

## Self-renewal of hemopoietic stem cells during mixed colony formation *in vitro*

(pluripotent/colony-forming unit-spleen/heterogeneity)

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**ABSTRACT** Replating experiments have shown that the self-renewal of pluripotent hemopoietic stem cells can be studied *in vitro* by clonal analysis techniques. The number of daughter stem cells detectable in individual primary clones produced *in vitro* varies markedly from one clone to another. These findings are consistent with a general model of stem cell differentiation in which the choice to self-replicate or not is ultimately determined at the single-cell level by a mechanism involving a random-event component that is intrinsic to the stem cell itself. Hemopoietic stem cells were identified by their ability to generate macroscopic-sized colonies having a visible erythroid component (i.e., gross red color) in standard methylcellulose assays containing medium conditioned by pokeweed mitogen-treated spleen cells and erythropoietin. In assays of replated primary or secondary colonies, inclusion of irradiated marrow-cell feeders was found to be an additional requirement. The mixed erythroid–megakaryocyte–granulocyte nature of colonies identified simply as macroscopic and erythroid was confirmed by cytochemical stains for lineage-specific markers. Marked variation in self-renewal was a feature of marrow stem cells both before and after maintenance in flask culture, although the overall self-renewal capacity exhibited by flask-cultured cells was  $\approx 5$ -fold higher. Variation in self-renewal was not correlated with primary colony size, which also varied over a wide range ( $0.2\text{--}9 \times 10^5$  nucleated cells per colony). Variation in stem cell self-renewal has been previously associated with hemopoietic stem cell proliferation *in vivo*. Its persistence *in vitro* in assays of dilute single-cell suspensions casts doubt on the significance of microenvironmental influences in directing stem cell differentiation.

Adult bone marrow is a typical cell-renewal system, consisting of stem cells and their differentiating progeny. The distinguishing property of a stem cell is its self-renewal function. Restated, a stem cell is a cell whose proliferative capacity has not yet been irreversibly restricted to a finite number of cell divisions. Virtually nothing is known about molecular changes that may accompany or underlie such restrictive events in any tissue. Attempts to elucidate the self-renewal process and its regulation have thus relied on less explicit biological endpoints. For example, in the murine hemopoietic system, individual colony-forming unit-spleen (CFU-S) cells, each capable of producing multiple cell lineages, can be detected by their ability to generate large mixed colonies in the spleens of irradiated recipients (1, 2). The evidence for CFU-S self-renewal derives from the demonstration that spleen colonies also contain CFU-S cells—i.e., progeny that have the original properties of unrestricted differentiation potential and spleen colony-forming ability.

Shortly after the spleen colony assay was described, the observation was made that the number of CFU-S cells present in individual spleen colonies was remarkably variable from one

colony to another (3–5). These results were considered to be at variance with those expected for a homogeneous population of stem cells subject to the same proliferative pressures during spleen colony formation. To explain the observed heterogeneity, Till *et al.* (6) proposed a model of stem cell proliferation in which the decision to self-renew involved a random-event component and the concept of a probability for self-renewal ( $P$ ) was introduced. However, no mechanism triggering self-renewal has yet been described, and it has therefore been difficult to visualize how its probability might be set. Further, it is not known whether extrinsic factors can influence this probability. Indeed, others have proposed that the heterogeneity seen in CFU-S cell production during spleen colony formation may be accounted for by variations in extrinsic factors due to local differences in the inductive properties of the splenic microenvironment (for review, see ref. 4).

Recently, several investigators have shown that hemopoietic colonies of mixed composition and single-cell origin can be obtained in cultures initiated with adult marrow cells (7–10). In this report, we show that, in methylcellulose cultures, the majority of colonies, simply identified by the dual criteria of macroscopic size and red color (hemoglobin content), are derived from pluripotent self-renewing stem cells. Using this assay, we have examined the extent of self-renewal exhibited by individual stem cells stimulated *in vitro* under relatively controlled conditions.

