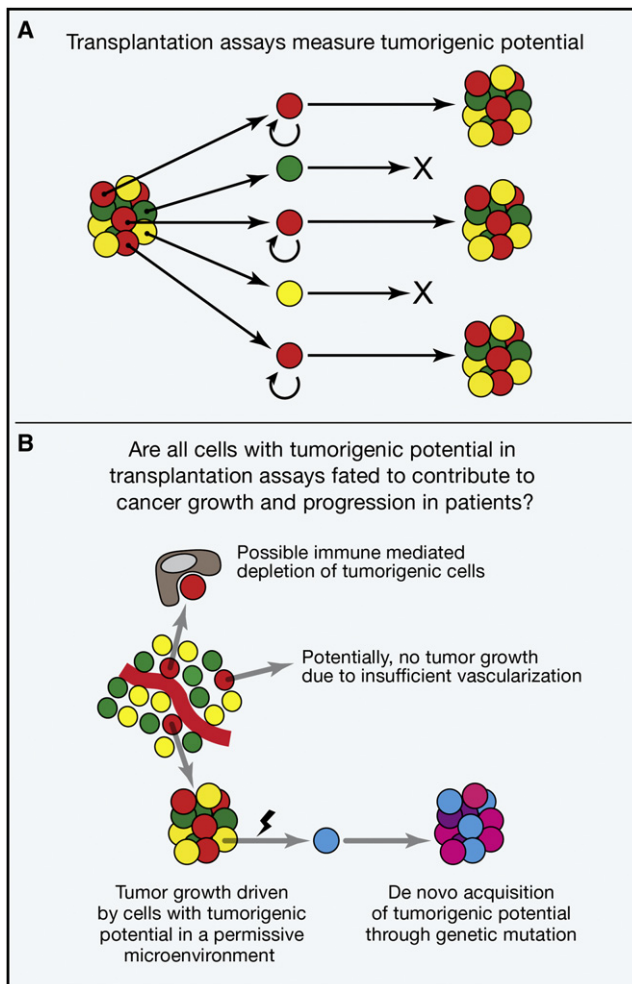
Figure 4. Fate versus Potential in Cancer
(A) Potential describes what cells can do in a permissive environment. The cancer stem cell model, and the transplantation assays (black arrows) on which it is largely based, address the potential of cancer cells to form tumors. (B) Fate reflects what cells actually do in a specific environment. In the context of cancer, the question is which cells are fated to contribute to tumor growth and disease progression in the patient. Many of the cells that have the potential to form tumors upon transplantation may not be fated to contribute to disease progression in a particular patient because they are not in a permissive environment. For example, some cancer cells undergo cell death due to hypoxia or immune effector activity. Some of these cells might have the potential to form a tumor if transplanted into another environment, but are fated to undergo cell death in the tumor environment in which they actually reside in the patient. There may also be cells that lack the ability to form a tumor upon transplantation but that are nonetheless fated to contribute to disease progression in the patient, such as if they acquire a new mutation that increases their proliferation. Transplantation assays only assess potential, not fate, in a patient and therefore should attempt to detect the full range of cells with the potential to form tumors. No transplantation assay mimics the environment within patient tissues and such assays are neither designed nor capable of assessing cell fate in patients. Consequently, very little is known about the spectrum of cells fated to contribute to tumor growth and disease progression in patients or the extent to which it overlaps with the spectrum of cells that can form a tumor upon transplantation.

(Buchstaller et al., 2012). We found that approximately 20% of mouse MPNST cells have the potential to form tumors, even when transplanted into fully immunocompetent mice. This suggests that tumorigenic cells may be common in a number

of solid cancers, though it is important to note that we have not tested whether MPNSTs are hierarchically organized, and therefore, it is unknown whether this cancer has a shallow hierarchical organization.

An important question is whether we have systematically underestimated the frequency of tumorigenic cells by using transplantation assays that have not been optimized to detect the full range of cells with the potential to form tumors. Work on human HSCs and AML has identified numerous assay improvements that increased estimates of stem cell frequency by orders of magnitude (Eppert et al., 2011; Kennedy et al., 2007; McDermott et al., 2010; McKenzie et al., 2005; Notta et al., 2010), but assays for tumorigenic cells from solid cancers generally have not been studied to the same extent. Enzymatic dissociation conditions, sorting conditions, transplantation site, the extracellular matrix environment, and the recipient mouse (sex and strain) all affect the ability to detect tumorigenic cells. Other undiscovered assay parameters may also be important. Estimates of tumorigenic cell frequencies may continue to increase in many cancers as assays improve, though tumorigenic cells will likely remain rare in some cancers despite such improvements (Eppert et al., 2011; Ishizawa et al., 2010).

