Different Stromal Cell Lines Support Lineage-selective Differentiation of the Multipotential Bone Marrow Stem Cell Clone LyD9

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Summary

An interleukin 3–dependent multipotential stem cell clone, LyD9, has been shown to generate mature B lymphocytes, macrophages, and neutrophils by coculture with primary bone marrow stromal cells. We report here that coculture with the cloned stromal cell lines PA6 and ST2 can support differentiation of LyD9 cells predominantly into granulocyte/macrophage colony-stimulating factor (GM-CSF)– and granulocyte (G)-CSF–responsive cells, respectively. However, these stromal cell lines were unable to support lymphopoiesis of LyD9 cells. The GM-CSF-dependent line, L-GM, which was derived from LyD9 cells cocultured with PA6 stromal cells, could differentiate into macrophages and granulocytes in the presence of GM-CSF. The L-GM line can further differentiate predominantly into neutrophils by coculture with ST2 stromal cells. The G-CSF-dependent line, L-G, which was derived from LyD9 cells cocultured with ST2 stromal cells, differentiated into neutrophils in response to G-CSF. Although the stromal cell–supported differentiation of LyD9 cells required the direct contact between LyD9 and stromal cells, a small fraction of LyD9 cells that were pretreated with 5-azacytidine could differentiate into neutrophils and macrophages without direct contact with stromal cells. These results indicate that different stromal cell lines support lineage-selective differentiation of the LyD9 stem cell and that 5-azacytidine treatment can bypass the requirement of direct contact with stromal cells, albeit with a lower frequency.

The totipotent bone marrow stem cell is defined as a cell that has the potential to reproduce itself as well as to give rise to cells of each lineage in the blood. During the course of differentiation, progeny of the totipotent stem cell gradually lose their capacity to differentiate into particular lineages, giving rise to multipotent stem cells that eventually become committed to a single cell lineage. Stem cells proliferate, differentiate, and mature in the presence of growth factors. The totipotent bone marrow stem cell has been difficult to maintain in vitro because a growth factor for the totipotent stem cell has not been isolated. On the other hand, molecular cloning of cDNAs encoding growth factors and lymphokines, and their large-scale production, have facilitated establishment of multipotent stem cell lines and progenitor cell lines cultured in vitro (1–3). IL-3 has been shown to support the growth of multipotent stem cells without induction of differentiation (4, 5). Several progenitor cell lines such as FDCP-mix A4 and 32D C13 can be induced to a specific lineage by a lineage-specific growth factor, although they generally proliferate and remain undifferentiated in media containing IL-3 (6, 7).

Another regulatory element of hematopoiesis is a stromal cell network in the bone marrow. This idea is supported by the finding that bone marrow stromal cells are a necessary requirement for a long-term bone marrow culture system, in which hemopoiesis of various cell lineages can be sustained without added growth factors (8, 9). Interestingly, the hemopoietic cells must be in direct contact with the stromal cells for proliferation and differentiation (10). Recently, stromal cell lines such as PA6 and ST2 (11–13) were shown to support differentiation of primary bone marrow cells into distinct lineage cells (14). Such functional diversity of stromal cells could be ascribed to different growth factors produced or differential cell adhesion molecules expressed by each stromal cell. Combination of appropriate growth factors derived from stromal cells and growth factor receptors expressed by progenitor cells might be a key element for the lineage-specific differentiation. In addition, an appropriate pair of cell adhe-

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sion molecules expressed on stromal and stem cells could be required for not only proliferation signal triggering but also specific growth factor/receptor expression.

The above experiments using primary bone marrow cells do not allow to distinguish (*a*) that a stromal cell line selectively expanded progenitors that were already committed and (*b*) that the stromal cell line supported differentiation of a multipotential stem cell in either stochastic or directive manner. It is therefore interesting to know whether different lineage cells differentiate from a single stem cell by coculture with different stromal cell lines. To address this question, we took advantage of an IL-3-dependent stem cell clone, LyD9. This clone has been shown to differentiate into multiple lineage cells by coculture with bone marrow stromal cells: B lymphocytes, neutrophils, and macrophages (15–18). Differentiation of LyD9 cells requires direct contact with primary stromal cells (16). We have found that two stromal cell lines, PA6 (11) and ST2 (13), are able to support differentiation of the LyD9 stem cell line predominantly into two different lineage cells: granulocyte/macrophage-colony-stimulating factor (GM-CSF)[1]-responsive and granulocyte CSF (G-CSF)-responsive cells, respectively.

Materials and Methods

Cell Lines. The procedure for the establishment and detailed characterization of the LyD9 cell clone has been described (15). It grows in RPMI 1640 supplemented with 10% (vol/vol) FCS, 50 μM 2-ME, 2 mM L-glutamine, and 5% WEHI 3B–conditioned medium. The LyD9 cell line was recloned in our laboratory. The ST2-S10 (ST2) and MC3T3-G2/PA6 (PA6) cell lines were established from the bone marrow of NZB mice and the preadipose tissue of calvaria of newborn mice, respectively (11, 13).

5-Azacytidine Treatment of LyD9 Cells. 5-Azacytidine treatment of LyD9 cells before coculture was carried out basically according to Castellazzi et al. (19). 5-Azazcytidine was added to 5 μg/ml in a 5-ml culture of LyD9 cells (10^6 cells/ml). The concentration of 5-azacytidine was diluted to half by the addition of an equal volume of fresh medium after 24 h of the culture. 2 d later, cells were washed to remove 5-azacytidine, cultured for 10 d, and used for induction.

Induction of Differentiation with Stromal Cell Lines. LyD9 cells (10^6) or 5-azacytidine-treated LyD9 cells (10^6) were cocultured on either ST2 or PA6 cells. To test the requirement of direct contact, cells were separated from stromal layers by a membrane chamber (pore size, 0.45 μm) (Millipore Continental Water Systems, Bedford, MA). The condition for coculture is the same as that for maintenance of LyD9 cells, except that WEHI 3B–conditioned medium is reduced to 0.7% for the minimal maintenance of the LyD9 cells. After a 1-d coculture, floating cells were removed, and adhering cells were further cultured for the induction of differentiation. PA6 and ST2 stromal cell layers were changed several times during the coculture, and the culture supernatant (0.7%) of WEHI 3B cells was supplied in coculture every third day. Every week thereafter, the cocultured LyD9 cells were harvested and washed, and then 10^6 cells were transferred to wells containing one of the following CSFs: GM-CSF (20 U/ml), macrophage CSF (M-CSF) (10% of the L cell culture supernatant), and G-CSF (100 U/ml). Viable cells were counted 10 d after the transfer to growth factor–containing medium.

