REVIEW ARTICLE

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Clinical and Therapeutic Implications of Cancer Stem Cells

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Almost all cancers arise in organs and tissues containing cells that have the ability, for the lifetime of the organism, to replicate in order to maintain and replace cells that are lost because of aging or damage. For example, the entire lining of the gut is replaced approximately every 7 days, and the skin epithelium is replaced once a month. With cell damage from factors such as toxins, ultraviolet radiation, or inflammation, cell loss and replacement are accelerated.[1,2]

Healing damaged tissue with the use of a large number of undamaged cells would seem to be the most efficient mechanism of repair. However, in tissues in which cancers normally arise, such as the skin, mammary glands, gut, brain, and blood, only a minority population of stem cells maintains and repairs the tissue, whereas most replication-competent cells have a limited life span. Why would such a seemingly inefficient mechanism of tissue homeostasis, in which only a few cells are ultimately responsible for fixing damage, be used by long-lived multicellular organisms? The answer most likely is to reduce the incidence of cancer. It is now clear that normal cells in tissue undergo mutations during cell division and exposure to agents that cause genotoxic stress.[3] Since cancer can arise from multiple oncogenic mutations, the odds of a cell accumulating all of the mutations needed for oncogenic transformation are drastically reduced if only a few replication-competent cells in a tissue survive for the duration of a person's life. Thus, in order to understand cancer, it is important to understand the underlying biologic features of stem cells and how these cells differentiate into the mature cells of a tissue.

The Biologic Features of Normal Somatic Stem Cells

Blood (hematopoietic) stem cells are the best-understood mammalian somatic stem cells because a huge effort was put into finding the cause of death from ionizing radiation created by the atomic bomb. Death from the minimum lethal dose of radiation is a result of blood-system failure. A seminal study by Becker et al. showed the existence of hematopoietic stem cells.[4] Subsequently, hematopoietic stem cells have been shown to constitute approximately 1 of 50,000 to 100,000 nucleated bone marrow cells,[5] and a single hematopoietic stem cell can regenerate the blood system in a lethally irradiated mouse (Fig. 1).

Although murine hematopoietic stem cells are largely quiescent and divide only four times during the lifetime of a mouse (approximately 2 years),[6] in times of stress, when increased production of blood cells is required, quiescent hematopoietic stem cells will replicate in order to supply the needed immune cells. When hematopoietic stem cells do divide, they can give rise to either another stem cell or to progenitor cells that eventually differentiate into mature cells such as red cells, platelets, granulocytes, and lymphocytes. Cell division that generates another

