Cancer Stem Cells—Perspectives on Current Status and Future Directions: AACR Workshop on Cancer Stem Cells

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Introduction

A workshop was convened by the AACR to discuss the rapidly emerging cancer stem cell model for tumor development and progression. The meeting participants were charged with evaluating data suggesting that cancers develop from a small subset of cells with self-renewal properties analogous to organ stem cells. Indeed, one critical question contemplated at the Workshop was whether tumors derive from organ stem cells that retain self-renewal properties but acquire epigenetic and genetic changes required for tumorigenicity or whether tumor stem cells are proliferative progenitors that acquire self-renewal capacity. Of course, both mechanisms may occur and may depend on the organ site. Either mechanism is different from the widely held notion that most cells in a tumor should be competent for tumor formation. If the cancer stem cell model is correct and if such cells retain the hallmarks of some tissue stem cells in being rare and entering the cell cycle infrequently, they could constitute a population that is intrinsically resistant to current therapies designed to kill cycling cells. The participants critically discussed the need for a precise definition of cancer stem cells, the requirement for new markers and more rapid and tractable *in vitro* and *in vivo* assays, and the need to develop drug screening strategies to selectively target cancer stem cells to generate therapeutics for this subpopulation of cells that could be resistant to classic treatments while possessing potent tumor-forming capacity.

The Cancer Stem Cell Hypothesis and Its Implications

In the cancer stem cell model of tumors, there is a small subset of cancer cells, the cancer stem cells, which constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and maintain the tumor. These cancer stem cells have the capacity to both divide and expand the cancer stem cell pool and to differentiate into the heterogeneous nontumorigenic cancer cell types that in most cases appear to constitute the bulk of the cancer cells within the tumor. If cancer stem cells are relatively refractory to therapies that have been developed to eradicate the rapidly dividing cells within the tumor that constitute the majority of the nonstem cell component of tumors, then they are unlikely to be curative and relapses would be expected. If correct, the cancer stem cell hypothesis would require that we rethink the way we diagnose and treat tumors, as our objective would have to turn from eliminating the bulk of rapidly dividing but terminally differentiated components of the tumor and be refocused on the minority stem cell population that fuels tumor growth. This explains why the cancer stem cell hypothesis is at the center of a rapidly evolving field that may play a pivotal role in changing how basic cancer researchers, clinical investigators, physicians, and cancer patients view cancer.

Cancer Stem Cells: An Old Idea Reemerging at an Important Time

It has long been known from light microscopic studies that both normal tissues and the tumors that develop within them comprise a heterogeneous collection of cell types, frequently including immune cells, a stroma consisting of various mesenchymal and endothelial cells, and a variety of normal or malignant cells specific to the tissue. Cells within the tumor often seem to correspond to different stages of development. Epithelial cancers, for example, typically contain cells exhibiting divergent nuclear morphologies and differentiation features. Prevailing explanations for the observed tumor cell heterogeneity include influences of the microenvironment and genomic instability that generate the genetic and epigenetic changes, which prevent faithful and accurate replication and transmission of stable genotypes and phenotypes. Such instability could also explain why tumors typically contain a subset of cells that are refractory to most treatments. However, an alternative (or complementary) emerging concept is that malignant cell populations may reflect the continuing operation of perturbed differentiation processes. Inherent to such a model is the formation of malignant populations consisting of a developmentally defined hierarchy of heterogeneous phenotypes derived from a small subset of "cancer stem cells."

Hematopoietic Stem Cells Have Led the Way

Human cells fulfilling the properties expected of drug-resistant cancer stem cells were initially isolated from blood cancers. Tritium-labeling studies conducted on a variety of blood cancers in the 1960s showed the existence of a subset of primitive-appearing cells with cycling properties different from the majority of tumor cells. These early tritium-labeling studies, coupled with genetic studies suggesting that many leukemias contained an immature cell population capable of generating postmitotic progeny, predicted the existence of a leukemic stem cell.

