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Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies

(hemopoietic stem cell/clonal cell culture/mixed hemopoietic colonies)

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ABSTRACT **Mouse marrow and spleen cells formed colonies consisting of 40–1,000 blast cells after 16 days of incubation in methylcellulose culture in the presence of medium conditioned by pokeweed mitogen-stimulated mouse spleen cells. These colonies could be distinguished from other hemopoietic colonies *in situ* by the complete absence of signs of terminal differentiation. Replating of these colonies (tentatively named stem cell colonies) revealed their self-renewal capacity and the extensive ability to generate secondary colonies, many of which were multipotential hemopoietic colonies. Some of the colonies revealed 100% replating efficiencies. Analyses of individual stem cells colonies revealed concurrent and high incidences of spleen colony-forming units and the macroscopic granulocyte–erythrocyte–macrophage–megakaryocyte colony-forming units (CFU-GEMM) in culture. Replating comparison between the stem cell colonies and GEMM colonies strongly indicated that the progenitors for the stem cell colonies are higher in the hierarchy of stem cell differentiation than are CFU-GEMM. Quantitation of stem cell colonies provides an assay for the class of primitive hemopoietic progenitors described here.**

Production in the bone marrow and destruction in the peripheral tissues of mature blood cells takes place throughout the lifespan of an animal. In order to account for the ever-continuing process of the production of mature blood cells, a class of cells was envisioned and termed pluripotent hemopoietic stem cells; this class must fulfill two major criteria—namely, capabilities for extensive self-renewal and production of progenies in all hemopoietic cell lineages. Two decades ago, a spleen colony method was developed by Till and McCulloch (1) for enumeration of a fraction of murine pluripotent hemopoietic stem cells (CFU-S, colony-forming units in spleen). Recently, multipotential hemopoietic progenitors became assayable in clonal cell cultures. Metcalf and his colleagues (2) and our group (3) described hemopoietic progenitors that are capable of differentiation in more than three cell lineages in culture of fetal mouse liver and adult mouse marrow cells, respectively. Subsequently, human multipotential hemopoietic progenitors that are capable of differentiation into granulocyte–erythrocyte–macrophage–megakaryocyte (GEMM) lineages were identified in cell culture (4). The exact stage of these hemopoietic progenitors in the hierarchy of hemopoietic differentiation has not been identified. Their overall self-renewal capacity, recently documented in detail by Humphries *et al.* (5), suggests that CFU-GEMM may be located close to CFU-S in the differentiation stages of hemopoiesis.

We recently identified a class of murine hemopoietic colonies with apparent restriction to granulocyte–macrophage–megakaryocyte (GMM) differentiation and yet with replating capabilities almost identical to those of GEMM colonies (6). Detailed characterization of the differentiation capabilities of the GMM colonies suggested a possibility that the hemopoietic stem cells that are high in the hierarchy of stem cell differentiation may not reveal terminal differentiation (e.g., hemoglobin synthesis) in standard incubation periods of primary culture. Our systematic replating and histochemical analyses of murine hemopoietic colonies containing only undifferentiated cells resulted in identification of a class of hemopoietic stem cells that appear to be more primitive than CFU-GEMM in the hierarchy of hemopoiesis. The colonies derived from these stem cells were tentatively designated as stem cell colonies.

MATERIALS AND METHODS

Clonal Cell Culture Methods. Ten to 15-week-old female BDF_1 mice were obtained from Simonsen's Laboratory, Gilroy, CA. Bone marrow and spleen cells were prepared in α medium (Flow Laboratories, Rockville, MD) as described (6). Methylcellulose culture was carried out by using a modification (6) of the techniques described by Iscove *et al.* (7). Unless otherwise specified, 1 ml of culture medium contained 2×10^4 nucleated bone marrow cells or 2×10^5 spleen cells, 2 units of partially purified human urinary erythropoietin with a specific activity of 840 units/mg of protein (kindly provided by Makoto Kawakita, Kumamoto University Medical School, Japan) and 10% (vol/vol) medium conditioned by pokeweed mitogen-stimulated spleen cells (PWM-SCM). Granulocyte–macrophage (GM) colonies (8), erythroid bursts (9), and megakaryocyte colonies were counted on day 8. Megakaryocyte clusters consisting of more than four cells were scored as colonies (10). Colonies considered to contain more than two cell lineages were scored as mixed colonies on day 10 and then individually picked and stained for determination of cellular composition. Mast cell colonies (11) and stem cell colonies were counted on day 16 of culture.