Establishment of the L-GM Line. LyD9 cells were maintained on a monolayer of PA6 stromal cells in the presence of 0.7% of WEHI 3B culture supernatant. 4 wk later, cocultured LyD9 cells were transferred to media containing GM-CSF (20 U/ml). GM-CSF-responding cells were maintained for >2 mo, and their morphology, cytochemical staining, surface phenotypes, and responsiveness to the growth factors were characterized. The L-GM line was maintained in media containing IL-3.

Establishment of the L-G Line. LyD9 cells pretreated with 5-azacytidine were cocultured on ST2 stromal cells as described above, and transferred after 4 wk to media containing G-CSF (100 U/ml). G-CSF-responding cells obtained were transferred to the medium containing a low concentration of IL-3 after we confirmed that cells became nonviable without G-CSF. Morphological and cytochemical analyses of L-G cells were performed after culturing in G-CSF for 10 d.

Antibodies and Flow Cytometry. The surface phenotype of L-GM cells was determined by immunofluorescence staining with antibodies as follows: FITC-labeled polyclonal goat anti-mouse IgM (Cappel Laboratories, Malvern, PA); FITC-labeled monoclonal anti-Ia (Meiji Institute of Health Science, Kanagawa, Japan); and 6B2 (anti-B220) (20), 2.4G2 (anti-Fcγ receptor) (21), M1/70 (anti-MacI) (22), and F4/80 (23) were detected by biotin-conjugated goat anti-rat IgG and FITC-avidin. FACS® (Becton Dickinson & Co., Mountain View, CA) was used for the analysis. Rabbit anti-GM-CSF antibody was provided by Toray Industries, Inc. (Tokyo, Japan). One unit of the antibody neutralizes 10 U of GM-CSF.

Characterization of LyD9 Cells and their Derivatives. May-Grünwald-Giemsa staining was used for morphological study. Staining of α-naphthylbutyrate and naphthol AS-D chloroacetate esterase was done by a commercial kit (Muto Chemical Company, Tokyo, Japan). To determine the phagocytic activity, L-GM cells were stimulated with 100 ng/ml PMA (Sigma Chemical, Co., St. Louis, MO) for 3 h. Cells were washed with the culture medium, and incubated with latex beads (0.8 μm; Sigma Chemical Co.) for 6 h. After removal of the medium, cells were washed several times with the medium without FCS, stained with May-Grünwald-Giemsa, and observed by a phase contrast microscope (×1000).

Growth Assay of L-GM and L-G. Proliferative responses of L-GM and L-G cells to each growth factor were determined as described previously (17). L-GM and L-G cells (4×10^4 cells/200 μl) were incubated in microtiter wells in the culture medium containing the growth factors indicated. The cultures in duplicate were incubated at 37°C. Cell proliferation was measured by [^{3}H]thymidine uptake during the last 6 h of the 48 h incubation.

Growth Factors. Purified human rIL-2 was provided by Takeda Pharmaceutical Company (Osaka, Japan). IL-3 was obtained from culture supernatant of WEHI 3B cells. IL-4 and IL-5 were obtained from X63Ag8 myeloma cells transfected with IL-4 and IL-5 cDNA, respectively (24–26). Purified human rIL-6 (27) was provided by Dr. T. Hirano (Osaka University). Murine rGM-CSF (28) was provided by Dr. T. Sudo (Biomaterials Research Institute Co., Kanagawa, Japan). Purified human rG-CSF (29) and human M-CSF were provided by Dr. S. Nagata (Osaka Bioscience Institute). PA6 cells (10^6) were layered on a plate in the same medium as that for LyD9 cells, except for the absence of IL-3. LPS (1 μg/ml; Sigma Chemical Co.) was added during last 3 d of the culture. The culture supernatant of PA6 cells was collected on day 5, and concentrated by a nitrocellulose membrane filter (0.45 μm; YM10; Amicon Corp., Danvers, MA).

[1] *Abbreviation used in this paper:* GM-CSF, granulocyte/macrophage colony-stimulating factor.

Results

Generation of Myeloid Cells from LyD9 Cells by Coculture with Stromal Cell Lines. Differentiation of LyD9 cells by coculture with the stromal cell line PA6 was investigated by morphological and cytochemical studies. A considerable proportion (~0.1%) of LyD9 cells cocultured with PA6 cells for 4 wk contained myeloid-like nuclei and became positive for myeloid-specific enzymes such as α-naphthylbutyrate esterase and myeloperoxidase (Fig. 1). The proportion of myeloid cells increased when LyD9 cells were pretreated with 5-azacytidine. Similar experiments using ST2 stromal cells did not yield a detectable number of myeloid cells. However, 5-azacytidine-treated LyD9 cells cocultured with ST2 cells produced a considerable portion (~0.1%) of myeloid cells (data not shown).

To characterize differentiated cells, an aliquot (10^6 cells) of LyD9 cells cocultured with the stromal cell line (either PA6 or ST2) was transferred every week to media containing either GM-CSF, G-CSF, or M-CSF. Viable cells were scored 10 d after the transfer to growth factor–containing media. As shown in Table 1, 4-wk coculture of LyD9 cells with PA6 cells generated a significant number of GM-CSF-responding cells and a small number of G-CSF-responding cells. Pretreatment of LyD9 cells with 5-azacytidine enhanced and accelerated the induction of LyD9 cells into the myeloid cells by PA6 stromal cells. By contrast, when PA6 cells were replaced by ST2 cells, no GM-CSF-responding cells were generated, whereas a small number of G-CSF-responding cells were produced (Table 1). 5-Azacytidine-treated LyD9 cells differentiated into G-CSF-responding cells more quickly and more efficiently by coculture with ST2 cells. Similar experiments with PA6 or ST2 cells did not give rise to M-CSF-responsive cells even after 4 wk of coculture.