Studies of acute myelogenous leukemia (AML) in the 1990s provide compelling evidence for the existence of a cancer stem cell

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subpopulation. Efforts to define the cell of origin of hematopoietic cancers were greatly enhanced by specific and quantitative assays, extensive lineage maps, and the availability of cell surface markers for distinct cell types comprising this system. For human AML, cancer stem cells were defined as those cells capable of regenerating human AML cell populations in irradiated transplanted nonobese diabetic (NOD)/severe combined immunodeficient (SCID) mice. The AML stem cells possessing this property were found to display a $CD34^+CD38^-$ cell surface phenotype, similar to that typical of normal human primitive hematopoietic progenitors. This suggested that the AML stem cells may have originated from normal stem cells rather than arising from more committed progenitors, although as will be discussed, this may not necessarily be the case for all cancer stem cells.

Definition: What Is a Cancer Stem Cell?

An accurate definition is critical to enable researchers working in the same or different systems to compare cells exhibiting a common set of properties. The consensus definition of a cancer stem cell that was arrived at in this Workshop is a cell within a tumor that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor. Cancer stem cells can thus only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor. The implementation of this approach explains the use of alternative terms in the literature, such as "tumor-initiating cell" and "tumorigenic cell" to describe putative cancer stem cells.

It must be emphasized that proliferation is not synonymous with self-renewal. A self-renewing cell division results in one or both daughter cells that have essentially the same ability to replicate and generate differentiated cell lineages as the parental cell. Stem cells have the ability to undergo a symmetrical self-renewing cell division, causing identical daughter stem cells that retain self-renewal capacity, or an asymmetrical self-renewing cell division, resulting in one stem cell and one more differentiated progenitor cell. In addition, it is thought that stem cells may divide symmetrically to form two progenitor cells, which could lead to stem cell depletion. Promoting this form of division would be a way to deplete the cancer stem cell population and may constitute an alternative strategy to inducing cell death to treat cancer.

Cancer Stem Cells in Solid Tumors

Evidence for the existence of cancer stem cells in solid tumors has been more difficult to obtain for several reasons. Cells within solid tumors are less accessible, and functional assays suitable for detecting and quantifying normal stem cells from many organs have not yet been developed. Therefore, the cell surface markers required to isolate such cells have not been identified. There has been some impressive work in this area recently, including the demonstration that single mouse mammary cells can be transplanted and reconstitute a complete mammary gland. Cells have also been isolated from human breast tumors that can cause breast cancer in NOD/SCID mice through serial transplantations, suggesting a capacity for self-renewal. These cells were $CD44^+$ $CD24^-$/low in eight of nine patients and established tumors in recipient animals when as few as one hundred cells were transplanted, whereas tens of thousands of breast cancer cells with a different marker set failed to induce tumors. Brain tumor stem cells that can produce serially transplantable brain tumors in NOD/SCID mice have also been isolated from human medulloblastomas and glioblastomas. These cells can be enriched by sorting for $CD133^+$, a marker found on normal neural stem cells, and the transplantation of one hundred $CD133^+$ tumor cells was sufficient to initiate the formation of a tumor in recipient animals. In contrast, no mice injected with the negative population developed brain tumors. More recently, cells have been isolated from human prostate cancer patients that can produce serially transplantable prostate tumors in NOD/SCID mice. Sorting for Hoechst dye–excluding side population (SP) cells and cells expressing CD44 allowed the isolation of a population enriched for cells with this ability. Together, these studies reveal that only a small subset of cells in several different tumor types is capable of tumor formation in such transplant assays. These data are consistent with the cancer stem cell hypothesis. Nonetheless, caution needs to be exerted when interpreting transplantation assays as described in the section on functional and phenotypic assays.

Clarifying the Concepts and Definitions

The term cancer stem cell has led to some confusion. Many interpret the term cancer stem cell to mean that such cells derive from the stem cells of the corresponding tissue. Cancer stem cells may indeed arise from normal stem cells by mutation of genes that make the stem cells cancerous, but this may not be the case in all tumors. For example, in blast crisis chronic myelogenous leukemia (CML), a committed granulocyte-macrophage progenitor may acquire self-renewal capacity and thus "reacquire" stem-like properties due to the effects of later mutations. It is conceivable that more differentiated cells can, through multiple mutagenic events, acquire the self-renewal capacity and immortality that typify cancer stem cells. In both of these examples, a differentiated cell, not the tissue stem cell, eventually evolves to become a full-blown cancer stem cell.