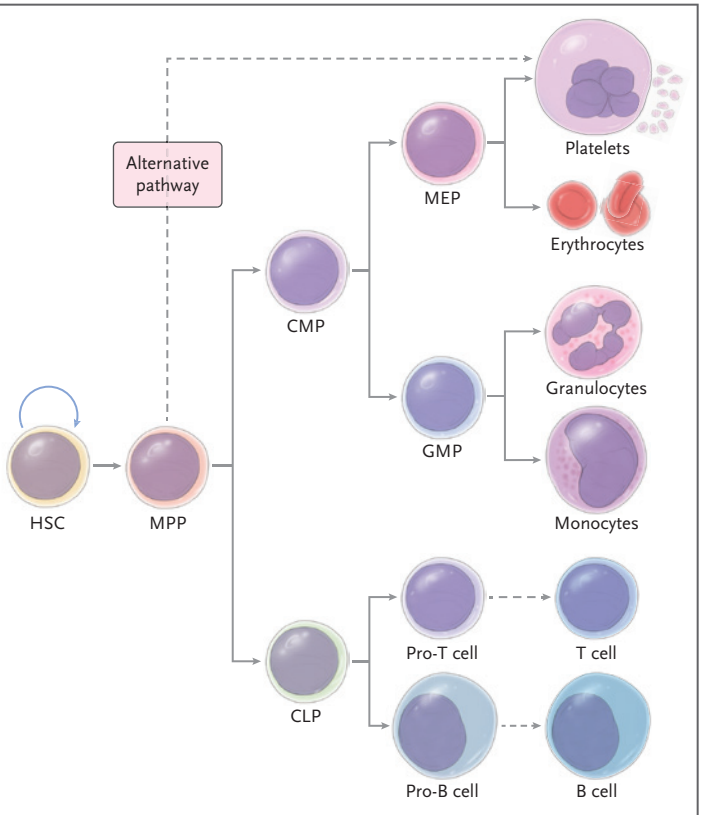

Figure 1. Classic Model of the Hematopoietic Differentiation Hierarchy.

An overview of hematopoietic stem-cell (HSC) development in a mouse is shown. The HSC can regenerate itself for the lifetime of the animal through a process of cell division called self-renewal. The HSC can also generate progeny such as multipotent progenitor (MPP) cells, which then progressively can give rise to more differentiated cell types. The differentiating progeny are unable to self-renew and have a limited life span. The dashed lines show a recently emerging alternative model in which lineage commitment (e.g., to making platelets) occurs early, even in the first differentiating cell division. CLP denotes common lymphoid progenitor, CMP common myeloid progenitor, GMP granulocyte–monocyte progenitor, and MEP megakaryocyte–erythrocyte progenitor.

stem cell is called self-renewal. Self-renewing cell division generates another stem cell with a similar capacity to proliferate and regenerate the blood system for the lifetime of an animal. However, when a stem cell generates a progenitor cell, with the exception of memory lymphocytes, the progenitor cells have limited replication capacity and cannot maintain hematopoiesis for extended periods.

Other tissues and organs in which cancer commonly develops, such as the skin, mammary glands, gut, prostate gland, liver, and even the brain, also contain stem cells.[7-12] The stem cells in each of these tissues appear to share some properties with hematopoietic stem cells in the sense that they persist for a lifetime, can expand to regenerate themselves after loss of stem cells because of exposure to toxins, and can migrate in the tissue to repair tissue injury. For example, with inflammation and activation of the innate immune system by toll-like receptor (TLR) signaling, stem cells of the gut and mammary glands are activated by the TLR signaling pathway to repair the tissue damage.[2] Normally, the majority of the stem cells in the mammary glands, brain, and skin are quiescent,[13,14] whereas a large number of leucine-rich repeat-containing G-protein–coupled receptor 5 (Lgr5)–positive stem cells of the gut are proliferating. Furthermore, like drosophila germ cells in which early but not late progenitor cells can reacquire stem-cell properties, some early progenitors in the gut reacquire expression of Lgr5.[15,16] Thus, stem cells in different tissues are not identical.

Since stem cells in different tissues are unique to that particular tissue and the cancers that arise from the same tissue, intensive investigation of stem cells in all tissues is of paramount importance to understand cancer and degenerative diseases of specific tissue types. Both cell-extrinsic and cell-intrinsic factors regulate the central stem-cell property of self-renewal in each tissue. Nurse cells provide a niche that produces factors such as Kit ligand for hematopoietic stem cells or Wnts and R-spondins (ligands for the β-catenin signaling pathway) for stem cells of the gut, brain, and liver.[17,18]

The primary difference between a stem cell and the other cells in a tissue is the epigenetic makeup (DNA methylation and chromatin modifications). It is not surprising that certain epigenetic regulators, transcription factors, and microRNAs play an important role in the self-renewal of stem cells.[19,20] For example, the chromatin modifier Bmi1 is absolutely required for self-renewal of hematopoietic stem cells, as well as for self-renewal of neural, mammary-gland, and prostate-gland stem cells.[21] Bmi1 functions by modifying histones and repressing the expression of genes regulating apoptosis ($p19^{Arf}$ and the Tp53 pathway), senescence ($p16^{ink4a}$), and differentiation (*Hox* genes) in stem cells but not in their differentiated progeny.[19,22] However, the expression of Bmi1 by stem cells is not sufficient for self-renewal and stem-cell maintenance. With differentiation, daughter cells also begin to ex-

press factors such as the microRNA miR-200, which targets Bmi1, and the Bmi1 antagonist Usp16.[23] Thus, stem cells are not defined solely by the expression of positive regulators of self-renewal but also by the lack of expression of genes that inhibit self-renewal in progenitor cells (Fig. 2).