Do Tumorigenesis Assays Test the Ability to Recapitulate Tumor Heterogeneity?
A fundamental element of the stem cell model is that cancer stem cells give rise to phenotypically diverse progeny that recapitulate the heterogeneity of the tumor from which they derive (Figure 1C). This is presumed to occur through epigenetic mechanisms akin to the differentiation of normal stem cells; however, nobody has experimentally confirmed that epigenetic differences distinguish tumorigenic from nontumorigenic cells. The differentiation of cancer stem cells into nontumorigenic progeny is commonly assumed to be the major driver of heterogeneity in cancers that follow the stem cell model; however, the degree of genetic heterogeneity in such cancers is unknown. Therefore, some of the phenotypic and functional differences among cancer cells that have been attributed to the differentiation of cancer stem cells might derive from genetic differences that arise through clonal evolution (Figure 5A).

Since genetic changes are irreversible and stochastic, no tumorigenic cell can recapitulate the genetic heterogeneity of the tumor from which it derives. If the degree of genetic heterogeneity within a tumor is low, then cancer stem cells may be able to largely recapitulate the heterogeneity of the tumors from which they derive by differentiating into nontumorigenic cells (Figure 5B). However, if there is extensive genetic heterogeneity, then every tumorigenic cell may form a genetically distinct tumor and no tumorigenic cell will recapitulate the heterogeneity of the primary tumor (Figure 5C). Increasing evidence suggests that there is more genetic heterogeneity within tumors than previously thought (Navin et al., 2011; Yachida et al., 2010). However, driver mutations are rare among the genetic changes observed in cancer, so the degree of genetic heterogeneity that influences cancer cell function may be more modest. Nonetheless, if there is widespread genetic heterogeneity, functional and phenotypic differences among cancer cells cannot be assumed to be driven by epigenetic hierarchies rather than genetic differences (Campbell et al., 2010; Ding et al., 2010).
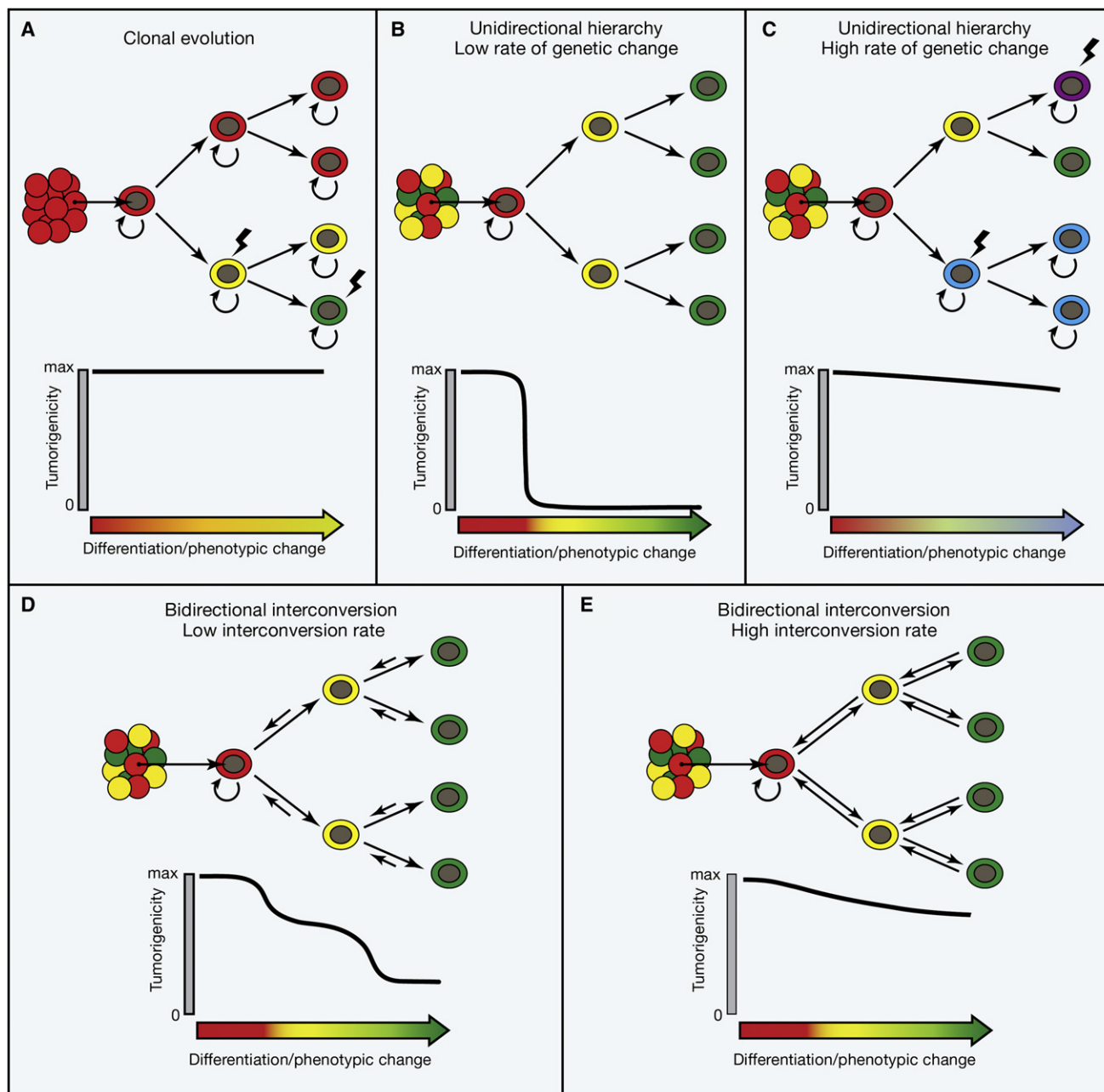
Figure 5. The Influences of Genetic Change and Reversible Transitions in Cell States on Hierarchical Organization in Cancer

