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Disparate differentiation in mouse hemopoietic colonies derived from paired progenitors

(hemopoietic stem cell/clonal cell culture/hemopoiesis)

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ABSTRACT **We analyzed the differentiation of murine hemopoietic colonies derived from paired progenitors in culture. Single progenitors were isolated by use of a micromanipulation technique from blast cell colonies cultured from the spleens of 5-fluorouracil-treated mice. Eighteen to 24 hr later, the paired progenitors were separated with a micromanipulator and cultured in methylcellulose medium containing erythropoietin and pokeweed-mitogen spleen cell conditioned medium. Six to nine days later, the two colonies derived from the paired progenitors were individually picked and differential counts were performed by using May–Grunwald–Giemsa stain. The abbreviations used here are n, neutrophil; m, macrophage; e, eosinophil; mast, mast cell; M, megakaryocyte; E, erythrocyte. Of a total of 387 pairs that could be evaluated, 68 were pairs of colonies consisting of dissimilar combinations of cell lineages such as m-nmmastEM, M-nmmastEM, nm-nmmastEM, nmmastM-nmmastEM, M-nmmastM, nmmast-nmmastM, nm-nmmastE, M-nmM, n-nmM, mM-nmM, m-nmmast, nm-nme, me-nm, mM-nm, n-ne, m-mmast, m-mM, M-nm, M-mM, E-nm, m-nm, M-m, etc. Thirty-nine were homologous pairs revealing identical lineage combinations such as nmmastEM, nmmastM, nmmast, mmastEM, nmEM, nme, nmM, mM, and nm lineages. However, in members of some of these pairs, the proportions of the individual cell lineages were significantly different. The remainder were pairs of single lineage colonies. Paired progenitors obtained from the stem cell colonies of normal mice also revealed homologous and nonhomologous expression of the cell lineages. Comparison of lineage expression in colonies derived from single progenitors with the sum of lineages expressed in pairs of colonies derived from single progenitors indicated that the diversity was not due to injury inflicted by micromanipulation. These observations provide experimental data in support of stochastic mechanisms of stem cell differentiation.**

Several models have been proposed for the mechanisms of differentiation of hemopoietic stem cells. These models include the hemopoietic inductive microenvironment (HIM) model of Trentin (1), the "stem cell competition" model that was advocated most recently by VanZant and Goldwasser (2), and the "erythroid-obligatory" model of Nicola and Johnson (3) that features orderly and sequential loss of lineage potentials. We have proposed that stem cell differentiation involves progressive and stochastic restriction in the lineage potentials of hemopoietic stem cells (4). This model is an extension of the earlier stochastic model for renewal of spleen colony-forming units by Till *et al.* (5), our observations on the self-renewal and commitment of progenitors for blast cell colonies in culture (6), and the stochastic model for stem cell differentiation to erythroid or granulocytic cell lines by Korn *et al.* (7). Our proposal gained support from our most recent observations (8) of mixed hemopoietic colonies revealing diverse combinations of cell lineages that are derived from single progenitors. Advancing the micromanipulation technique one step further, we have now succeeded in analysis of the differentiation of hemopoietic colonies derived from the two progeny of a single parent cell. The results clearly documented dissimilar patterns of differentiation in the two daughter cells and provided strong evidence for the stochastic mode of stem cell differentiation.

MATERIALS AND METHODS

Cell Preparations. Ten to 15-week-old female BDF_1 mice were obtained from Simonsen's Laboratory (Gilroy, CA). We administered 5-fluorouracil (5-FUra) (Adria Laboratories, Columbus, OH) at a dosage of 150 mg/kg of body weight through the tail vein (9). Spleen cells were harvested 4 days after the 5-FUra injection and single-cell suspensions were prepared from pooled spleens of three to five mice. In some experiments, spleens were harvested from normal mice (10).

Blast Cell Colony Assay. Methylcellulose culture was carried out by using a modification (10) of the technique described by Iscove *et al.* (11). One milliliter of culture medium contained 6.0×10^5 spleen cells from 5-FUra-treated mice or 2.0×10^5 spleen cells obtained from normal mice, α medium (Flow Laboratories), 1.2% methylcellulose (Fisher Scientific), 30% fetal bovine serum (Flow Laboratories), 1% deionized bovine serum albumin (Sigma), 0.1 mM 2-mercaptoethanol (Eastman), 2 units of partially purified human urinary erythropoietin with a specific activity of 370 units/mg of protein (kindly provided by Makoto Kawakita, Kumamoto University Medical School, Japan) and 10% (vol/vol) spleen cell conditioned medium prepared with pokeweed mitogen (12).

