

Heterogeneity of Neoplastic Stem Cells: Theoretical, Functional, and Clinical Implications

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Abstract

Accumulating evidence suggests that human cancers develop through a step-wise, but nonlinear process of cellular diversification and evolution. Recent mutational analyses indicate that this process is more complex and diverse than anticipated before whole-genome sequencing methods were readily available. Examples are also emerging now of genetically abnormal clones of cells that have acquired mutations with known oncogenic potential but, nevertheless, may show no manifestations of malignant change for many years. To accommodate these diverse realities, we suggest the term neoplastic refer to clones of cells that have any type of somatic aberrancy associated with an increased propensity to become malignant, and the derivative term neoplastic stem cell be adopted to identify the cells responsible for the long-term maintenance of such clones. Neoplastic clones would thus include those that never evolve further, as well as those that eventually give rise to fully malignant populations, and all stages in between. The term cancer stem cells would then be more appropriately restricted to cells generating subclones that have established malignant properties. More precise molecular understanding of the different stem cell states thus distinguished should contribute to the development of more effective prognostic and therapeutic tools for cancer diagnosis and treatment. *Cancer Res*; 73(3); 1037–45. ©2012 AACR.

Introduction

Cancer means different things to biologists, clinicians, and patients. Nevertheless, there is a common assumption that a key property of most cancers is a potential for permanent uncontrolled growth that will cause premature death of the host if the cancer is not adequately treated. It is also widely accepted that cancers represent abnormal outgrowths from a single initial cell whose progeny then sequentially accumulate additional intrinsic changes that confer the properties of deregulated proliferation, invasive behavior, and faulty or blocked differentiation (1). Increasing evidence indicates that

these changes result from alterations to the genome and epigenome, acquired as part of a random, multistep process in cells that must already possess or can reactivate a permanent growth potential (2–6). The multistep nature of this process explains why most cancers, particularly at early stages in their development, reflect many features of the normal tissue in which they arise.

Recent studies have introduced a new view on cancer development. Next generation DNA sequencing methods indicate that the cellular DNA repair machinery is unable to keep up with the background rate of errors that accumulate in the genomes of cells of multicellular organisms, most of which are likely to prove biologically inconsequential (4, 7–12). In addition, there is growing evidence of changes in the molecular mechanisms that regulate stem cell differentiation and its control during normal development and aging, as well as in response to wounding, infections, and other perturbations of normal physiology (13–19). Because of the self-renewal capability of stem cells (20–22), unrepaired changes in the genomes of these cells can be retained in their amplified progeny. If a change is deleterious or able to provoke an effective immune response, the altered cells will not persist. However, with increasing age and an associated decline in immunocompetence, not all stem cells are likely to be eliminated in this way. As a result, many tissues will accumulate stem cells with somatic lesions, some of which may be pro-oncogenic even though they do not cause an overt change in the properties of the cells in which they first appear or in their expanding progeny (2, 4, 8, 23, 24). Appreciation of these issues has begun to undermine historic black and white distinctions between normal cells and transformed cells based on an assessment of

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their karyotypes, morphology, and behavior. The concept of accruing mutations and epigenomic changes with time (age) is consistent with the general observation that the incidence of overt malignancies increases with age (25). A more recent study has shown that the frequency of mutations in hematopoietic cells of aging individuals is a significant risk factor in their development of leukemia (26).

Biologic Foundation and Implications of the Terms "Clonal", "Neoplastic," and "Malignant"

Most cancers are clonal, that is, they derive from only one of the many trillions of cells present in the human body that are already tissue specified (Table 1). However, as normal tissues are also composed of self-sustaining clones, the term "clonal" on its own is not particularly useful as an indicator of a neoplastic process. The concept of a neoplastic clone implies permanence as well as the potential to develop into an overt cancer. Thus, evidence of a clonal population of cells, particularly if documented only once, is not sufficient on its own to infer an underlying malignant process. Even the apparent permanence of a neoplastic clone does not necessarily indicate that it will generate an overt neoplasm. Neoplastic populations of cells and their related clinical states may remain stable and even unchanged for decades (27, 28). Indeed, analysis of twins (29) and studies of "normal" individuals (30, 31) are providing evidence that undiagnosed (clinically asymptomatic) neoplastic states are more common than previously recognized. It is also important to note that a clone does not have to be obviously enlarged to be categorized as neoplastic, as the extent of growth perturbation may be quite subtle.

