"Destemming" Cancer Stem Cells

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Cancer stem cells have been variously defined as cells within a cancer that have the exclusive ability to self-renew and to differentiate into the heterogeneous lineages of cancer cells that comprise the tumor. Interest in cancer stem cells is currently high, arising from recent reports identifying cell surface markers that can be used to sort such cells from primary human tumors. However, use of the term cancer stem cell may be misleading. A better term might be cancer-initiating cells because it remains to be demonstrated that cancer stem cells have the properties that define normal stem cells, including multipotency and the ability to undergo asymmetric and symmetric divisions. Many properties of cancer stem cells remain unclear, particularly the stability of their phenotype. These uncertainties must be considered in the development and testing of compounds targeted against putative cancer stem cells. Tumors apparently contain very few cancer stem cells, so that when tests of compounds targeted to such cells are designed, short-term response trials may not be informative and long-term trials must be planned, particularly if the drugs could also kill normal stem cells.

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Cancer stem cells have been defined as "a small subset of cancer cells within a cancer that constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor" (1). Elucidating the biologic properties of cancer stem cells may provide new insights into the factors that drive tumor initiation and progression and may help to develop novel methods to overcome drug resistance, to improve therapeutic efficacy, and to develop novel cancer treatments with low systemic toxicity (1). Despite these prospects, however, it would be unfortunate if new drugs that target solid tumors were developed on the basis of current experimental observations on cancer stem cells and brought to clinical trial before more is known about cancer stem cells.

Traits of Normal Stem Cells and Cancer Stem Cells

A normal healthy stem cell is characterized by its unequivocal self-renewal capability that is sustained by symmetric and asymmetric cell divisions (2). In symmetric cell divisions, both daughter cells retain the same phenotype. In asymmetric cell divisions, each daughter cell inherits distinct cytoplasmic components, including latent transcription factors that are capable of differentially regulating gene expression in the two cells. A normal stem cell is typically multipotent—a property that is manifested as the capability to differentiate into different cell phenotypes that frequently cross lineages and embryonic germ layers. The current definition of a cancer stem cell does not include multipotency; in fact, the extent to which cancer stem cells share the characteristics of normal stem cells, including stable self-renewal properties, is unclear. Thus, although the term cancer stem cell may be catchy, it is somewhat misleading and appropriate only in a limited sense. A functional definition that includes terms such as "tumor-initiating cell" or "cancer-initiating cell," as proposed by some groups (3–6), would be more appropriate at this time. Even these terms can be misinterpreted because the cancer-initiating cells isolated from a clinically detectable tumor may have substantial genetic differences from the initial transformed cells that originated the tumor (1,7).

Recent attempts to define the gene expression profile of putative cancer stem cells (by use of cell surface markers discussed below) have led to the definition of a prognostic gene signature in breast carcinoma (8). Similar gene profiling analyses have been performed on cancer stem cells isolated from other tumor types (9,10). Doubts remain, however, about the uniqueness of the gene expression pattern obtained from isolated tumor cell subpopulations that have been reported to correspond to putative cancer stem cells because they can apparently vary among tumors of the same histopathologic type (11). Also, as discussed below, the markers used to isolate the putative cancer stem cells are not unique to cancer stem cells and are used only to enrich the isolated tumor cell subpopulation for such cells. In addition, whether the proposed cancer stem cells are able to self-renew by exploiting similar asymmetric cell divisions and are multipotent, as are normal stem cells, remains to be demonstrated. Future studies might, for instance, assess whether a carcinoma-derived cancer-initiating cell

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can regenerate a different type of tumor (e.g., of mesodermal origin) when it is transplanted into an environment that is nonpermissive for carcinoma development but permissive for mesenchymal tumor development. Alternatively, such studies might investigate the relationship between genetic instability (and the accompanying genetic and epigenetic changes) involved in neoplastic transformation (12) and regulation of the complex machinery responsible for asymmetric division.