### MATERIALS AND METHODS

**Mice.** Mice used were adult (C57BL/6  $\times$  C3H/He) $F_1$  hybrids (from Biobreeding Laboratories, Ottawa, Ont., Canada, or Charles River Breeders, Canadian Breeding Farms, St. Constant, PQ, Canada).

**Assays.** Unless otherwise stated, primary colonies for analysis were obtained from the nonadherent fraction of 2-week-old flask cultures set up with adult B6C3F $_1$  marrow cells and maintained as described (9). By this manipulation, it was routinely possible to obtain 10–20 well-isolated macroscopic erythroid colonies per  $2\text{--}5 \times 10^4$  cells plated in each assay culture. Other advantages of flask-cultured marrow over fresh adult marrow (i.e., decreased numbers of committed granulopoietic and erythroid precursors) have been discussed (11).

Primary colonies were grown in  $\alpha$  minimal essential medium (Connaught Laboratories, Willowdale, Ont., Canada)/0.8% methylcellulose/30% fetal calf serum (lot 29101111, Flow Laboratories, McLean, VA)/1% deionized bovine serum albumin (Sigma)/0.1 mM 2-mercaptoethanol, containing step III erythropoietin at 2.5 units/ml (Connaught) unless otherwise stated

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Abbreviations: PWM-SCCM, medium conditioned by pokeweed mitogen-stimulated spleen cells; CFU-S, colony-forming unit-spleen (cells).

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and a predetermined optimal concentration medium conditioned by pokeweed mitogen-stimulated spleen cells (PWM-SCCM) (2.5% and 0.3–0.6% for assays of fresh and flask-cultured marrow, respectively; see refs. 9, 11, 12). Cell suspensions constituted 9% of the culture volume, and 1.1 ml of the complete mixture was plated per 35 mm Petri dish. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air.

Erythroid colonies achieving macroscopic size after 9 days of incubation represent a subclass of all late appearing colonies containing erythroid cells (of burst-forming unit-erythroid origin) and can also be readily distinguished from purely granulopoietic colonies (of colony-forming unit-culture origin) in cultures examined without fixation or staining. Individual macroscopic erythroid colonies were taken into sterile, finely drawn out Pasteur pipettes by using an inverted microscope and then gently dispersed in small volumes (0.3–0.6 ml) of 2% fetal calf serum in  $\alpha$  medium. Secondary colony assays used the same culture conditions as primary colonies with two modifications:  $5 \times 10^5$  irradiated [1500 rads (1 rad =  $1.0 \times 10^{-2}$  gray), 280-keV x-rays] fresh marrow cells were included in each assay culture and 1.25% PWM-SCCM was used.

CFU-S were assayed by intravenous injection of cell suspensions into irradiated recipients (850 rads, 280-keV x-rays). After 9 days, spleens were removed and fixed in Bouin's solution, and macroscopic spleen colonies counted.

## RESULTS

**Optimization of Replating Conditions.** Previous experiments suggested that even though assays of the nonadherent cell fraction of flask cultures showed a linear colony–cell dose–response curve down to 4000 cells per 1-ml culture (12), addition of irradiated marrow cells might be required to achieve maximal plating efficiency in replating studies (11). Data from two experiments showing that the number of macroscopic erythroid colonies developing in secondary assays of pooled, resuspended primary colonies was greatly increased when irradiated marrow cells were also present are given in Table 1;  $5 \times 10^5$  irradiated cells per ml of culture appeared sufficient to achieve a maximum enhancing effect. This requirement for irradiated cells could not be replaced by simply increasing the concentration of PWM-SCCM (i.e., to > 0.3%). Rather, in conjunction with irradiated cells, up to 5% PWM-SCCM sometimes improved secondary colony formation (data not shown). Increasing the concentration of unpurified PWM-SCCM > 5% resulted in decreased hemoglobin production (colonies were paler in color and cultures showed decreased <sup>59</sup>Fe incorporation into heme over a 24-hr period) so that any possible increase in number of mixed colonies could not be detected.