Preparation of Conditioned Media. Serum-free PWM-SCM was prepared by using a modification of the method described by Guilbert and Iscove (12). After being washed three times with α medium, spleen cells were incubated at 2×10^6 cells per ml in a 1:1 mixture of α medium and modified Ham's F_{12} medium (Flow) containing a 1:300 dilution of pokeweed mito-

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Abbreviations: CFU, colony-forming units; CFU-S, colony-forming units in spleen; GM, granulocyte–macrophage; GMM, granulocyte–macrophage–megakaryocyte; GEMM, granulocyte–erythrocyte–macrophage–megakaryocyte; PWM-SCM, medium conditioned by pokeweed mitogen-stimulated spleen cells.

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gen (GIBCO), 0.1 mM α-thioglycerol (Sigma), 25 mM Hepes (Flow), 0.1 μM sodium selenite (Sigma), 1% deionized bovine serum albumin, 60 μg of human transferrin (Sigma) per ml, 0.45 μM $FeCl_3$ (Mallinckrodt), 16 μg of soybean lecithin (Sigma) per ml, and 9.6 μg of cholesterol (Calbiochem) per ml for 7 days.

Staining. Individual colonies were lifted from the methylcellulose medium by using a 3-μl Eppendorf pipette under direct microscopic visualization and collected in Eppendorf microcentrifuge tubes containing 1 ml of phosphate-buffered saline. After being washed two times with phosphate-buffered saline, the samples were immediately spun in a cytocentrifuge (Cytospin; Shandon Southern, Elliott, IL) at 800 rpm for 5 min and processed for May–Grunwald–Giemsa and specific staining.

Replating Experiments. On day 16 of primary, secondary, and tertiary cultures, stem cell colonies were individually lifted from the methylcellulose medium and suspended in 0.2 ml of α medium. After gentle pipetting, samples were then divided into two aliquots: one half to be processed for morphologic examination of constituent cells and the other half to be added individually to 0.9 ml of methylcellulose culture medium containing 10% PWM-SCM and 2 units of erythropoietin in the culture dishes. The mixture was again thoroughly mixed by gentle pipetting. Cultures of the replated cells were incubated and colonies were scored in the same manner as in the primary cultures. Colony size was determined by direct cell counting *in situ* with ×200 magnification on an inverted microscope when colonies consisted of less than 300 cells. When colonies contained more than 300 cells, counting was carried out on the stained Cytospin preparations.

For simultaneous spleen colony and replating analyses, individual stem cell colonies were collected in tubes containing 3 ml of α medium, washed twice, and resuspended in 1 ml of α medium. The colony size was estimated *in situ*. The samples were then divided into two aliquots: one half to be used for replating experiments and the other half to be assayed for CFU-S. Macroscopic GEMM colonies were collected into tubes containing 3 ml of α medium, washed twice, and resuspended in 1 ml of α medium. These samples were then divided into three aliquots: 0.2 ml to be used for cell counting, 0.4 ml to be used for replating experiments, and 0.4 ml to be injected into syngeneic recipient mice for the CFU-S assay.

Spleen Colony Assay. Aliquots from the stem cell and GEMM colonies were injected intravenously into syngeneic recipient mice that had been irradiated with 850 rads (8.5 grays) at 1,450 roentgens (0.374 coulomb/kg)/min, using a ^{137}Cs irradiator (Gammator model M-38; Isomedix, Parsippany, NJ). Spleens from the recipient mice were harvested 9 days later, fixed in Bouin's solution, and examined for macroscopic spleen colonies.

