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Experimental Hematology

Experimental Hematology 2009;37:775–783

Problems in the promised land: Status of adult marrow stem cell biology

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(Received 5 May 2009; revised 5 May 2009; accepted 7 May 2009)

Long-term engrafting marrow hematopoietic stem cells have been considered to be a quiescent stem cell in G_0. However, there are contradictory reports on this point in the literature, showing marked variability of results over time and between mice. Furthermore, there are circadian rhythms for stem cells and progenitors. In general, most studies have not taken stochastic variability or circadian rhythms into account. In addition, stem cell purification has represented the present gold standard in stem cell research. However, evidence exists that the stem cell separations leave behind most stem cells and are not random. Thus, purified stem cells may not be representative of the stem cells in the unseparated marrow cell population. The epitope-based purification of stem cells may have misled the stem cell field. Lastly, there are interesting published studies indicating that the irradiated marrow microenvironment might be toxic to marrow stem cells, limiting self-renewal capacity, and that quantitative engraftment occurs in nonablated mice. These considerations suggest that in carrying out stem cell studies, attention needs to be directed to the appropriate number of repeat experiments, to circadian rhythms, to possible purification skewing of results, and to the most appropriate transplant assay model. © **2009 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Concepts

Adult marrow stem cells remain to be fully defined, and there are issues about the basic biology of these cells that need to be addressed. These concepts include the stochastic variability of the system, impact of circadian rhythms, whether the reported purified stem cells are representative of the original stem cell population in whole unseparated marrow, whether clonal studies are informative, and whether the irradiated or nonablated mouse is the proper assay model for the stem cell.

We are in a very exciting scientific period. We can now do things that seemed impossible a few years ago. Reverse transcription polymerase chain reaction, gene cloning, animal cloning, embryonic stem cells, induced pluripotent stem cells, inhibitory RNAs, in vivo imaging, knockin and knockout mice, and a wide variety of molecular magic with microarrays and proteomics. Add to this fluorescence-activated cell sorting (FACS) of variously labeled stem cells and a host of second messenger insights. We are in the era of molecular-targeted drugs, with Gleevec (Novartis Pharmaceuticals Corporation, Parsippany, NJ, USA) leading the pack.

Much progress also appears to have been made in the field of adult stem cell biology. Stem cells have been purified to homogeneity using a variety of techniques, but focusing on antibody binding to cell surface epitopes and FACS [1–25]. These approaches result in highly purified stem cell subsets with a high potential for in vivo repopulation and in vitro high-proliferative potential colony-forming cell (HPP-CFC) growth. Studying these purified stem cells, molecular control mechanisms continue to be reported with very complicated transcriptional networks [26] and gene expression patterns [27–29], while the number of hormone regulators continues to expand.

Testing basic assumptions

It remains important to test basic assumptions. The adult stem cell field is, of course, dependent on the definition of the stem cell and its subsequent study. There are certain assumptions here, e.g., that the phenotype of the individual stem cell is relatively stable and can be defined using a set of surrogate markers. In this case, it is important to realize that a specific separation of stem cells, while resulting in phenotypically homogeneous cells based on the separative parameters, gives

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0301-472X/09 $–see front matter.
doi: 10.1016/j.exphem.2009.05.003

functionally heterogeneous populations of cells. Thus, $CD34^+$ or elutriated Lin^-Kit^+ cell populations [30,31] make up a diverse population of cells with multilineage potential that can be further subsetted on the basis of Sca-1 antigen and rhodamine 123 dye efflux [32,33] or CD38 expression [31]. An analysis of stem cell targets chosen on the basis of rhodamine or Hoechst 33342 dye staining alone or in combination with Sca-1 and/or c-kit expression have been shown to contain overlapping cohorts with differing proliferative histories, engraftment potentials, and gene expression patterns [34–36]. In addition, it has been assumed that the purified stem cell population is representative of the whole stem cell population in the starting unseparated bone marrow. It may not be. An additional critical assumption is that repopulation of the irradiated mouse is a valid gold standard assay for the stem cell, but this may represent a stem cell toxic microenvironment, and the most appropriate assay may be the nonablated normal mouse. Finally, a temporal consistency is assumed, such that the stem cell does not change characteristics on a day-to-day or season-to-season basis. Extant data on circadian rhythms [37–40] and on noncircadian temporal variation in stem cell numbers and cell-cycle status [41] challenge this assumption.