Relationship Between Normal Stem or Progenitor Cells and Cancer-Initiating Cells

Stem and/or progenitor cells (i.e., immature cells that are precursors of specific maturation lineages) have been identified in virtually all primary epithelial tissue types and/or organs, but it is currently unclear whether cancer-initiating cells exhibit asymmetric division—the phenomenon that is primarily responsible for the accentuated self-renewal and pluripotent capability of normal stem cells. In fact, although genes associated with the phenotype of normal stem cells (i.e., stemness) and with self-renewal are now starting to be examined in putative cancer stem cells (13), it is unclear which genes are part of the intricate gene networks regulating the stemness and self-renewal of cancer stem cells. A recent study in *Drosophila* (14) has shown that, if asymmetric division is perturbed by mutating controlling genes (e.g., numb or pros), normal neuroblasts start behaving as cancer cells by showing a dysregulated cell cycle, resistance to apoptosis, and the ability to grow into a tumor mass when transplanted. These results support the hypothesis that disruption of asymmetric division during uncontrolled proliferation of somatic stem cells may lead to cancer (15). A corollary investigation (16) demonstrated that misexpression of Oct-4 (a master regulator of stemness in epithelial cells) causes pronounced intestinal dysplasia in a doxycycline-dependent transgenic murine model. Thus, evidence is gradually emerging that genetic alterations that disturb key stem cell features and affect the cell cycle are linked to the induction of neoplastic transformation and that neoplastic cells with these alterations are clearly distinct from normal stem cells.

Related investigations also provide experimental support to the hypothesis that tumors may originate from the transformation of a progenitor and/or stem cell. For example, soft-tissue sarcoma cells and bone marrow–derived mesenchymal progenitor cells share a common phenotype (17,18), and the progenitor cells can be converted experimentally into Ewing's sarcoma cells by transfecting them with a construct encoding the oncogenic fusion protein ETS–FLI-1 (19). Furthermore, a conditional transgenic mouse model in which the chimeric synovial sarcoma fusion protein SYT–SSX is misexpressed was used to show that synovial sarcomas originate from myogenic precursors (20). Although these findings suggest that a property of mesenchymal tumors is that they are derived from specific immature cells, at least one investigation (21) showed convincingly that even epithelial tumors (such as those of gastrointestinal tract) may arise from progenitor cells of nonepithelial origin. These results raise the possibility that small subsets of neoplastic cells in a carcinoma may originate from different cell lineages and different pathways than most of the other neoplastic cells in a carcinoma.

It is still not known whether cancer-initiating cells preserve their original molecular traits as the tumor grows. As many gene mutations accumulate in a tumor, different populations of cancer cells with diverse phenotypes can form (22–24), even when all cells originate from a single clone (25), indicating that cancer-initiating cells probably do not remain stable throughout tumor progression. For example, studies in mice that used conditional expression of certain oncogenes (e.g., Myc or Wnt-1) in tumor cells (26,27) found that tumors will initially regress when the oncogene expression is inactivated but that some tumors will recur even in the absence of the oncogene expression, indicating that additional genetic (or epigenetic) changes appear to have occurred in a portion of the tumor cells to allow tumor recurrence.

Exposure of cells to hypoxia, a condition that characterizes various tumor lesions, supports the notion that cancer-initiating cells are unstable (28). After exposure of neuroblastoma and breast carcinoma cells to hypoxia, the expression of genes associated with differentiation is reduced and the expression of genes associated with early embryonal stages of development is increased (29). Such hypoxic cells thus have a phenotype that resembles a more primitive "stem cell–like" phenotype. Exposure to hypoxia has also been reported (30) to reversibly arrest preadipocytes in an undifferentiated state and to maintain the expression of pref-1, a key stem and/or precursor cell gene that negatively regulates adipogenic differentiation. Exposure of a medulloblastoma cell line to hypoxia has been reported (31) to increase the expression of CD133, a putative stem cell marker. Thus, putative cancer-initiating cells within a tumor may change their phenotype as the tumor grows, and solid tumors may contain a hierarchy of cells, in which different cell populations have different capacities for tumor repopulation, as observed in leukemia (32). Drugs directed against a certain phenotype of cancer-initiating cells thus may not be effective against all cancer-initiating cells in a tumor. Clonal analyses of cancer-initiating cells for drug sensitivity in experimental and naturally occurring tumor lesions could be used to investigate this potential limitation.