Establishment of GM-CSF- and G-CSF-dependent Cell Lines from LyD9 Cells. LyD9 cells that had been cocultured with PA6 stromal cells were cultured in media containing GM-CSF for >4 wk, and thereafter, grown cells were maintained as a cell line called L-GM. Similarly, the L-G line was generated from 5-azacytidine-treated LyD9 cells by coculture with the ST2 stromal line, followed by subsequent selection in G-CSF-containing medium. L-GM and L-G cells grew in the presence of GM-CSF and G-CSF, respectively (Fig. 2). However, the growth response of L-G cells to G-CSF was tran-

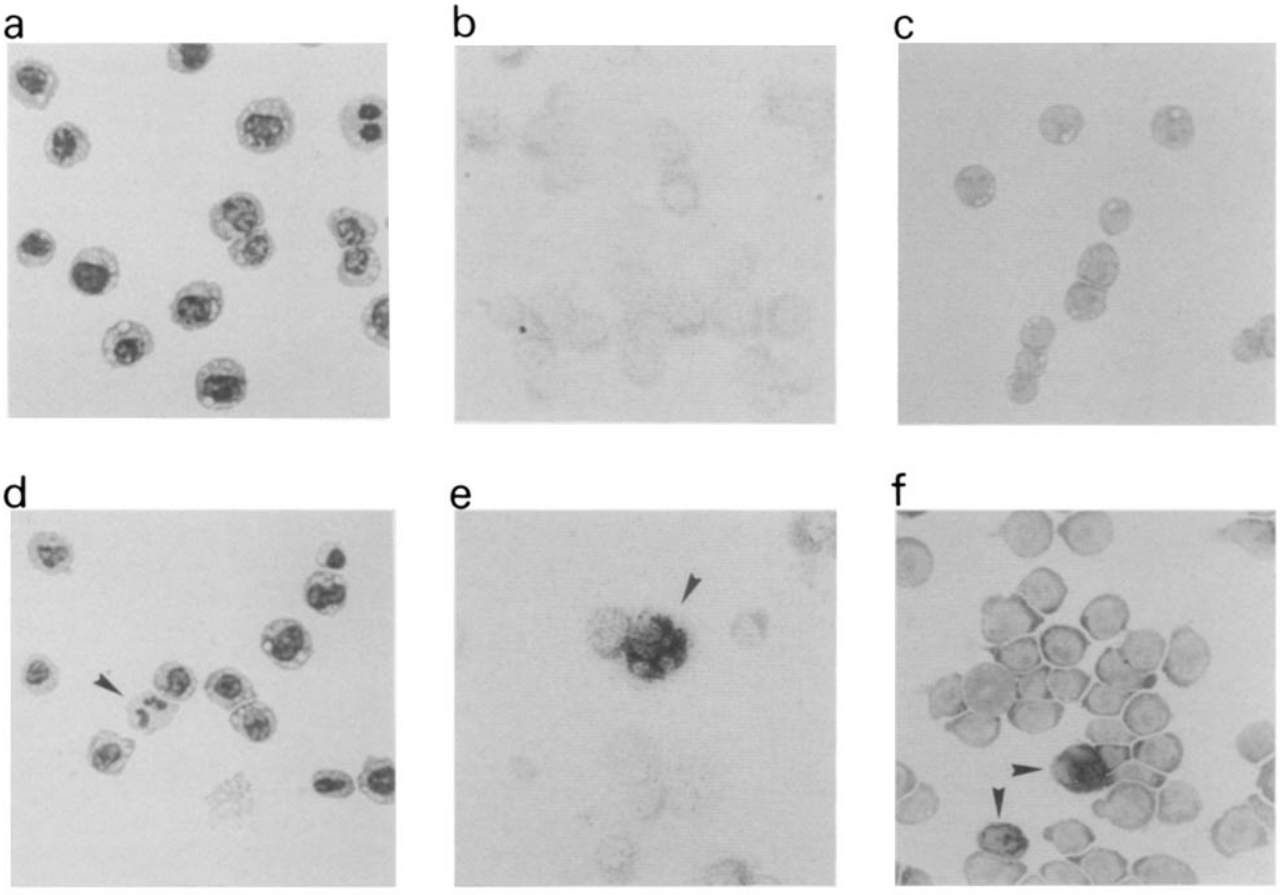

Figure 1. Morphological and cytochemical analyses of original LyD9 and cocultured LyD9 cells. Original LyD9 (*a–c*) and cocultured LyD9 (*d–f*) cells were analyzed morphologically by May-Grünwald-Giemsa staining (*a* and *d*), and cytochemically with α-naphthylbutyrate esterase (brown) and naphthol (AS-D) chloroacetate esterase (blue) (*b* and *e*), and myeloperoxidase (*c* and *f*). Pictures of LyD9 cells cocultured with PA6 cells for 4 wk are presented representatively. Arrowheads indicate typical myeloid cells (×200).

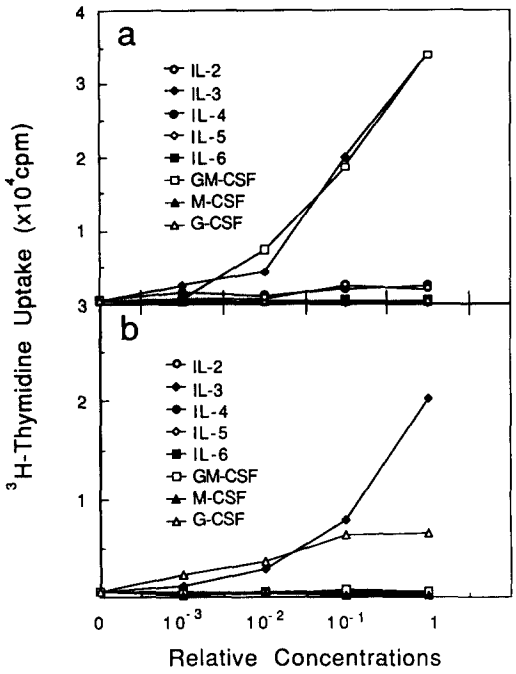

Figure 2. Growth stimulation of L-GM and L-G cells by growth factors. [^{3}H]Thymidine incorporation into L-GM (*a*) and L-G (*b*) cells was measured in the presence of growth factors indicated. Concentrations of growth factors indicated on the horizontal line as 1 were as follows: IL-2, 10 U/ml human rIL-2; IL-3, 20% of WEHI-3B culture supernatant; IL-4 and IL-5, 20% of culture supernatant of X63Ag8 myeloma cells transfected with IL-4 and IL-5 cDNAs, respectively; IL-6, 100 U/ml human rIL-6; GM-CSF, 50 U/ml rGM-CSF; M-CSF, 100 ng/ml rM-CSF; and G-CSF, 100 U/ml rG-CSF. These concentrations of growth factors give maximal growth response to each positive control cell. L-GM and L-G cells cultured without growth factors (indicated on the horizontal line as 0) incorporated 668 and 488 cpm, respectively. The data represent the mean of duplicate cultures.

sient. L-GM and L-G cells proliferated in response to IL-3, thus, they were maintained in IL-3-containing media. No other growth factors examined could induce proliferation of L-GM and L-G cells.