RESULTS

Colony Formation. When mouse bone marrow and spleen cells were cultured in the presence of erythropoietin and PWM-SCM for over 10 days, most of the colonies showed signs of terminal differentiation—i.e., the red of hemoglobin, large megakaryocytes, and granulocytes recognizable by their polygonal shape. A macroscopic GEMM colony seen on day 10 of culture is shown in Fig. 1*A*. When culture was continued beyond 2 weeks, some colonies completely disintegrated, and almost all colonies showed signs of cell degeneration. On day 16 of culture, only three types of colonies revealed no signs of degeneration—namely, hemopoietic stem cell colonies, mast cell colonies, and GMM colonies. Individual stem cell colonies consisted of 40–1,000 loosely arranged, occasionally clumped, homogeneous populations of round cells with no signs of terminal differentiation (Fig. 1*B*). The incidence of the stem cell colonies was 0.95 ± 0.8 per 2×10^4 marrow cells and 2.1 ± 0.9 per 2×10^5 spleen cells. Mast cell colonies presented in Fig. 1*C* and

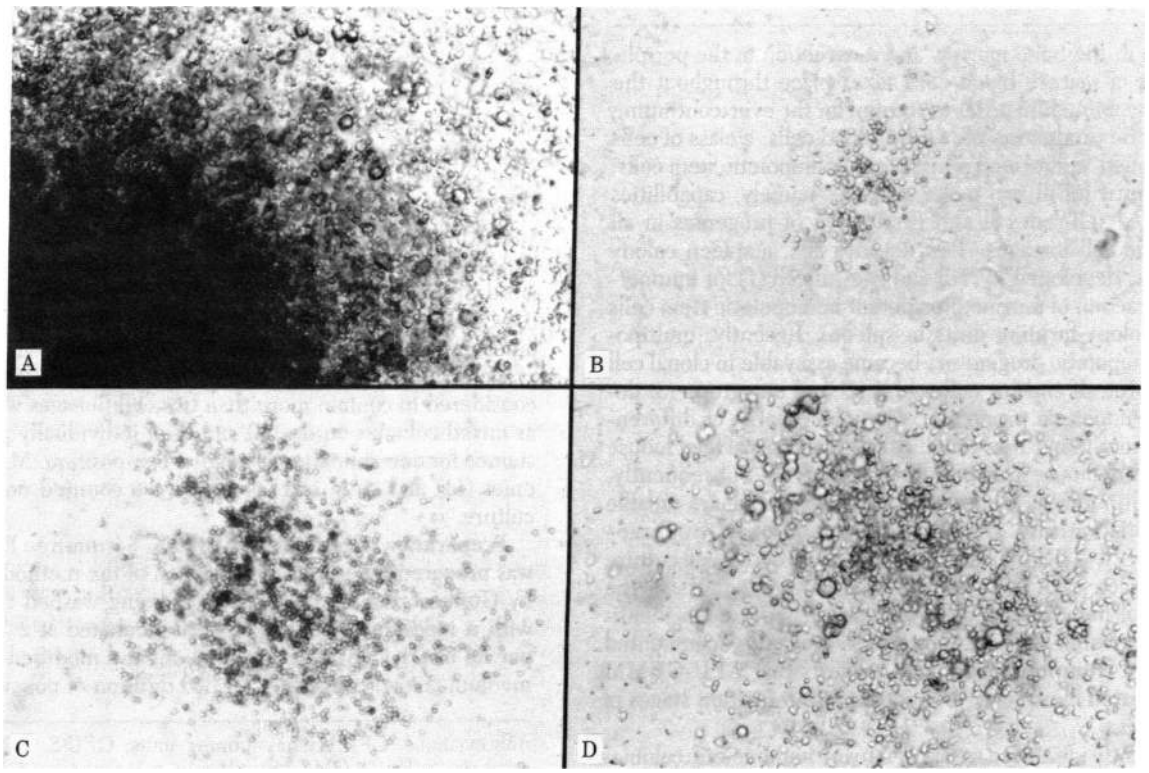

FIG. 1. Different classes of hemopoietic colonies presented at the same magnification (×50). (*A*) Day 10 macroscopic GEMM colony; (*B*) day 16 stem cell colony, consisting of cells with no signs of terminal differentiation; (*C*) day 16 mast cell colony consisting of round and highly refractile cells; (*D*) GMM colony observed on day 16 of culture revealing the presence of large cells (megakaryocytes).