These issues are particularly relevant in understanding the subclonal diversity that characterizes many fully malignant lesions by the time they first become clinically apparent. The defining features of malignancy are perturbed differentiation and invasive properties as well as deregulation of mechanisms that control normal stem cell proliferation, self-renewal, and survival (Table 2). The multistep nature of the oncogenic

process that results in the acquisition of these abnormal properties anticipates that they will occur as part of a larger and increasingly nonlinear evolution, exacerbated by an erosion of genomic and/or epigenomic stability. Dominant and overtly malignant clones would thus be expected to commonly develop on a background of numerous related, but biologically and genetically distinct subclones with variable premalignant and malignant features (Fig. 1). Whole-genome sequencing studies of cells taken from different sites within the same tumor mass and/or at different sites or times from the same patient have recently provided additional evidence of this type of process (32–34). Thus, features ascribed to malignant populations are generally restricted to one or a few dominant subclones in the sample and fail to reflect the full diversity of the tumor due to the limited nature and size of the samples that can be accessed and the methodologies routinely used to analyze them (28).

Early Phases of Premalignant Neoplastic Clone Development

In the early phases of the development of a neoplastic clone, the minimal changes acquired typically seem to have little impact on its morphologic, immunophenotypic, or transcriptional features. This is consistent with the idea that the mechanisms that determine the normal unidirectional differentiation of the tissue and establish its hierarchical structure are likely to be relatively unperturbed. The chronic phase of chronic myeloid leukemia (CML) is a classic example of such a neoplastic condition in which most features of normal blood cell production are retained by the members of the neoplastic clone, although their output is deregulated (35, 36). However, if the chronic phase is not treated effectively, progression to a blast phase that resembles an aggressive (malignant) myeloid or lymphoid leukemia inevitably occurs. This is usually accompanied by the acquisition of new mutations believed to perturb the persisting mechanisms that regulate chronic phase stem cells or their early downstream differentiating progeny

Table 1. Indicators of clonal populations in early-stage cancer

Indicator	Examples
Abnormal cell morphology	Cell atypia, dysplasia, and immaturity
Accumulation of cell products in biologic fluids (serum/plasma)	Paraproteinemia, "tumor markers"
Aberrant immunophenotypes	CD5 ⁺ B cells, CD25 ⁺ mast cells, abnormal granulocytic and monocytic cells in low-risk MDS
Detection of disease-related fusion genes or point mutations ^a	<i>BCR/ABL</i> , <i>KIT D816V</i> , mutations in <i>RAS</i> or <i>BRAF</i> , <i>TcR</i> RR, <i>Ig</i> RR, <i>JAK2 V617F</i> , <i>FIP1L1/PDGFR</i>
Abnormal mRNA patterns in genome-wide screens (NGS/exome-sequencing)	Diagnostic algorithms are currently being developed for various neoplasms
Indirect molecular demonstration of monoclonality	Differentially methylated X-linked genes polymorphisms, <i>G6PD</i> , <i>HUMARA</i>

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; HUMARA, *human androgen receptor* gene; NGS, next generation sequencing; RR, gene rearrangement; *TcR*, *T cell receptor* gene; *Ig*, *immunoglobulin* gene.

^aGerm line controls (buccal swap, nails, or hair) or quantification may confirm that the aberration is a somatic defect.