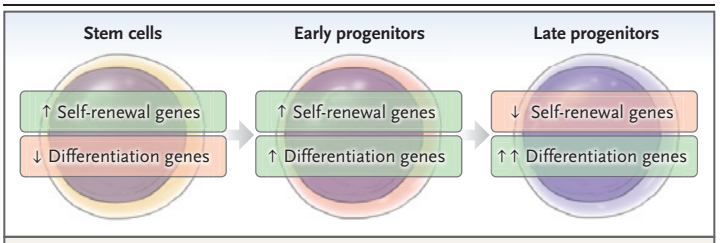

Figure 2. Positive and Negative Regulation of Stem-Cell Self-Renewal.
HSCs can regenerate themselves by expressing genes that are necessary for self-renewal, and they lack expression of genes that induce cell death, cellular senescence, and differentiation. The first differentiated progeny (early progenitors) often express many genes that positively regulate self-renewal, but they also turn on genes that prevent self-renewal and promote differentiation. Their progeny (late progenitors) turn off self-renewal genes and turn on differentiation genes. As cells differentiate, the number of differentiation genes increases (double arrows).

STEM CELLS AND CANCER INITIATION

Stem cells are the longest-living cells in many tissues, and multiple mutations must accumulate in cancer. Thus, the initial oncogenic mutations probably occur in stem cells. The evaluation of patients with acute myeloid leukemia (AML) who were survivors of exposure to atomic-bomb radiation in Japan provided early evidence that this is the case. Examination of normal hematopoietic stem cells in patients who had been cured of leukemia revealed that a significant number of hematopoietic stem cells carried an *AML1-ETO* mutation; this suggests that the original preleukemic mutation occurred in hematopoietic stem cells.[24] Single-cell sequencing studies involving patients with AML showed that normal hematopoietic stem cells carried one or two of the mutations in the frank AML blasts.[25] This finding is also consistent with a model showing that cancer arises from sequential mutations accumulating in tissue stem cells.[26] The discovery of clonal hematopoiesis provided the ultimate proof of the stem-cell mutation model.[27-30] The risk of leukemia is 1% per year among patients with mutations leading to clonal hematopoiesis.

There is evidence in both mice and humans that the initial oncogenic mutations in solid tumors occur in stem cells.[11,31-33] In genetic studies involving models of brain cancer, skin cancer, and colon cancer in mice, single oncogenic mutations in either stem cells or more differentiated progeny indicated that mutations in stem cells lead to cancer.[11,31-35] Furthermore, as in AML, human glioblastoma mutations are seen in normal neural stem cells in the subventricular zone, away from the primary tumor; this suggests that as in mouse models and human AML, the initial mutations in human glioblastoma tumors occur in stem cells.[36]

Although it is clear that in many cancers the initial oncogenic mutation or mutations occur in a stem cell, the subsequent mutation that ultimately results in the first fully transformed cancer cell that gives rise to the cancer might occur either in a stem cell or in an immature progenitor cell.[22,26,37-41] Why can the final oncogenic transformation occur in progenitor cells? The initial oncogenic mutations frequently involve epigenetic regulators that control stem-cell functions.[2,25,27-30,42] A combination of several critical mutations that together activate self-renewal programs and also inactivate senescence and apoptosis programs can confer long-term proliferation capacity to early progenitor cells (Fig. 3).[22] Examples of the former include activating mutations of the telomerase (*TERT*) promoter in multiple types of cancer[36,43] and the epigenetic regulator *TAL1* promoter in AML.[44] Examples of the latter include inactivating mutations of tumor suppressor genes such as *Tp53* and *CDKN2a*.[45-47] The latter mutations can then confer uncontrolled proliferation and expansion properties to the cells.[22]

