Clonal and systemic analysis of long-term hematopoiesis in the mouse

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We have analyzed the temporal in vivo fate of 142 individual stem cell clones in 63 reconstituted mice. Long-term sequential analyses of the four major peripheral blood lineages, obtained from animals engrafted with genetically marked stem cells, indicate that developmental behavior is primarily a function of time. As such, the first 4–6 months post-engraftment is characterized by frequent fluctuations in stem cell proliferation and differentiation behavior. Gradually, a stable hematopoietic system emerges, dominated by a small number of totipotent clones. We demonstrate that single stem cell clones are sufficient to maintain hematopoiesis over the lifetime of an animal and suggest that mono- or oligoclonality may be a hallmark of long-term reconstituted systems. A model is proposed, wherein lineage-restricted differentiation and dramatic clonal flux are consequences of mechanisms acting on an expanding pool of totipotent cells and are not indicative of intrinsically distinct stem cell classes.

[*Key Words*: Hematopoiesis; stem cells; clonal analysis; developmental behavior]

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Hematopoiesis is a complex program of cellular differentiation that yields at least eight cell lineages in a continuous and regulated fashion (for review, see Metcalf 1988). At the center of this system is a population of stem cells endowed with the ability to self-renew, as well as to differentiate into mature cell types. The in vivo developmental and proliferative properties of such cells have been inferred by functional assays involving transplantation of marked cell populations into radiation-ablated or genetically deficient mice (for review, see Dexter and Spooncer 1987). The existence of totipotent stem cells able to clonally contribute to all mature blood cell populations has been established (Abramson et al. 1977); however, the inherent limitations of classical techniques have not permitted a definition of developmental events occurring at early times in the clonal proliferation of totipotent cells.

Other studies have defined classes of progenitor cells with limited in vitro developmental and self-renewal potential (Metcalf 1984). These cells are considered to be closely linked to mature cell populations and, as such, define the opposite end of a hematopoietic hierarchy, in which a segregation of developmental potential accompanies a decrease in self-renewal capacity (Ogawa et al. 1983). These in vitro studies have also suggested that developmental decisions of self-renewal and lineage commitment may be governed by stochastic mechanisms (Korn et al. 1973; Nakahata et al. 1982). Other studies have indicated that specific microenvironments are instrumental in determining stem or progenitor cell behavior (Trentin 1970; Dexter et al. 1976).

More recent in vivo studies have employed retroviral-mediated gene transfer to efficiently and randomly mark the entire spectrum of stem cells (Dick et al. 1985; Keller et al. 1985; Lemischka et al. 1986). The distribution of proviral markers in mature tissues identifies distinct types of stem cell developmental behavior. These include totipotent lineage contribution, as well as a variety of contributions to individual lineages or subsets of lineages. Such lineage-restricted behavior may represent intrinsically distinct classes of stem cells, the influence of the host environment, or the result of stochastic mechanisms acting on unrestricted totipotent stem cells.

Previous studies have also demonstrated that the clonal contribution of individual stem cells to mature hematopoietic tissues can change with time (Mintz et al. 1984; Lemischka et al. 1986; Snodgrass and Keller 1987; Capel et al. 1989). These fluctuations have been interpreted as a reflection of previously proposed clonal succession models of stem cell utilization (Kay 1965; Micklem et al. 1983, 1987; Mintz et al. 1984). However, because of the limited scope of these approaches and their inability to sample the system sequentially, these studies may simply reflect a system not at steady-state or particular post-reconstitution demands.

Taken together, these experimental difficulties necessitated a long-term, sequential lineage-specific analysis in deriving an accurate developmental and proliferative fate map of stem cell behavior. Consequently, in the

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present studies, we have developed a system and applied techniques to permit such an in vivo evaluation of stem cell behavior.

Results

We analyzed the behavior of 142 hematopoietic stem cells in the context of 63 recipients for periods of 4–16 months. Our basic strategy employed transplantation of retrovirally "marked" stem cells into lethally irradiated adult mice. Because of random retroviral integration properties, each virally transduced stem cell is uniquely marked. On engraftment of such marked stem cells, their developmental and proliferative behavior is measured by Southern blot analysis of the distribution and molarity of proviral markers in DNA obtained from mature hematopoietic tissues. Similarly, fluctuation in stem cell behavior can be identified by the variation of markers over time.

Temporal analysis strategy

The first step in the experimental strategy is the in vitro infection of hematopoietic tissue with recombinant retroviruses. In the following studies, adult bone marrow obtained at different stages of a post-5-fluorouracil (5-FU) regeneration period, as well as mid-gestation (day 14) fetal liver, were used as stem cell sources. A series of newly constructed retroviruses (shown in Fig. 1) was used to provide markers in most experimental animals. These are more stable (i.e., not able to move or reinfect cells in vivo following a single cycle of infection and reverse transcription) than previously employed viruses. A series of in vitro tests (see Materials and methods) demonstrated a 50- to 100-fold reduction in viral transmission subsequent to an initial infection of cells. More importantly, marker virus was not detected in the sera of 16 animals reconstituted with infected cells.

Genetically transduced (marked) cell populations were harvested and engrafted into lethally irradiated syngeneic adult recipient animals. A 20-fold range of cell inocula was used. To examine the developmental behavior and temporal dynamics of individual stem cell clones, it was necessary to sample mature hematopoietic tissues in discrete lineages at multiple times. Techniques were devised to isolate granulocytes, monocyte–macrophages, T lymphocytes, and B lymphocytes for Southern blot analysis, from a small sample of peripheral blood (150 μl = 5–10% of total blood volume; see Fig. 2). These four lineages represent >90% of the nucleated cell types present in peripheral blood (Russell and Bernstein 1966).

The overall temporal analysis strategy is as follows: Total peripheral blood was sampled initially at 4–6 weeks postengraftment. Animals judged to be reconsti-

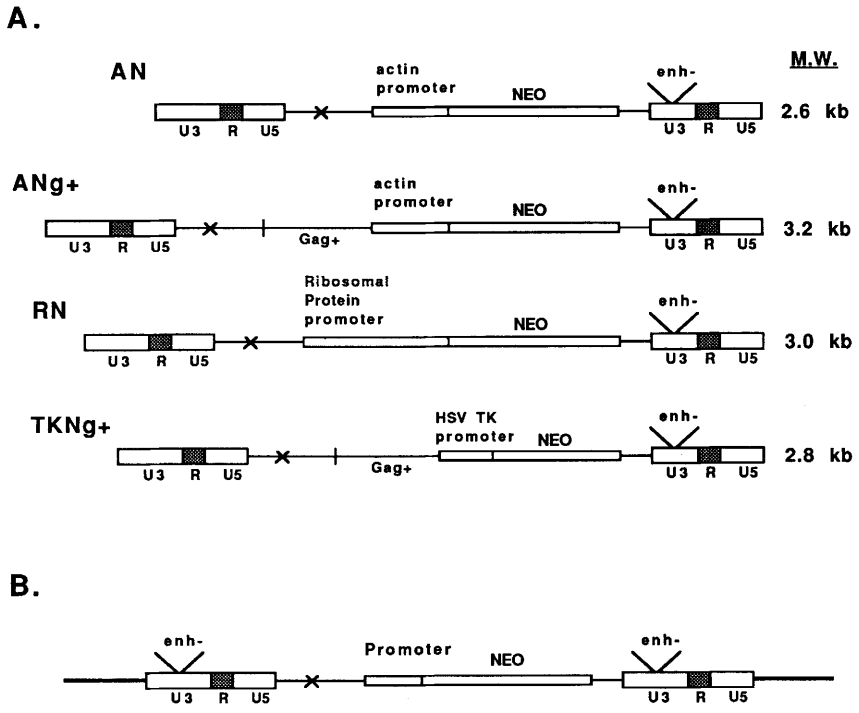
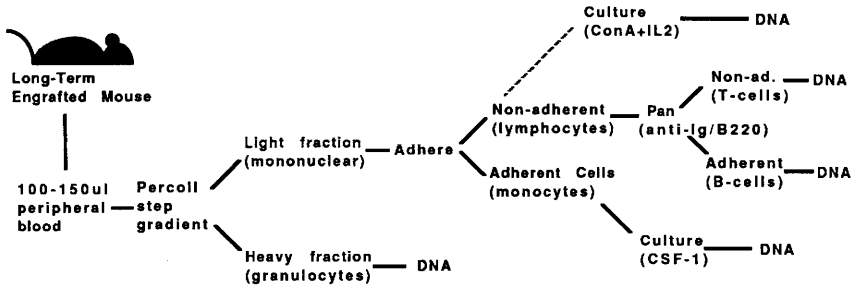