Cytochemical studies were performed to characterize the differentiation properties of the L-GM and L-G lines cultured in GM-CSF and G-CSF, respectively (Fig. 3). About one-third of the L-GM cells that had been cultured in the presence of GM-CSF had bent nuclei with a small nuclear-cytoplasmic ratio (Fig. 3 *a*). L-GM cells also were positive for α-naphthylbutyrate esterase and myeloperoxidase (Fig. 3, *b* and *c*). Only a few neutrophils (<0.01%) were found in L-GM cells. L-GM cells showed phagocytic activity when stimulated with PMA (Fig. 3 *d*). A few percent of the L-GM cells appeared as mature macrophages even when cultured in IL-3 for 1 mo. By contrast, the majority of L-G cells that had been cultured for 10 d in the presence of G-CSF became neutrophils with typical lobulated nuclei and naphthol AS-D chloroacetate esterase–positive granules (Fig. 3, *e* and *f*).

The surface phenotype of L-GM cells was consistent with that of myeloid lineage cells. As shown in Fig. 4, L-GM cells expressed more Mac 1, F4/80, and Ia antigens than LyD9 cells. Expression of the B220 and Fcγ receptor antigens on L-GM was as high as that on LyD9 cells. L-GM cells did not express IgM. Surface antigens of the L-G line could not be studied because of the strong auto-fluorescence of this line.

L-GM Cells Can Differentiate into Neutrophils. When L-GM cells were further cocultured with ST2 cells in the presence of 25 μg/ml LPS for 1 mo, ~30% of the cocultured L-GM cells had the morphological and cytochemical properties of neutrophils (Fig. 5). Similar experiments with another GM-CSF-dependent derivative of LyD9, K-GM (17), yielded similar results (data not shown). Since monocyte/macrophage lineage cells appeared in L-GM and K-GM cells that had been cultured with GM-CSF, these results indicate that both L-GM

Table 1. *Generation of GM-CSF and G-CSF-responding Cells by Coculture of LyD9 Cells with Stromal Cell Lines*

Pretreatment of LyD9 cells	Period of coculture	PA6		ST2	
		+ GM-CSF	+ G-CSF	+ GM-CSF	+ G-CSF
	wk				
None	2	0	0	0	0
	3	<100	<100	0	<100
	4	6×10^5	700	0	500
5-Azacytidine	2	10^3	300	0	200
	3	4×10^6	4×10^3	0	8×10^3
	4	5×10^7	6×10^4	500	2.5×10^5

LyD9 cells with or without 5-azacytidine pretreatment were cocultured with either ST2 or PA6 stromal cell line for the weeks indicated, and 10^6 LyD9 cells were transferred to the medium containing GM-CSF (20 U/ml) or G-CSF (100 U/ml). 10 d after the transfer, viable cells were counted. Same sets of the experiment with M-CSF or without growth factor addition did not give rise to any survived cells (data not shown). Aliquots taken at 1-wk coculture did not yield any viable cells.

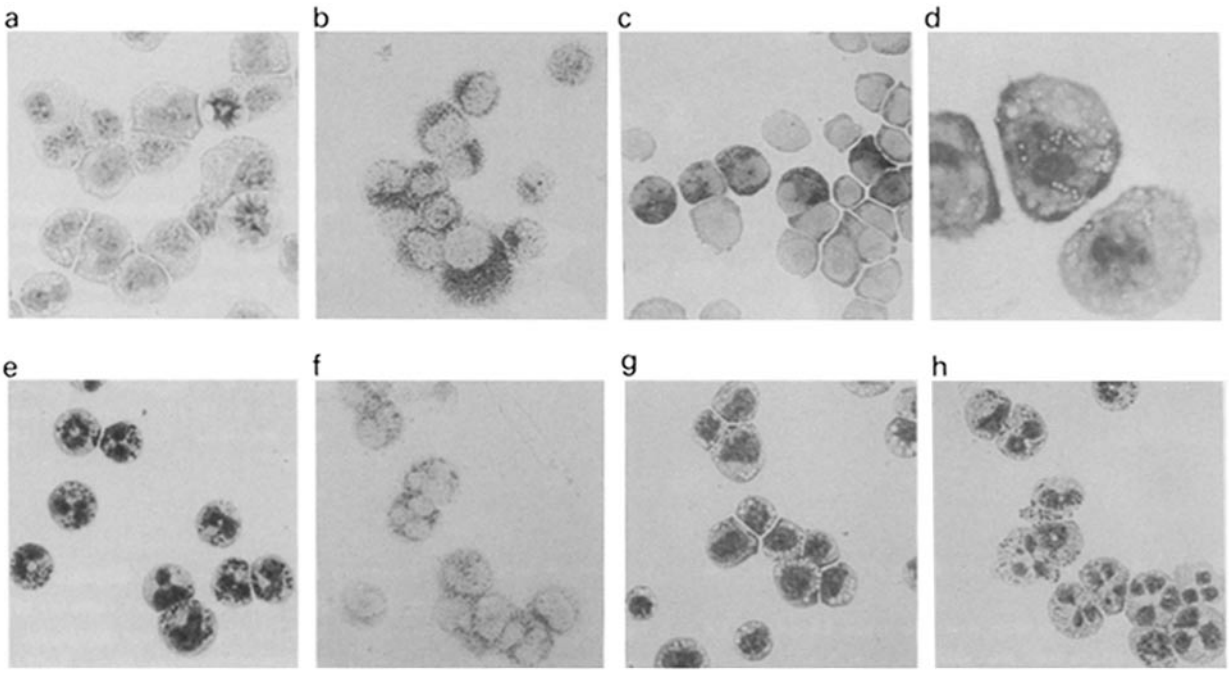

Figure 3. Morphological and cytochemical analyses of differentiated L-GM and L-G cell lines. May-Grünwald-Giemsa staining of L-GM (*a*) and L-G (*e*) cell lines are shown. L-GM (*b*) and L-G cell lines (*f*) were analyzed cytochemically with both α-naphthylbutyrate esterase (brown) and naphthol AS-D chloroacetate esterase (blue). Expression of myeloperoxidase (*c*) and functional phagocytosis (*d*) of latex beads in the L-GM cell line were examined. Morphology of the L-G cell line maintained with IL-3 (5% of WEHI 3B–conditioned medium) for 1 mo is shown in *g*. The IL-3-cultured L-G cells were transferred to the medium containing G-CSF (100 U/ml), cultured for 10 d, and the morphology was examined (*h*).