Culture of Paired Progenitors. The technique used for single-cell manipulation was similar to the one described previously (8). On day 7 of culture of spleen cells from 5-FUra-treated mice (9) and on day 16 of culture of normal cells (10), blast cell colonies consisting of 20–100 cells were identified, lifted from the methylcellulose medium by use of a 3-μl Eppendorf pipet under microscopic visualization, and individually suspended in 0.1 ml of α medium. Each sample was then added to 0.9 ml of methylcellulose medium in a second 35-mm culture dish and the dish was agitated gently in order to disperse the colony. A single cell was transferred to a third 35-mm culture dish containing 1 ml of methylcellulose medium by use of a fine Pasteur pipet (with a diameter of approximately 30 μm) attached to a micromanipulator with motorized adjustments (M-10M) (Hacker Instruments, Fairfield, NJ). Approximately one-third of the cells from a dispersed single blast cell colony could be easily identified and

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Abbreviations: 5-FUra, 5-fluorouracil; n, neutrophil; m, macrophage-monocyte; e, eosinophil; mast, mast cell; E, erythrocyte; M, megakaryocyte; Bl, blast cell.

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were transferred to a third dish (8). After confirmation of the presence of a single cell in each culture dish, incubation was carried out at 37°C in 5% CO_2 in an air atmosphere. When doublets developed from single progenitors 18–24 hr later, they were separated in the 35-mm dishes by micromanipulation and allowed to develop into two separate colonies. In certain instances, each of the doublets was individually placed into a newly prepared 35-mm dish by micromanipulation. On days 6–9 of incubation, when the secondary colonies appeared to have matured, individual colonies were lifted from the methylcellulose medium, suspended in 0.2 ml of α medium, and divided into two aliquots. One half of the sample was spun in a Shandon cytocentrifuge and stained with May–Grunwald–Giemsa stain for differential counting (13), and the other half was replated to identify the presence of unexpressed lineage potentials in the secondary colonies (12). The presence of cholinesterase activity in megakaryocytes was demonstrated by the method of Jackson (14). The size of small colonies (<500 cells) was estimated *in situ* and the size of larger colonies by use of a counting chamber.

RESULTS

Single hemopoietic progenitors were obtained from blast cell colonies derived from spleen cells of 5-FUra-treated mice. A total of 1240 single cells were individually transferred to second dishes. Eighteen to 24 hr later, doublets were observed in 500 dishes. The doublets were separated by micromanipulation and were allowed to form colonies. Three-hundred and eighty-seven doublets produced pairs of colonies and 68 of these pairs of colonies consisted of dissimilar combinations of cell lineages. We chose 22 representative pairs and have presented the size and differential counts of the individual colonies in Table 1. The abbreviations of the cell lineages are based on the recommendations of a Workshop in a Uni-

Table 1. Cell number and differential counts of colonies derived from paired daughter cells of 5-FUra-treated mice that reveal disparate lineage combinations