Table 1. Number of macroscopic erythroid colonies in assays of replated primary colonies as a function of number of irradiated marrow cells added

Experiment	Irradiated fresh marrow cells, no. per dish			
	0	$5 \times 10^5$	$1 \times 10^6$	$2 \times 10^6$
1	0.25 ± 0.25	6.3 ± 1.0	5.8 ± 0.95	2.5 ± 0.5
2	0.75 ± 0.50	4.3 ± 1.3	5.3 ± 0.75	ND

Nine-day-old macroscopic erythroid colonies developing in primary assays of flask-cultured marrow were picked and pooled (32 in experiment 1, 50 in experiment 2). The equivalent of one primary colony was plated in each 1.1-ml secondary assay culture. Values are mean ± SEM of macroscopic erythroid colonies determined from four replicate assay cultures.

**Continuing Stem Cell Self-Renewal Through Two Generations of Colony Formation *in Vitro*.** Macroscopic erythroid colonies obtained in assays of replated primary colonies were further characterized with respect to size, number of cell lineages represented, and content of cells capable of tertiary macroscopic erythroid colony formation. Results from two experiments are summarized in Table 2. Secondary macroscopic erythroid colonies ranged in size from  $6 \times 10^4$  to  $10^6$  cells per colony. This is comparable with the primary colonies from which they derived ( $2 \times 10^4$  to  $9 \times 10^5$  cells per colony, see Fig. 2). Secondary colonies also resembled primary colonies in their content of acetylcholinesterase-positive megakaryocytes (13) and benzidine-positive erythroblasts (14) (experiment 1, Table 2). No attempt was made to identify specifically cells of the granulocyte series in secondary colonies.

In both experiments, some of the secondary colonies tested (12 of 49) gave rise to macroscopic erythroid colonies in tertiary assays. Sixteen of these were examined cytologically (6 of the 7 obtained in experiment 1 and 10 of the 67 obtained in experiment 2). Again, all contained numerous acetylcholinesterase-positive megakaryocytes and benzidine-positive erythroblasts. In 7 of these 16 tertiary colonies, the presence of numerous myeloperoxidase-positive cells (15) (chiefly of monocyte morphology) was also noted. These results indicate that the proliferation of cells retaining both pluripotency and high proliferative capacity persists through at least two generations of colony formation and that concomitant differentiation of other progeny along at least three pathways occurs during both of them.

However, with serial replating, a decrease both in the proportion of colonies exhibiting self-renewal and in the average extent of self-renewal per 9-day-old colony was observed. (Compare the 20% and 31% positive secondary colonies shown in Table 2 with the 48% positive primary colonies shown in Fig. 1. Compare also the 0.4 and 2.3 tertiary colonies per secondary colony shown in Table 2 with the  $\approx 5$  secondary colonies per

Table 2. Properties of second- and third-generation macroscopic erythroid colonies

Property	Experiment 1	Experiment 2
Nucleated cells in secondary colonies, no.	$7.4 \times 10^4$ to $1 \times 10^6$ (20 colonies)	$5.9 \times 10^4$ to $1.2 \times 10^6$ (29 colonies)
Secondary colonies yielding tertiary macroscopic erythroid colonies, no.	4 of 20 (20%) (mean, 0.4; range, 0–4)	8 of 29 (31%) (mean, 2.3; range, 0–23)
Morphology of secondary colonies	21 of 21 with erythroid cells and megakaryocytes*	ND
Tertiary colonies yielding fourth-generation macroscopic erythroid colonies, no.	ND	0 of 25
Morphology of tertiary colonies	6 of 6 with erythroid cells and megakaryocytes; 2 with myeloperoxidase-positive cells	10 of 10 with erythroid cells and megakaryocytes; 5 with myeloperoxidase-positive cells

Nine-day-old colonies were individually assayed for secondary colony formation in the usual manner. A random selection of the resultant secondary and subsequent tertiary colonies were analyzed after 11 or 12 days of incubation. ND, not done.

\* Myeloperoxidase staining not done.