Figure 1. Retroviral markers. (*A*) The design of four retroviral marker vectors used in the current experiments. The individual viruses, labeled AN, ANg$^+$, RN, and TKNg$^+$, all share an enhancer deletion (enh$^-$) in the 3′ LTR, a selectable drug-resistance gene (neo), and a point mutation (×) that inactivates the viral splice donor. The Neo^r gene is expressed from three different internal promoters appropriately labeled. An additional feature in the ANg$^+$ and TKNg$^+$ viruses is a portion of the viral *gag* gene (Gag$^+$). The molecular weight of the unit length provirus is indicated to the *right* of each marker. (*B*) The structure of a provirus derived from the constructs in *A*. Note the duplication of the enhancer deletion to the 5′ LTR.



Lineage	Recovery	Purity
T-Cells	200,000 cells	80-90%
B-Cells	80,000 cells	80-90%
Granulocytes	100,000 cells	80-90%
Monocytes	500,000 cells	80-90%

Figure 2. Peripheral blood lineage fractionation. (*Top*) Flow chart of the fractionation strategy designed to obtain pure populations of granulocytes, monocyte–macrophages, T lymphocytes, and B lymphocytes from (150 μl) peripheral blood. Below the diagram is a table representing typical cell yields and purities.

tuted by genetically marked stem cells were subsequently examined at 6- to 8-week intervals, for periods of 4–16 months, using the lineage fractionation procedure. In the following sections, we present detailed temporal analyses of individual reconstituted systems that illustrate the major themes observed.

Long-term analysis: Stability of stem cell clones

Initially, we chose a long post-engraftment period prior to analysis because it was of primary interest to determine whether the variety of previously observed stem cell behaviors was a permanent hallmark of the hematopoietic system (Lemischka et al. 1986; Snodgrass and Keller 1987; Capel et al. 1989). Figure 3, A–D shows typical examples of mice, in which detailed temporal analyses began at 3–4.5 months post-transplantation. The animals were analyzed with respect to the number and contribution of stem cell clones (labeled NEO in Fig. 3A–D). Quantitation of each sample relative to a single-copy gene (the endogenous IL-3 gene) is shown (labeled IL3). Animal A (Fig. 3) was reconstituted with bone marrow. Peripheral blood lineages were sampled sequentially (numbers 2–6 above the lanes identify time points) for 1 year. This recipient was then sacrificed, and a variety of hematopoietic tissues were analyzed. In addition, bone marrow cells were used to reconstitute a secondary set of four irradiated recipients.

Each DNA sample is digested with restriction enzyme *DraI*, which does not cleave within the proviral marker. Thus, each band represents a unique integration site. All visible stem cell clones are totipotent, as demonstrated by identical proviral integrants in all lymphoid and myeloid tissues (Fig. 3A, NEO). Furthermore, temporal analysis demonstrates no change of the stem cell clones (stability) observed in this animal. In addition, the myeloid and lymphoid blood tissues from the four secondary recipients display the identical clonal composition. As shown by the numerical values below the panel in Figure 3A, most, if not all, of the mature cells derive from the transduced stem cell clone(s). Thus, at most, two clones or possibly even one clone possesses the proliferative and developmental ability to permanently and continuously support the hematopoietic system.

The mouse analyzed in Figure 3B is an example of a bone-marrow-reconstituted animal in which a single stem cell clone is contributing to virtually all of the hematopoietic tissue. In addition, mouse B was also analyzed for host contribution to the peripheral blood. This was facilitated by transplanting female bone marrow into males and measuring the male-specific contribution to the cells with a Y-chromosome-specific probe (described previously in Lemischka et al. 1986). As shown by the numbers below each lane in Figure 3B, the maximum host contribution is 20%. This corroborates the single-copy gene (IL-3) quantitation data, which demonstrate that a single marked clone gives rise to the bulk of each tissue. We have observed cells with such remarkable reconstituting ability at approximately equal frequencies in all post-5-FU bone marrow samples assayed (~15%).

Animals C and D (Fig. 3) were reconstituted with fetal liver cells. A small number of totipotent clones contributes stably to 50–100% of each tissue sample. Despite the fetal origin of these cells, their behavior in the context of an adult animal appears very similar to the behavior of adult bone-marrow-derived counterparts. The only type of clonal instability detected in these animals is seen in Figure 3D, lane M4. The retroviral copy number decreased ~50% in monocyte–macrophages be-

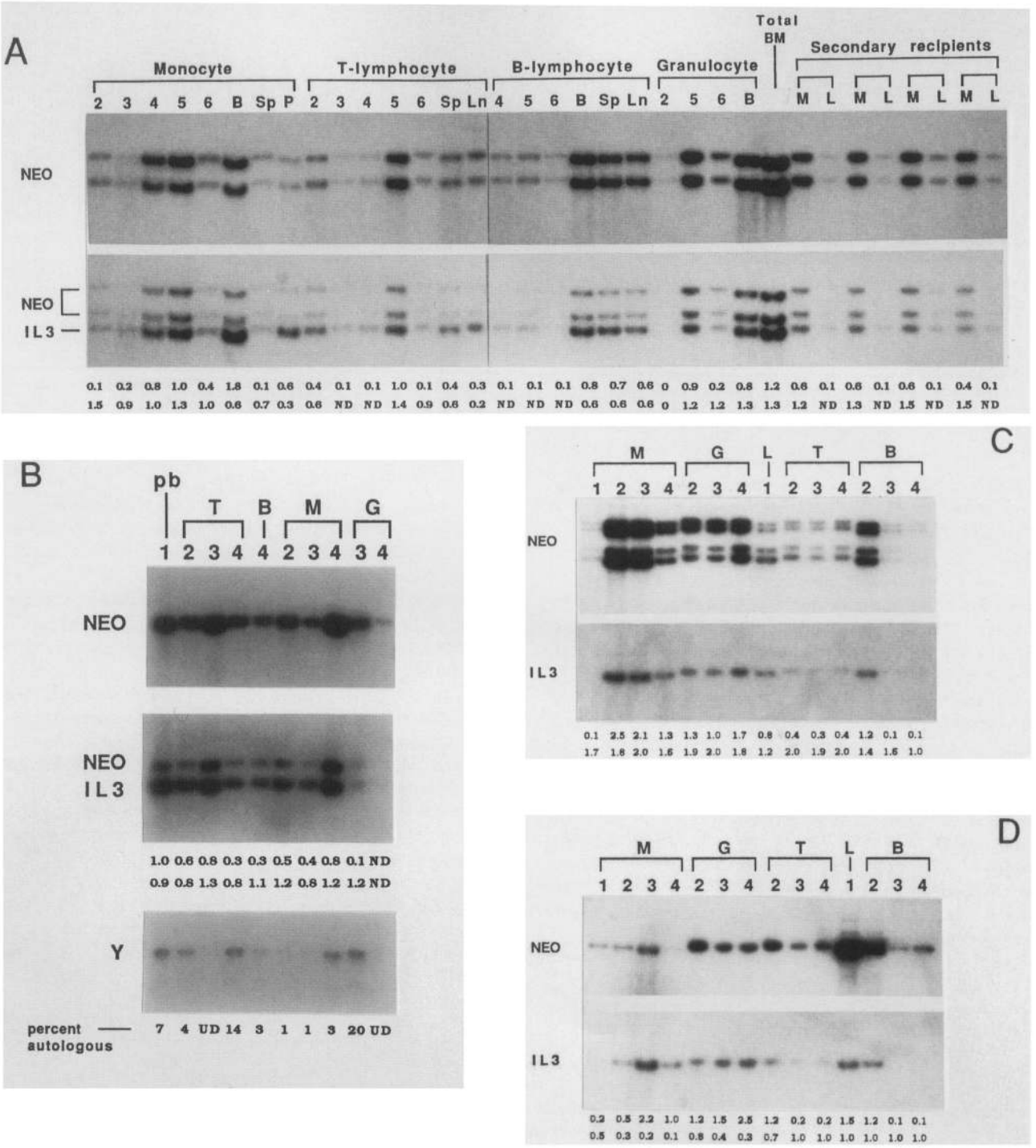


Figure 3. (*See following page for legend.*)

tween time points 3 and 4 (Fig. 3D). Interestingly, the proviral integrant and, thus, the stem cell clone remained stable in the other three lineages.