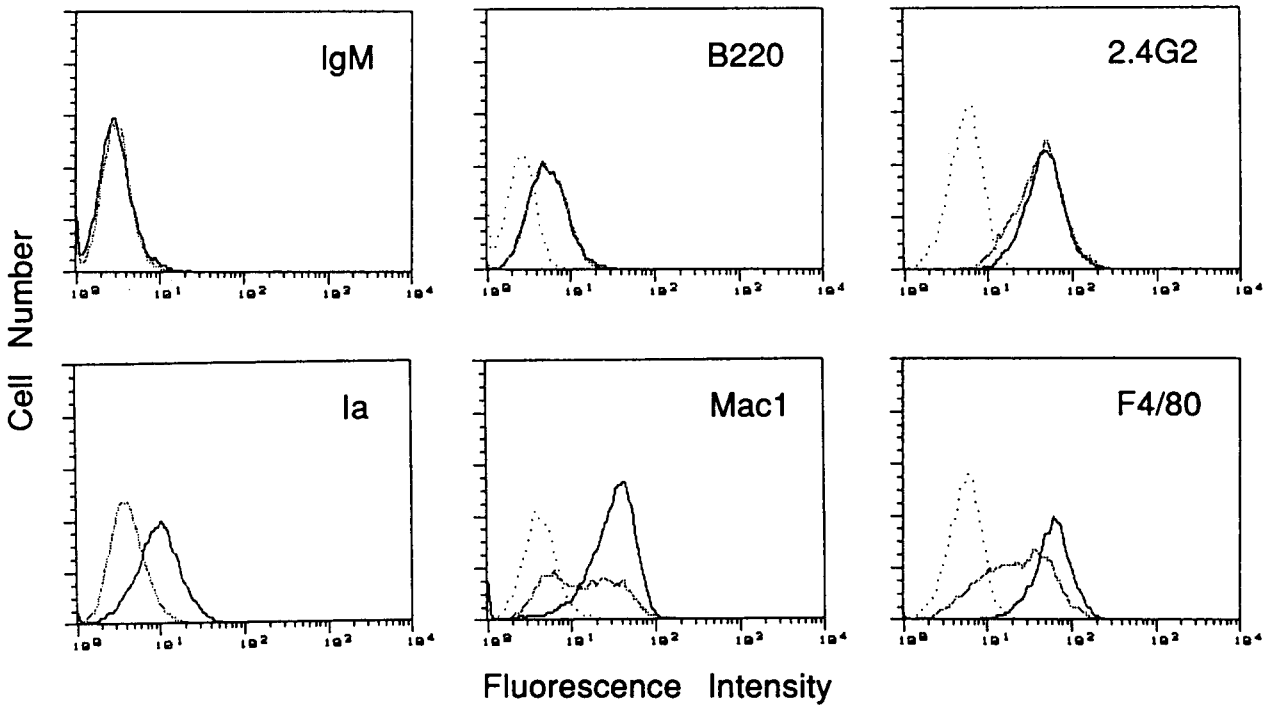

Figure 4. Surface phenotype of L-GM cells. Expression of surface antigens on L-GM cells was examined by immunofluorescence staining, and flow cytometry with mAbs was indicated (see Materials and Methods). Staining profiles of noninduced LyD9 cells were also included (·····). Negative controls were L-GM cells stained with the second-step reagent only (– – –). Data of L-GM cells were also shown (———).

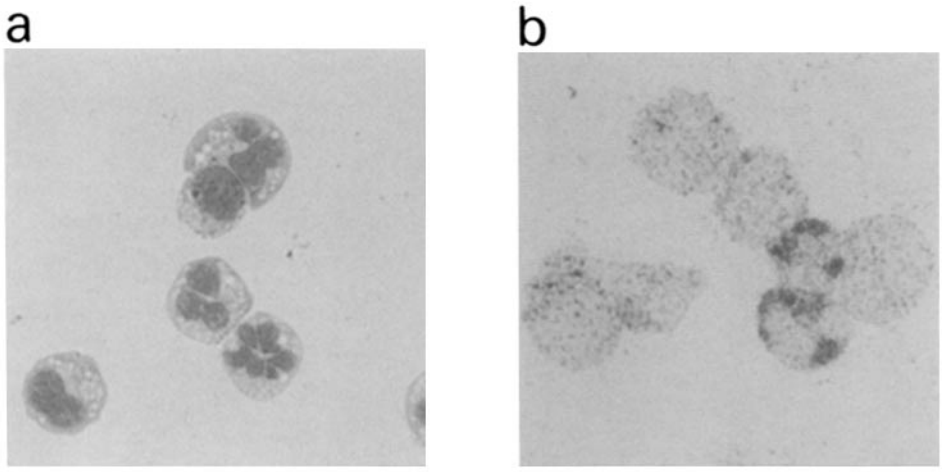

Figure 5. Differentiation of L-GM cells into granulocytes. L-GM cells were cocultured with ST2 cells in the presence of 25 μg/ml LPS. After 1 mo of the coculture, the morphology of induced L-GM cells was analyzed by May-Grünwald-Giemsa staining (*a*), and their expression of α-naphtylbutyrate and naphthol AS-D chloroacetate esterase were examined cytochemically (*b*) (×400).

and K-GM cell lines are progenitors of neutrophils and macrophages.

G-CSF Induces Differentiation and Eventual Death of L-G Cells. L-G cells proliferated and differentiated into neutrophils in the presence of G-CSF, but gradually ceased to proliferate and the majority of them died within 2 wk. L-G cells grew almost indefinitely when cultured in medium containing IL-3. However, the morphological characteristics of neutrophils disappeared in the IL-3 containing medium in 1 mo (Fig. 3 *g*). Neutrophil characteristics such as lobular nuclei were regained by transferring L-G cells into G-CSF-containing medium (Fig. 3 *h*). The recovery of L-G cells is not due to the reversal of maturation but rather to the growth of the remaining immature L-G cells, because careful limiting dilution experiments indicate that mature granulocyte clones derived from L-G cells by G-CSF only rarely proliferated in response to IL-3. It is likely that not all the L-G cells mature within 2 wk in the G-CSF-containing media, and surviving immature L-G cells proliferated again in the presence of IL-3.