Reliability of the Current Markers and Isolation Protocols for Cancer Stem Cells

Several cell surface markers (e.g., CD24, CD44, CD133, CD166) and dye efflux assays (that are primarily based on the differential release of incorporated Hoechst dyes or rhodamine-121 through overexpressed or overactivated drug transporters in the cell membrane) have been used to sort populations of putative cancer-initiating cells from cultured tumor cell lines or resected tumor specimens (3,6,11,33–42). As described in many of these papers, cancer-initiating cells sorted in these ways display apparent self-renewal capabilities in vivo; i.e., the sorted cells can generate a tumor mass in mice each time that they are transplanted into a new mouse. Although cell culture manipulations and animal models may reveal characteristics that could be compatible with certain cancer-initiating cell features, a major concern is the lack of a gold standard for the unambiguous identification of cancer-initiating cells. The use of in vivo transplantation is problematic because the results of this assay can be highly heterogeneous, depending upon the transplantation conditions (43), and because

any transplantation of cancer-initiating cells into a normal tissue site does not recapitulate the environment that these cells encounter in a naturally occurring cancer (44). Furthermore, when examining the in situ distribution of proposed markers for cancer stem cells (such as CD44 or CD24 for breast, prostate, colon, or pancreatic carcinomas; CD133 for glioblastomas or colon, prostate, or kidney carcinomas; CD20 for melanoma; CD166 for colon carcinomas or cytokeratins for several carcinomas), no defined localization patterns have been discovered that reliably identify only cancer-initiating cells in tumors.

Another uncertainty in published data that are derived from in vivo assays of putative (immuno)sorted cancer-initiating cells is the discrepancy in the numbers of sorted versus unsorted cells that are required to initiate a tumor mass. This numerical discrepancy is illustrated in Fig. 1 and is based on results derived from one of the most comprehensive datasets in the literature (3). These data represent combined results from 17 different human xenograft colon carcinomas (six primary tumors and 11 liver metastatic tumors) from which cells that did or did not express CD133 were sorted by use of a magnetic bead technique. [All tumors in that study expressed some level of CD133; however, a recent report has indicated that not all colon carcinomas may express CD133 (11).] Although combining the results from a number of tumors masks the variability between the tumors, the expected sigmoid relationship was obtained when the number of cells injected was plotted against the proportion of tumor masses initiated (45). Approximately 200 sorted CD133-positive colon cancer–initiating cells were required to generate tumors in 50% of injection sites when the cells were sorted for CD133 positivity, whereas approximately 60 000 unsorted cells were required to generate tumors in 50% of the injection sites. On average, approximately 12% of the cells in the unsorted population were positive for the CD133 antigen, so that approximately 7000 CD133-positive cells in the unsorted population were required to induce tumors when a large excess of non–cancer-initiating (CD133-negative) cells was also injected.

There are several possible explanations for this discrepancy in the numbers of sorted and unsorted cells required to form a tumor. O'Brien et al. (3) suggested that this discrepancy might be explained if CD133-negative cells adversely influence the growth

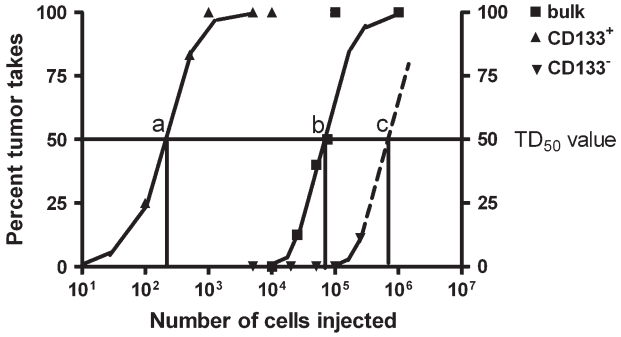

Fig. 1. Tumor formation by sorted and unsorted cells. The percentage of injection sites (in this experiment, the kidney capsule of immune-deprived mice) in which tumors were established is expressed as percent tumor takes. Values were obtained from 17 primary or metastatic human colon carcinoma cells that were either unsorted (bulk) or sorted by the expression of the surface marker CD133. The numbers of cells necessary for a tumor to form in 50% of the injection sites (TD_{50} value) are as follows: **a** = approximately 200 cells; **b** = approximately 60 000 cells, and **c** = approximately 700 000 cells. The mean percentage of CD133-positive cells in the tumors was 11.8% (range = 1.8%–24.5%). Data are from O'Brien et al. (3).

of the sorted cancer-initiating cells. If this interpretation is correct, it would certainly complicate the interpretation of results from such transplantation assays in terms of the "effective" number of stem-like cells in tumor lesions. However, inhibition of tumor growth is inconsistent with results from many other studies [e.g., (46–48)] of spontaneous tumors transplanted in syngeneic animal models that found that adding lethally irradiated tumor cells to the injected cell population reduced, rather than increased, the number of cells required to cause tumor growth.