We have been forced to address these assumptions by recent problems in reproducing extant work in the field of stem cell biology. We have carried out a series of studies that have led us to a continuum theory of stem cell biology. Our and others observations indicate that the adult marrow stem cell phenotype changes reversibly with cell-cycle passage [42–51] and that stem cells are intrinsically a cycling population [52–55]. In continuing studies on this subject, we utilized the elegant approach of Passegue and colleagues [24] in studying G_0, G_1, and $S/G_2/M$ phases of stem cell cycle to address our continuum studies. We were intrigued by their observations that all long-term hematopoietic stem cells (HSC) were in G_0. Our first study simply sought to reproduce this separation of long-term hematopoietic stem cells (LT-HSC) by FACS based on pyronin and Hoechst supravital staining. We followed the described methods exactly, with the exception of using FLK2 instead of Thy 1.1 in the separation. This has been reported [10] to give an equivalent LT-HSC separation. Our first studies, in contrast to those of Passegue et al. [24], showed almost 50% of LT-HSC in $S/G_2/M$ at 24 weeks postengraftment with similar numbers in G_0. A subsequent experiment, however, failed to reproduce the first, showing most LT-HSC in G_0 and, furthermore, similar separations of whole bone marrow with pyronin and Hoechst have indicated that LT-HSC is distributed equally between G_0/G_1 and $S/G_2/M$. In parallel studies on homing, we separated LT-HSC and short-term HSC, finding that engraftment of ST-HSC persisted in both out to 27 weeks, although short-term HSC had lower levels of repopulation than LT-HSC. What do these differences mean? Is the data from one laboratory wrong or are the methods not adequately reproduced. Is rare cell contamination one explanation? We believe an individual experiment is probably the right result for that experiment at that moment in time. The result of each experiment being valid. This, of course, suggests that assumptions of stability of stem cell phenotype may be in error and that the specific manner of carrying out adult stem cell experiments may need to be reconsidered.

Reconsideration: Variability of results of stem cell studies as to incidence and cell-cycle phase over time and between individual mice

Two previous studies from our laboratory indicated potential problems with variability of results [40,56]. Variation of granulocyte-macrophage colony growth between experiments was a common theme and initially attributed to a vague entity termed *biologic variability*. To minimize such variation, we routinely pooled cells from multiple mice, usually five, for the granulocyte-macrophage progenitor assays, but comparing control levels of granulocyte-macrophage colony-forming cells (GM-CFC) per cell number or tibia, using pooled cells, there was still marked variability in colony numbers. Even more striking was the variability in cell-cycle status of these cells between different experiments. This prompted a statistical evaluation of the validity of cell-cycle suicide techniques. Analyzing a total of 66 experiments in which hydroxyurea suicide of GM-CFC was carried out, it was found that with three separate experiments and a "true value" of 23% kill, that the actually observed values were $<10\%$ in 17% of the cases and $>40\%$ in 10% of the samplings [56]. Results with tritiated thymidine suicide confirmed this variability. These data demonstrated the variability of progenitor numbers and cycle suicide values between separate experiments.