The term tumor-initiating cell can also cause confusion. In some of the seminal studies in this field, the term "cancer initiating" has been used to refer to the ability of these cells to initiate tumors when transplanted. The tumor-initiating cell is often used to mean the cell that causes a tumor (or leukemia) in xenograft models of human cancer. Some have extrapolated that the cell that initiates a tumor xenograft is the same as the cell that received the first oncogenic "hits" in the patient. It is clear that the cancer stem cell capable of forming a tumor at one point in time might change during the progression of the disease. Thus, a tumor may be initiated by a set of mutations leading to transformation of one cell type, but progressive mutations occurring during the evolution of the tumor may result in the acquisition of stem cell properties by a second cell type at a later time (Fig. 1). The stem cells within an individual tumor may constitute a moving target, that is, the cells that drive growth at one point in time may not be identical to those doing so at another stage in tumor evolution or during metastasis. Furthermore, the genetic and epigenetic instability that are fundamental properties of tumor biology can induce cellular heterogeneity within the stem and nonstem cell populations of the tumor. Evidence was given that specific oncogenes or mutations could play a significant role in determining the target cell that eventually becomes malignant. Animal models will be useful for understanding the origins of cells with the properties expected of cancer stem cells and when and where they arise during cancer initiation and progression.

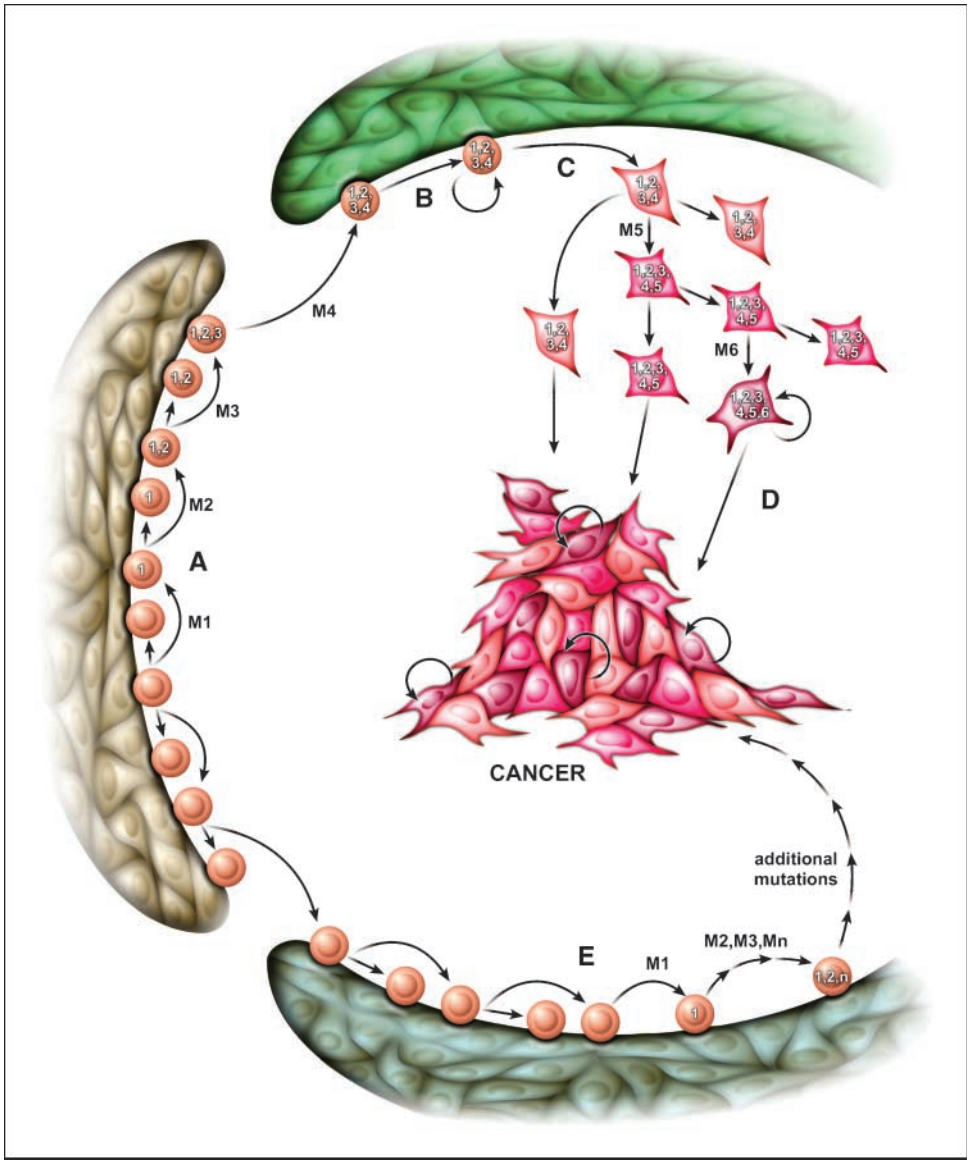

Figure 1. Some potential mechanisms by which tissue stem cells generate cancer. Several potential routes by which mutations can accumulate in stem cells to generate cancer, based on data presented at the AACR Cancer Stem Cell Workshop. *A,* normal cells divide within a niche (*green*) and can accumulate mutations over time. *B,* stem cells may have acquired mutations that enable them to survive and self-renew within an alternative niche. This may enable a population of mutant stem cells to expand in a new region and/or in the environment of different supporting cells (i.e., an alternate niche). Alternatively, mutant stem cells may have acquired the ability to induce the proliferation of niche cells to allow for expansion of the niche to accommodate the mutant stem cells. *C,* mutations within the stem cells may enable them to proliferate in the absence of a niche, but they would require additional mutations to undergo self-renewal (*D*). A related possibility is that mutated stem cells undergo a differentiation program but retain proliferative potential. Acquisition of