Table 2. Terminology and axiom-definitions for neoplastic cells and related conditions

Operative term	Definition	Criteria
Clonal cells	Persistent <u>or</u> nonpersistent cells that express a molecular lesion that is not detected in the germline in the same patient; umbrella term that includes other cells listed below.	Molecular lesion ^a , somatic; cells may or may not be detectable by flow cytometry or by histology/IHC.
Neoplastic cells	Persistent clonal cells expressing a molecular lesion that is not detectable in the germline in the same patient. May produce a histologically defined premalignant condition or an overt malignancy.	Molecular lesion ^a , somatic; persistent cells; may or may not be detectable by flow cytometry or by histology/IHC.
Premalignant neoplastic condition	Produced by neoplastic cells and: clinical significance and/or prognosis remain largely uncertain at diagnosis ^b and: criteria for a malignant condition are not yet met.	Molecular lesion ^a , somatic and persistent and: defined neoplastic lesion detected by histology/IHC or/and by flow cytometry (indolent type)
Malignant cells	Neoplastic cells that produce a histologically defined malignancy (cancer).	Usually invasive morphology and histology

Abbreviation: IHC, immunohistochemistry.

^aDisease-related lesion that is detectable by polymerase chain reaction, sequencing, conventional cytogenetics, or FISH.

^bExamples: see Table 3.

(3, 16, 37). A stepwise progression to acute myeloid leukemia (AML) can also frequently be followed in other myeloproliferative neoplasms (MPN) and myelodysplastic syndromes (MDS) (38–40). Even in the case of *de novo* AML, the malignant clone still may show phenotypic and biologic evidence of a residual persisting hierarchy, although no cells show morphologic features of differentiation (41–43).

Numerous examples also exist of long-lived, neoplastic clones that show minimal changes from the normal human tissues in which they are found and from which they presumably arise (Table 3). Monoclonal gammopathy of undetermined significance (MGUS) is an example of a premalignant hematopoietic neoplasia that may or may not progress to an overt malignancy, that is, multiple myeloma (7, 24). In solid tissues, neoplastic clones that show altered properties but may not evolve to overt (invasive) malignancies are also well documented, for example, in the gastrointestinal tract (4, 19, 44), mammary gland (45, 46), prostate (47), endometrium (48), lung, and skin (8) (Table 3). In all of these conditions, transformation to an overt cancer with invasive and/or metastatic activity is now considered to be accompanied by the accumulation of additional oncogenic mutations.

Normal, Neoplastic, and Cancer SCs

Most normal tissues are composed primarily of specialized cells that must undergo some rate of replacement throughout life. Evolution has dealt with this challenge by programmat-

ically uncoupling the mechanisms that allow cells to divide indefinitely and those involved in imposing a specialized program of biochemical activities within them. This process has led to the creation of tissue hierarchies in which durable self-renewal capability is usually restricted to cells that have a defined but latent differentiation potential (the stem cells), and the output of expanded numbers of differentiated progeny is accomplished by a series of cell divisions that precede the terminal execution of a unidirectional differentiation program (13, 14, 49).

The concept of cancer stem cells (CSC) derives from 3 observations. The first is the fact that malignant clones usually display some features of their tissue of origin. The second observation is the permanence of malignant populations, implying that the population as a whole must be sustained by a subset of cells that possess or reversibly acquire a "self-renewal competent" state. The third observation is the multiplicity of rare events that are typically accumulated before a malignant population is detectable (2, 23, 50–52). The fact that these events are rare is supported by the many years found to separate the first events and the appearance of a derivative malignant cell population (53, 54). However, unless an initial change deregulates cell output, the clone it produces is unlikely to comprise sufficient progeny to ensure that at least one undergoes a further compounding change. These considerations have led to the idea that many of the molecular mechanisms responsible for the hierarchic organization of a tissue are