Culture no.	Colony no.	Day of harvest	Cells per colony	Differential counts,† %							Colony type
				n	m	e	mast	E	M	Bl	
1	a	7	350		100.0						m
	b		25,000	2.4	10.4		8.8	77.2	1.2		nmmastEM
2	a	7	34						100.0		M
	b		2,000	7.6	26.0		2.0	60.8	1.2	2.4	nmmastEM
3*	a	7	2,700	5.4	94.6						nm
	b		1,300	18.4	50.0		20.4	4.4	6.0	0.8	nmmastEM
4	a	8	1,500	59.2	16.8		18.4		4.4	1.2	nmmastM
	b		2,000	75.2	6.4		4.4	8.0	5.0	1.0	nmmastEM
5	a	7	58						100.0		M
	b		3,000	54.0	9.2		24.8		8.0	4.0	nmmastM
6	a	8	500	41.5	46.5		12.0				nmmast
	b		2,500	29.6	48.0		20.4		1.2	0.8	nmmastM
7	a	7	100	6.5	93.5						nm
	b		500	69.5	6.5		15.0	9.0			nmmastE
8	a	7	28						100.0		M
	b		400	70.0	21.0				9.0		nmM
9	a	8	95	100.0							n
	b		500	92.0	6.0				2.0		nmM
10	a	8	70		98.2				1.8		mM
	b		200	4.0	94.0				2.0		nmM
11	a	7	500		100.0						m
	b		3,000	44.6	37.4		18.0				nmmast
12*	a	7	700	79.2	20.8						nm
	b		1,300	60.0	24.8	15.2					nme
13	a	8	100		91.0	9.0					me
	b		2,500	15.2	84.8						nm
14	a	8	7		57.1				42.9		mM
	b		600	52.0	48.0						nm
15	a	7	2,000	100.0							n
	b		3,000	92.0		8.0					ne
16	a	7	300		100.0						m
	b		400		80.0		20.0				mmast
17*	a	7	800		100.0						m
	b		300		97.6				2.4		mM
18	a	7	7						100.0		M
	b		300	3.0	97.0						nm
19*	a	7	19						100.0		M
	b		26		25.0				75.0		mM
20	a	7	10,000					100.0			E
	b		500	58.0	42.0						nm
21*	a	7	350		100.0						m
	b		800	18.0	82.0						nm
22	a	7	39						100.0		M
	b		600		100.0						m

*See *Results*.
†Differential counts were performed on 500 cells, unless smears had fewer cells.

versity of California at Los Angeles Symposium (15): n, neutrophil; m, monocyte-macrophage; e, eosinophil; mast, mast cell; E, erythrocyte; M, megakaryocyte; Bl, blast cell. The remaining 46 nonhomologous pairs of colonies consisted of 5 m-nmmastEM, 2 M-nmmastEM, 2 nmmastM-nmmastEM, 1 nmmast-nmmastM, 2 nm-nmEM, 2 m-nmmast, 1 nm-nmmast, 1 M-nM, 1 mast-nmast, 28 m-nm, and 1 M-m colonies. As is shown in Table 1, marked difference in the size and cell composition of the pairs was seen. To detect unexpressed lineage potentials (12), all colonies listed in Table 1 were replated. Only some of the nmmastEM colonies formed secondary colonies but they did not disclose potentials that had not been expressed previously. *In situ* appearance and May–Grunwald–Giemsa smears of the colonies from culture 1 are presented in Fig. 1. Table 1 shows the various patterns of disparity we observed. In many cases, the lineages expressed in one of the pairs were inclusive of those expressed in the other. For example, in cultures 1, 2, 5, 8, 9, 11, 15–17, 19, and 21, one of the paired progenitors produced a single lineage colony while its counterpart produced a multi- or oligolineage colony. In other cultures, both cells of the pair formed multi- or oligolineage colonies sharing more than one constituent lineages (e.g., cultures 3, 4, 6, 7, 10, and 12). In cultures 13 and 14, the pairs of bilineage colonies shared one common lineage. In cultures 18, 20, and 22, the pairs of colonies did not share a common lineage.

The observations presented in Table 1 suggest several ways in which commitment to single lineages may take place. Culture 1 shows that commitment to the monocyte-macrophage lineage may take place during one cell division of a multipotential parental cell. Culture 11 illustrates that oligopotential progenitors may yield monocyte-macrophage progenitors after one cell division. Cultures 16, 17, 21, and 22 indicate the ways in which bipotential progenitors divide once and produce monocyte-macrophage progenitors. Cultures 2, 5, 8, 18, 19, and 22 reveal that commitment to the megakaryocyte lineage may also occur during single cell divisions of progenitors differing in numbers of potencies. Lineage expression in cultures 9 and 15 suggest that neutrophil progenitors may be produced in a similar manner. At this time, we are not certain whether or not such rules apply to other hemopoietic lineages.

In our previous report, we identified oligopotential progenitors capable of expression of diverse combinations of lineages. Cultures 3, 4, 6, 10, and others in Table 1 demonstrate that the mechanism for production of these oligopotential progenitors is through progressive restriction in lineages. Culture 20 is a special case in which the parental cell that was presumably capable of nmE differentiation produced progeny committed to nm and E lineages. These observations strongly indicate that the stochastic principle applies not only to the types but also to the numbers of lineage potentials.