A separate problem is the consideration of heterogeneity between individual mice as to stem cell values. In general, pooled cells have been utilized in stem cell studies to minimize biologic variability, but this removes the essential characteristic of individual variation between mice. Colony-forming unit spleen (CFU-S), the original stem cell assay [57] was extensively studied, and it was generally found to be relatively quiescent, with S-phase values of $\leq 10\%$ [58–62]. A number of studies showed higher S-phase values for CFU-S ranging from 16% to 48% [56,63–73]. Our own work showed varying results from no killing with hydroxyurea or tritiated thymidine to killing rates up to 25% [56]. The work by Necas and Znojil [41] is particularly informative. They determined the number of CFU-S and the fraction synthesizing DNA in individual normal mice of several inbred strains, and data obtained during a period of 5 years was subjected to analysis of variance. Large differences were shown to exist in the number of CFU-S in the femoral bone marrow of individual mice measured on the same day. These differences were greater if measurements were performed on different days. The

fraction of DNA synthesizing CFU-S was, on average, 30% in these normal mice, but the range of measurements on both the same and different days was 0% to 60%. The authors measured CFU-S from day 7 to day 12 and found similar results. Others who also found significant variations were cited in this article. Now, admittedly, the CFU-S assay has been regarded by many as a poor representative of the primitive bone marrow renewal stem cells, but considering our cell-cycle data and the continuum concept of stem cell regulation, we would propose that, in fact, it is a very good clonal assay for renewal stem cells. These data indicate large stochastic variations in stem cell phenotype in marrow from individual mice or marrow studied at different points in time. Several features may underlie these variations. The Necas studies were carried out at the same point in time each day, but circadian rhythms could still provide an explanation. Necas and Znojil had proposed that there might be bursts of CFU-S proliferation over time. These observations need to be considered in the context of the continuum theory. This concept of bursts of CFU-S foreshadows our continuum model, where the stem cell continuously and reversibly changes phenotype tied to cell-cycle phase. These observations could explain dramatic shifts in stem cell phenotype. Simple random variations unrelated to this might also be operative. At any rate, these data suggest that in studying marrow stem cells, multiple experiments need to be carried out over time, and that individual mice as well as pooled cells from groups of mice should be studied. Use of pooled cells, of course, obscures the intrinsic heterogeneity of stem cell values between mice. These considerations also raise questions of whether the status of LT-HSC can be adequately determined using a limited number of experiments during a limited time frame. This, in turn, raises questions of the validity of molecular studies on LT-HSC separated from a limited number of cell pools in a limited number of experiments.

Figure 1 is a graphic representation of results in which a mean from pooled cells is obtained vs data from individual mice. The assumption for the mean data is that the true S-phase data would be 30%, but a look at the second set representing true data from individual mice gives a very different picture. We would contend that knowledge of a mean value, as represented by data from individual mice or from pooled cells from individual mice, and an appreciation of the individual heterogeneity of results are both important for our understanding of the stem cell system.

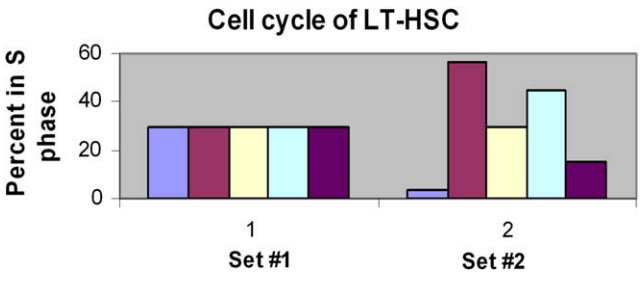

Figure 1. Set #1 represents assumed values of mice in which pooled cells were assayed (30% in S phase), while Set #2 represents the actual data from individual mice (mean values: 30%). LT-HSC = long-term hematopoietic stem cells.

Circadian considerations

Another explanation for the variability of stem cell results could be the effect of circadian rhythms on these results. The existence of circadian rhythms within the hematopoietic system has been recognized since the late 1940s [74], but has generally been ignored in studies on stem cell biology, in part, because these studies are so difficult to carry out. Differentiated cell types and different progenitor classes, including 8-day CFU-S, CFU–granulocyte macrophage, burst forming units-erythrocyte, CFU-erythrocyte, and CFU–granulocyte-erythroid-macrophage-monocytes, have been shown to exhibit distinct circadian rhythms and to show seasonal variations [37–39]. In addition, bone marrow susceptibility to cytotoxic drugs has been shown to vary according to circadian and seasonal rhythms [75]. Daily rhythms of biologic activities of plants and animals are a universal phenomena [76], but applying this knowledge to stem cell biology has not been routinely carried out. We investigated circadian rhythms of engraftable stem cells (10 weeks), studying 6 circadian times (hours after light onset or HALO) 4 hours apart of male B6.SJL mice engrafted into C57BL/6 J mice, the latter having been exposed to 100 cGy whole body irradiation [40]. This model is essentially a competitive transplant model in which the infused marrow cells compete against the residual host cells. These studies showed marked and significant nadirs at HALO 8 and 24, in the context of these experiments 6:00 AM and 2:00 PM. These shifts were in the range of three to fourfold for 10-week engraftable stem