PREMALIGNANT MUTATIONS IN STEM CELLS

Since oncogenic mutations cause clonal hematopoiesis, it is not surprising that patients with these mutations have a very high risk of leukemia. However, it is surprising that patients with clonal hematopoiesis have an increased risk of other conditions, including cardiovascular disease and type 2 diabetes mellitus. Clonal hematopoiesis can lead to increased coronary atherosclerosis and calcification. Several of these

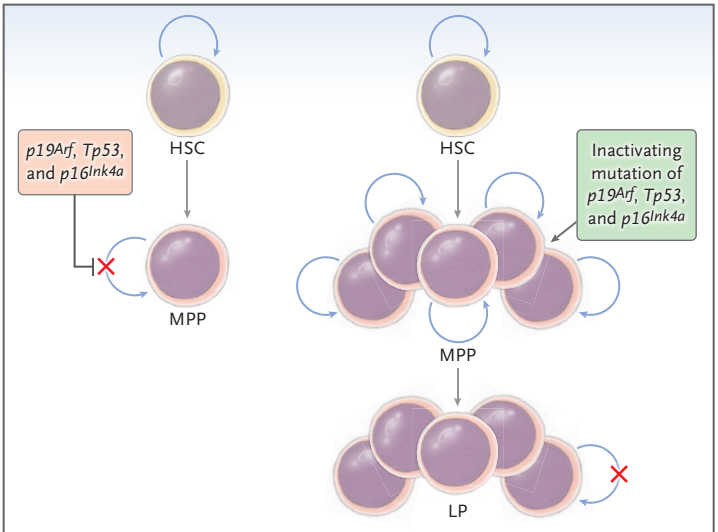

Figure 3. Molecular Mutations Conferring Stem-Cell Function to Early Progenitor Cells.

In normal hematopoiesis (left), expression of tumor-suppressor genes such as *Tp53*, which is mutated in some cases of clonal hematopoiesis, prevents MPP cells from self-renewing. Mutation of three tumor-suppressor genes (right) enables early but not late MPP cells to acquire the cardinal stem-cell function of self-renewal. This confirms the hypothesis that the more differentiated a cell becomes, the more mutations are necessary for the cell to gain uncontrolled replication. LP denotes late progenitor.

mutations occur in genes such as *IDH1|2*, *DNMT3a|b*, and *TET2*, which have been linked to stem-cell regulation.[48] In mice, mutation of *tet2* (a common epigenetic regulator mutation seen in clonal hematopoiesis) increases the incidence of atherosclerosis. This is probably a result of perturbed function of monocytes and macrophages caused by *tet2* mutation.[27,28] Mutation of *jak2*, another common mutation associated with clonal hematopoiesis, promotes thrombosis.

Thus, perturbations of normal immune functions of progeny of hematopoietic stem cells can contribute to other pathologic conditions. Genomic sequencing of normal cells in other tissues suggests that premalignant mutations might accumulate in other tissue stem cells. In glioblastoma, the otherwise normal subventricular zone stem cells in the brain contain oncogenic mutations seen in frank cancer. During aging, apparently normal cells of the esophagus, skin, mammary glands, and probably other tissues can contain some mutations that are seen in cancers arising in those tissues. It is reasonable to postulate that if mutations occur in the stem cells in those tissues, then these stem cells could be premalignant cells that are prone to the development of cancer, as is the case in clonal hematopoiesis.[49,50]

CANCER STEM CELLS AND PROGNOSIS

Cancer stem cells are present in many myeloid leukemias and solid tumors, including glioblastoma and breast, colon, and skin squamous-cell cancers.[51] The initial isolation of solid-tumor cancer stem cells was based on assays of samples of human breast cancer cells that had been transplanted into immunodeficient mice.[52] These assays showed that in eight of nine tumors, only a minority of cancer cells had stem-cell properties. However, there was early skepticism regarding the existence of cancer stem cells, since differences in the mouse and human microenvironment or the transplantation assay itself could have led to erroneous conclusions.