additional mutations in the proliferative progenitors would then be required to enable them to self-renew. *E,* normal stem cells may be exposed to a niche that has itself undergone modifications. Self-renewing divisions in the aberrant niche may then select for specific types of mutations within the stem cells, which are the precursors of cancer. The cancer shown is composed of a heterogeneous cell population that could be generated by the self-renewing divisions of the mutated cancer stem cell along with its "differentiated" cell types that comprise the tumor. Various mutated proliferative progenitors could also contribute to the tumor along with the self-renewing, differentiation competent progenitor that is the cancer stem cell. Both models are compatible with clonal origin of most tumors as all cells shown derive from a common stem cell ancestor.

Normal tissue stem cells are dependent on interactions with adjacent stromal cells that comprise a specialized microenvironment or niche, which is necessary for the maintenance of stem cell identity and self-renewal capacity (Fig. 1). Similarly, in some malignancies, tumor growth is also thought to depend on a dynamic interaction with adjacent stromal cells that compromise the tumor niche. As tumors grow, the niche may change. It is reasonable to surmise that the stem cells of a tumor may also evolve with changing cues in their microenvironment, including infiltration of immune cells and activation of inflammatory responses. In addition, from studies of the *Drosophila* germ cell niche shown at the Workshop, it is possible that cancer stem cells will signal to their niche to allow it to expand as the cancer stem cells proliferate.

Cancer Stem Cell Assays

One of the most frequent errors made in defining stem cells is generalizing results obtained in studies of stem cells in one organ. Although stem cells in different adult tissues share the fundamental properties of self-renewal and the ability to differentiate into a diversity of mature cell types, stem cells in different organs can differ significantly from one another. Thus, properties that are useful for the identification and characterization of stem cells in one tissue are frequently not shared with the stem cells in a different organ. This is likely to also be true for cancer stem cells isolated from different tumor types.

Self-renewal and lineage capacity are the hallmarks of any stem cell. Therefore, as with normal stem cells, assays for cancer stem cell activity need to be evaluated for their potential to show both self-renewal and tumor propagation. The gold standard assay that fulfills these criteria is serial transplantation in animal models, which, although imperfect, is regarded as the best functional assay for these two critical criteria.