Short-term analysis: fluctuation of stem cell clones

In an effort to gain insight into stem cell clonal behavior at early post-reconstitution intervals, we initiated the temporal analysis at earlier times after engraftment (1–2 months). Stem cell behavior during this interval was substantially different from that described above. Representative examples are shown in Figure 4, which presents the Southern blots from three bone-marrow-reconstituted (I–III) and three fetal-liver-reconstituted mice (IV–VI). In addition to totipotent clones, instances

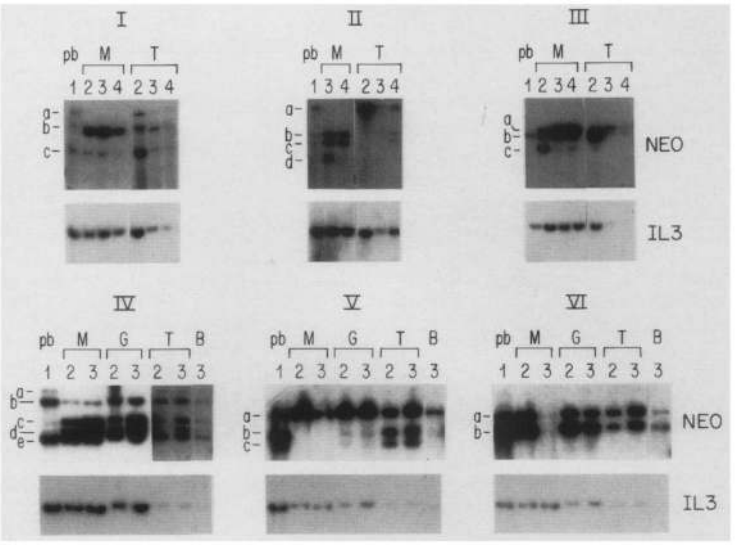


Figure 4. Short-term clonal fluctuation. (*I–III*) Temporal analysis of bone-marrow-reconstituted recipients, in which the first four time points are shown. (pb) Unfractionated peripheral blood; (M) monocytes; and (T) T lymphocytes. Numbers above each lane designate the temporal time point. Individual clones (integrants) are designated *a–d* at *left* of each Southern blot. Below NEO is the same blot, reprobed with IL-3 for quantitation. (*IV–VI*) Similar analyses of fetal-liver-reconstituted animals, in which the first three time points are shown. Abbreviations are the same as above, with the addition of B lymphocytes (B) and granulocytes (G). In *IV*, a longer exposure of lanes T2, T3, and B3 is spliced into NEO. Temporal sampling points for *I*: (*1*; pb) 1.25 months; (*2*) 4.5 months; (*3*) 6 months; (*4*) 7.25 months. For *II–VI*: (*1*; pb) 1–1.75 months; (*2*) 2.25–2.75 months; (*3*) 3.75–4.25 months; (*4*) 5–5.5 months.

of myeloid- and lymphoid-restricted clonal contribution were observed, for example, clone a (time points 3 and 4) for animal II and clones b and c (time points 2 and 3) for animal V (Fig. 4). These clones are largely confined to the T-cell lineage and represent the most common lineage-restricted behavior that we have observed. Less frequently, myeloid (macrophage)-restricted contribution was observed, as represented in animal II, clone d, and in animal III, clone c (Fig. 4).

Multiple examples of clonal instability were also observed in this short post-engraftment interval. One frequently observed phenomenon was the loss of a clone between the first and second time points. We refer to this type of rapid reduction in stem cell activity as clonal extinction. The vast majority of such extinction events occur at relatively early post-engraftment times. Lymphoid- and myeloid-restricted integrants (clones) also display gradual and rapid extinction behavior, respectively (Fig. 4, cf. animal II, clone a, between time points 2 and 4, 3–4 month interval; cf. II, clone d, and III, clone c, between time points 2 and 3, 1.5–2 month interval). Finally, as noted previously in Figure 3, we find examples of a monocyte-specific reduction/extinction (mouse VI, cf. time points 2 and 3; Fig. 4). As before, the marked stem cell contribution to the other three lineages remains stable.

A novel theme observed in these studies is a reduction/extinction of some clones accompanied by an increase in other clones. We term this behavior clonal equilibration. Clones b–d in mouse IV are an example of this type of phenomenon (Fig. 4). Clone b diminishes as the contribution of clones c and d increases. Moreover, it can be seen that the relative increase in clones c and d is first evident in myeloid tissues (time point 2) and at the subsequent time point in lymphoid tissue (time point 3). Similarly in animal III, clone c undergoes a large reduction in contribution between the first and second fractionation points, with a concurrent increase in clone a (Fig. 4; more pronounced initially in monocytes). The above examples of lineage restriction and clonal decreases or increases are most readily detected during early post-reconstitution intervals.

Figure 3. Long-term clonal behavior of engrafted hematopoietic stem cells. Southern blot analysis of four representative animals (*A–D*) that were monitored for at least 10 months. For each, there is an analysis of retroviral marker content (NEO probe), as well as data for an autosomal marker (IL3). In each case, the DNA samples were cleaved with restriction enzyme *Dra*I, which does not cut in the proviral marker; therefore, each band represents a unique integration event. The samples are grouped by lineage, as follows, for blood tissues: (pb) peripheral blood; (T) T lymphocytes; (B) B lymphocytes; (M) monocytes; (G) granulocytes; and (L) lymphocytes (i.e., both T and B). The numbers above the lanes indicate temporal time points. Below each lane are two sets of numbers. The *top* row is the relative DNA content, the *bottom* row is the estimated retroviral copy number (see Materials and methods). (*A*) Mouse reconstituted with 8.5×10^5 bone marrow cells from a donor treated 6 days pre-sacrifice with 5-FU. Temporal analysis via peripheral blood sampling was performed for 14 months. Subsequently, the mouse was sacrificed to obtain hematopoietic tissues and to generate secondary hosts (see Materials and methods). Tissue designations are (B) bone marrow, (Sp) spleen, (P) peritoneum, and (Ln) lymph node. Samples from secondary recipients are peripheral blood, fractionated for myeloid (M) and lymphoid (L) tissue. (ND) No data. Numbers above temporal sample lanes correspond to the following times post-engraftment: (*1*) 3.5 months [*B*, *C*, and *D* (data not shown)]; (*2*) 6.5 months; (*3*) 8 months; (*4*) 9.75 months; (*5*) 11.75 months; (*6*) 13.75 months. (*B*) Mouse reconstituted with bone marrow (3.6×10^5 cells from a day-6 5-FU donor) and temporally monitored by peripheral blood analysis. In addition to NEO and IL-3 data, analysis for autologous contribution, using a Y chromosome-specific probe is also shown. Below each lane of the Y probe is the percentage of total tissue derived from autologous tissue. (UD) Undetectable. Numbers above temporal sample lanes correspond to the following times post-engraftment: (*1*; pb) 4.25 months; (*2*) 7.25 months; (*3*) 8.75 months; (*4*) 1.25 months. (*C* and *D*) Two animals reconstituted with fetal liver tissue and temporally analyzed as in *A* and *B*. The animal represented in *C* received 2.5×10^6 cells, and animal D received 4.1×10^6 cells. Temporal samplings are 2.75 months (*1*), 5 months (*2*), 7 months (*3*), and 8.75 months (*4*).