Of the 387 cultures, 319 resulted in pairs of colonies expressing the same combinations of cell lineages. Thirty-nine cultures produced homologous pairs of multilineage colonies, including 3 pairs of nmmastEM colonies, 6 pairs of nmmast colonies, 24 pairs of nm colonies and one pair each of nmmastM, mmastEM, nmEM, nme, nmM, and nM colonies. Although the lineage combinations were the same, the proportions of the individual cell lineages were not identical between the members of a pair. Some of the examples are shown in Table 2. Culture 2 consisted of a pair of nmmastM colonies. However, one of the colonies presented a signifi-

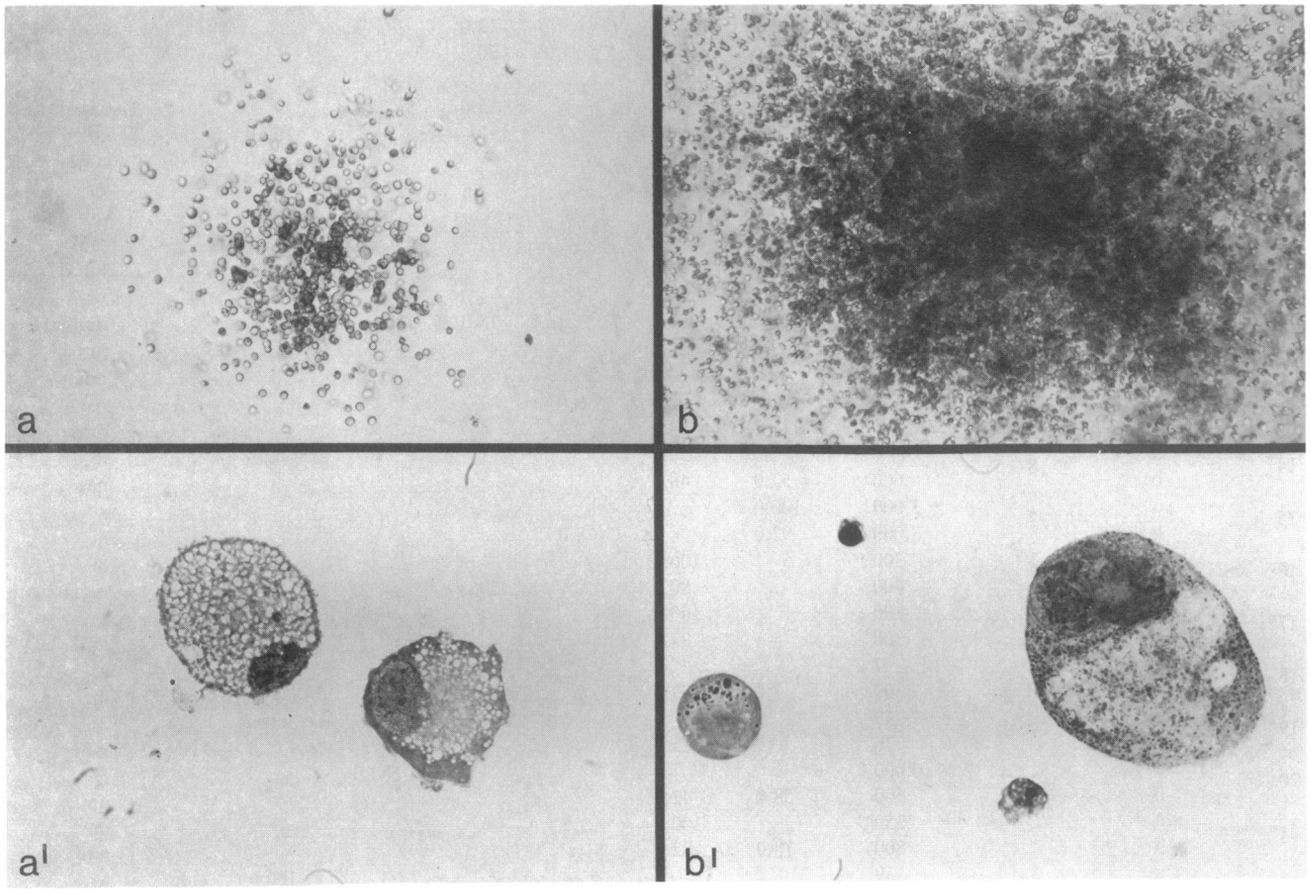

FIG. 1. Appearance of colonies derived from paired progenitors and portions of their companion slides that we stained with May–Grunwald–Giemsa stain (culture 1, Table 1). (*a*) Macrophage colony, (*a'*) slide; (*b*) a multilineage colony, (*b'*) a portion of the slide revealing a mast cell, two erythroblasts, and a megakaryocyte (from left to right).