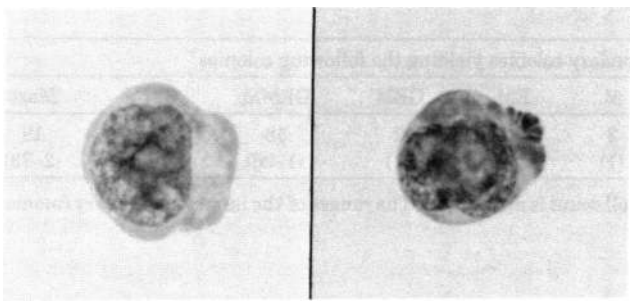

FIG. 2. Two representative cells constituting stem cell colonies. (May–Grunwald–Giemsa stain; ×1,500). The cells reveal immature nuclei and absence of cytoplasmic differentiation.

described elsewhere (11) could be distinguished from stem cell colonies by the refractile nature of individual cells and dark-to-black hue of the aggregates of mature mast cells. GMM colonies presented in Fig. 1*D* and characterized in a separate report (6) were distinguishable from stem cell colonies by the presence of large megakaryocytes in the colonies. When the stem cell colonies were individually lifted from the methylcellulose medium and stained with May–Grunwald–Giemsa, only blast cells such as those presented in Fig. 2 were seen. The blast cells were negative with benzidine, toluidine blue, and alcian blue–safranin staining and staining for acetylcholinesterase and myeloperoxidase.

Replating Experiments. Confirmation of the extensive self-renewing ability of the progenitors of the stem cell colonies and

Table 1. Results of replating experiments of stem cell colonies

Colony no.*	Total cell count	Number of secondary colonies per ½ primary colony†										Total replating efficiency, %
		S	B	GM	M	EM	GEM	GEMM	GMM	Mast	Total	
1	119	1	0	3	3	22	5	18	6	2	60	101
2	50	0	0	2	2	0	0	7	1	0	12	48
3	118	0	0	10	0	0	0	12	2	0	24	41
4	201	4	0	0	0	4	0	64	13	3	88	88
5	263	2	0	0	0	7	3	124	10	5	151	115
6	398	0	16	155	0	5	4	25	10	0	215	108
7	200	0	0	0	0	0	0	52	0	40	92	92
8	50	0	0	4	0	0	0	6	4	2	16	64
9	916	5	9	11	6	41	45	245	102	41	505	110
10	334	2	7	56	3	2	6	47	21	10	154	92
11	51	0	0	0	1	2	0	3	1	0	7	27
12	352	0	0	24	4	0	0	5	4	89	126	72
13	210	0	3	5	0	0	0	55	4	8	75	71
14	138	1	6	3	1	9	1	21	5	3	50	72
15	276	1	6	4	1	8	2	107	10	3	142	103
16	82	2	0	0	0	1	0	41	2	1	47	115
17	205	1	0	8	0	1	0	78	5	0	93	91
18	354	1	17	37	1	47	7	41	13	2	166	94
19	42	0	0	0	1	0	0	3	2	5	11	52
20	406	4	8	20	1	9	12	78	34	44	210	103
21	226	2	0	2	1	12	1	78	25	0	121	107
22	181	0	5	4	2	18	6	28	4	1	68	75
23	43	2	1	2	0	0	0	13	8	0	26	121
24	938	0	46	28	6	110	20	154	24	15	403	86
25	103	0	0	2	0	0	0	20	4	0	26	50
26	45	1	0	0	0	0	0	9	0	0	10	44
27	83	0	0	0	0	0	0	19	0	0	19	46
28	129	0	0	23	0	4	0	33	2	10	72	112
29	54	0	0	1	0	2	0	3	1	0	7	26
30	500	1	31	33	3	38	1	25	2	80	214	86
31	232	2	7	56	3	2	6	47	21	10	154	133
32	41	0	0	5	0	0	0	5	1	0	11	54
33	118	0	0	10	0	0	0	12	2	0	24	41
34	40	0	0	9	3	0	0	0	5	0	17	85
35	454	10	0	0	0	51	5	90	15	44	215	95
36	206	2	3	22	2	14	3	37	14	2	99	96
37	208	0	0	25	3	0	0	10	15	55	108	104
38	492	0	31	3	3	38	1	25	2	0	103	42
39	50	0	0	4	0	3	0	3	4	2	16	64
40	209	0	16	45	0	0	3	39	6	10	119	114
41	134	2	0	0	0	0	0	36	8	0	46	69
42	218	2	3	12	0	10	0	30	5	0	62	57
43	99	0	0	2	2	0	0	33	0	0	37	75
	Mean 223											Mean 80

* Stem cell colonies 1–14 were derived from marrow cells and the remaining colonies (nos. 15–43) were harvested from spleen cell cultures.