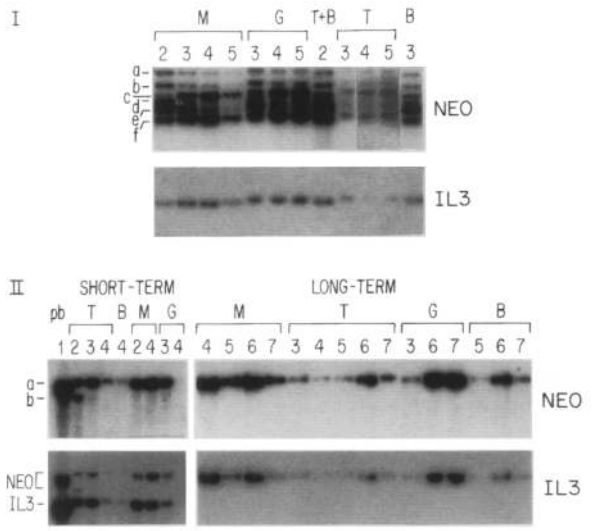


Figure 5. Clonal equilibration. (*I*) Temporal analysis of a fetal-liver-reconstituted mouse. Lanes T4 and T5 in NEO are spliced to show a longer exposure. Abbreviations are as in Fig. 4. Temporal sampling points are 1 month (*1*) [*II* (data not shown)], 2.5 months (*2*), 4.5 months (*3*), 6.5 months (*4*), and 8.75 months (*5*). (*II*) Short- and long-term temporal analysis of a bone-marrow-reconstituted mouse. Each NEO is aligned such that clone *a* is juxtaposed. The short-term quantitation data are represented as a combined NEO and IL-3 analysis, whereas the long-term quantitation data are the IL-3 analysis alone. Abbreviations are the same as in Fig. 4. Temporal sampling points are (*1*) 1.5 months (*1; pb*), 2.5 months (*2*), 4 months (*3*), 5.75 months (*4*), 7 months (*5*), 9.5 months (*6*), and 11.5 months (*7*). The band representative of the IL-3 gene is not aligned between the short- and long-term groups.

Short- and long-term analysis: clonal equilibration as a function of time

Figures 3 and 4 show examples of stem cell contributions to hematopoietic lineages after relatively long (>4 months) and short (1–5.5 months) times. These analyses demonstrate two dramatically different classes of stem cell behavior and suggest that clonal stability occurs over time, with a small number of initially fluctuating totipotent clones eventually dominating and remaining stable. To test this hypothesis directly, stem cell clones were examined in the same animals at both early and late times post-engraftment. Figure 5 shows the Southern blots from two animals that were sampled beginning at 4–5 weeks post-engraftment. The sampling period was then continued sequentially for 9–12 months.

Animal I (Fig. 5) displays a relatively complex spectrum of totipotent stem cell clones. In examining the two earliest time points (numbers 2 and 3), fluctuations in stem cell contribution were observed as before. The relative intensity of clones d and e decreases substantially, whereas that of clone c (and, to a lesser extent, clone f) increases. Subsequent time points then appear to be stable, with clones c and f as the dominant totipotent clones (Fig. 5, numbers 4 and 5).

In animal II (Fig. 5), early after engraftment (1.5 months), there are two clones (a and b) present in unfractionated blood; however, by the second time point (6 weeks later), clone b is visible only in T lymphocytes. At subsequent sampling points, clone b drops permanently below detectable levels. Conversely, clone a in animal II is totipotent and present at stable levels for all time points assayed (numbers 1–7; 1.5–11.5 months). As in previous examples, (Fig. 3), the mature progeny derived from totipotent stable clones (Fig.5, I, c and f; II, a) constitute ~50–100% of the total tissue in each sample (numerical data not shown). Also noteworthy in animals I and II (Fig. 5) is a reduction over time in the number of clones that function in a given animal. This is accompanied by the gradual emergence or continued stability of a small number of clones that continue to function for lifelong time periods.

Table 1 summarizes the types of clonal behavior observed and the frequency with which each event was detected. These data are divided according to (1) the source of stem cells (i.e., bone marrow or fetal liver donor tissue), and (2) their behavioral category. Each event is representative of an individual stem cell clone and is based on a minimum of three time points. The single most prevalent category (stable behavior: totipotent equal, 27.5% overall) consists of clones that contribute to an equal fraction of all lineages in a stable fashion (over 4–16 months). A spectrum of overall contribution, ranging from 5% or 10% to nearly 100% of total tissue was observed.

We also note stable clones (totipotent disparate) that contribute at unequal levels to different lineages. Most clones in this category (L > M) preferentially contribute to lymphoid tissue (8.5% overall). Less frequent (2% overall) are totipotent clones that contribute preferentially to myeloid tissue (M > L). The totipotent short-term category includes those clones that have been monitored for only two time points, and these data demonstrate that totipotent clones may also assume stable developmental behavior beginning at early post-engraftment times. Collectively, 52% of all stem cell clones analyzed contribute in a stable fashion to the mature cell populations.

Unstable behavior in Table 1 is defined as increases or decreases in the contribution of individual stem cell clones over two or more time points. A coordinate lymphoid and myeloid decrease in the contribution of a totipotent clone was observed in 2% of the analyzed clones. Much more frequent were decreases in either the myeloid or lymphoid lineages (20.5% of the time). A totipotent clone was observed to decrease specifically in myeloid contribution with a frequency of 15.5%. Clonal decrease in lymphoid cell types with concurrent stability in myeloid cells was not observed (Table 1). All lymphoid-specific decreases occurred in stem cell clones that were initially detected only in lymphoid cell types. Collectively, unstable behavior was detected in 48% of the clones assayed. Decreases in contribution of a stem cell to a lineage over time occurred in 33% of clones, whereas increases are observed in 15% of the unstable clones. Interestingly, the frequency of coordinate lym-

Table 1. *Dynamic behavior of individual stem cell clones*

	Bone marrow		Fetal liver		Total	
	clones	frequency (%)	clones	frequency (%)	clones	frequency (%)
Stable behavior						
totipotent equal	21	35.0	18	22.0	39	27.5
totipotent disparate						
L > M	6	10.0	6	7.5	12	8.5
M > L	1	1.5	2	2.5	3	2.0
totipotent short-term	NA	NA	20	24.5	20	14.0
Unstable behavior						
decrease: totipotent (coordinate) L + M	2	3.5	1	1.0	3	2.0
totipotent (myeloid) L + M → L + m	7	11.5	15	18.5	22	15.5
(lymphoid) L → 1	7	11.5	0	0.0	7	5.0
total to F1	5	8.5	10	12.0	15	10.5
Increase: totipotent (coordinate) L + M	9	15.0	7	8.5	16	11.5
myeloid to totipotent (M → M + L)	2	3.5	3	3.5	5	3.5
Total	60 (28 mice)		82 (35 mice)		142	

The frequencies of different types of behavior detected for individual stem cell clones are presented with respect to bone marrow or fetal liver tissue source. For example, of all bone-marrow-derived clones, 21 (35%) display stable behavior. Also presented is overall frequency of clones in a particular category. Each type of behavior was scored by examining at least three temporal time points. Stable behavior denotes clones whose quantitative contribution does not change over time. The totipotent equal category represents clones that contribute to all lineages, and the extent of contribution to each lineage is equal. The totipotent disparate category describes clones that contribute to different lineages stably but at quantitatively different levels [either lymphoid (L) > myeloid (M), or M > L]. Totipotent short-term is a special category representing animals that have only been temporally sampled twice; however, the clones scored are totipotent and stable out to 10–12 weeks post-engraftment. (NA) Not applicable. Unstable behavior is divided according to temporal increase or decrease. A totipotent coordinate L + M fluctuation is one observed to occur simultaneously in lymphoid and myeloid cell populations. A totipotent myeloid L + M → L + m decrease denotes diminishment of a totipotent clone in myeloid contribution with stability in lymphoid contribution. The lymphoid (L → 1) decrease category represents clones that initially appear restricted to the T-cell lineage and diminish over time. The category of myeloid to totipotent increase (M → M + L) defines clones initially visible only in myeloid cell types and that subsequently enhance their contribution to include lymphocytes.

phoid–myeloid increase in the contribution of a totipotent clone is nearly six times greater than the frequency of coordinate decrease.