Direct Contact with Stromal Cells Is Required for Myelopoiesis from LyD9 Cells. We then tested whether direct contact of LyD9 cells to the stromal cell lines is required for myelopoiesis, as previously described for lymphopoiesis from LyD9 cells by coculture with primary bone marrow stromal cells (16). We cultured LyD9 cells with the stromal cell lines (PA6 or ST2) in two chambers separated by a nitrocellulose membrane (0.45 μm). Each week during this coculture, an aliquot of LyD9 cells (10^6 cells) was transferred to media containing either GM-CSF or G-CSF, and cell numbers were determined 10 d later. As shown in Table 2, no GM-CSF-responding cells and very few, if any, G-CSF-responding cells appeared even after 4 wk of the coculture of LyD9 cells with the stromal cell lines without direct contact. These results clearly indicate that direct contact with stromal cells is required for differentiation of LyD9 cells into myeloid cells.

5-Azacytidine-treated LyD9 Cells Can Differentiate without Direct Contact with Stromal Cells. In contrast, PA6 cells could induce 5-azacytidine-treated LyD9 cells to differentiate into

Table 2. *Generation of GM-CSF and G-CSF-responding Cells by the Culture of LyD9 Cells and Stromal Cell Lines in Separate Membrane Chambers*

Pretreatment of LyD9 cells	Period of coculture	PA6		ST2	
		+ GM-CSF	+ G-CSF	+ GM-CSF	+ G-CSF
	wk				
None	2	0	0	0	0
	3	0	<100	0	<100
	4	0	<100	0	<100
5-Azacytidine	2	<100	200	0	<100
	3	10^5	300	0	200
	4	10^6	1.5×10^3	<100	2.5×10^3

Experiments were done as described in Table 1, except that LyD9 and stromal cells were cultured in separate membrane chambers (pore size, 0.45 μm).

GM-CSF-responding cells without direct contact (Table 2). Much smaller but significant numbers of G-CSF-responding cells were also induced. Under the same conditions, ST2 stromal cells could support differentiation of G-CSF-responding cells but not of GM-CSF-responding cells. The 5-azacytidine treatment of LyD9 cells before the coculture allowed generation of GM-CSF- and G-CSF-responding cells without direct contact with stromal cells, but the lineage specificity of the stromal cells was not altered by this treatment.

We then tested whether the culture supernatant of PA6 cells is able to induce differentiation of LyD9 cells into myeloid cells. As shown in Table 3, 5-azacytidine-treated LyD9 cells were induced to differentiate into GM-CSF-dependent cells by preincubation with supernatant from PA6 cells. After screening with known growth factors, we found that GM-CSF itself could replace the supernatant of PA6 cells. Anti-GM-CSF antibody could inhibit proliferation of GM-CSF-responding cells from 5-azacytidine-treated LyD9 cells by the PA6 supernatant. GM-CSF mRNA was found in PA6 and ST2 cells by PCR (data not shown).

Table 3. *GM-CSF Can Induce GM-CSF-responsive Cells from Azacytidine-treated LyD9 Cells*

Incubation with:		
Growth factors	Anti-GM-CSF antibody	GM-CSF-responding cells
	U/ml	
PA6 supernatant		
50%	0	6.8×10^5
100%	0	7.0×10^5
GM-CSF		
5 U/ml	0	3.7×10^5
10 U/ml	0	7.2×10^5
PA6 supernatant		
50%	0.4	0
100%	0.4	0
GM-CSF		
5 U/ml	4	0
10 U/ml	4	0
None	0	0

5-Azacytidine-treated LyD9 cells were preincubated with various amounts of growth factors with or without anti-GM-CSF antibody. The culture supernatant of PA6 was concentrated to 10% of its original volume, and added to the culture as indicated by final concentrations of the original. WEHI 3B–conditioned medium was added to 0.7% to all the cultures to maintain the viability of LyD9 cells. After 1 wk of preincubation, 2×10^6 5-azacytidine-treated LyD9 cells were transferred to the medium containing 20 U/ml GM-CSF. Total viable cells were counted after a 10-d culture in the presence of GM-CSF. LyD9 cells without 5-azacytidine pretreatment gave rise to neither GM-CSF- nor G-CSF-responding cells at the same condition as above.

We estimated the frequency of GM-CSF-responding cells in 5-azacytidine-treated LyD9 cells by culturing these cells in methylcellulose. As shown in Table 4, only two to six cells out of 10^6 5-azacytidine-treated LyD9 cells formed colonies in the presence of GM-CSF, whereas 55–80% of the LyD9 cells formed colonies in the presence of GM-CSF, whereas 55–80% of the LyD9 cells formed large colonies in the presence of IL-3. It is, therefore, likely that the 5-azacytidine treatment of LyD9 cells produced a small fraction of cells that could bypass at least a part of the function of PA6 stromal cells.

Discussion

We have established a well-defined in vitro system for myeloid differentiation from a multipotent stem cell clone. This system includes the stem cell clone LyD9 and the cloned stromal cell lines PA6 and ST2. LyD9 cells cocultured with the PA6 and ST2 stromal cell lines differentiated predominantly into GM-CSF- and G-CSF-responsive cells, respectively. The GM-CSF-responsive (L-GM) and G-CSF-responsive (L-G) lines were obtained by coculture of LyD9 cells with PA6 and ST2 stromal cells, respectively. L-GM cells differentiated into macrophages and less frequently into neutrophils in response to GM-CSF, while L-G cells differentiated into neutrophils in response to G-CSF. The L-GM line further differentiated into neutrophils by coculture with the ST2 stromal cell line. Although 5-azacytidine-treatment of LyD9 cells stimulated the differentiation frequency into myeloid cells, the lineage specificity determined by the stromal cell lines did not change. Coculture of LyD9 cells with the PA6 and ST2 stromal lines did not generate either B lymphocytes or M-CSF-responsive

Table 4. *Frequency of GM-CSF-responding Cells in 5-Azacytidine-treated LyD9 Cells*

	No. of colonies/10^6 LyD9 cells	
Growth factors	Total	Large colonies
None	0	0
	0	0
	0	0
	0	0
GM-CSF (30 U/ml)	2	0
	6	4
	2	1
	4	4
IL-3 (30 U/ml)	5.5×10^5	5.5×10^5
	8.1×10^5	8.1×10^5

5-Azacytidine-treated LyD9 cells (10^6) were cultured in 2.2% methylcellulose containing RPMI 1640–20% FCS media (1 ml) with growth factors indicted at 37°C under 7.5% CO_2. 7 d after incubation, the number of cell colonies (≥30 cells) was counted under a phase-contrast microscope. Colonies containing >200 cells were scored as large colonies.

cells, even when various growth factors such as IL-4, IL-5, IL-7, and M-CSF were added. Takeda et al. (18) reported that LyD9 cells can be induced to B lymphocytes by coculture with the RPO.10 stromal cell line. These results indicate that different stromal cell lines allow the selective growth of particular hematopoietic lineages from a stem cell line, although we cannot determine whether commitment was made in a stochastic or directive manner.