† S, stem cell colonies; B, erythropoietic bursts; GM, granulocyte–macrophage colonies; M, megakaryocyte colonies; EM, erythrocyte–megakaryocyte colonies; GEM, granulocyte–erythrocyte–macrophage colonies; GEMM, granulocyte–erythrocyte–macrophage–megakaryocyte colonies; GMM, granulocyte–macrophage–megakaryocyte colonies; Mast, mast cell colonies.

Table 2. Results of replating experiments of secondary stem cell colonies

Number of colonies analyzed	Total cell count	Number of secondary colonies yielding the following colonies*								
		S	B	GM	M	EM	GEM	GEMM	GMM	Mast
25	161 ± 68	6 (1–2)	1 (1)	13 (1–23)	2 (1)	3 (2–4)	2 (1)	18 (1–26)	8 (1–7)	19 (2–73)

The primary stem cell colonies were derived from spleen cell cultures. Total cell count is mean ± SD. The ranges of the number of tertiary colonies per ½ secondary stem cell colonies are given in parentheses.
* Abbreviations of the types of colonies are as in Table 1.

their ability to generate a large number of secondary colonies, including mixed hemopoietic colonies, was provided by replating experiments. The results of analyses of 43 stem cell colonies are presented in Table 1. Twenty out of the 43 colonies (47%) produced secondary stem cell colonies, revealing the self-renewal capacity of their progenitors. All colonies yielded secondary colonies, particularly GEMM colonies, with very high replating efficiencies. Some of the primary stem cell colonies revealed almost 100% replating efficiency of secondary colonies. Higher than 100% replating efficiencies seen in some colonies are probably due to technical errors in estimation of cell numbers.

Twenty-five secondary stem cell colonies were again individually picked and analyzed for the content of progenitors for tertiary colonies. The results are presented in Table 2. All colonies yielded tertiary colonies at various but high (mean = 28%) replating efficiencies. Five out of the 25 colonies again reproduced tertiary stem cell colonies. Of interest was the increase in the incidence of mast cell colonies over the first replating experiments.

When 10 tertiary stem cell colonies were analyzed for the progenitor content by replating studies, 1 colony reproduced a stem cell colony. Eight out of the 10 colonies revealed progenitors for differing classes of colonies, including GEMM colonies.

Analysis of CFU-S and Other Progenitor Incidences in Individual Stem Cell Colonies. Next we examined CFU-S and progenitors for hemopoietic colonies simultaneously in individual stem cell colonies. The individual colonies were lifted from the methylcellulose and, after washing, divided into two aliquots: one for analysis of CFU-S and the other for determination of the types and number of CFU in culture. The results are presented in Table 3. Nine out of 10 colonies produced spleen colonies in various numbers. Some colonies (e.g., colonies 2, 6, and 7) revealed exceedingly high efficiencies for spleen colony formation. If we accept the f number (seeding efficiency for CFU-S) to be 0.17 (13), the numbers of CFU-S in colonies 2, 6, and 7 are estimated to be 12, 47, and 29, respectively. These numbers corresponded closely to those of GEMM colonies in each stem cell colony. In addition, the total replating efficiencies in culture were 100%, 58%, and 42% for colonies 2, 6, and 7, respectively. These results indicated significant overlap between CFU-S and CFU-GEMM.

Analysis of CFU-S and Other Progenitor Incidences in GEMM Colonies. We also carried out identical analysis on GEMM colonies. The results summarized in Table 4 demonstrated very low incidences of CFU-S and other hemopoietic progenitors in the GEMM colonies. None of the 10 GEMM colonies produced stem cell colonies upon replating.