Figure 6 documents the basic theme of temporal stabilization/equilibration of clonal activity, displaying the relative contributory activity of all stem cell clones (monitored for >5 months) that were observed to undergo temporal fluctuation (not included are clones that extinguish completely). Instability was observed to occur predominantly during the first 4–6 months post-engraftment. After 5 months, continued fluctuation was observed in only 27.5% of the clones monitored. After 6.5 months, this frequency decreased to 7%. In addition, only a single case of fluctuation, initiated after 1–2 months post-engraftment, was observed (see clone marked with asterisk).

Taken together, the data summarized in Table 1 and Figure 6 demonstrate an early post-engraftment time of fluctuation in stem cell utilization, followed by long-term stability.

Discussion

These studies represent the first continuous analysis of hematopoietic clonal dynamics. To facilitate these analyses, we modified previous protocols, using retroviruses to mark stem cells in four critical ways. First, because proviral integrants reflect stem cell behavior only if the marker virus is stable (unable to reinfect cells in vivo), a new set of immobile viruses was used to permit accurate clonal studies. Second, different adult and fetal stem cell sources were engrafted into adult recipients to gain possible insight into the relative importance of stem cell intrinsic, as well as environmental, properties in the ultimate behavior of the hematopoietic system. Third, a range of cell doses was used to avoid a limiting-dilution engraftment of stem cells. Fourth, and key to the present studies, was the development of a substantially more benign strategy for temporal analysis than previously employed invasive techniques such as partial splenectomy (Snodgrass and Keller 1987). Aside from the extensive

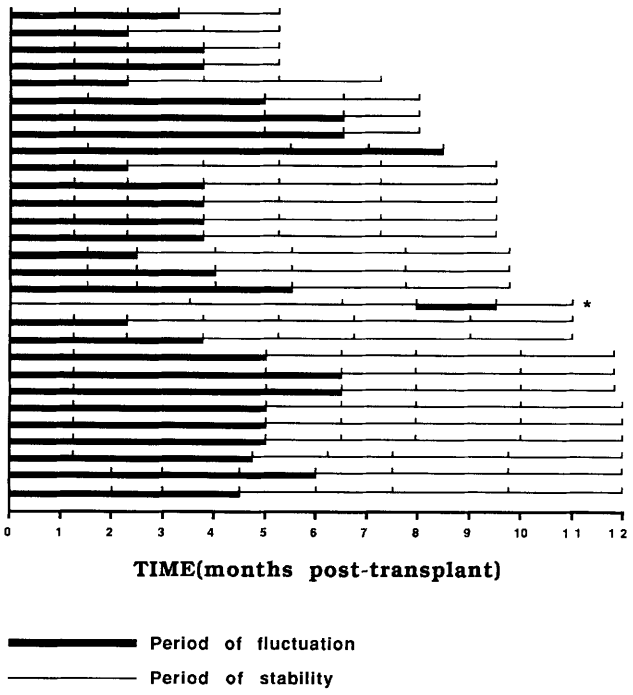


Figure 6. Temporal dynamics of stem cell fluctuation. Stem cell clones were analyzed with respect to their contributory activity as a function of time. Each horizontal line represents the behavior of an individual stem cell clone, where the thick regions indicate periods of fluctuation (either increasing or decreasing) and the thin regions indicate periods of stable contribution (assayed by quantitation of Southern blot data). The short vertical marks along the top of each line designate the sampling points for each clone. The overall length of each line indicates the total period of analysis (months). The zero time point indicates the time of engraftment. Fluctuation in clonal contribution was defined as a change of ≥20% (increase or decrease) as a function of time. Intervals of flux were defined as the earliest and latest points at which variation was evident.

hematopoietic stress likely to be induced by major surgical procedures, evidence exists for the seeding of spleen and thymus with highly clonogenic precursor cells (Till and McCulloch 1961; Ezine et al. 1984). Therefore, analysis of a particular organ segment may not be indicative of overall clonal composition. Because of its circulating nature, peripheral blood should have no geographical bias with respect to clonality. Moreover, due to their short peripheral half-lives (1–2 days) (van Furth and Cohn 1968; Erslev and Gabuzda 1985), the myeloid subpopulations are a particularly good indicator for the proliferative activity of individual stem cell clones.

Permanent hematopoiesis is often derived from a small number of stable totipotent stem cell clones

When viewed from 4 to 16 months post-reconstitution, the predominant hallmarks of the blood system are stability and simplicity, in that hematopoiesis often derives entirely from a small number of continuously functioning totipotent stem cell clones. No compelling evidence for continuously functioning nontotipotent stem cell clones was found. Stable long-term lineage-restricted contribution was limited to lymphoid populations. Because of the long half-life of mature T-cell subpopulations (Freitas et al. 1986), such lineage-restricted contribution need not be indicative of a self-renewing T-restricted stem cell. Similar lineage-restricted contribution to short-lived myeloid cell lineages was not observed; thus, it appears that reconstituted hematopoietic systems are typically derived from few totipotent stem cell clones. In addition, because identical clonal behavior was characteristic of all stem cell sources, the prevalent determinant of stem cell function may be the host environment. This may reflect a saturable and small number of microenvironmental niches capable of supporting the proliferation and differentiation of such cells. Furthermore, our data suggest that a single totipotent stem cell in an initially engrafted population is sufficient for permanent blood formation. Taken together with previous limiting-dilution studies (Boggs et al. 1982; Harrison et al. 1988), these data indicate that reconstitution should theoretically be possible with single physically isolated stem cells (Visser et al. 1984; Spangrude et al. 1988).

Clonal progeny of totipotent stem cells can represent from several percent to 100% of every lineage. Given the range with which a totipotent clone can contribute to mature tissue, stem cell activity may be viewed as a quantitative continuum and not as an absolute quiescent versus active state. The observation made here of a novel transiently contributing clone after long post-engraftment times (see Fig. 6) directly demonstrates the presence of minimally active or dormant stem cells. It is therefore likely that clones exist whose progeny represent minimal (undetectable fractions) numbers of peripheral blood cells. Thus, permanently reconstituted hematopoietic systems do not generally utilize the entire engrafted population of stem cells, either at single points in time or in a sequential fashion over long time periods.

Permanent hematopoietic stability is frequently preceded by a period of clonal fluctuation

In contrast to the present experiments, previous studies have described discrete classes of stem cells and have suggested that the initially engrafted stem cells are sequentially utilized (Lemischka et al. 1986; Snodgrass and Keller 1987; Capel et al. 1989). In the studies presented here, similar examples of restriction, disparate lineage contribution, and clonal fluctuation were observed. However, these phenomena were nearly always transient and occur, almost exclusively, in the first 4–6 months following engraftment. Thus, a return to normal levels of mature hematopoietic tissue (4 weeks post-transplant; van Bekkum and de Vries 1967) is not indicative of stability at the stem cell level.