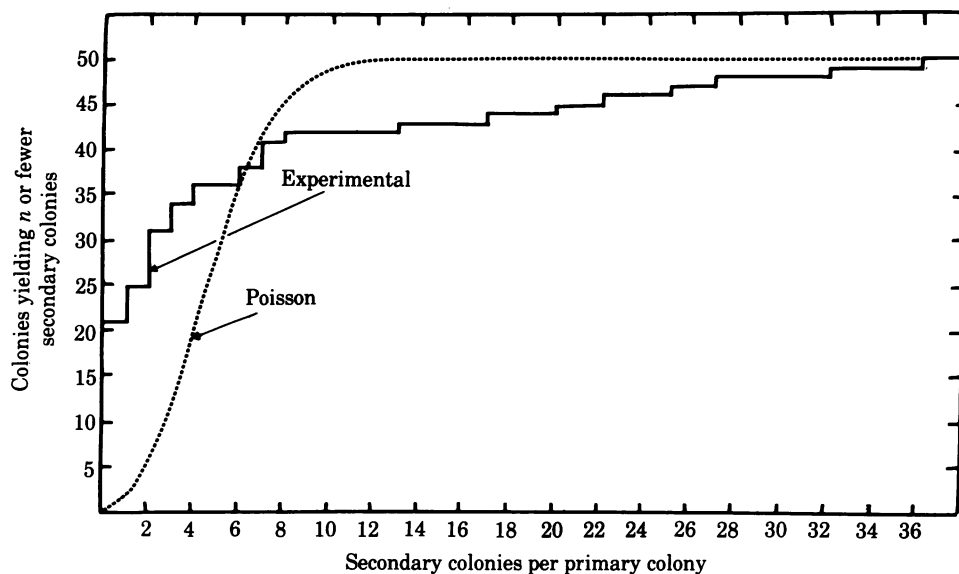


FIG. 1. Cumulative distribution of number of 9-day-old primary macroscopic erythroid colonies yielding  $n$  or fewer secondary macroscopic erythroid colonies on replating (—) and distribution expected on the basis of  $\bar{n} = 5.3$  with variation solely due to sampling error and hence described by Poisson statistics (.....). Sixty percent of the primary colony was plated in the secondary assay; total stem cells per colony is thus  $n/0.6$ . Total primary colonies = 50. SD = 9.0.

primary colony shown in Table 1 and Fig. 1.) By the third generation of colony formation, self-renewal was no longer demonstrable. Thus, of 25 tertiary colonies replated (Table 2, experiment 2), none yielded further colonies that achieved macroscopic size. A similar phenomenon for CFU-S cells proliferating *in vivo* has been widely documented (16). Whether the decrease seen *in vitro* is due to the operation of similar or different mechanisms is not known.

**Heterogeneity in Stem Cell Self-Renewal in Primary Colonies Derived from Flask-Cultured Marrow Cells.** Stem cell self-renewal in individual colonies derived from 2-week-old flask cultures was also assessed. The solid line in Fig. 1 shows

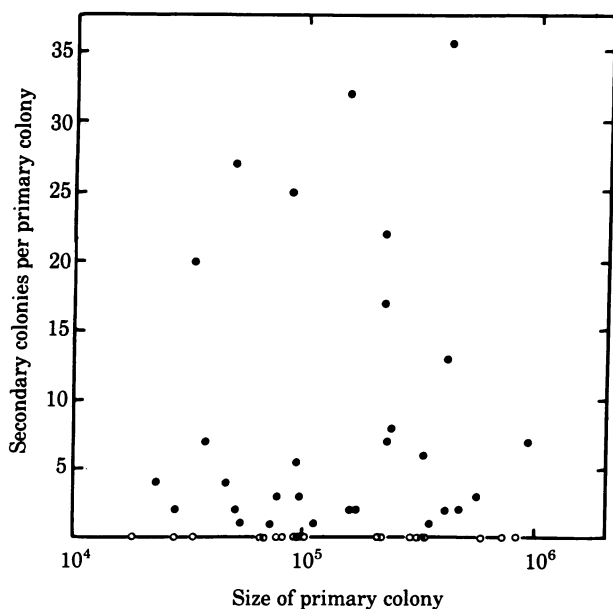


FIG. 2. Scatter plot of size (total nucleated cells per primary macroscopic erythroid colony) versus stem cell content (as represented by the yield of secondary colonies on replating). Data are for the 50 replated colonies shown in Fig. 1.  $\circ$ , zero stem cell content;  $\bullet$ , nonzero stem cell content.