Table 2. Cell number and composition of colonies derived from paired progenitors of 5-FUra-treated mice that reveal the same combinations of cell lineages

Culture no.	Colony no.	Day of harvest	Cells per colony	Differential counts,* %							Colony type
				n	m	e	mast	E	M	Bl	
1	a	7	2000	30.4	62.0		4.0	3.2	0.4		nmmastEM
	b		3000	52.0	26.0		8.0	12.4	1.6		nmmastEM
2	a	7	400	42.5	5.0		37.5		15.0		nmmastM
	b		600	87.0	7.0		2.0		3.0	1.0	nmmastM
3	a	7	100	5.0	94.0		1.0				nmmast
	b		1500	48.6	37.0		14.4				nmmast
4	a	7	300	1.0	97.0	2.0					nme
	b		900	17.0	76.5	6.5					nme
5	a	7	300	6.4	93.6						nm
	b		200	86.0	14.0						nm

*Differential counts were performed on 500 cells, unless smears had fewer cells.

cant number of mast cells while the other contained only 2.0% mast cells. The percentages of neutrophils were also substantially different. Culture 3 revealed differences not only in the percentages of mast cells but also in colony size. Two-hundred and eighty cultures revealed pairs of single-lineage colonies of identical types, including 257 pairs of macrophage colonies, 17 pairs of mast cell colonies, and 6 pairs of megakaryocyte colonies. In some instances, there were significant differences in the size of the colonies between members of the same pair.

It was possible that the lineage expressions in the nonhomologous pairs of colonies reflect aberrations during late stages of colony formation rather than the lineage potentials of the paired progenitors. To exclude this possibility, we observed the time course of colony formation *in situ* from progenitors. *In situ* estimates of the cell number and expression of specific lineage characteristics [such as the red color of erythrocytes, large and refractile nature of megakaryocytes, and polygonal shape of granulocytes (9)] of the two colonies were serially recorded. As reported previously (9), the signs of differentiation in the pure macrophage and megakaryocyte colonies could be recognized as early as day 3 of incubation, and it was possible to distinguish these colonies from their companion multilineage colonies. These observations supported our interpretation that the disparate lineage expression in the pairs of colonies reflects the nonhomologous potentials of the paired progenitors.

It was also possible that micromanipulation inflicted damage to the progenitors and caused the discordant expression of lineages in the pairs of colonies. To exclude this possibility, we compared the lineages expressed in colonies that were derived from single progenitors with the sum of the lineages expressed in the paired colonies. Earlier in this section, we described a total of 1240 single cells from blast cell colonies that were individually transferred to second dishes by micromanipulation. Of these, 500 formed doublets, which were again separated by micromanipulation and allowed to form pairs of colonies. The remaining 740 dishes revealed one cell, no cells, or more than two cells. These dishes were left in the incubator without additional micromanipulation and many revealed single colonies. On day 7 of incubation, individual colonies were harvested and the differential counts were determined by May–Grunwald–Giemsa staining. When we compared the frequencies of the expression of diverse combinations of lineages in the single colonies to the frequency of pairs of colonies that jointly expressed the same lineage combinations, we found that the relative frequencies of lineage expression were very similar in the two groups. For example, in both groups approximately two-thirds (65.3 and 66.5%, respectively) revealed monocyte-macrophage differentiation and only about 4% (3.6 and 4.4%, respectively) revealed nmmastEM expression. The relative incidences of nm expression were 17.8% (single colonies) and 13.7% (paired colonies). These results revealed that additional micromanipulation did not alter lineage expression by the progenitors and suggested that disparate lineage expression in the pairs of colonies reflects intrinsic properties of the paired progenitors.