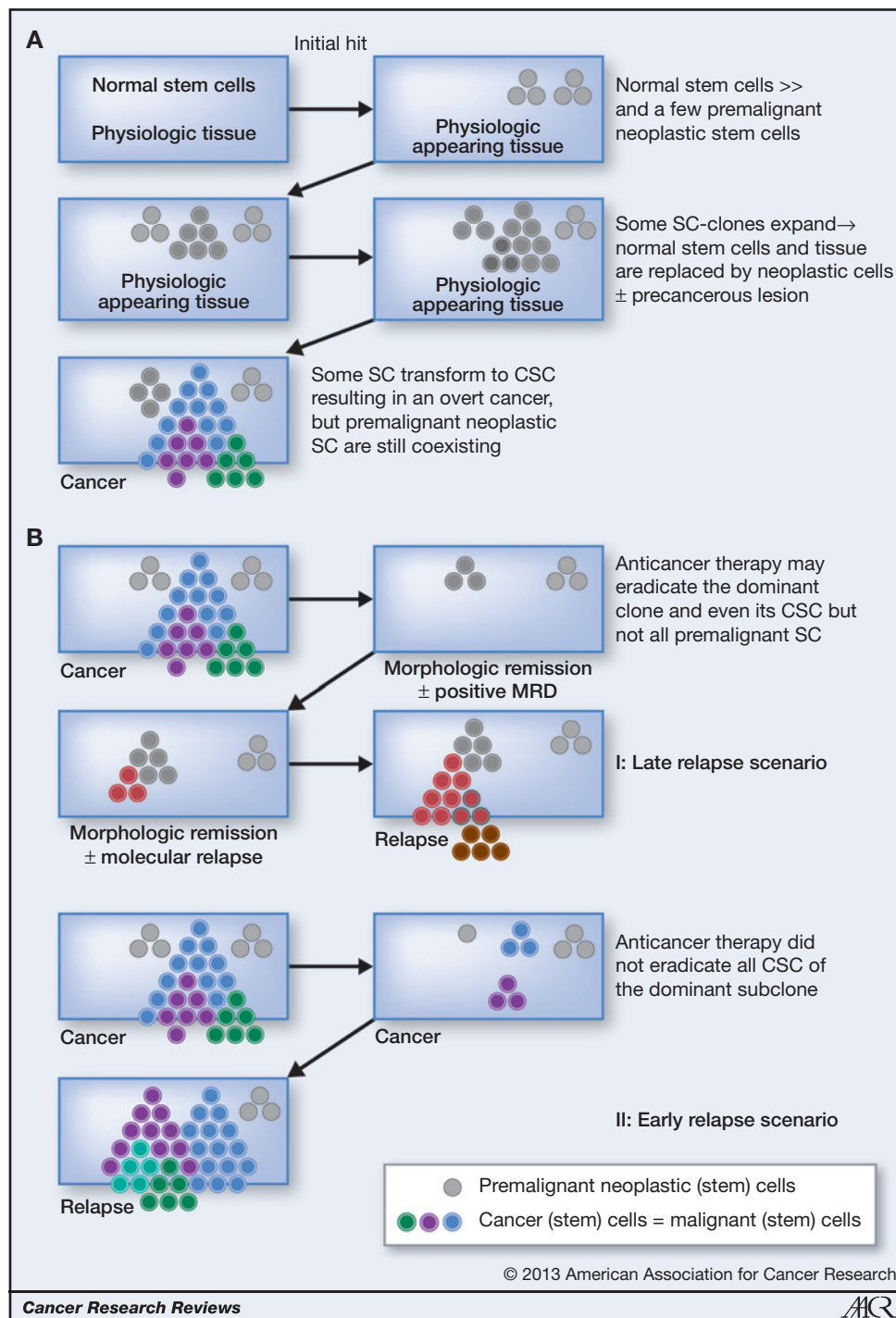


Figure 1. A, proposed model of evolution of Neo-SCs. An initial transforming event converts a normal stem/progenitor cell into a Neo-SC. By definition, Neo-SCs have self-renewing capacity and the potential to generate a persistent clone. These cells are referred to as premalignant (or preleukemic) Neo-SCs when they produce small-sized subclones (yellow cells) and have not yet acquired all of the properties required to be classified as malignant. During the subsequent phases of oncogenesis, slowly expanding subclones may arise from early subclones, but even if slowly expanding, these newly formed subclones may not yet have any immediate cancer-initiating potential. In fact, many of them may never produce an overt malignancy because they can be controlled by the natural immune system and environment of the organ in which they reside (blue boxes). Later, after an unpredictable time, one or more cells may accumulate a sufficient number of hits that are oncogenic (indicated by color-changes) and enable the cells to generate an overt malignancy. These cells are fully malignant Neo-SCs (also called cancer stem cells or leukemic stem cells). However, the other premalignant subclones and their Neo-SCs remain and continue to evolve. The term Neo-SCs is thus an umbrella term used to define the total collection of premalignant and malignant Neo-SCs. B, effects of anticancer therapies on Neo-SCs. Anticancer therapy may eliminate some or even most of the rapidly expanding subclones and their Neo-SCs. Resulting remissions may be durable, at least for some time. However, as long as premalignant Neo-SCs were not eradicated, the disease may relapse from these Neo-SCs after a certain (unpredictable) latency period (late relapse scenario). If fully malignant Neo-SCs were not (all) eradicated by the anticancer therapy, an early relapse will inevitably follow (early relapse scenario). MRD, minimal residual disease.