the cumulative distribution of secondary macroscopic erythroid colony numbers obtained in replates of 50 primary colonies assayed concurrently. In this experiment, 60% of each individual colony suspension was replated, the remainder being used for nucleated cell counts. Twenty-nine of the 50 primary colonies yielded at least one secondary macroscopic erythroid colony. The maximum number in a single assay was 36 (or 60, had the entire primary colony been replated). Overall, a mean of 5.3 macroscopic erythroid colonies per assay was obtained—i.e., 8.8 per primary colony. This value is similar to that obtained in previous experiments (Table 1). However, in contrast to the data shown in Table 1, the SD (9.0) and coefficient of variation (1.7) for replicate colonies (rather than replicate assays) were significantly larger ( $P < 0.001$ ) (17) than those expected from sampling error alone and hence described by Poisson statistics. The cumulative distribution expected for a Poisson distribution having a mean of 5.3 is shown by the dotted line in Fig. 1. Marked departure of the experimental distribution from that predicted by Poisson statistics is clearly evident.

The relationship between the number of secondary colonies found in each of the 50 primary colonies and the size of the primary colony is shown in Fig. 2. Although the size of primary colonies varied over a 50-fold range (from  $1.8 \times 10^4$  to  $9.2 \times 10^5$  nucleated cells per colony); there was no correlation between this parameter and the extent of stem cell renewal [Spearman's rank correlation coefficient = 0.06 (17)].

Individual 9-day-old primary colonies were also assayed for CFU-S cells (12). Table 3 shows the results from two separate experiments in which a total of 146 primary colonies were examined. Marked variation was also a feature of CFU-S numbers and, again, there was no apparent correlation with primary colony size (size range for colonies containing none or one or more CFU-S were  $0.1\text{--}3.2 \times 10^5$  and  $0.3\text{--}2 \times 10^5$ , respectively).

**Self-Renewal in Primary Colonies Derived from Fresh Marrow Cells.** Preliminary experiments suggested that maximum numbers (5–10 per dish) of well-isolated macroscopic erythroid colonies were obtained in 1.1-ml cultures containing  $5 \times 10^4$  fresh marrow cells at 2.5% PWM-SCCM and erythropoietin at 1 unit/ml. A total of 49 primary colonies generated in such assays were individually replated after 9 days of growth

Table 3. CFU-S content of individual 9-day-old macroscopic erythroid colonies derived from flask-cultured marrow

Experiment	Colonies assayed	CFU-S per colony		
		Mean	SD	Range
1	69	1.7	3.0	0-16
2	77	0.9	3.1	0-18

Individual macroscopic erythroid colonies were picked and assayed for CFU-S as described in *Materials and Methods*.

(two experiments). The number of secondary macroscopic erythroid colonies obtained is shown in Table 4. In both experiments, 25-30% of the primary colonies assayed yielded secondary colonies and an overall mean value of one secondary colony per primary colony was obtained. In the more extensive experiment, the data were also sufficient to document heterogeneity in the extent of stem cell self-renewal among individual colonies. In this experiment, primary colony size was also determined; again no correlation with self-renewal was found (data not shown).

### DISCUSSION

The present studies provide direct evidence for hemopoietic stem cell self-renewal in a completely *in vitro* colony assay system. An assessment of stem cell self-renewal by replating rather than by spleen colony formation offers a significant quantitative advantage. Thus, although 9-day-old macroscopic erythroid colonies contained CFU-S cells at an incidence 5- to 10-fold higher than previously reported for colonies *in vitro* (18, 19), on average, only one spleen colony was obtained per primary colony injected (Table 3; ref. 12). In contrast, the average number of *in vitro* secondary macroscopic erythroid colonies per primary colony was  $\approx 5$ . Assessment of stem cells by replating experiments was, however, attendant on the discovery of the importance of using irradiated marrow cells in secondary assay cultures. The 5-fold difference in numbers of colonies obtained by *in vitro* and *in vivo* methods corresponds closely to the assumed seeding efficiency of CFU-S cells (3), thus accounting for the similarity in estimates of self-renewal determined by the two methods—i.e., at least two or three and up to at least five or six self-renewal divisions completed in the first 9 days of colony growth. These values are also similar to previous estimates for CFU-S cell self-renewal in 8- to 9-day-old spleen colonies (3, 5). It should be noted that macroscopic erythroid colony numbers in assays of fresh marrow account for only 10% of the total CFU-S population, a discrepancy that remains unexplained.