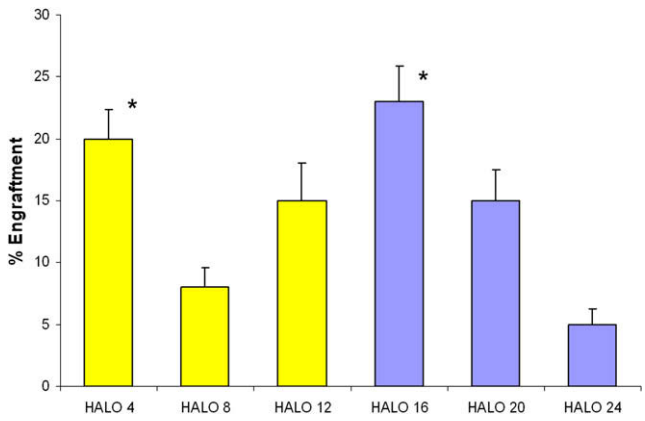

Figure 2. Mean percent engraftment of marrow from three separate experiments carried out in July. These data represent three different experiments with a total of 15 donor mice per time point. $*p < 0.0004$ from hours after light onset (HALO) 8 and 24. From D'Hondt L, McAuliffe C, Damon J, et al. Circadian variations of bone marrow engraftability. J Cell Physiol. 2004;200:63–70, reprinted with permission.

Table 1. Circadian rhythm of hematopoietic cells

Engraftment into nonablated mice at 10 weeks
Numbers of HPP-CFC and total progenitors
Cell-cycle status of HPP-CFC and total progenitors

HPP-CFC = high-proliferative potential colony-forming cell.

cells (Fig. 2), and similar circadian changes were seen with HPP-CFC and total GM-colonies but at HALOs 12 and 24. Cycle status of the progenitors showed approximately threefold increased killing peaks at HALOs 8 and 24 ($p < 0.002$ from HALO 4 and 16, respectively).

We also saw a seasonal shift. These results were obtained in July, but when studies were repeated in February, there was only one nadir at HALO 8. In separate studies, we did not find a circadian rhythm to host engraftability [40]. These studies are summarized in Table 1. They indicate that one should anticipate marked shifts in stem cell phenotype at 4 to 8-hour time intervals during the day and that seasonal shifts are also probable. These shifts could account for much of the variability observed in various stem cell studies, especially with regard to cell-cycle status.

Clonal studies vs populations studies, where is the truth? Are highly purified stem cells representative of the true stem cell population?

Perhaps the major advance in adult stem cell biology of the recent years has been the utilization of different monoclonal antibodies to various cell surface epitopes to subset marrow stem cells using FACS. Has this really represented an advance? Certainly the technology applied here is very impressive. In general, marrow separations have involved a density step to get rid of red cells and granulocytes and application of antibodies to mature cell antigens with iron tags and magnetic separations to obtain a lineage-negative population of marrow cells. This lineage-negative population is then labeled with a variety of monoclonal antibodies (with fluorescent tags) recognizing various "stem" cell antigens and the hematopoietic stem cell isolated by FACS [5–25]. A variation on this theme is to stain lineage-negative cells with the supravital dyes Hoechst 33342 or rhodamine 123 and separate low-staining cells [1–4]. Other less popular separations include elutriation and recovery of cells from a previous in vivo cell infusion [77], or use of aldehyde dehydrogenase or levels of reactive oxygen species as separative probes [78,79].