Table 3. Examples of premalignant conditions and malignant derivatives

Disease	Premalignant early lesion	Early neoplasm	Overt cancer/malignancy	Advanced/resistant cancer
MDS	IDUS/ICUS	Low-risk MDS	High-risk MDS	sAML
CML	early CP	CP	AP	BP/BC
SM	MMUS	ISM	ASM	MCL
MM	MGUS	Smoldering MM	MM	PCL
CLL	CBL	Low-risk CLL	High-risk CLL	Resistant CLL
Colon Ca	Adenoma	Adenomatosis	Colon Ca	Resistant/meta Ca
Bronch SCC	BSM	CIS/BIN	Bronchial SCC	Resistant/meta SCC
Endomet Ca	EGD	SEIC/SEIN	Endomet Ca	Resistant/meta Ca
Prostate Ca	PH	PIN	Prostate Ca	Resistant/meta Ca
Me	Nevus	Me <i>in situ</i>	Me	Resistant/meta Me

Abbreviations: AP, accelerated phase; ASM, aggressive systemic mastocytosis; BC, blast crisis; BIN, bronchial intraepithelial neoplasm; BP, blast phase; Bronch SCC, bronchial squamous cell carcinoma; BSM, bronchial squamous metaplasia; Ca, carcinoma; CBL, chronic B lymphocytosis; CIS, carcinoma *in situ*; CLL, chronic lymphocytic leukemia; CP, chronic phase; EGD, endometrial glandular dysplasia; Endomet Ca, endometrial carcinoma; ICUS, idiopathic cytopenia of unknown significance; IDUS, idiopathic dysplasia of unknown significance; ISM, indolent systemic mastocytosis; MCL, mast cell leukemia; Me, melanoma; Meta, metastasis-forming; MM, multiple myeloma; MMUS, monoclonal mast cells of unknown significance; PCL, plasma cell leukemia; PH, prostate hyperplasia; PIN, prostatic intraepithelial neoplasia; sAML, secondary AML; SCC, squamous cell carcinoma; SEIC, serous endometrial intraepithelial noninvasive carcinoma; SEIN, serous endometrial intraepithelial neoplasm; SM, systemic mastocytosis.

still intact in early- and intermediate-stage premalignant clones.

In light of all of these considerations, we suggest the updated definitions for different types of (stem) cells provided in Table 2. These definitions emphasize restricting the use of neoplastic to sustained clonal cell populations within tissues that display phenotypic changes and/or genetic mutations associated with clinically established aberrant growth (including, but not requiring, that these be associated with an oncogenic process). Accordingly, the term neoplastic stem cells (Neo-SC) would identify all cells that sustain clones, including premalignant Neo-SCs and malignant Neo-SCs. At the same time, the umbrella term Neo-SCs would not automatically imply that any of the clonal constituents have malignant potential or pose a clinical concern. On the other hand, the clonal outputs of individual normal stem cells are rarely either persistent or dominant under homeostatic conditions, whereas Neo-SCs often produce clones that exhibit both of these features, in addition to their variable likelihood of further progression toward a malignant state. Examples of readily observed progression of a clinically recognized premalignant neoplastic clone include colon adenoma, various categories of *in situ* "cancers", MGUS with plasmacytosis, and early chronic phase CML (Table 3).