The observed increases or decreases in clonal contribution display a range of kinetic and lineage-specific differences (see Table 1). Clones observed to extinguish

completely and rapidly (within 6–8 weeks) may represent transiently functioning totipotent stem cell clones or myeloid-restricted cells, whose short-lived progeny would not be detectable at later times. We have observed initially totipotent clones, whose progeny, at subsequent time points, are only visible in the T-cell population. Over time, such T-restricted contribution diminishes further, consistent with a gradual turnover of a mature long-lived cell population. The converse situation of initially totipotent and subsequently myeloid-restricted clones is not observed.

An intriguing case of extinction of stem cell activity is specific to the macrophage lineage. This phenomenon suggests that in vivo, the regulation of this lineage can be independent of the granulocyte lineage and may therefore involve monopotential intermediates and not bipotential progenitors such as CFU-GM (Metcalf 1984). Granulocyte-specific extinction is not observed nor are there obvious examples of extinction specific to the lymphoid cell populations accompanied by persistence of the given stem cell clone in the myeloid lineages. However, a coordinate decrease in both myeloid cell populations has been observed. Taken together, these observations suggest that in vivo developmental decisions by a totipotent clone may progress in a relatively ordered fashion. The initial loss of macrophage contributory behavior may be the earliest indicator for the extinguishment of a totipotent stem cell clone. Given that the contribution of a totipotent stem cell clone to discrete cell lineages proceeds through an intermediate class(es) of committed progenitors or restricted stem cells, the rapid disappearance of stem cell progeny in the macrophage lineage directly suggests that such restricted stem cells are not capable of extensive self-renewal.

Hematopoietic stability is the result of an emergence and dominance of few stem cell clones

An additional class of fluctuation behavior observed at early times post-engraftment is clonal enhancement (increases). The identification of clones contributing to major cell populations only after 2.5–3 months following engraftment demonstrates that a stem cell can remain functionally dormant and that not all stem cells assume major hematopoietic roles in a concurrent fashion. In fact, the stem cell clones whose contributory activity is relatively slow in manifestation are frequently destined to remain stable over long time periods. Stem cell clones that expand their lineage contribution from one lineage to additional lineages have also been observed. Such clones contribute first to myeloid tissues and subsequently to both myeloid and lymphoid tissues (Table 1).

Collectively, the above observations indicate that clonal fluctuation is a reflection of hematopoietic disequilibrium. Our data indicate that disequilibrium is generally evident for the first 4–6 months post-engraftment. At later times, the system appears to achieve a dynamic steady state, and very little fluctuation (i.e., changes in clonal stem cell contribution) is observed. Therefore, variance in clonal behavior is a function of time and, thus, may depend more on systemic equilibrium than on intrinsically different classes of stem cells. In some cases, an additional characteristic is the emergence of a small number of clones that ultimately dominate the clonal profile. The outgrowth of dominant clones appears to occur simultaneously with the extinction of lesser clones. Thus, systemic equilibration may involve the gradual selection of a subset of engrafted totipotent clones.

A model for the temporal behavior of engrafted totipotent stem cell clones and the reconstituted hematopoietic system

By definition, the clonal proliferation of uncommitted stem cells is accompanied by self-renewal or commitment decisions. Analysis of mature hematopoietic tissues at single points in time can only measure stem cell commitment; however, self-renewal activity can be revealed by the temporal behavior of a given stem cell clone.

Discrete clonal markers can only be detected in substantially large fractions of a cell population; therefore, the mechanisms that mediate either clonal stability or shifts are likely to function directly on highly clonogenic cells. We have presented evidence that the vast majority of clones in our analyses are totipotent; thus, an attractive hypothesis is that these cells are uncommitted, that is, can self-renew. The temporal segregation of unstable and stable clonal behavior is consistent with the action of stochastic commitment versus self-renewal mechanisms on an expanding pool (clone) of target stem cells. The temporal behavior of stem cells can be interpreted in the context of a simple model, presented in Figure 7. Stable behavior would be a reflection of a relatively large pool of clonally related stem cells and, therefore, a significant amount of prior self-renewal (Fig. 7A). Stem cells undergoing an initial period of extensive self-renewal thus appear minimal in lineage-repopulating activity during this interval. However, such expanded (self-renewed) stem cell clones would subsequently dominate long-term hematopoiesis. Because clonal expansion is exponential, a dominant (large) clone of stem cells could result from a subtle initial proliferative or self-renewal advantage. Extinction, as well as lineage-restricted behavior of particular stem cell clones, would only be observed when the target cell population was small enough so that all members of a given clone could be statistically expected to either self-renew or commit to a program of differentiation (Fig. 7C). A numerically small clone of stem cells would also account for disparity, enhancement, or fluctuations in lineage contribution. Dramatic disparity observed at short post-reconstitution times would be a simple consequence of small numbers of clonally related uncommitted stem cells upon which mechanisms governing developmental decisions operate (Fig. 7B). A gradual clonal expansion of such cells (during the first 4–6 months postengraftment)