Till et al. [80] suggested that their studies on CFU-S indicated that "relevant control mechanisms were operative at the level of populations rather than single cells." They further proposed that the behavior of individual stem cells was analogous to that of individual radioactive nuclei. Populations of nuclei give decay with a highly predictable half-life, but it was impossible to predict exactly when an individual nucleus will undergo radioactive decay. This seems a reasonable view of the stem cell populations today and should urge caution to those focused on the study of highly purified marrow stem cells.

In previous work on engraftment of marrow stem cells into nonablated mice, we showed that whole marrow engrafted quantitatively [81–87]. The validity of the nonablated transplant model was established. We determined the total marrow cellularity in BALB/c male mice (530 million cells) and then calculated the theoretical engraftment if 40 million infused marrow cells were added to marrow or, alternatively, if infused marrow cells replaced marrow in female BALB/c recipient mice [87]. The theoretical engraftments for replacement or addition were 7.5%

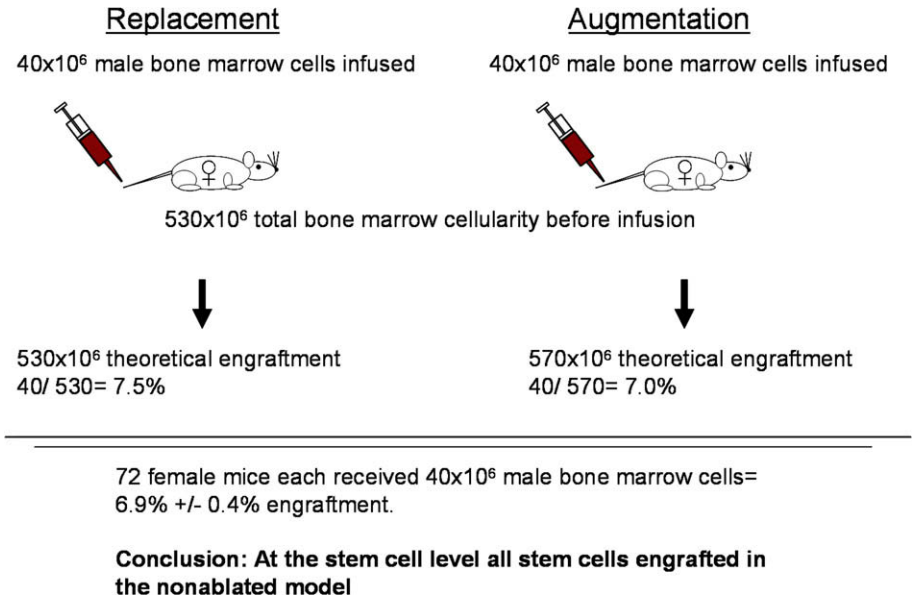

Figure 3. Engraftment into nonablated mice.

Table 2. Lineage-negative rhodamine/Hoechst dull stem cell recovery

No. of LRH	No. transplanted mice	% recovered from original marrow
3,000	9	3.6
2,600	12	6.4
5,500	3	3.3
10,000	3	0.8
10,000	5	2.6

LRH = Lineage-negative rhodamine/Hoechst.
Engraftment determined at 6 weeks to 6 months in nonablated host mice [data from 88].

and 7.0%, respectively. This model assumed that all marrow stem cells home and engraft in marrow only and that total marrow cells in a nonmanipulated cell population correlated directly with the number of stem cells in that population. In a series of 72 experiments in which 40 million male BALB/c cells were infused into nonablated female BALB/c mice (controls for different experiments), we had an engraftment rate of 6.9% ± 0.4% (Fig. 3).