Importantly, just as normal tissue integrity is continuously maintained by multiple clones of differentiating stem cells, a similar biology is thought to apply to abnormal clones that display a proliferative behavior that is greater than that typical of the cells from which they arise. Thus, conditions that elicit the distinguishing regenerative potential unique to the normal stem cells of a given tissue can also frequently be used to detect the stem cells that maintain perturbed clones, be they neoplastic but not (yet) malignant or fully malignant. Accordingly,

"propagating ability," either *in vitro* or *in vivo* (in xenografted immunodeficient mice), is also not necessarily sufficient to discriminate between normal stem cells, nonmalignant or premalignant Neo-SCs, and malignant Neo-SCs (28). While this potential source of confusion is widely appreciated among experimentalists, a nomenclature that recognizes and attempts to eliminate this difficulty, as proposed in this article, has not yet been generally agreed upon. The underlying issues are also of importance as assays for different types of Neo-SCs are becoming increasingly deployed to identify new agents with anticancer potential and prognostic markers able to predict the likely effectiveness of such agents. It is also important to note that neither Neo-SCs nor CSCs must derive by genetic alterations in normal tissue stem cells. They may also develop from immature progenitor cells that reacquire self-renewal and thus stem cell properties during transformation.

Early Types of Neo-SCs May Have Features Distinct from CSCs

The recent development of new and powerful methods for whole-genome analyses at multiple levels down to single nucleotides has rapidly escalated the many types of cancers investigated in this way. The data already accrued have greatly enlarged our appreciation of the typically large scale of genome diversification that commonly occurs even before a given neoplasm becomes symptomatic. Indeed, the extent of the changes documented has suggested that the majority of mutations present are biologically neutral "passenger mutations," reflecting genomic evolution and/or instability of the cells without other biologic significance (5, 6, 55). As a consequence, inferring which mutations have "driver" activity and under what conditions, has become a major challenge.

The selective outgrowth of neoplastic clones and subclones ultimately relies on the acquisition by the Neo-SCs responsible for clonal maintenance of new properties that perturb normal control mechanisms and give the clonal cells deregulated growth properties. Although this may not lead to the immediate generation of fully malignant CSCs, deregulated growth is one of their key features. Deregulated growth of the clone may be achieved by changes that confer or enhance self-renewal, proliferation, and/or a survival advantage to the initial stem cells or their derived non-stem cell progeny. In solid tissues, the acquisition of deregulated invasive properties is another key feature of CSCs. Importantly, none of these would be anticipated to necessarily increase the rate of mutations or epigenetic changes. Thus, progression to a more aggressive (malignant) state would be expected to depend on the increased number of potential target cells in which additional events would then be more likely. Such changes would also be expected to be accelerated by deregulation and/or overt mutation of the DNA synthesis and repair machinery, or other mechanisms that could contribute to a heightened genomic instability [e.g. control of reactive oxygen species (ROS)].

In summary, early types of Neo-SCs may differ markedly in their biology, epigenetic features, and mutational profiles when compared with fully malignant Neo-SCs (CSCs). We have recently suggested that early Neo-SCs be designated as pre-malignant (28). Nonmalignant Neo-SCs (or Neo-SCs with unknown malignant potential) are more cumbersome alternative terms, although they may better convey the important concept of uncertain oncogenic potential.

Interestingly, there is a growing evidence that the molecular mechanisms that control the "stem cell state" are not as irreversible as historically assumed for both "normal" (56–58) and malignant cell populations (59–62). Thus, the possibility that Neo-SCs can transiently lose and then regain stem cell properties is one that needs to be entertained in future studies of the prognostic significance of new biomarkers and the long-term therapeutic effects of new agents.

Detection and Characterization of Early-Stage Neo-SCs

With the exception of a few examples, little is known about the numerical, phenotypic, and functional diversity of Neo-SCs that do not have malignant properties. This is likely due to both, their poor accessibility and lack of knowledge about how to detect and isolate them. One of the best exceptions are the Philadelphia chromosome-positive (Ph⁺)/*BCR-ABL*⁺ Neo-SCs from patients with early chronic phase CML, although even these have been notoriously elusive to study because of their low frequency (63–65). In other examples of nonmalignant neoplastic lesions, like the MPNs or colon adenomas, primary Neo-SCs have also been proven difficult to maintain *in vitro* or *in vivo* (66, 67).