The lineage specificity determined by the stromal lines may depend on cell adhesion molecules and growth factors produced by the stromal cells. Since both PA6 and ST2 cells are able to produce GM-CSF and M-CSF (our unpublished data), other growth factors may be involved in determination of different lineage specificity by the PA6 and ST2 stromal cells. ST2 stromal cells can generate G-CSF-responsive cells from L-GM as well as LyD9 cells. ST2 stromal cells may generate G-CSF-responsive cells from LyD9 cells theoretically via GM-CSF-responsive cells. However, the efficiency of generation of G-CSF-responsive cells from the L-GM line by ST2 cells was not high enough to explain the absence of GM-CSF-responsive cells in ST-2-cocultured LyD9 cells. Thus, the pathway involved in generating G-CSF-responsive cells by ST2 cells does not seem to overlap with that generating GM-CSF-responsive cells by PA6 cells. Another type of stromal cells should be responsible for generation of M-CSF-responsive cells like LS-1 cells (17).

In our previous system, using a primary culture of bone marrow stromal cells, direct contact with stromal cells was required for the proliferation and differentiation of LyD9 cells (16). This conclusion was supported in the present system using stromal cell lines because the membrane chamber experiments practically abolished the generation of GM-CSF- and G-CSF-responding cells (Table 2). However, the present study has shown that the cell contact requirement is circumvented by 5-azacytidine treatment of LyD9 cells, though the numbers of GM-CSF-responding cells decreased considerably without the contact (Tables 2 and 3). 5-Azacytidine treatment is known to activate several important genes involved in differentiation (30, 31). Since 5-azacytidine treatment of LyD9 cells allowed generation of GM-CSF-responding cells, it is likely that stromal cell contact induces transcription of a set of genes that are activated by 5-azacytidine treatment. As the 5-azacytidine-treated LyD9 cells gave rise to GM-CSF-responsive cells, genes activated by this treatment may include those for (*a*) the GM-CSF receptor, (*b*) the cytoplasmic signal transduction pathway of the GM-CSF receptor, and (*c*) the regulatory proteins for target genes activated by GM-CSF. In fact GM-CSF receptor expression is required at least because the GM-CSF receptor was not expressed on the LyD9 cells by the binding assay (32). Comparison of these two cells, i.e., LyD9 cells and GM-CSF-responsive derivatives, will provide important clues to understanding which genes need to be activated for the lineage commitment.

We previously reported the establishment of several different myeloid progenitor cells derived from the LyD9 cell (17). The K-5 cell line proliferates without differentiation in the presence of either IL-5 or IL-3 (33). K-5 cells differentiate into neutrophils and macrophages by coculture with PA6 stromal cells. K-GM and L-GM cells proliferate in the presence of either IL-3 or GM-CSF. Both cells can be induced to differentiate into neutrophils when cocultured with ST2 stromal cells. LS-1 cells grow in the presence of any of the four growth factors: IL-3, GM-CSF, G-CSF, and M-CSF (17). L-G cells grow in the presence of IL-3 and differentiate in response to G-CSF. These derivatives can be classified into two groups: (*a*) those that differentiate by coculture with stromal cells (K-5, K-GM, and L-GM), and (*b*) those (LS-1 and L-G) that can be induced to differentiate by growth factors.

Transfer of several IL-3-dependent cell lines from IL-3 to a different CSF results in the induction of terminal differentiation. FDCP-mix (4, 7), NFS-60 (34), and 32D C13 (6) cell lines respond to GM-CSF, erythropoietin, and G-CSF, respectively. All these lines are distinct from LyD9 but rather similar to its committed derivatives like LS-1, L-GM, K-GM, and L-G cells because the above three lines are committed to either myeloid or erythroid lineages, whereas LyD9 cells are not committed to either the myeloid or the lymphoid lineage (17). Coculture of LyD9 cells with PA6 and ST2 stromal cells will thus provide a useful system to dissect molecular mechanisms of commitment to the myeloid lineage.