(A) According to the clonal evolution model, many cancer cells have tumorigenic potential (circular self-renewal arrows) and heterogeneity arises through stochastic genetic/epigenetic changes (lightning bolt). Changes in cell phenotype are not necessarily associated with changes in tumorigenic potential.

(B) For cancers that follow a stem cell model in which only the cells at the top of the hierarchy retain tumorigenic capacity, the differentiation of these cells into nontumorigenic progeny creates tumor heterogeneity. Differentiation is associated with a loss of tumorigenic potential.

(C) For cancers with a high rate of genetic change, clones of cells within the hierarchy depicted in (B) may acquire tumorigenic potential as a consequence of new mutations. Phenotypic changes are sometimes associated with changes in tumorigenic potential and sometimes not. Note that it would be difficult to experimentally distinguish this model from the model in (A).

(D) A cancer that is hierarchically organized according to the cancer stem cell model but in which nontumorigenic cells can inefficiently revert to higher levels of the hierarchy. In this case, tumorigenic cells could be enriched or depleted using markers but "nontumorigenic" cells from the bottom of the hierarchy would always retain some tumorigenic capacity due to their ability to revert to tumorigenic states.

(E) A hierarchically organized cancer in which cells readily and reversibly interconvert between tumorigenic (red) and nontumorigenic (yellow and green) states. Note that it would be difficult to experimentally distinguish case (E) from case (C). In cancers that are genetically unstable or subject to efficient reversible cell transitions, the cancer stem cell model may be untestable, because it may be difficult to experimentally distinguish from cancers in which there is no hierarchy but where heterogeneity arises through clonal evolution.

In cancers that follow the stem cell model, cancer stem cells would be expected to undergo genetic change over time. Consistent with this, leukemia-initiating cells in B-ALLs undergo clonal evolution (Anderson et al., 2011; Notta et al., 2011), though these studies did not test whether the B-ALLs were hierarchically organized according to the cancer stem cell model. In cancers that do not follow the cancer stem cell model, genetic change would also occur over time and would be predicted to introduce phenotypic and functional heterogeneity. Thus, the cancer stem cell and clonal evolution models can be interacting, or independent, sources of heterogeneity depending on the cancer.