The existence of cancer stem cells was further questioned because of experiments that in retrospect were shown to have flaws. Examples of these flaws include conflation of the expression of a particular gene with a cell that was a stem cell, failure to understand that environmental signals can induce epigenetic changes that change the markers that a stem cell expresses, use of cell lines instead of primary mouse or human cells for experiments, and lack of awareness that oncogenic mutations can lead to two or more cell populations that can function as stem cells in a tumor.

Nevertheless, modern in vitro organoid culture assays, experiments involving genetic lineage tracing of both mouse and human cancers, and — most important — in vivo microscopy of mouse breast tumors have all confirmed that the initial transplantation experiments with breast, brain, and skin squamous-cell and basal-cell cancers were interpreted correctly. In many of the aforementioned solid tumors, only a minority population of the cancer cells had the ability to maintain a tumor, and they retained the potential to generate more stem cells as well as terminally differentiated progeny.[53] The in vivo microscopy investigation of mouse tumors showed that the majority of the breast cancer cells were differentiated and had limited or no ability to proliferate. The cancer stem cells were a minority that could give rise to clones of continuously growing tumors. Notably, the long-term clonogenic potential was maintained only

in a fraction of the progeny, whereas the rest had lost this capacity; this finding underscores an inherent hierarchy.[53]

Epigenetic programs that are used by normal stem cells to regulate cell differentiation, long-term proliferation (self-renewal), tissue repair, and differentiation are corrupted in cancer. This results in the continuous expansion of cells with long-term growth potential. Nonetheless, in many tumors, only a small subset of the cancer cells has the ability to self-renew at any point in time. Martin et al. performed studies that exploited the expression of different alleles of the X-linked gene *G6PD* to show that in chronic myeloid leukemia (CML) and in many cases of AML, postmitotic cancer cells are derived from the leukemic clone.[54] These cells are called leukemic (cancer) stem cells.[54] Since certain mutations can confer stem-cell properties on a previously non–self-renewing cell population, the cancer stem cells can ultimately arise from an early progenitor cell.[37] Thus, the term "cancer stem cell" is a functional definition and does not necessarily designate a cancer cell that is a transformed normal stem cell. Cancer stem cells that are derived from a progenitor cell do depend on activation of normal stem-cell pathways[39,55,56] and inactivation of pathways that inhibit stem-cell self-renewal.[22] Even in cases in which leukemia is derived from an early progenitor cell, most of the leukemic blasts generated by the leukemic stem cells are cells with limited or no proliferation capacity.[37,39]

Furthermore, the more mutations that a cancer cell undergoes, the greater the chance that pathways that promote self-renewal will be activated and pathways that inhibit self-renewal will be inactivated. This is consistent with observations in the evolution of CML to blast crisis as well as in triple-negative breast cancer and melanoma.[11,26,27,31-33] Cancer cells use normal stem-cell self-renewal for long-term proliferation and tissue-repair pathways for invasion[26]; this suggests that the stem-cell frequency in a cancer would correlate with prognosis. This is indeed the case with breast cancer, colon cancer, and AML.[51]

CANCER STEM-CELL BIOLOGIC FEATURES AND THE CLINIC

A major question about the cancer stem-cell field is whether it has any clinical relevance. Data generated in the past few years indicate that it does. For example, using a new bioinformatics approach, Dalerba et al. found that caudal-type homeobox transcription factor 2 (CDX2) was a biomarker that could be used to quantify the number of undifferentiated, primitive cells in colon cancer.[57] Approximately 4 to 7% of colon cancers are made up of more than 95% CDX2-negative immature colon cancer cells. As predicted, in the study by Dalerba and colleagues, patients with CDX2-negative stage II colon cancer had a very poor prognosis. These results were initially questioned, but another group of researchers later reported that their results were consistent with those of Dalerba and colleagues.[58]