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References

1. Dexter, T.M., and E. Spooncer. 1987. Growth and differentiation in the hemopoietic system. *Annu. Rev. Cell Biol.* 3:423.
2. Metcalf, D. 1989. The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. *Nature (Lond.).* 339:27.
3. Nicola, N.A. 1989. Hemopoietic cell growth factors and their receptors. *Annu. Rev. Biochem.* 58:45.
4. Dexter, T.M., J. Garland, D. Scott, E. Scolnick, and D. Metcalf. 1980. Growth of factor-dependent hemopoietic precursor cell lines. *J. Exp. Med.* 152:1036.
5. Ihle, J.N., L. Rebar, J. Keller, J.C. Lee, and A.J. Hapel. 1982. Interleukin 3: possible roles in the regulation of lymphocyte differentiation and growth. *Immunol. Rev.* 63:5.
6. Valtieri, M., D.J. Tweardy, D. Caracciolo, K. Johnson, F. Mavilio, S. Altmann, D. Santoli, and G. Rovera. 1987. Cytokine-dependent granulocytic differentiation: regulation of proliferative and differentiative responses in a murine progenitor cell line. *J. Immunol.* 138:3829.
7. Heyworth, C.M., T.M. Dexter, O. Kan, and A.D. Whetton. 1990. The role of hemopoietic growth factors in self-renewal and differentiation of IL-3-dependent multipotential stem cells. *Growth Factors.* 2:197.
8. Dexter, T.M., T.D. Allen, and L.G. Lajtha. 1977. Conditions controlling the proliferation of haemopoietic stem cells *in vitro*. *J. Cell. Physiol.* 91:335.
9. Whitlock, C.A., and O.N. Witte. 1982. Long-term culture of B lymphocytes and their precursors from murine bone marrow. *Proc. Natl. Acad. Sci. USA.* 79:3608.
10. Bentley, S.A. 1981. Close range cell:cell interaction required for stem cell maintenance in continuous bone marrow culture. *Exp. Hematol. (NY).* 9:308.
11. Kodama, H-A., Y. Amagai, H. Koyama, and S. Kasai. 1982. Hormonal responsiveness of a preadipose cell line derived from newborn mouse calvaria. *J. Cell. Physiol.* 112:83.
12. Nishikawa, S-I., M. Ogawa, S. Nishikawa, T. Kunisada, and H. Kodama. 1988. B lymphopoiesis on stromal cell clone: stromal cell clones acting on different stages of B cell differentiation. *Eur. J. Immunol.* 18:1767.
13. Ogawa, M., S. Nishikawa, K. Ikuta, F. Yamamura, M. Naito, K. Takahashi, and S-I. Nishikawa. 1988. B cell ontogeny in murine embryo studied by a culture system with the monolayer of a stromal cell clone, ST2: B cell progenitor develops first in the embryonal body rather than in the yolk sac. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1337.
14. Kincade, P.W., G. Lee, C.E. Pietrangeli, S.-I. Hayashi, and J.M. Gimble. 1989. Cells and molecules that regulate B lymphopoiesis in bone marrow. *Annu. Rev. Immunol.* 7:111.
15. Palacios, R., and M. Steinmetz. 1985. IL-3 dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes *in vivo*. *Cell.* 41:727.
16. Kinashi, T., K. Inaba, T. Tsubata, K. Tashiro, R. Palacios, and T. Honjo. 1988. Differentiation of an interleukin 3-dependent precursor B-cell clone into immunoglobulin-producing cells *in vitro*. *Proc. Natl. Acad. Sci. USA.* 85:4473.
17. Kinashi, T., K. Tashiro, K. Inaba, T. Takeda, R. Palacios, and T. Honjo. 1989. An interleukin-4-dependent precursor clone is an intermediate of differentiation pathway from an interleukin-3-dependent precursor clone into myeloid cells as well as B lymphocytes. *Int. Immunol.* 1:11.
18. Takeda, S., S. Gillis, and R. Palacios. 1989. *In vitro* effects of recombinant interleukin 7 on growth and differentiation of bone marrow pro-B- and pro-T-lymphocyte clones and fetal thymocyte clones. *Proc. Natl. Acad. Sci. USA.* 86:1634.
19. Castellazzi, M., P. Vielh, and S. Longacre. 1986. Azacytidine-induced reactivation of adenosine deaminase in a murine cytotoxic T cell line. *Eur. J. Immunol.* 16:1081.
20. Coffman, R.L., and I.L. Weissman. 1981. B220: a B cell-specific member of the T200 glycoprotein family. *Nature (Lond.).* 289:681.
21. Unkeless, J.C. 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med.* 150:580.
22. Springer, T., G. Galfre, D.S. Secher, and C. Milstein. 1979. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. *Eur. J. Immunol.* 9:301.
23. Austyn, J.M., and S. Gordon. 1981. F4/30, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* 11:805.
24. Kinashi, T., N. Harada, E. Severinson, T. Tanabe, P. Sideras, M. Konishi, C. Azuma, A. Tominaga, S. Bergstedt-Lindqvist, M. Takahashi, F. Matsuda, Y. Yaoita, K. Takatsu, and T. Honjo. 1986. Cloning of complementary DNA encoding T-cell replacing factor and identity with B-cell growth factor II. *Nature (Lond.).* 324:70.
25. Noma, Y., P. Sideras, T. Naito, S. Bergstedt-Lindquist, C. Azuma, E. Severinson, T. Tanabe, T. Kinashi, F. Matsuda, Y. Yaoita, and T. Honjo. 1986. Cloning of cDNA encoding the murine IgG1 induction factor by a novel strategy using SP6 promoter. *Nature (Lond.).* 319:640.
26. Karasuyama, H., and F. Melchers. 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4, or 5, using modified cDNA expression vectors. *Eur. J. Immunol.* 18:97.
27. Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S.-I. Kashiwamura, K. Nakajima, K. Koyama, A. Iwamatsu, S. Tsunasawa, F. Sakiyama, H. Matsui, Y. Takahara, T. Taniguchi, and T. Kishimoto. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature (Lond.).* 324:73.
28. Kajigaya, S., T. Suda, J. Suda, M. Saito, Y. Miura, M. Iizuka, S. Kobayashi, N. Minato, and T. Sudo. 1986. A recombinant murine granulocyte/macrophage (GM) colony-stimulating factor derived from an inducer T cell line (1H5.5) Functional Restriction to GM progenitor cells. *J. Exp. Med.* 164:1102.
29. Nagata, S., M. Tsuchiya, S. Asano, Y. Kaziro, T. Yamazaki, O. Yamamoto, Y. Hirata, N. Kubota, M. Oheda, H. Nomura, and M. Ono. 1986. Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. *Nature (Lond.).* 319:415.
30. Constantinides, P.G., P.A. Jones, and W. Gevers. 1977. Functional striated muscle cells from non-myoblast precursors following 5-azacytidine treatment. *Nature (Lond.).* 267:364.
31. Darmon, M., J.-F. Nicolas, and D. Lamblin. 1984. 5-azacytidine is able to induce the conversion of teratocarcinoma-derived mesenchymal cells into epithelial cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:961.
32. Kinashi, T., K.H. Lee, M. Ogawa, K. Tohyama, K. Tashiro, R. Fukunaga, S. Nagata, and T. Honjo. 1991. Premature expression of the macrophage colony-stimulating factor receptor on a multipotential stem cell line does not alter differentiation

lineages controlled by stromal cells used for coculture. *J. Exp. Med.* 173:1267.

33. Tohyama, K., K.H. Lee, K. Tashiro, T. Kinashi, and T. Honjo. 1990. Establishment of an interleukin-5-dependent subclone from an interleukin-3-dependent murine hemopoietic progenitor cell line, LyD9 and its malignant transformation by autocrine secretion of interleukin-5. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1823.
34. Hara, K., T. Suda, J. Suda, M. Eguchi, J.N. Ihle, S. Nagata, Y. Miura, and M. Saito. 1988. Bipotential murine hemopoietic cell line (NFS-60) that is responsive to IL-3, GM-CSF, G-CSF, and erythropoietin. *Exp. Hematol. (NY).* 16:256.