This surprisingly high rate of engraftment indicated that virtually all stem cells engrafted in the nonablated mouse and that engraftment in the nonablated host was determined by simple competition between host and donor cells (more on nonablated marrow to come). Thus, the nonablated transplant model would appear to monitor all the engraftable stem cells, while engraftment into lethally irradiated hosts, representing essentially an amplification system, cannot be utilized to measure the levels of engraftment at the stem cell level. We will expand on these comments later. However, we review this here to indicate the validity of the nonablated transplant model. In studies on engraftment of highly purified lineage-negative rhodamine/Hoechst low stem cells into this nonablated mouse model, we found multilineage long-term engraftment [88]. However, these studies demonstrated that the engraftment capacity of the final purified stem cell product was far less than that of the whole unseparated marrow from which the cells were purified. Our studies indicated that only from 0.8% to 6.4% of the engraftable stem cell capacity of the starting bone marrow was recovered in the purified stem cell fraction (Table 2). These separations are not random and, thus, these data indicate that the purified cells are probably not representative of the starting stem cell population, although they are certainly highly purified. This concept is illustrated in Figure 4.

These results with the lineage-negative rhodamine/Hoechst stem cells undoubtedly hold for the other separations. These results could represent either a simple loss of stem cells with each separative step or variations in recovery of cells that support stem cell function, but are not in themselves stem cells. In any case, data indicate that the elegant antibody separations could have led investigators to the study of stem cell populations, which may not be relevant to normal biology. Thus, at the very least, studies of the function of purified stem cells should also include studies of unseparated marrow in order to be sure of biologic relevance. Furthermore, it is worth noting that a continuum model of stem cell biology would indicate that stem cell purification could never be definitive because the phenotype of the stem cell will be changing constantly.

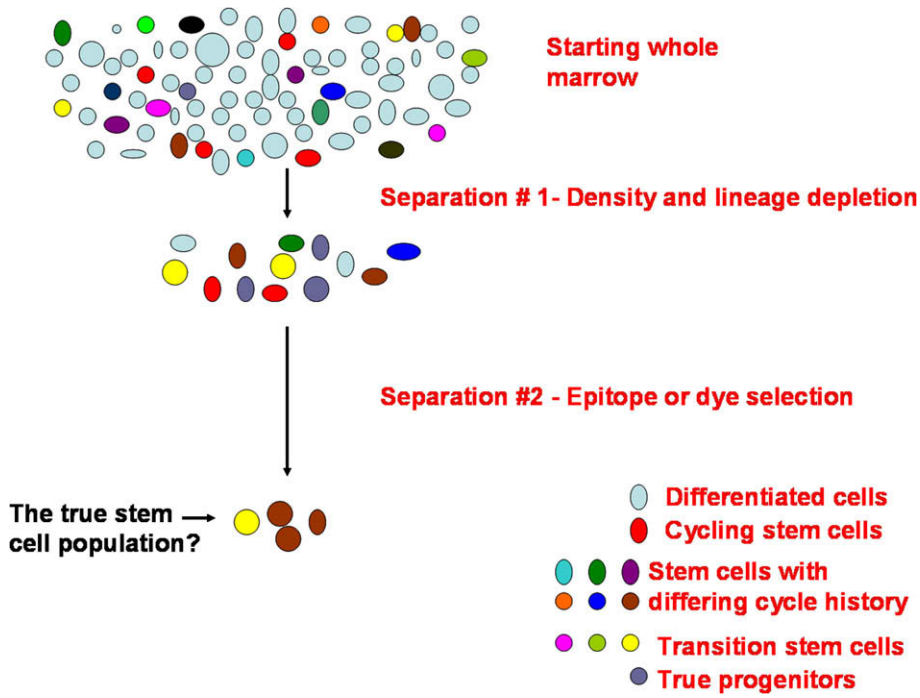

Figure 4. Theoretical stem cell purification resulting in a highly purified, but nonrepresentative stem cell population.