In transplantation assays, cells are xenografted into an orthotopic site of immunocompromised (typically NOD/SCID) mice that are assayed at various time points for tumor formation. To show self-renewal, cells then must be isolated from the tumors and

grafted into a second recipient animal. Issues complicating transplantation assays include potential effects of the grafting site. It is known that normal stem cells can be highly dependent on signals from the surrounding stroma for function, and it is not clear what the effect may be on separating cancer stem cells from any supporting cells during the course of the assay. Experiments using mixed populations of normal and breast tumor cells in mice have shown that combining tumor cells with normal fibroblasts increases latency and decreases tumor take, whereas combining them with carcinoma-associated fibroblasts has the opposite effect. Conversely, nontumor cells placed next to tumor stroma can become independently tumorigenic, possibly due to stroma-induced genetic or epigenetic instability. The number of cells needed to form a tumor can also be affected by the addition of irradiated feeder cells or the use of Matrigel; for feeder cells, by orders of magnitude.

Interpretation of transplantation assays is also complicated by the possibility that the cells that can recapitulate the tumor might have a greater ability to survive in the host; the extraordinarily high level of genetic and epigenetic changes that take place within most cancer cells, in some cases, as high as 1,000 daily, may allow some cells to generate diverse cell types not because they are stem cells per se but because of their genetic/epigenetic instability. Still, although serial transplantation assays remain the best developed method to date for identifying cells with the properties expected of cancer stem cells, more sophisticated, precise, and simpler assays are likely to emerge as the field develops.

In vivo assays are the gold standard for identifying stem cells; however, as serial transplantation experiments with animal models can take 6 months or more, high-throughput screens for lead compounds will be difficult, if not impossible, using animal models. Therefore, the development of reliable surrogate assays would significantly enhance drug development.

An ideal *in vitro* assay would be (*a*) quantitative; (*b*) highly specific, measuring only the cells of interest; (*c*) sufficiently sensitive to measure candidate stem cells when present at low frequency; and (*d*) rapid. Several *in vitro* assays have been used to identify stem cells, including sphere assays, serial colony-forming unit (CFU) assays (replating assays), and label-retention assays. Studies have also been done with the goal of determining genetic signatures that define cancer stem cells. However, each of these methods has potential pitfalls that complicate interpretation of the results. All three groups reporting the isolation of normal breast stem cells indicated that individual breast stem cells do not form spheres by themselves using conditions that are permissive for the formation of neurospheres or mammospheres. Furthermore, the rapidity of sphere development in many systems makes it unlikely that they arose from single cells solely through clonal expansion. The difficulty of distinguishing the relative contributions of aggregation and proliferation in sphere formation poses a major impediment to their use to construct lineage maps. Serial CFU assays have been used to identify cells with increased proliferative potential, but their activity must be confirmed by a clonal *in vivo* assay. Indeed, in the hematopoietic system, selecting for CFU in semisolid medium usually identifies progenitors and not stem cells. This may be due to the need for the niche to provide the requisite signals for both self-renewal and proliferation.

Additional technical issues make stem cell isolation and functional assays challenging. Although flow cytometry offers a sensitive, specific, and robust method of cell isolation and purification, some of its technical limitations make its application to stem cell purification challenging. For example, even when using advanced sorting techniques to distinguish single cells from aggregates, doublets (cells sticking to one another) can still occasionally sort together and need to be eliminated. Thus, microscopy is needed to show that single cells were indeed isolated. It is very difficult to make viable single-cell suspensions of solid tissues, such as brain and epithelial tissues cells. Thus, techniques for dissociating cells must be carefully developed. Most flow cytometers are typically set up to sort blood components using small diameter streams at high pressures. These conditions are often not tolerated by larger, more fragile cells found in many organs. Therefore, diameters of the liquid stream and sorting pressures frequently must be optimized for cells isolated from solid tissues. In addition, although phenotypes based on markers often use terms, such as high, middle, low, and nonexpression, to describe the properties of the sorted cells for each marker, these terms are subjective and can vary depending on the method used for cell preparation, how the gates are set, and the antibody preparation used. Thus, cells marked as one phenotype by one group may exhibit another phenotype in other hands. This could be remedied by availability of standardized antibodies and by consistent calibration of each batch of fluorescently labeled antibodies. At this point in time, such quality control needs to be done by the individual investigator. Because of these technical challenges, it takes the typical neophyte several months of training in an experienced stem cell laboratory to even begin to master flow cytometry isolation of cancer stem cells.