Another issue concerns the extent to which existing assays reliably test phenotypic heterogeneity. Some have suggested that melanomas have intrinsically different populations of tumorigenic and nontumorigenic cells that can be distinguished based on ABCB5 (Schatton et al., 2008) or CD271 (Civenni et al., 2011; Boiko et al., 2010) expression. However, neither of these markers correlate with the frequency of tumorigenic cells, and in our hands, small numbers of cells that are positive or negative for ABCB5 or CD271 have a similar capacity to form tumors and to recapitulate the phenotypic heterogeneity of the tumors from which they derive (Quintana et al., 2010). We also identified 20 other markers that are heterogeneously expressed by human melanoma cells and transplanted just 10 cells that were either positive or negative for each of these markers into NOD/SCID IL2Rγ^{null} mice (Quintana et al., 2010). All subpopulations exhibited a similar capacity to form phenotypically heterogeneous tumors. Thus, we have subdivided melanomas from many patients into almost 50 subpopulations of cells based on differences in marker expression and have not found any subpopulation that lacks the ability to form a phenotypically heterogeneous tumor.

The simplest interpretation of our data (Quintana et al., 2010) and other data (Pinner et al., 2009; Roesch et al., 2010) is that melanoma cells are phenotypically plastic, reversibly turning on and off markers. In contrast, Civenni et al. reported that both CD271$^+$ and CD271$^-$ melanoma cells formed tumors in NOD/SCID IL2Rγ^{null} mice but that the tumors formed by CD271$^-$ cells were not heterogeneous for CD271 expression and that only CD271$^+$ cells formed tumors in NOD/SCID mice (Civenni et al., 2011). They speculated that the difference relative to our results reflected different dissociation conditions. In our hands, both dissociation methods yield similar results with CD271$^+$ and CD271$^-$ melanoma cells each forming heterogeneous tumors that can be serially passaged, irrespective of whether they are transplanted in to NOD/SCID or NOD/SCID IL2Rγ^{null} mice (E. Quintana, U. Eskiocak, and S.J.M., unpublished data); however, if differences in enzymatic dissociation methods in solid cancers can sometimes generate reproducible differences in the phenotype of tumorigenic cells, this would illustrate the complexity of reproducing "cancer stem cell" markers.

If there are certain cells that reproducibly form tumors in NOD/SCID IL2Rγ^{null} mice and reproducibly fail to form tumors in NOD/SCID mice, what would this mean? Are some human cells more able to evade rejection by mouse immune cells? If so, does this have any physiological relevance for these cells in humans? If some tumorigenic cells are more able to generate phenotypically heterogeneous progeny than others, what does this mean? Could this reflect genetic differences among the cells (Campbell et al., 2010; Ding et al., 2010) rather than hierarchical epigenetic differences? Ultimately, a cell that has the potential to form a tumor in any assay has the potential to contribute to disease progression in a patient and cannot be ignored during therapy.

Uncertainty in Cancer Stem Cell Markers

In some cases, it has proven difficult to confirm markers that originally appeared to robustly distinguish tumorigenic from nontumorigenic cells; for example, CD133 was reported as a marker of tumorigenic brain cancer cells, and even large numbers of CD133-negative brain tumor cells were reported to lack the ability to form tumors (Bao et al., 2006; Singh et al., 2004). On this basis, gliomas and medulloblastomas were concluded to follow a cancer stem cell model. Yet subsequent studies found tumorigenic activity among both CD133$^+$ and CD133$^-$ brain tumor cells (Beier et al., 2007; Chen et al., 2010; Joo et al., 2008; Wang et al., 2008). These discrepancies could reflect differences among patients, methodological differences among laboratories, or ascertainment/tumor selection bias that led early studies to overestimate the robustness of markers. Work with other markers continues to support the conclusion that gliomas and medulloblastomas follow a cancer stem cell model (Read et al., 2009; Son et al., 2009; Ward et al., 2009); however, to confirm the existence and identity of cancer stem cells, it is necessary to identify markers that reproducibly distinguish tumorigenic and nontumorigenic cells, at least in specific subsets of patients.

Tumorigenic ovarian cancer cells have been reported to be enriched in the CD44$^+$ (Alvero et al., 2009), CD44$^+$CD117$^+$ (Zhang et al., 2008b), and CD133$^+$ (Curley et al., 2009) subpopulations of ovarian cancer cells. However, when Stewart et al. evaluated a large cohort of ovarian cancers, they were unable to find CD44$^+$CD117$^+$ cells in most ovarian cancers and the CD44$^+$CD117$^+$ cells they did find were depleted for tumorigenic activity (Stewart et al., 2011). CD133 enriched tumorigenic ovarian cancer cells in some cases and not in other cases. CD133 expression changed on some tumorigenic cells during passaging (Stewart et al., 2011). This suggests that CD133 only marks ovarian cancer stem cells under defined conditions in some patients and that the hierarchical organization of some ovarian cancers is not stable. It remains uncertain what fraction of ovarian cancers follow the stem cell model.