Subsequent large studies have also confirmed that such patients with both microsatellite instability–positive (mutations in DNA-repair pathway genes, particular those genes involved in mismatch repair) and microsatellite stability (no mutations in DNA-repair pathway genes) CDX2–negative stage II colon cancer tumors have a poor prognosis.[59] Of potential clinical interest, unlike the majority of patients with stage II colon cancer, patients with stage II CDX2-negative colon cancers appeared to benefit from adjuvant chemotherapy in a multivariate analysis. Although this was a retrospective study and patients did not initially undergo randomization according to CDX2 expression, CDX2 was the only new variable analyzed in clinical studies of closely matched patient cohorts. It is possible that the biologic features of cancer stem cells may inform treatment decisions in other cancers.

Normal stem cells and their cancer stem-cell counterparts protect themselves from toxins and genotoxic stress through multiple mechanisms, including increased expression of DNA-repair pathways, expression of antiapoptotic genes, and maintenance of a low reactive oxygen species (ROS) environment.[1,60-62] Many therapeutic agents, including radiation and some chemotherapeutic agents, kill cells by increasing the levels of ROS. Both normal and malignant stem cells protect themselves from ionizing radiation and certain chemotherapy drugs by reducing ROS levels induced by these treatments.[1] The *KEAP1–NRF2* pathway is a major regulator of ROS, and mutations of this pathway in lung cancer make the cells resistant to radiation. Most patients who have stage II or stage III lung cancer with *KEAP1–NRF2* mutations have a relapse of their disease after radiation therapy[63]; this suggests

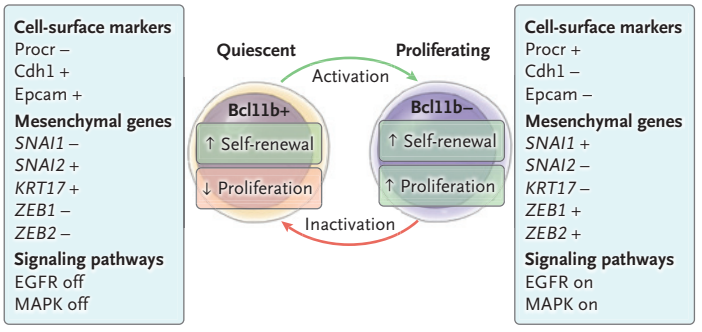

Figure 4. Normal Stem-Cell Heterogeneity.

Bcl11b regulates the quiescence of normal mammary-gland stem cells. Environmental signals can transiently induce proliferation of cells, but the cells can reenter a quiescent state. Many cell-surface markers and signaling pathways are different in quiescent cells and proliferating cells. Thus, environmental signals can alter the marker expression and signaling pathways of stem cells. Different diagnostic markers are indicated for quiescent cells and proliferating stem cells. Furthermore, the drug sensitivity and resistance profiles of quiescent cells and proliferating cells can differ. EGFR denotes epidermal growth factor receptor, Epcam epithelial-cell adhesion molecule, and MAPK mitogen-activated protein kinase.

that this group of patients should receive alternative therapies.

Because cancer stem cells are often inherently partially resistant to some standard therapies, there has been an intensive effort to identify agents that effectively target these cells. Many putative agents to target cancer stem cells have failed in clinical trials.[64] There are several reasons for such failures. Some agents were developed on the basis of activity against cells that were incorrectly identified as stem cells. Another reason for failure is that many researchers have thought of cancer stem cells as a single, uniform population of cells and have not considered that both normal and malignant stem cells have a response to environmental signals and differ in the pathways that they use (Fig. 4). For example, in the mammary gland, quiescent cells and proliferating stem cells each have a partially mesenchymal phenotype, but they can differ in the cell surface and in the individual mesenchymal cell markers that they express.