The apparent disparity may have been an artifact of culture. For example, it was possible that the plated single cells released autostimulating factors and that there were concentration gradients of these factors that affected the subsequent maturation of colonies. In order to exclude this possibility, in some of the cultures designated with asterisks in Table 1, each of the paired progenitors was plated in a fourth, newly prepared, dish. Disparate differentiation was again observed.

Finally, we entertained the possibility that 5-FUra might have caused irreversible damage to the progenitors and thereby caused the discordant expression of the lineages. Consequently, we obtained paired progenitors from stem cell colonies of normal mice (10) and analyzed their differentiation in culture. Both homologous and nonhomologous pairs of colonies were seen. In Table 3, examples of homologous and nonhomologous differentiation in the pairs of colonies are presented.

DISCUSSION

We have presented evidence that differentiation in colonies derived from paired hemopoietic progenitors can be extremely variable. The time course observation of the colony formation from the paired progenitors revealed that this discordance within a pair is seen at the early stages of colony formation and suggested that it reflects the intrinsic potentials of the paired progenitors. It is unlikely that the disparate expression of lineages was caused by damage inflicted by micromanipulation since an additional step of micromanipulation did not add heterogeneity in lineage expression. Further, it was not due to a mutagenic effect of 5-FUra, since studies using paired progenitors obtained from normal mice also revealed nonhomologous expressions of lineages. We also negated the possible effects of endogenously produced humoral factors by replating each of the paired daughter cells into a new dish. These observations strongly indicated that the discordant expression of lineages reflects the intrinsic potentials of the paired progenitors.

Previously, we documented oligopotential progenitors capable of expressing diverse combinations of cell lineages (8). Data presented in this paper suggested that these oligopotential progenitors could be produced from multipotential progenitors through progressive restriction in lineage potentials. In this paper, we also presented evidence that monopotential progenitors may be derived directly from multipotential and oligopotential progenitors. The stochastic principle seems to

Table 3. Cell number and compositions of colonies derived from paired progenitors of normal mice

Culture no.	Colony no.	Day of harvest	Cells per colony	Differential counts,* %						Colony type
				n	m	e	mast	E	M	
1	a	7	500	5.0	95.0					nm
	b		2,000	20.4	62.0		6.4	10.0	1.2	nmmastEM
2	a	7	2,500	3.4	82.0		5.0	7.4	2.2	nmmastEM
	b		3,000	18.4	46.4		8.0	25.6	1.6	nmmastEM
3	a	7	80		100.0					m
	b		1,000	70.0	26.0	4.0				nme
4	a	7	2,500	32.0	68.0					nm
	b		1,200	31.6	59.2			9.2		nmE
5	a	7	700		100.0					m
	b		300		10.0			90.0		mE
6	a	7	2,000	22.4	77.6					nm
	b		14,000	32.0	68.0					nm
7	a	7	200		100.0					m
	b		300	12.0	88.0					nm

*Differential counts on 500 cells, unless smears had fewer cells.

apply not only to the types of lineages but also to the numbers of lineage potentials. A large number of the paired progenitors produced colonies revealing the same lineages or the same combinations of lineages. However, there were discordances among some of the apparently homologous pairs. Both differential counts and colony sizes differed significantly between members of some of the pairs. Proliferative potentials of single lineage progenitors may also be under stochastic control mechanisms.

Individual lineages were not expressed at the same frequency. For example, of the 319 homologous pairs of colonies, 257 were pairs of monocyte-macrophage colonies. Among the nonhomologous pairs as well, the monocyte-macrophage lineage was identified most frequently. This predominance of the monocyte-macrophage lineage, which is in agreement with our previous observation (8), may be due to several factors. It may be the result of a culture artifact. For example, the levels of various humoral factors for each cell lineage in the conditioned medium may be different. Macrophages may be most resistant to the adverse conditions in the artificial culture system. Alternatively, the predominance of macrophage expansion may reflect the physiological properties of the progenitors. It is possible that committed macrophage progenitors can divide more times than the progenitors for other lineages. Observations of the primitive macrophage progenitors with a high proliferative potential by Bradley *et al.* (16) in a different culture system may support this premise. Whether or not physiological skewing exists in the pattern of lineage expression, our observations are consistent with the notion that differentiation of hemopoietic stem cells is a stochastic process.

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