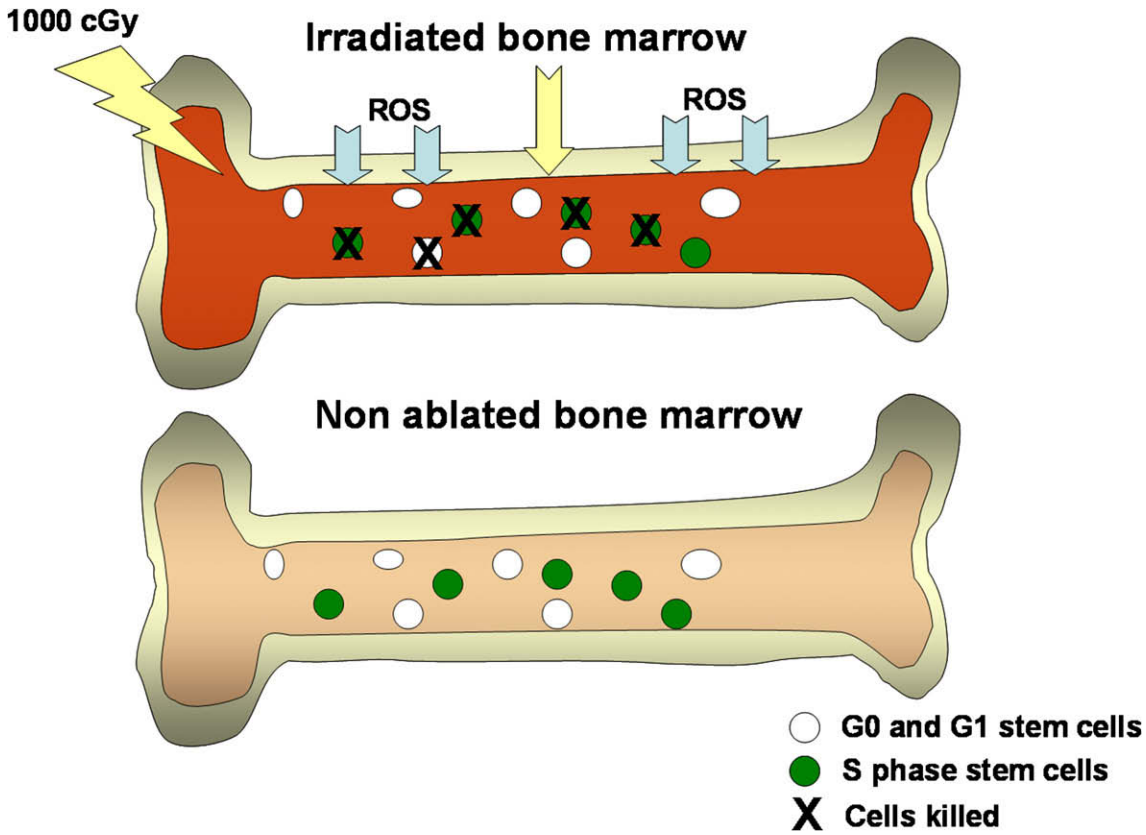

Figure 5. Top bone irradiated with 1,000 cGy and bottom not exposed to irradiation. ROS = reactive oxygen species; X = cell death.

The work of Sieburg et al. [89], indicating the 16 types of repopulation kinetics for HSCs, would also indicate that the goal to isolate one specific stem cell type may be futile and end up in providing misleading information.

We noted that our studies indicating a loss of stem cells with purification were carried out in nonablated mice. Whether similar results would be seen in the more traditional model of irradiation ablation of murine hosts is an open question. However, use of different engraftment models raises another potential problem.

Stem cell engraftment models and gold standards: Which is the preferable model for assay of long-term engrafting stem cells; the nonablated or the irradiated mouse?