The well-documented phenomenon of marked variation in CFU-S content of individual spleen colonies has been generally accepted as evidence of heterogeneity in the extent of self-renewal expressed by individual stem cells proliferating *in vivo*. The mechanisms responsible for this heterogeneous behavior

Table 4. Self-renewal in individual macroscopic erythroid colonies derived from fresh marrow

Experiment	Primary colonies replated, no.	Primary colonies yielding secondary colonies, no.	Secondary colonies formed, no.		
			Mean	SD	Range
1	10	3	0.6	1.3	0-4
2	39	10	1.2	3.1	0-14

Nine-day-old macroscopic erythroid colonies from assays of fresh marrow were individually replated in the usual manner. Sixty percent of the primary colony was plated in the secondary culture.

are not understood. Several models have been proposed. One stresses the concept of intrinsic factors such that the decision to self-renew is subject to a probability  $P$  (6). A contrasting model emphasizes the deterministic role of extrinsic factors. According to this latter model, the marked variation in CFU-S content of individual spleen colonies is explained by local variations in the "instructive" capacity of splenic stromal cells (i.e., the hemopoietic inductive microenvironment). If this latter model were correct, the heterogeneity observed for stem cell production *in vivo* would not be expected to persist *in vitro*, where microenvironmental variations can be assumed to be negligible. The present studies failed to confirm this prediction; marked heterogeneity in stem cell self-renewal was clearly evident in every experiment in which *in vitro* colonies were assayed individually. Furthermore, this variation was not attributable to errors inherent in the assay procedures used. This suggests that ultimately the decision to self-renew is determined by a mechanism intrinsic to the stem cell itself and that the role of extrinsic factors in influencing the self-renewal behavior of stem cells at the single-cell level is more permissive than directive in nature.

The possibility of preexisting differences in the self-renewal capacity of stem cells forming colonies *in vitro* was also considered. Evidence suggesting that there may be a hierarchy of stem cells *in vivo* with decreasing self-renewal capacities has been obtained from a number of systems (20-25). In the present experiments, we tested the possibility that self-renewal capacity might be correlated with the size of colony assayed. The results showed no correlation between these two parameters. On the other hand, the average degree of stem cell self-renewal seen in colonies derived from fresh marrow was considerably less (a factor of  $1/5$ ) than that in assays of marrow cells from 2-week-old flask cultures. This latter finding suggests that the replating assay can detect differences in the probability for self-renewal characteristic of different stem cell populations.

Persistence of self-renewal during secondary colony formation makes it possible to look for continuing high or low self-renewal in the progeny of stem cells that exhibit high or low self-renewal capacity initially. In the serial replating studies described in Table 2, two of the colonies analyzed gave comparable numbers of secondary colonies (23 and 18). Of these, five and four, respectively, were again replated. The numbers of tertiary colonies obtained were 0, 0, 0, 0, and 1 and 0, 0, 10, and 23. These data, albeit preliminary, failed to reveal a subclass of stem cells having a consistently high self-renewal capacity. More extensive family histories on a larger number of individual stem cells offers an approach to the problem of discriminating definitively between variations in self-renewal due to inherent heterogeneity in the population of stem cells assayed and variations due to the mechanism that determines whether or not self-renewal will occur. It will also be of interest to use the type of experiment described in this paper to investigate factors that influence the probability for self-renewal. Finally, the present studies should serve as an impetus and guide to the development of analogous methodologies for human stem cells, where the assessment of stem cell self-renewal behavior will be limited, of necessity, to results obtainable from *in vitro* replating experiments.

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