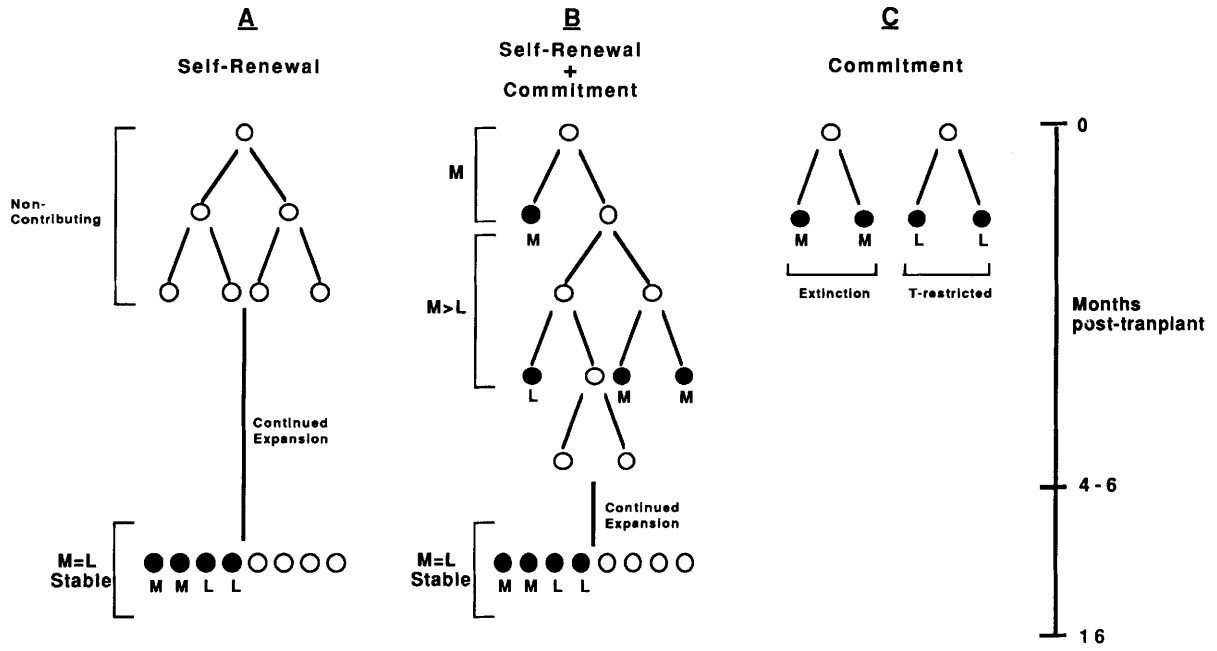


Figure 7. A model of engrafted totipotent stem cell behavior. The three general categories of clonal behavior are illustrated: (*A*) stable contribution as a consequence of self-renewal; (*B*) disparate/fluctuating contribution as a result of self-renewal and commitment; (*C*) decreasing/restricted contribution as a result of commitment. (○)Totipotent or uncommitted stem cells; (●) committed progenitors. (M) Myeloid committed; (L) lymphoid committed. Committed progenitors are not self-renewing; however, they are likely to be highly clonogenic. At *left* or beneath each category (in brackets) are the classes of observed behavior; at *right* is the time post-engraftment. The designations are the same as in Table 1. (*A*) Continuous stable contribution is a consequence of an initial period of self-renewal (designated by an expansion of ○) shortly after engraftment. Such clones would appear to be noncontributing during this time (prior to our first sampling point). Subsequent stochastic commitment of stem cells from an expanded pool of uncommitted cells gives rise to equal and stable lineage contribution. (*B*) Unequal or fluctuating contribution of a stem cell clone is observed in situations where commitment decisions occur in parallel with significant self-renewal. As discussed in the text, such clones would initially display restricted (M only) or disparate (M > L) contribution. Gradual expansion of the uncommitted pool of stem cells results in an equilibration of contribution during the first 4–6 months postengraftment. (*C*) Decreasing or restricted contribution occurs in situations where either stochastic mechanisms or the demands of a radiation-ablated system result in stem cell commitment, without significant self-renewal. Under these circumstances, stem cell commitment to the myeloid lineage yields a decrease/extinction of a given clone because of a short myeloid cell half-life. Commitment along both myeloid and lymphoid lineages, or the lymphoid lineage alone, yields a persistence of lymphoid cell types due to the long-lived nature of some T lymphocytes. Additional details of these phenomena are discussed in the text.

results in the homogenization of apparent contribution, assuming a stochastic commitment process. Interestingly, the occurrence of more subtle disparities at much longer times suggests that the steady-state pool of highly clonogenic cells is not exceedingly large.

In summary, these studies have defined the temporal behavior of individual stem cell clones and, more importantly, present both short and long-term perspectives of systemic hematopoietic clonal dynamics. The general theme of clonal fluctuation evolving into stability permits a re-evaluation of many previous studies and a more rational design of future experiments. We argue that primitive totipotent stem cell behavior is governed by a stochastic mechanism; however, the readout of such mechanisms may depend more on temporally distinct systemic demands and/or on an ordered progression of exact commitment decisions. Therefore, a fate map of stem cell commitment is only valid when viewed kinetically. Similarly, clonal succession models of stem cell utilization must be viewed in the context of our current data, which suggest that hematopoietic equilibrium is reached by a selective expansion of few initially engrafted stem cells. Thus, a means to introduce unique markers into individual members of stem cell clones during the period of in vivo equilibration/proliferation is necessary.

Materials and methods

Cells and viruses

The viruses were derived from either ψ2 or ψCRE packaging cell lines by standard procedures (Mann et al. 1983; Danos and Mulligan 1988). The viral titers were typically 1×10^6 NEO^r CFU/ml. Producer lines were maintained in α minimal essential medium (MEM), with 10% calf serum (Hyclone) and penicillin/streptomycin. All viruses carry a deletion that spans the Moloney murine leukemia virus (MoMuLV) *Pvu*II to *Xba*I sites in the U3 region of the 3′ long terminal repeat LTR; (Cone et al. 1987). During reverse transcription, this enhancer deletion is duplicated to the 5′ LTR, thereby rendering the virus defective for the synthesis of genomic RNA. Viruses designated *gag*$^+$

contain the 5′ portion of the viral *gag* gene (gift of D. Miller). This region has been shown to increase retroviral titer 5- to 10-fold (Bender et al. 1987). In addition, all viruses carry the neomycin phosphotransferase gene, driven by an internal promoter. The AN and ANg$^+$ viruses use the chicken β-actin promoter (Seiler-Tuyns et al. 1984), whereas the TKNg$^+$ virus employs the herpes simplex virus (HSV) thymidine kinase promoter, and the RN virus uses the mouse L32 ribosomal protein promoter (Dudov and Perry 1984). In each case, the promoters are derived from constitutively expressed housekeeping genes. Supernatants from virus producer cells, as well as blood samples from reconstituted animals, were assayed for the transmission of both NEO^r virus and transferable helper virus function. NEO^r virus was assayed using standard infection of NIH-3T3 cells to quantitate virus content. Helper function was tested by infection of the 3T3-116 indicator cell line (Danos and Mulligan 1988), followed by quantitation of mobilized virus carrying the histidinol dehydrogenase (HisD) gene (Hartman and Mulligan 1988). The presence of HisD virus was assayed by infection of NIH-3T3 cells and selection for resistance to L-histidinol (4 mM, Sigma). In addition to selecting for HIS^r, both the infected 3T3-116 cells and the subsequently infected NIH-3T3 cells (i.e., the target cells for infection with supernatant from the 116 cells) were also selected for NEO^r. Because helper activity varied no more than twofold between different virus producer lines, differences in the quantity of NEO^r virus detected in 116 supernatants provides a measure of the efficacy of the enhancer deletion, when compared to an intact LTR virus. For our enhancer-deleted producer lines, we observed a 50- to 100-fold decrease in the quantity of NEO^r virus present in 116 supernatant, in comparison to the intact LTR murine sarcoma virus (MSV DHFR Neo) described previously (Williams et al. 1984).

To test blood samples for helper activity, 100 μl of serum was used to infect 2×10^4 to 4×10^4 3T3-116 cells by standard procedure. Forty-eight hours later, supernatant was harvested and used to infect naive NIH-3T3 cells. These cells were then cultured for an additional 48 hr, followed by selection in L-histidinol. In addition, supernatants from 3T3-116 cells were used to infect ψCRE cells and ψCRIP cells (an amphotropic packaging line) to amplify initially released HisD indicator virus. Forty-eight hours later, supernatants from the ψCRE and ψCRIP infections were used to infect naive packaging cells of the opposite host range (i.e., ψCRE supernatants onto ψCRIP cells and ψCRIP supernatants onto ψCRE cells), to further amplify HisD mobilization (Danos and Mulligan 1988). Finally, after an additional 48 hr, supernatants from secondary ψCRE- and ψCRIP-infected cells were used to infect NIH-3T3 cells, and these were subsequently selected for the presence of the HisD indicator virus. Of 16 serum samples tested, only 1 had any detectable helper activity, as assayed by HisD mobilization. Nonetheless, no sera, including the sample in which helper was evident, showed any detectable NEO^r virus.

Infection of hematopoietic tissue

All mice were of the C3H/HeJ strain background and were obtained from Jackson laboratories. To obtain donor bone marrow, female mice (aged 7–12 weeks) were injected with 5-FU (150 mg/kg), 2, 4, 6, or 8 days prior to sacrifice. The cells were plated onto monolayers of virus-producing fibroblasts at a concentration of 3×10^6 to 5×10^6 cells/10-cm dish. Virus-producing cells were seeded one day previous at a concentration of $\sim 5 \times 10^5$ cells per plate. Coculture was performed as described (Lemischka et al. 1986), using recombinant IL-3 (10–15 U/ml) (gift from Frank Lee, DNAX; and Biogen) in place of WEHI-3B supernatant. Hematopoietic cell harvest and engraftment into lethally irradiated syngeneic male mice were performed as described (Lemischka et al. 1986). Cell doses ranged from 0.1×10^6 to 2.5×10^6 cells per recipient.

To obtain fetal liver cells, donor females were mated, and the time of vaginal plug discovery was scored as day 0. Preliminary experiments had demonstrated a high transduction efficiency for fetal liver. On day 14 of gestation, donors were sacrificed (cervical dislocation) and fetal liver was dissected in Iscove's modified Dulbecco's medium (IMDM; with 5% fetal calf serum and 3% BSA). Livers were homogenized by repeated passages through a 10-ml pipette, followed by filtration through sterile wire mesh (75-μm pore size). Cells were plated at 3×10^6 to 5×10^6 cells/10-cm dish on virus-producing fibroblasts. Coculture was done in 10 ml of αMEM with 8% calf serum, 10% fetal calf serum (FCS), 0.5% BSA (Boehringer–Mannheim), 10–15 U/ml recombinant IL-3 (a generous gift of John McKearn, Monsanto), 5 ng/ml of IL-1- α (gift from P. Lomedico, Hoffman-LaRoche), and 2 μg/ml Polybrene (Sigma). After 40–48 hr, fetal liver cells were harvested and processed for injection into lethally irradiated syngeneic recipients. Some recipients received reconstituted complete fetal liver cell populations, previously fractionated on the basis of expression of the antigen AA4.1 (McKearn et al. 1984). Inocula sizes ranged from 2.5×10^6 to 1.5×10^7 cells per animal. One initial experiment utilized MSV-DHFR-NEO (Williams et al. 1984); however, all subsequent studies employed the viral markers described in Figure 1.