Cancer stem cell markers have proven difficult to broadly confirm in a number of solid cancers, raising questions about whether we have overestimated the number of cancers that follow this model and making it difficult to study the biology of these cells. Alternatively, it is possible that many cancers are hierarchically organized but that there is considerable diversity among patients in terms of the markers that distinguish tumorigenic from nontumorigenic cells. This is a key issue that must be resolved and which may require approaches other than the traditional dependence upon cell surface markers. For example, the separation of live cancer cells based on functional measures, such as signaling pathway activation (Vermeulen et al., 2010), might reduce phenotypic variability among tumorigenic cells. Unfortunately, the genetic approaches currently required to do this are only possible in mice where the use of inbred genetic

backgrounds and targeted mutations already reduce variability in cancer models relative to what is observed in patients.

Do Nontumorigenic Cells Sometimes Form Tumorigenic Cells?

Recent studies have suggested that the differentiation of cancer stem cells into nontumorigenic cells may be reversible (Chaffer et al., 2011; Gupta et al., 2011). In culture, immortalized human mammary epithelial cells (HMECs) undergo an epithelial-to-mesenchymal transition (EMT) following sustained expression of the transcription factors Snail or Twist, silencing of E-cadherin, or exposure to TGF-β (Mani et al., 2008; Morel et al., 2008; Scheel et al., 2011). This EMT was interpreted as conferring stem cell properties upon normal or transformed epithelial cells in culture, partly because the cells acquired a $CD44^+CD24^-$ phenotype, similar to breast cancer stem cells. The idea that cancer cells might reversibly transition between epigenetically defined tumorigenic and nontumorigenic states is appealing, partly because mechanisms that generate reversible heterogeneity can confer therapy resistance (Roesch et al., 2010; Sharma et al., 2010). However, it is not clear whether CD44 and CD24 consistently distinguish tumorigenic from nontumorigenic cells in cultured cell lines or whether nontumorigenic cells acquire tumorigenic potential by EMT in breast cancers in vivo.

The idea that nontumorigenic cancer cells could sometimes revert to having tumorigenic capacity is plausible given that restricted progenitors dedifferentiate into stem cells in certain normal tissues. In the *Drosophila* testis and ovary, spermatogonia and cytocytes dedifferentiate into germline stem cells under certain circumstances (Brawley and Matunis, 2004; Kai and Spradling, 2004). In mouse testis, spermatogonial progenitors can also dedifferentiate into spermatogonial stem cells (Barroca et al., 2009). So far this dedifferentiation has only been observed in normal tissues at low frequencies or under restricted circumstances. Nonetheless, if nontumorigenic cancer cells revert to a tumorigenic state at an appreciable rate in certain cancers, then this would undermine the ability to distinguish tumorigenic from nontumorigenic cells as even the "nontumorigenic" cells would be expected to form tumors at some level.

It is not clear whether the cancer stem cell model would effectively describe cancers in which there are tumorigenic and nontumorigenic states that can reversibly interconvert. If cells in the nontumorigenic state only convert to the tumorigenic state under restricted circumstances or with low efficiency, then it may still be possible to identify markers that distinguish populations that are enriched or depleted for tumorigenic capacity (Figure 5D). However, if cells in the nontumorigenic state convert to the tumorigenic state with high efficiency, it should not be possible to distinguish tumorigenic from nontumorigenic cells, potentially rendering the cancer stem cell model untestable in such cancers (Figure 5E). New models may be required to describe the heterogeneity in such cancers.

Cancer Stem Cells and Metastasis

Metastasis requires cells from a primary tumor to detach, invade the vascular or lymphatic system, migrate to distant sites, extravasate, then proliferate extensively and recruit new vasculature (Nguyen et al., 2009). There is much discussion regarding the metastasis of cancer stem cells, though nobody has actually tested whether the cells that are enriched for tumorigenic activity in transplantation assays are also enriched for the capacity to metastasize under physiological conditions. A fundamental question is whether metastatic potential is confined to a single population of tumorigenic cells or whether it arises stochastically through genetic or epigenetic changes that occur in many cancer cells without regard to their competence to form tumors prior to the stochastic change.