DISCUSSION

In this report, we have described a class of hemopoietic stem cells that reveal extensive self-renewal capacities and very high incidences for early hemopoietic progenitors. The self-renewal capabilities of the progenitors for the stem cell colonies were documented by demonstration of fourth-generation stem cell colonies in a total incubation period of 64 days. There were remarkably high incidences (occasionally 100%) of early hemopoietic progenitors such as CFU-GEMM and CFU-GMM in these colonies. These observations strongly indicated a very primitive nature of the precursors for the stem cell colonies. There has been indirect evidence for the heterogeneous nature and the hierarchy of hemopoietic stem cells (14–18). In this model, the early stem cells will be less committed to differentiation and have a higher self-renewal capacity than the stem cells in lower states in the hierarchy, which would be more committed to differentiation and have a lower self-renewal potential. The former population of stem cells has been named "pre CFU-S" cells by some investigators (15). The results presented here support this concept and suggest that the progenitors for the stem cell colonies are higher in the stem cell hierarchy than CFU-GEMM for the following reasons. (*i*) Cells constituting the stem cell colonies possess slower proliferative capacities than

Table 3. Results of CFU-S assays and replating experiments of individual stem cell colonies

Colony no.	Total cell count of primary colony	CFU-S content per ½ primary colony*	Number of secondary colonies per ½ primary colony†								
			S	B	GM	M	EM	GEM	GEMM	GMM	Mast
1	350	2	1	0	6	0	5	0	24	3	0
2	54	2	0	3	2	0	1	1	15	5	0
3	480	1	0	0	0	0	3	0	15	2	0
4	181	0	0	0	3	0	2	0	9	1	0
5	472	1	0	0	17	2	3	2	18	0	0
6	163	8	1	0	0	0	0	0	38	8	0
7	194	5	0	0	9	2	3	2	19	3	3
8	108	1	0	0	3	3	0	0	8	2	0
9	99	2	0	0	2	2	0	0	33	0	0
10	200	1	0	5	11	0	5	3	16	2	3

Spleen cells were cultured at 2×10^5 cells per ml.
* Background spleen colonies were 0 ± 0 (5 mice).
† Abbreviations of the types of colonies are as in Table 1.

Table 4. Results of CFU-S assays and replating experiments of individual GEMM colonies

Number of colonies	Total cell count of primary colonies	Number of primary GEMM colonies yielding the following colonies*									
		CFU-S	S	B	GM	M	EM	GEM	GEMM	GMM	Mast
10	220,000 ± 250,000	1 (1)	0	6 (1–8)	5 (1–17)	3 (2–3)	3 (1–5)	2 (1–2)	4 (1–4)	1 (1)	2 (1–5)

GEMM colonies were harvested from the same cultures as those for the stem cell colonies in Table 3. Total cell count is mean ± SD. The ranges of the number of secondary colonies per 2/3 primary GEMM colonies are given in parentheses.
* Abbreviations of the types of colonies are as in Table 1.

those forming GEMM colonies, because on the average only 223 cells were produced after 16 days of culture. In contrast, the macroscopic GEMM colonies attained an average size of 2.2×10^5 cells after 10 days in culture. This extremely slow proliferation of cells in stem cell colonies is in agreement with the general concept in hemopoiesis that the very early stem cells are quiescent in their proliferative activities. (*ii*) Whereas cells in GEMM colonies revealed the signs of terminal differentiation in 10 days of culture, those in stem cell colonies showed no such differentiation after longer periods in culture. (*iii*) Whereas stem cell colonies yielded a large number of secondary GEMM colonies, GEMM colonies did not produce stem cell colonies and yielded only a few secondary GEMM colonies in the replating experiments. The latter information on the frequency of secondary GEMM colonies in our experiments is in agreement with the results reported by Metcalf *et al.* (19) and Humphries (5). These results strongly indicated that the progenitors for the stem cell colonies are more primitive than CFU-GEMM.

The very low incidence of CFU-S in GEMM colonies in our analyses corresponds with that observed by Metcalf *et al.* (19). Our observations differ slightly from those of Humphries *et al.* (5), who have examined CFU-S incidences in GEMM colonies derived from marrow cells after incubation for 2 weeks in flask cultures. Although preliminary in nature, our simultaneous analyses of CFU-S and CFU-GEMM suggested that there may be significant overlaps between these progenitors. If CFU-S and CFU-GEMM indeed prove to represent overlapping populations of stem cells, the progenitors for the stem cell colonies may be located higher than CFU-S in the hierarchy of stem cell differentiation. Characterization of the stem cell colonies and their progenitors may provide further insight into the mechanisms of very early processes of stem cell renewal and commitment.

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