Mechanisms of Disease Progression: Role of Molecular Lesions and Epigenetic Deregulation

Little is known about the factors that regulate the progression of cancer, although these are known to have disease-specific time frames. One factor is related to the frequent

observation of mutations in or deregulated functionality of genes that normally help to maintain genomic stability in clones that have achieved malignant properties (68, 69). If relevant changes are accrued early, these would be expected to affect the rapidity with which a malignant subclone becomes apparent. Thus, one important factor is genomic instability, which may be triggered by both mutations and epigenetic changes. For example, genetic instability may result from unfaithful/inefficient DNA repair and an increased production of ROS that may lead to oxidative DNA damage and thus, contributes to genetic instability (70, 71). On the other hand, progression to a fully malignant state may occur rapidly if the initial mutations are capable of eliciting the required changes.

Role of Epigenetic Regulation in Disease Progression and CSC Evolution

Now there is also emerging evidence that perturbations in genes that regulate the epigenomic status of cells make a common and important contribution to the evolution of malignant cells (10, 28). Although this is not a surprising finding, little is known about how and why this occurs. Some of the oncogenic lesions accumulating in neoplastic cells during disease progression may trigger focal hypermethylation of certain tumor suppressor genes and thereby contribute to epigenetic disturbances (72). Other lesions may lead to abnormal chromatin methylation or acetylation. Finally, epigenetic changes may depend on the influence of the tumor microenvironment. Indeed, even fully malignant cells are not usually capable of autonomous growth or survival, but remain responsive to environmental cues that regulate their cycling activity, oxygenation, accessibility to diffusible drugs, migratory activity, and other key features.

Relevance to the Clinic

The development of improved methods to detect, isolate, and characterize "early" (pre-malignant) Neo-SCs before they have evolved to acquire malignant properties has clinical as well as biologic importance. First, these cells may play a role in cancer relapses, particularly in which the latter are delayed or not anticipated (28). In line with this possibility, late relapses often behave differently and exhibit different patterns of genomic alteration and (epi) genetic properties as compared with initially detectable malignant cells. One explanation for such a finding would be the possibly slow but likely expansion of still viable, but quiescent pre-malignant Neo-SCs or CSCs with other mutations that were present before the treatment was started at a level *in vivo* that was below the limit of detection. Even if a remission-induction therapy was effective in eliminating all the CSCs present, some pre-malignant Neo-SCs may possess or have acquired new mechanisms that make them treatment-resistant. An example of Neo-SCs that have different mutations from malignant clones believed to have a common ultimate origin is provided by the *JAK2* V617F+ MPNs, in which secondary AMLs have been found to lack the original *JAK2* mutation (73). Similar observations have been made in other types of leukemias and even in CML, although in the latter example, the occurrence of a Ph-negative blast phase is a rare event (74, 75). Given the mutagenic potential of many

of the antineoplastic drugs in widespread use, they might also be anticipated to accelerate the production of new CSCs from surviving premalignant Neo-SCs. Thus, improved characterization of premalignant Neo-SCs, and strategies to detect and eliminate them, could lead to more effective (curative) anticancer therapies.

Conclusions

The CSC hypothesis has important clinical implications and is already being used to develop and anticipate new treatment approaches that will be more effective against solid tumors as well as leukemias. At the same time, there is a growing appreciation of the complexity of how malignant populations develop and the nonlinear evolutionary process that describes their emergence and divergence. Multiple lines of evidence indicate that this often involves initial changes that alter the growth properties of normal tissue stem cells leading to the production of slowly enlarging but nonmalignant neoplastic clones with at least a partially preserved hierarchic structure and from which CSCs may later develop from different stages within this perturbed hierarchy. There is thus an important need to distinguish the different molecular states of cells that sustain transformed populations during their evolution and learn more about the mechanisms that dictate their different abnormal properties

and likelihood of continued malignant transformation. Such knowledge may lead to the development of more specific, durable, and even preventive individualized anticancer strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Development of methodology: P. Valent

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Valent

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Valent, C. Eaves

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