Of clinical relevance, the signaling pathways, which are active in the cycling and quiescent stem cells, can differ.[13] For example, in the skin and other tissues, there are quiescent and cycling populations of stem cells. The proliferating stem cells are dependent on Wnt signaling, whereas the quiescent ones are not.[65] Thus, an agent targeting Wnt signaling for the treatment of skin cancer might eliminate the cycling stem cells but not the quiescent cancer stem cells. In colon cancer in mice, colon cancer stem cells, as defined by the expression of Lgr5, can be regenerated by other cancer cells; this suggests a reason for the failure of therapies directed against Lgr5-positive cells. The mouse data were consistent with previous data from human transplantation that defined populations of both LGR5-positive and LGR5-negative cells that are capable of generating tumors.[18,66] In experiments involving colon cancer in humans, analysis of transplantation and single-cell gene expression showed that defined populations of LGR5-positive and LGR5-negative cells could regenerate tumors as well as LGR5-negative cancer cells that did not give rise to tumors. Thus, it may be possible to use the single-cell gene-expression profiles of each tumorigenic cell population to design patient-specific treatment strategies to ensure that all of the tumorigenic cell populations are targeted.[66,67]

Another major reason for treatment failure is that normal stem cells and cancer stem cells are both targeted by a particular therapeutic agent. For example, there has been great excitement about drugs that target the bromodomain and extraterminal (BET) family of epigenetic readers of histone acetylation (the BET pathway) as a way to target genes, such as the oncogene *MYC*, that depend on the pathway for expression. However, normal gut stem cells and many cancer stem cells are targeted by agents that inhibit the BET pathway.[68] In some circumstances, maximally effective doses of BET pathway inhibitors could be equally toxic to normal gut stem cells and to cancer stem cells, resulting in severe gastrointestinal toxicity. Thus, these agents may or may not achieve a favorable therapeutic index in the clinic.

Recently, the Food and Drug Administration approved three new drugs that can target cancer stem cells. Vismodegib is a hedgehog inhibitor that targets at least a subset of cancer stem cells in basal-cell carcinoma.[69,70] Ivosidenib, an inhibitor of the gene encoding isocitrate dehydrogenase 1 (*IDH1*), is approved for the treatment of patients with relapsed leukemia. Mutant *IDH1* causes accumulation of (*R*)-2-hydroxyglutarate, which blocks histone demethylation and cell differentiation.[48] Venetoclax increases ROS in leu-

kemic stem cells. Previous agents that target ROS pathways in cancer stem cells, such as L-*S*,*R*-buthionine sulfoximine, also target normal stem cells, resulting in toxicity that limits their clinical use. Recently, Jordan and colleagues found that the BCL2 inhibitor venetoclax selectively killed AML stem cells in a significant number of patients.[71] A clinical study involving elderly patients with a poor prognosis and a very poor response to standard therapy showed that at least 60% of the patients receiving combination therapy with venetoclax had complete remissions. Some of these remissions were prolonged.[72] These studies showed the therapeutic efficacy of an agent that effectively targets cancer stem cells.

The biologic features of stem cells are also relevant to immune therapies.[62,73,74] Since stem cells are essential for the repair of tissue damage, it is not surprising that they express regulatory factors to protect themselves from immune damage. Hematopoietic stem cells, and probably other stem cells, express CD47 to protect themselves from phagocytosis by macrophages. CD47 provides a "do not eat me" signal to macrophages. Cancer cells can exploit CD47 to protect themselves from macrophage attack. In a recent study involving patients with treatment-resistant non-Hodgkin's lymphoma, a CD47 inhibitory antibody restored sensitivity to rituximab in 50% of patients, and 36% had a complete response.[75]

THE FUTURE

The translation of cancer stem-cell biologic research to the clinic has great promise but is in its infancy. Furthermore, several issues remain to be solved in order to realize its full potential. First and foremost is the need to better define the molecular and cellular biologic features of normal stem cells and stem cells in the tumors that arise from various tissues. It is clear that some but not all oncogenic mutations can confer stem-cell properties to a partially differentiated cell. As evidenced by venetoclax, ivosidenib, vismodegib, and the CDX2 studies, cancer stem-cell biologic research could inform new vulnerabilities of the cell to both known and new therapies. Since oncogenic mutations can eliminate the reliance of a cancer stem cell on a particular gene for survival,[13,22] it is evident that successful eradication of cancer stem cells will depend on combination therapies that target multiple cancer stem-cell pathways. Therapeutic agents must target both quiescent and prolifer-