An alternative explanation for the discrepancy between the number of sorted and unsorted cells required to form a tumor may be related to a low level of immunity against the tumor cells present in the hosts. In this case, a small number of cells may not be sufficient to generate an immune response capable of rejecting the tumor, an intermediate number of tumor cells may generate an immune response that is sufficient to reject the tumor, and a large number of tumor cells may overwhelm the immune system so that the tumor cells survive and proliferate. This phenomenon has been

Table 1. Relationship between the number of human tumor cells sorted on the basis of specific marker expression and tumor formation in immunodeficient mice*

Tumor type	CIC markers† (% of tumor cells expressing marker)	No. of unsorted cells required for tumor take‡	No. of sorted cells expressing CIC markers for tumor take§	No. of cells expressing CIC markers in unsorted population	Transplantation site	Ref.
Breast	$CD44^+/CD24^{+/-}$ (11%–35%)	$>10^4$	200	$\sim10^3$	Mammary gland	54
Brain	$CD133^+$ (6%–29%)	$>10^5$	100	$\sim10^4$	Brain	53
Brain	$CD133^+$ (2%–3%)	$2–4 \times 10^4$	500–1000	$\sim10^3$	Brain	35
Colon	$CD133^+$ (0.7%–6.1%)	$>10^6$	$<3 \times 10^3$	$\sim2 \times 10^4$	Subcutaneous	6
Colon	$CD133^+$ (2%–25%)	6×10^4	200	$\sim7 \times 10^3$	Kidney capsule	3
Pancreas	$CD133^+/CD24^+/ESA^+$ (0.2%–0.8%)	$\sim6 \times 10^3$	100	~30	Pancreatic tail	36

* There are considerable uncertainties in some of the numbers presented in this table due to the limitations of the data in the relevant papers. CIC = cancer-initiating cell; Ref. = reference; + = positive; – = negative; ESA = epithelial surface antigen (also known as EpCAM).

† These markers were used for sorting putative cancer-initiating cells.

‡ Approximate number of unsorted cells from the tumor required to initiate tumor growth upon transplantation.

§ Approximate number of sorted cells (putative cancer-initiating cells) from the tumor required to initiate tumor growth after transplantation.