Temporal analysis and peripheral blood fractionation

At 4–5 weeks post-engraftment, 100 μl of blood was taken from recipient mice via capillary puncture of the retro-orbital sinus. Samples were processed by standard procedure (Maniatis et al. 1982) to yield genomic DNA representative of the total nucleated cell population. Six to 12 weeks later and, subsequently, at 6- to 8-week intervals, 150-μl aliquots of peripheral blood were obtained from individual recipients. Each sample was fractionated into individual hematopoietic lineages, using the following procedure: Blood was diluted to ~1.0 ml with physiologically buffered saline (PBS) and layered onto a two-step isotonic Percoll (Pharmacia) gradient (steps of 1.078 and 1.090 g/ml). Samples were centrifuged at 850 *g* for 25 min. Using this procedure, the red blood cells pellet, granulocytes band at the 1.078/1.090 g/ml interface, and mononuclear cells remain at the 1.078 g/ml step. Mononuclear cells and granulocytes were collected individually and washed twice with 10 ml of PBS plus 0.5% FCS. Granulocytes were processed to yield genomic DNA, using a variation of the agarose block procedure described by Poustka et al. (1987). Cells were resuspended in 35 μl PBS, mixed with an equal volume of 1% low-melt agarose (Seaplaque, FMC), and cast into two blocks per sample (done on ice to rapidly solidify blocks). The blocks were cast in a custom-made Plexiglas mold with internal dimensions of $0.18 \times 0.18 \times 0.07$ inches for each well. Each cell sample was transferred to a digestion buffer consisting of 2 mg/ml proteinase K, 1% Sarkosyl, and 0.1 M EDTA and incubated at 50°C for 48 hr. Blocks were then rinsed in 20–30 ml of TE and incubated in 10 mM Tris (pH 7.5), 1 mM EDTA, and 40 μg/ml phenylmethylsulfonyl fluoride (PMSF) for 1 hr at 50°C. Finally, samples were rinsed with an additional 20–30 ml of TE. (At this point, the blocks can be digested with restriction enzyme or stored in 0.2 M EDTA at 4°C). Mononuclear cells, from the light density fraction, were incubated for 2–3 hours in IMDM with 10% FCS and 20% L-cell conditioned medium (LCM). (The monocytes adhere to the tissue culture plastic, whereas lymphocytes remain in suspension.) After incubation, the non-

adherent mononuclear cells were harvested and transferred to 1.5-cm petri dishes coated with 10 μg/ml of goat anti-mouse immunoglobulin (gift of T. Manser). Cells were "panned" as described previously (Wysocki and Sato 1978), to separate B and T lymphocytes. The adherent B lymphocytes were lysed directly on the panning dish and processed by standard procedures to yield the B-cell DNA sample. Nonadherent T lymphocytes were processed via the agarose block procedure described above. In some cases, the earliest T-lymphocyte DNA samples were derived by first culturing the T cells in RPMI, 10% FCS, IL-2 (20 U/ml), and concanavalin A (3 μg/ml) for 3–5 days. Adherent monocytes were cultured (IMDM, 10% FCS, and 20% LCM) for 6–10 days, and genomic DNA prepared by standard procedure. Purity of the granulocyte and lymphocyte populations was assessed by immunofluorescence analysis, using lineage-specific monoclonal antibodies. Granulocytes and B lymphocytes were stained with antibodies RB6-8C5 and RA3-6B2, respectively (gift of R. Coffman, DNAX). T lymphocytes were stained with the αThy-1.2 antibody, 30-H12 (Becton-Dickinson). In each of the assayed populations, cells were 80–90% positive for the lineage-specific antibody. Monocyte–macrophages were identified by morphological characteristics and growth response to LCM. Cells in the monocyte–macrophage fraction were 80–90% pure after 6–10 days of culture.

Hematopoietic tissue fractionation

After obtaining a final blood sample, animals were sacrificed and hematopoietic tissues harvested. Peritoneal macrophages were obtained by rinsing the abdominal cavity with 2–4 ml PBS, followed by culture in IMDM with 10% FCS and 20% LCM for 3–5 days. Bone marrow was harvested and divided into three samples. The first sample was used to reconstitute lethally irradiated secondary recipients (2×10^6 to 5×10^6 cells per animal). The second sample was depleted of monocytes and B cells by serially adhering the sample to a tissue culture dish, followed by an anti-mouse immunoglobulin panning dish. The depleted sample was considered to be largely granulocytic, whereas the adherent cells were cultured to obtain bone marrow macrophages and the B cells were directly processed for genomic DNA. The final sample was prepared simply as total bone marrow. Lymph nodes were separated into B- and T-lymphocyte-enriched populations by panning cell suspensions on anti-mouse immunoglobulin dishes. Cell suspensions from spleen were subjected to the same panning procedure to yield B and T lymphocytes, as well as an adherence step to derive splenic macrophages (cultured as for bone marrow macrophages). Also, a portion of spleen was set aside for a total spleen DNA sample. In some cases, spleen cells were cultured in RPMI, 10% FCS, IL-2 (20 U/ml), and concanavalin A (3 μg/ml) to obtain T-lymphocyte samples.

Southern blot procedure

Restriction digest and electrophoresis were also carried out according to standard procedure (Maniatis et al. 1982). Samples cast in agarose blocks were rinsed (if previously stored in EDTA) with 20–30ml TE and equilibrated in 1× restriction buffer for 30–45 min. The buffer was then replaced with 1× buffer plus restriction enzyme and incubated at the appropriate temperature overnight. Following digestion, the blocks were electrophoresed, along with standard digests. After electrophoresis, all gels were transferred to nylon membrane (Zeta-bind, CUNO) and cross-linked using the ultraviolet light procedure described by Church and Gilbert (1984). Limiting quantities of DNA necessitated the use of high specific activity probes prepared by nick translation, using all four high specific activity (3000 Ci/mmole) [^{32}P]dNTPs (Lemischka et al. 1986). Hybridization was performed using conditions originally described by Reed (1988). A hybridization solution consisting of 0.36 M NaCl, 20 mM $NaPO_4$, 2 mM EDTA, 1% SDS, 0.5% nonfat milk, 10% dextran sulfate, and 500 μg/ml denatured herring sperm DNA was used. Hybridization was performed at 65°C for 12–14 hr. Inside the hybridization bags, the membranes were sandwiched between two pieces of Schleicher & Schuell 589-WH paper to help reduce background (Reed 1988).

Probes were linear fragments of NEO, IL-3 cDNA (Yokota et al. 1984), or a Y chromosome-specific probe (pY2; Lamar and Palmer 1984), all gel-purified as described (Vogelstein and Gillespie 1979). Quantitation was performed exactly as described previously (Lemischka et al. 1986). Briefly, membranes were probed sequentially with a retroviral marker-specific probe (NEO), an autosomal probe (IL-3), and a host-specific probe (pY2, only used for bone-marrow-reconstituted recipients). [Normalization of DNA content by densitometric analysis of IL-3 signal, allows quantitation of NEO-specific signal (also by densitometry).] Differences in IL-3 and NEO probe-specific activity were compensated by analysis of a marker sample that consisted of a cloned 3T3 line carrying a single NEO virus. Quantitation of host-derived DNA was performed as described (Lemischka et al. 1986).

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