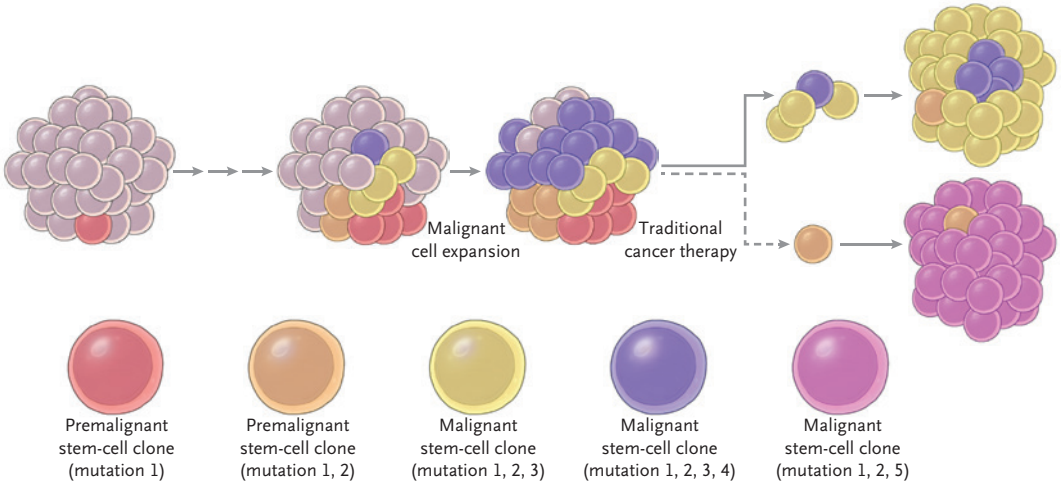

Figure 5. Model of Leukemia Relapse.

Many patients with acute myeloid leukemia (AML) have HSCs that harbor one or two premalignant mutations. Eventually, these HSCs undergo a mutation leading to overt AML. During the leukemia evolution, additional mutations can occur, leading to clonal evolution of the cancer. In cases of therapy failure, relapse may occur because one or more of the leukemia stem-cell clones was not eliminated. There is less biologic complexity in premalignant clones, which are also resistant to therapy, than in malignant ones. The development of diagnostic tests and therapeutic agents against premalignant stem cells could lead to a marked reduction in illness and death.

An audio interview with Dr. Clarke is available at NEJM.org

ating cancer stem cells, cancer stem-cell populations arising from specific mutations, and cells that have acquired mutations that eliminate dependence on a single stem-cell pathway.

It is obvious that tumors that contain large numbers of mutations are likely to result in complexities in targeting cancer stem cells. The investigation of relapses in AML provides insights into potential ways to tackle the problem. Relapses in AML involve multiple mechanisms, such as failure to eliminate all the different leukemic stem-cell clones in the tumor[39] (Fig. 5). Preleukemic hematopoietic stem cells, in which the same cells seen in clonal hematopoiesis often persist after therapy, could be another source of treatment failure. Just as in patients with clonal hematopoiesis, one could speculate that these cells could give rise to late relapses at a frequency of approximately 1% per year. Since there is less complexity in the molecular and cellular biologic features of premalignant cells, if therapies could be identified to eliminate these preleukemic cells and presumably premalignant (yet otherwise normal) stem cells in many solid tumors before a frank cancer arises, treatment could be more effective. In the future, combinations of small-molecule inhibitors, biologic agents, and immunotherapies against cancer stem-cell targets may enter the clinic and improve patient outcomes.

Disclosure forms provided by the author are available with the full text of this article at NEJM.org.

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