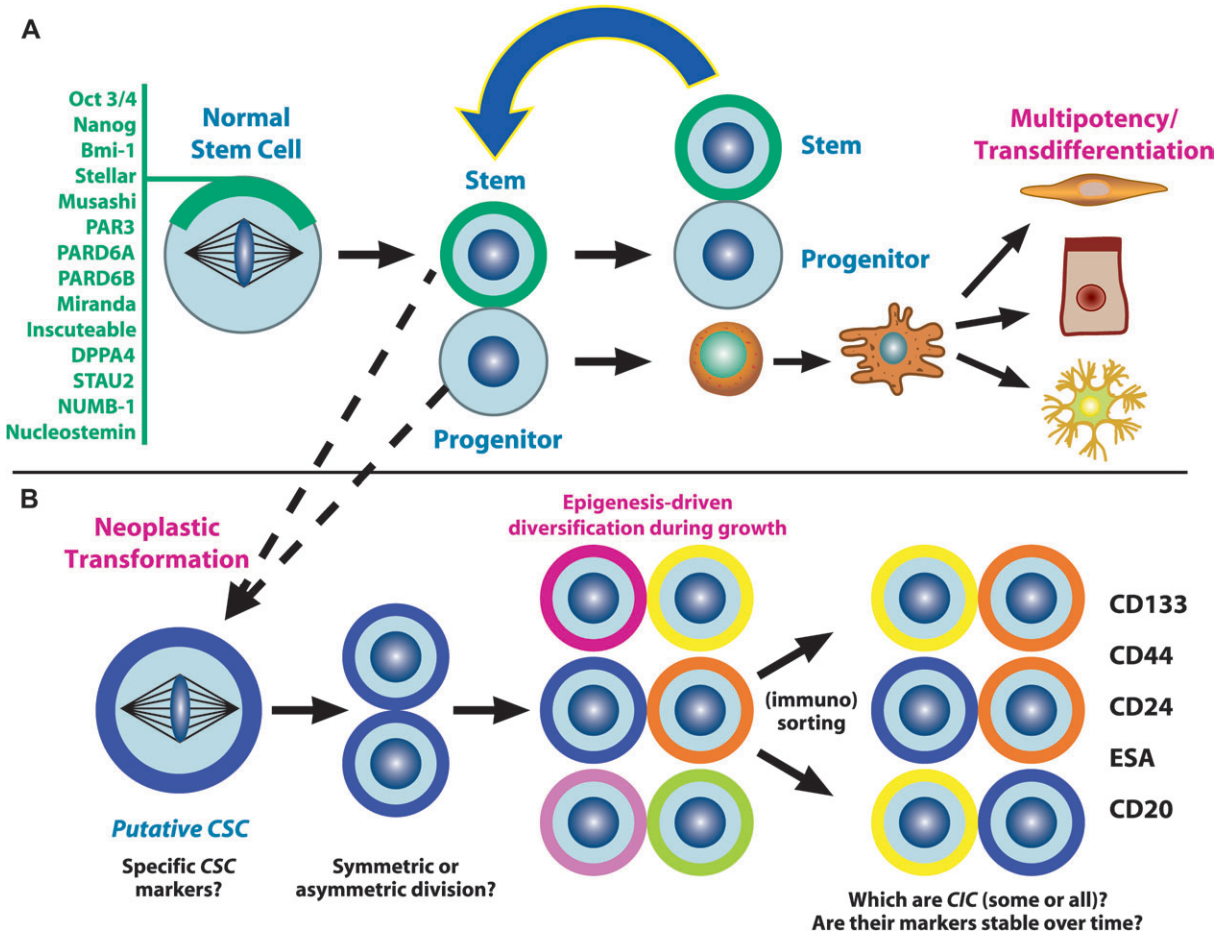

Fig. 2. Features of normal stem cells and cancer-initiating cells. **A**) Normal stem cells of the adult organism have the capacity of extensive self-renewal through primarily asymmetric cell division, which results in two distinct daughter cells. It is believed that adult stem cells may also undergo some symmetric division if needed to reconstitute the adult stem cell population. Numerous genes have been implicated in the behavior of stem cells, including the asymmetric division and self-replicating capacity, and some of these genes are listed at the left. These genes are not necessarily expressed simultaneously in all stem cells. Normal stem cells often give rise to progenitor cells that exhibit a multipotent differentiation potential or, in some cases, the capability to transdifferentiate across embryonic germ layer derivations (exemplified by differentiation into an epithelial cell, a muscle cell, or a nerve cell, as shown to the right). **B**) Cancer-initiating cells (CICs) are not known to undergo asymmetric division, and only a few of the genes associated with stemness (i.e., the phenotype of normal stem cells) or asymmetric cell division have thus far been identified in putative CICs. During amplification by symmetric division, CICs are susceptible to epigenetic changes and may thereby generate mixed populations of tumor cells (indicated by different colors), only some of which may resemble parental cells. Increasing experimental evidence supports the hypothesis that tumor cells form by neoplastic transformation of stem and/or progenitor cells from a tissue or an organ. Presently unanswered questions include whether specific markers for putative CICs exist, whether current (immuno)sorting strategies can isolate CICs that have uniform properties, and whether such properties are retained by CICs over the course of tumor growth. **Spindle-shaped cell** = smooth muscle cell; **middle cell** = epithelial gland cell; **lower cell** = astrocyte. **Colored circles** around the cells represent different phenotypes: **no outer ring** = progenitor cell; **dark green outer ring** = normal stem cell; **blue** = putative cancer stem cell; **other colors** = various phenotypes developing as the cells grow that may be attributed to epigenetic-driven diversification. CSC = cancer stem cell.

observed in animal models and is termed "sneaking through" (49–51). In syngeneic animal models transplanted with spontaneously arising tumors that were not immunogenic, sneaking through was not observed (52). However, as noted above, adding many lethally irradiated cells to the injected cell suspension still reduced the number of tumor cells required to induce a tumor (46–48).