Cancer Stem Cell Markers

It is clearly not sufficient to define a stem cell based solely on surface markers in the absence of linking marker expression to a self-renewal assay. None of the markers used to isolate stem cells in various normal and cancerous tissues is expressed exclusively by stem cells. For example, CD133 was used to successfully enrich for brain tumor stem cells, but it is also present on normal brain stem cells and on many nonstem cells in various tumors and tissues. The same is true for other commonly used markers, such as CD44, Sca1, and Thy1. In fact, the vast majority of cells that express these markers are not stem cells. In addition, markers used to identify stem cells from one organ are frequently not useful for identifying stem cells in other tissues: Sca-1 is useful for the identification of murine blood stem cells, but it is not consistently expressed by murine mammary duct stem cells. Furthermore, just because a marker can be used to identify stem cells from a particular organ does not mean it will work in all other contexts. For example, placing stem cells in culture can drastically alter their marker expression. Thus, describing the markers presently used to identify stem cells from one tissue as a "stemness" marker when investigating a potential stem cell population in a different tissue is misleading. The marker may or may not be useful for identifying stem cells from the other organ or tumor type.

Another phenotype used to distinguish cells is their presence within the SP fraction defined by Hoechst dye efflux properties. However, as with cell surface markers, possession of a SP phenotype is not a universal property of stem cells, and in some tissues, the SP fraction may not contain the stem cells. Experiments that identified normal breast stem cells by their ability to generate mammary glands in cleared fat pads showed that the

majority of these cells are not included within the SP fraction. Possible toxicity of the dye to cells that do not exclude it should also be considered as a caveat to interpreting functional assays of SP cells. As with other markers used to identify certain types of stem cells, marker expression must be linked with a functional assay. Because of this complicating factor, it is safer to first isolate stem cells using other methods and then ask whether that particular stem cell population is indeed included within the SP.

Label retention (bromodeoxyuridine incorporation) studies have also been proposed as way of identifying stem cells. This method is based on the assumption that normal or cancer stem cells either spend long periods not cycling or undergo an "immortal strand" DNA replication and therefore preserve the labeled state for an extended period. However, neither event is the case for stem cells in every organ or tissue. In fact, both normal and malignant breast stem cells appear to be cycling and so much more needs to be known about the regulation of cancer stem cell cycling behavior before assays based on this property can be relied on for purposes of identifying stem cells. Furthermore, it is known that not all stem cells are label retaining and not all label-retaining cells are stem cells. Thus, as with any other potential stem cell marker, one must link the property with a stem cell functional assay. In other words, label-retaining cells must be shown to regenerate the tissue *in vivo*.

Genetic and Epigenetic Signatures of ''Stemness''

Work has begun in several systems to develop genetic signatures that typify stem cells. Several genes and signaling pathways, including *Bmi-1, Tie-2, Shh, Notch*, and *Wnt/β-catenin*, have been shown to have important regulatory functions for some stem cells. However, as these genes frequently operate in other cell types, they cannot be called "stemness" genes. Microarray and genome-wide techniques can be applied to detect trends in genetic and epigenetic "blueprints" for cancer stem cells, but to identify true signatures, pure populations are necessary. This is especially true for cells expected to be rare, such as cancer stem cells, whose expression signature would be swamped by the majority of nonstem cells in a whole tumor sample. Even after a cancer stem cell signature from a particular type of tumor is identified, one cannot assume that a given signature is useful for identifying cancer stem cells in a different tumor type unless validated by a functional assay (such as an *in vivo* self-renewal assay as it is the most definitive at this point in time).