Hematopoietic stem cell existence and their characteristics have been defined in lethally irradiated hosts and long-term multilineage hematopoietic cell repopulation in lethally irradiated mice has been deemed to be the "gold standard" assay for a marrow hematopoietic stem cell. This assay is an amplification assay, as can be seen by observations of single hematopoietic cell repopulation of lethally irradiated mice. As noted here, this characteristic does not allow determination of the efficiency of engraftment at the stem cell level, the readouts here are differentiated hematopoietic cells, and these will be the same whether 1 or 1,000 marrow stem cells are engrafted, although the model can be utilized to compare the efficacy of different cell populations. Engraftment into nonablated mice is not an amplification system, but represents essentially competition between donor and recipient host stem cells. The studies cited here indicate that engraftment of stem cells contained in whole marrow populations is very efficient and, in fact, appears to be quantitative. The only puzzle here is that at times it appears to be too good. The lethally irradiated mouse transplant model has the advantage that a relatively small number of transplanted cells results in engraftment, and it has been assumed that irradiation is needed to "open spaces or niches" for stem cell occupancy. Our studies in the nonablated mouse disprove this concept and, in fact, at the stem cell level, it appears that homing and engraftment into irradiated mice is inferior to that seen in nonablated mice [90]. It is clear that irradiation damages the marrow environment, but it has been erroneously assumed that this was favorable to engraftment. Myelotoxic treatment results in a markedly abnormal marrow microenvironment. The endothelial barrier evidences damage [91] and irradiation $>1{,}000$ cGy leads to irreversible damage to the marrow sinus endothelium [92]. There is evidence that passage through an irradiated (or cyclophosphamide-treated) environment may impair self-renewal [93].

Table 3. Factors effecting stem cell study outcomes

Cell to cell variability
Mouse to mouse variability
Stochastic variability
Circadian rhythm
Purified stem cells vs whole marrow
Nature of engraftment host—irradiated or nonablated
Continuum cell-cycle–related phenotype fluctuations
Microvesicle phenotype modulation

In support of this are studies that showed that, in dogs, normal erythrocyte cells had a shorter lifespan in irradiated hosts than in normal hosts [94] and that, in mice, bone marrow proliferation was reduced after a single passage through an irradiated host [95,96]. Furthermore, chromosomal instability was observed in the progeny of normal hematopoietic cells after exposure to irradiated cells, and this was felt to be a result of inflammatory cells derived directly from the exposed hematopoietic cells [97,98]. Cellular redox state is a critical component of stress-induced cellular responses, and inherent in these responses are reactive oxygen species (ROS), which inflict direct cellular damage in addition to acting as intracellular second messengers. The ROS include superoxide (0.2^{-}), hydrogen peroxide (H_2O_2), hydroxyl radical (HO), and peroxyl (ROO). Radiation is a critical generator of ROS, and there is evidence that high ROS is damaging to marrow stem cells. Jang and Sharkis [79] showed that N-acetylcysteine, an antioxidant, was able to restore HSC function in the ROS^{high} stem cell population. All together these observations suggest that irradiation may damage stem cells by generating ROS, and it is possible that such damage might act selectively in different phases of stem cell cycle (Fig. 5).

Thus, a number of questions exist about studies of basic marrow stem cell biology that need to be addressed. Should whole marrow from individual mice rather than pooled cells be the subject of study? Do circadian effects have to be determined in stem cell studies? Does one need to carry out repeated experiments over time (at least four to six) in order for studies to be interpretable? Do we need to determine whether purified stem cells are representative of the whole stem cell population? Do we need to ascertain whether studies in the relatively nontoxic environment of nonablated host mice will give fundamentally different results from studies in irradiated hosts? These considerations then need to be viewed in the light of recent observations in the controversial field of stem cell plasticity, suggesting that cell-derived microvesicles, especially from injured tissue, may be able to alter the phenotype of marrow stem cells toward that of the tissue of origin for the microvesicles [99–102].

The large number of factors effecting stem cell study outcomes are outlined in Table 3. These considerations indicate that the cell-cycle status (and other phenotypical characteristics) of marrow stem cells need to be studied with careful attention to stochastic variability; circadian rhythms; nature of the stem cells in whole marrow; and influence of the marrow microenvironment. We conclude that the complexity of the marrow stem cell system is impressive and needs further fundamental study.

Acknowledgments

This work was supported by National Institute of Health (Bethesda, MD), National Center for Research Resources (5P20RR018757) and by the National Institute of Health, National Heart, Lung, and Blood Institute (7R01HL073749). No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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