Other in vivo transplantation studies (6,35,36,53,54) with relevant available data (Table 1) have reported similar discrepancies between sorted and unsorted cells, except for two involving brain or pancreatic tumors (for example, compare the numbers of sorted and unsorted cells expressing cancer-initiating cell markers required to cause tumor growth in Table 1) (35,36). In those two studies, reasonably good agreement was found between sorted and unsorted cells for the minimal number of CD133-positive cells or CD44-positive, CD24-positive, epithelial surface antigen–positive cells required to establish tumors. However, the data used to generate Table 1 are much less extensive than those used to generate Fig. 1, and consequently, there are considerable uncertainties in the estimates in Table 1. In addition, the markers presently used to sort putative cancer-initiating cells (including CD24, CD44, and CD133) are not specific for cancer-initiating cells because they are also expressed on normal cells (1). The authors of the studies discussed above (6,35,36,53,54) acknowledge that the sorting technologies used merely enriched for subpopulations that contain, but are not exclusively composed of, cancer-initiating cells.

More stringent clonal analyses and demonstration of tumors being formed by a single transplanted cancer-initiating cell are needed to firmly establish the ability of a single such cell to regenerate a neoplasm. Such proof should probably be similar to the gold standard adopted for normal stem cells; i.e., one single transplanted hematopoietic stem cell can reproduce the entire hematopoietic system in the recipient or a single transplanted breast stromal stem cell can generate a full mammary gland (55). Such evidence has been obtained for cancer-initiating cells in leukemia by lentiviral tagging of human acute myelogenous leukemia cells and the observation of individual clones present in NOD–SCID mice after serial transplantation of the tagged cells (32). Such information is not yet available for cancer-initiating cells in solid tumors.

Conclusions and Comments About Testing Novel Therapeutic Approaches

The many unresolved issues concerning the nature of cancer-initiating cells hamper the full understanding of their unique identity and their potential as specific therapeutic targets (Fig. 2). It is still unclear whether all cancer-initiating cells in any one tumor express the same molecular phenotype and whether they retain the same features throughout tumor growth. It is also uncertain whether the experimental procedures for identifying and characterizing cancer-initiating cells are sufficiently specific, especially when it is not immediately clear why the numbers of cancer-initiating cells required to establish a tumor differ so greatly between cells selected for the putative cancer-initiating cell marker and unselected cells. A "niche paradigm" (i.e., stem cells reside in a specialized microenvironmental location within a tissue that is optimal for their growth and maintenance as stem cells) is still poorly delineated for cancer-initiating cells (56,57), and very little is known about how microenvironmental components may affect these cells.

In practical terms, these uncertainties converge to the question of whether cancer-initiating cells and other tumor cells have different therapeutic sensitivities and whether they may constitute a moving, or even an evanescent, target for therapeutic approaches. Recent studies (5,35) have proposed that cancer-initiating cells may be more resistant to irradiation than other cells in the population. There are also reports (4,58–60) that cancer-initiating cells can be more resistant to chemotherapeutic drugs because of increased expression of antiapoptotic proteins or increased expression of the ATPase drug efflux pump ABCG2/5. However, more research is needed to establish both the initial sensitivity of cancer-initiating cells to various cancer treatments and whether their sensitivity to these treatments changes over time.

Further investigations on the origin and detailed properties of cancer-initiating cells should increase our understanding of tumor initiation and progression. For the optimal development of new anticancer drugs that are based on the cancer stem cell concept, current ambiguities on the nature of putative cancer-initiating cells and difficulties inherent to therapeutically targeting a small fraction of cells in a neoplasm should be taken into consideration. If anticancer drugs targeted to cancer-initiating cells are developed, their effectiveness will probably not be determinable by the current (short-term) approaches that involve testing for tumor regression but rather will require long-term approaches that involve combination treatments. Similarly, long-term studies will likely be required to assess whether long-term toxicities are also associated with depletion of stem cell pools in normal tissue. The importance of depleting the stem cell pool is well recognized for "renewal tissues," such as the bone marrow or gastrointestinal mucosa, but our knowledge of the importance of the stem cell pool in maintenance of "nonrenewal" tissues is currently very limited.

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