The development of *in vitro* assays, although critical, is at an early stage, and the results of all *in vitro* work must be examined rigorously and ultimately validated *in vivo*. Therefore, any method used to identify putative stem cells must be verified and followed by functional assays, preferably a gold standard *in vivo* assay, before claims about "stemness" can be made. It should be a goal of the field to develop cell surface marker and gene activity profiles that can be used reliably to identify cancer stem cells. At this time, the presence of particular markers or gene expression signatures associated with other stem cell populations, normal or cancer derived, is not sufficient to label a given population of cells as cancer stem cells without confirmation by functional assays. In addition, analysis and interpretation of these data are limited by the purity of the cell population in a given system; results from a mixed population constitute an average and not a specific signature. Furthermore, use of gene inactivation to eliminate "stemness" or gene activation to engender "stemness" would be required to functionally link any marker to stem cell identity. Although the isolation of markers correlated with stem cells can aide in stem cell isolation, the identification and isolation of genes that are functionally significant for "stemness" would constitute an important step forward and could provide valuable targets for drug development.

Implications for Cancer Therapy: Opportunities and Challenges

The cancer stem cell hypothesis posits that cancer stem cells are a minority population of self-renewing cancer cells that fuel tumor growth and remain in patients after conventional therapy has been completed. The hypothesis predicts that effective tumor eradication will require obtaining agents that can target cancer stem cells while sparing normal stem cells. Experimental evidence in human AML suggests that, compared with the bulk population of leukemic blasts, the leukemia stem cells are relatively resistant to conventional chemotherapeutic agents. Although it has been speculated in solid tumors that conventional agents kill the nontumorigenic cancer cells while sparing the cancer stem cells, this has not been proven. There are other models of drug resistance consistent with the existence of cancer stem cells that could explain relapse, including the classic view of mutation and selection.

The moving target nature of cancer stem cells may present a challenge in the clinic. To achieve effective implementation of new therapies, physicians will require methods of determining the type (or types) of cancer stem cells present in a given patient's tumor. Work involving 150 CML patient peripheral blood and bone marrow samples is encouraging in that patients in blast crisis all exhibited an expansion of the granulocyte-macrophage progenitor population, which included the fraction displaying stem cell properties. Therefore, it seems reasonable to expect that tumors sharing a similar pathology may also share common features in their cancer stem cell populations, which would facilitate diagnosis and the application of appropriate treatments. This point, however, needs to be borne out by further study.

It is important that agents directed against cancer stem cells discriminate between cancer stem cells and normal stem cells. This will require identification of realistic drug targets unique to cancer stem cells. The identification of such targets and the development of anticancer agents will require a fuller understanding of normal stem cell biology as well as the genetics and epigenetics of tumor progression. There is some indication that such an approach can be successful. For example, stem cells isolated from AML patients display differences from normal hematopoietic stem cells.

There has also been some success identifying agents effective against leukemia stem cells. Conventional anthracycline agents show synergy with proteasome inhibitors against AML stem cells, reducing viability *in vitro* dramatically. The novel agent parthenolide, isolated from Mexican medicinal plants and shown to be a potent nuclear factor-κB inhibitor, promotes apoptosis of AML stem cells and inhibits tumor development in NOD/SCID mice. Mutation of the Janus-activated kinase 2 (JAK2) kinase is found in many patients with the blood disorder Polycythemia Vera, and JAK2 inhibitors display efficacy against the cancer stem cells from these patients, although individual responses vary significantly.

Summary and Future Directions

Participants in the AACR Workshop agreed that, to move the cancer stem cell field forward, multiple assays need to be validated

for as many putative stem cell populations as possible. Cells should be interrogated by multiple methods, including functional assays, marker analysis, and analysis of genetic and epigenetic signatures. More accurate and standardized reagents are needed, particularly for the cell surface markers used for sorting.

Participants also expressed the frequent difficulties in obtaining sufficient quantities of patient tissues. It was noted that patients are often willing to supply tissue, but the current regulatory climate presents an obstacle for many. Joint efforts by the research and patient advocacy communities are needed to overcome these regulatory barriers.

Clearly, there is much excitement and momentum in this important field. Investigation of cancer stem cells offers the possibility of generating novel targets that could overcome issues of drug resistance, improve therapeutic efficacy, and make cancer treatment more successful and perhaps even curative while obviating systemic toxicity. The AACR will form a task force to discuss developments in this field to help identify and eliminate bottlenecks and to expedite progress in this promising area through focused scientific meetings and other mechanisms.

Appendix A. Participant list

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