Some studies have suggested that subpopulations of tumorigenic pancreatic cancer (Hermann et al., 2007) and colon cancer (Pang et al., 2010) cells are enriched for the capacity to metastasize. However, the relationship between tumorigenic cells and metastasis has not yet been addressed systematically by examining all of the cancer cells that can be found in circulation and their tumorigenic capacity. Therefore, it remains uncertain whether nontumorigenic cancer cells have a similar capacity to disseminate as tumorigenic cells, or whether nontumorigenic cells from a primary tumor can sometimes acquire tumorigenic capacity after migrating to a new environment.

It has been proposed that cancer cells acquire metastatic potential by undergoing an EMT (Kalluri and Weinberg, 2009). As cancer cells lose their epithelial characteristics, they lose intracellular adhesions and polarity while acquiring more mesenchymal features such as the ability to migrate, invade, and resist apoptosis (Thiery et al., 2009). EMT has been proposed as a requisite step for breast cancer metastasis (Yang et al., 2004) and induction of an EMT also confers upon cultured cells a surface marker phenotype ($CD44^+CD24^-$) similar to tumorigenic breast cancer cells (Mani et al., 2008; Scheel et al., 2011). If cancer cells that have no tumorigenic capacity in transplantation assays can acquire this potential by undergoing an EMT, then any cancer cell could acquire metastatic potential.

Although the cancer stem cell model and work on EMTs focus on epigenetic differences between tumorigenic and nontumorigenic cells, there is good evidence that irreversible genetic mutations also confer metastatic potential (Vogelstein et al., 1988). In pancreatic cancer and medulloblastoma, genetically distinct subclones initiate metastatic disease (Campbell et al., 2010; Wu et al., 2012; Yachida et al., 2010). This raises the question of whether only cancer stem cells are competent to acquire genetic changes that confer metastatic potential or whether nontumorigenic cells can acquire genetic changes that confer both tumorigenic and metastatic potential. These fundamental questions remain to be studied in primary tumors in vivo, so the implications of the cancer stem cell model for metastasis are unresolved.

Conclusions and Future Directions

- Some cancers are hierarchically organized into undifferentiated cells that can drive disease progression and differentiated cells with less capacity to drive disease progression, consistent with the cancer stem cell model.
- Cancers that exhibit this kind of hierarchical organization have functional differences among undifferentiated and differentiated cancer cells that affect response to therapy and prognosis.

- There remain a number of uncertainties that make it difficult to assess whether many, or few, cancers follow the stem cell model. Some "cancer stem cell" markers have proven difficult to confirm, and tumorigenic cell frequencies can sometimes increase dramatically as a result of changes in assay conditions.

- Many studies have assumed that markers discovered in earlier studies were universally able to distinguish tumorigenic from nontumorigenic cells, even in independent patient cohorts or in cultured cell lines. Given the heterogeneity that is evident among patients and the context dependence of some markers, "cancer stem cell" markers should be confirmed in functional assays in each patient or under each experimental circumstance in which they are used.

- It remains unclear to what extent we have systematically underestimated tumorigenic cell frequencies by using assays that are not optimized for the engraftment of transplanted cells. In some cases, the underestimates may be modest and may not affect conclusions. In other cases, cancers thought to have only rare tumorigenic cells may actually have common tumorigenic cells.

- It will be necessary to systematically assess the degree to which changes in assay conditions affect the spectrum of cancer cells that can form tumors. If a cancer cell has the potential to proliferate extensively in any assay, then it has the potential to contribute to disease progression, and it is perilous to ignore that cell during therapy.

- It will be important to fate map cells within tumors thought to follow the cancer stem cell model to test whether only small populations of phenotypically distinct cells actually promote tumor growth and disease progression.

- Few studies have assessed the degree of genetic heterogeneity among cancer cells within the same tumor or the extent to which this causes phenotypic and functional differences. In some cancers, genetic heterogeneity may confound the assumption that heterogeneity arises from hierarchical epigenetic differences, rendering the cancer stem cell model difficult to test.

- It will be important to test whether primary tumors in vivo contain cells that reversibly transition between tumorigenic and nontumorigenic states. The cancer stem cell model may not effectively describe cancers in which the efficiency of interconversion is high.

- It will be necessary to distinguish cancers that follow the stem cell model from those that do not to avoid testing agents that target specific subpopulations of cancer cells in patients who have no chance of benefitting from them.

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