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23. The cells (2 × 10^5 cells in a six-well microculture plate) treated with EGF or IFN-γ were incubated with [^{3}H]thymidine (5 μCi/ml) for 6 hours. Cells were then washed twice with phosphate-buffered saline, harvested onto the glass filters, and added to vials containing scintillation fluid for liquid-scintillation measurement of ^{3}H incorporation.
24. A two-layer soft-agar system was prepared by plating 0.5% agar in DMEM containing calf serum (20%) in 60-mm dishes [A. W. Hamburger and S. E. Salmon, *Science* **197**, 461 (1977)]. Cells were passed through a 25-gauge needle and then suspended in a 0.35% agar solution in DMEM enriched with calf serum (20%). The final concentration of cells in 0.35% agar solution was 2.5 × 10^4 cells/ml in 2 ml (5 × 10^4 cells). Plates were examined immediately after plating, to ensure that a single-cell suspension in agar had been achieved, and were then incubated. All specimens were plated in triplicate. DMEM (0.2 ml) containing calf serum (10%) was added to each well. IFN-γ was added to the final plating mixture just before plating (final concentration, 100 ng/ml), but not to control plates. IFN-γ was then added daily after plating to maintain the effect. Colonies were counted (a colony was defined as a new round aggregate of 50 or more cells) on day 8 with a phase-contrast inverted microscope.
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In Vitro Development of Primitive and Definitive Erythrocytes from Different Precursors

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During mouse embryogenesis the production of "primitive" erythrocytes (EryP) precedes the production of "definitive" erythrocytes (EryD) in parallel with the transition of the hematopoietic site from the yolk sac to the fetal liver. On a macrophage colony-stimulating factor–deficient stromal cell line OP9, mouse embryonic stem cells were shown to give rise to EryP and EryD sequentially with a time course similar to that seen in murine ontogeny. Studies of the different growth factor requirements and limiting dilution analysis of precursor frequencies indicate that most EryP and EryD probably developed from different precursors by way of distinct differentiation pathways.

Erythropoiesis originates in the yolk sac, then migrates to the fetal liver during mouse embryogenesis. EryP and EryD, which are produced in the yolk sac and the fetal liver, respectively, have distinct morphological and biochemical characteristics (*1*, *2*). Whether these two cell types develop from a single common hematopoietic precursor or not has been the subject of controversy (*3*, *4*). To address this question, we used the in vitro differentiation induction system of embryonic stem (ES) cells to hematopoietic cells (*5*).

Two waves of erythroid cell production were observed when D3 ES cells were cocultured with OP9 stromal cells (Fig. 1A) (*5*–*8*). The first wave of erythropoiesis appeared at day 6 of the induction, and all of the day 7 erythroid lineage cells were large-nucleated cells morphologically identical to EryP (Fig. 2A). The number of erythroid lineage cells suddenly decreased at days 8 and 9 to less than one-fifth of that at day 7. Subsequently, the second wave of erythroid lineage cells appeared around day 10 with a peak at day 14; these cells were small-nucleated erythroblasts or enucleated mature blood cells morphologically identical to EryD (Fig. 2B). In agreement with the report that EryP contain not only embryonic ζ- and ε-globin but also adult α-globin, whereas EryD contain only adult α- and β-globins (*9*), day 7 erythroid lineage cells were positive for staining with antibodies against embryonic as well as against adult hemoglobins, whereas day 14 erythroid cells were positive for staining with antibodies against adult hemoglobin only (Fig. 2, C to F) (*10*–*12*). Expression of ζ-, α-, and ε-globin mRNA in day 7 erythrocytes

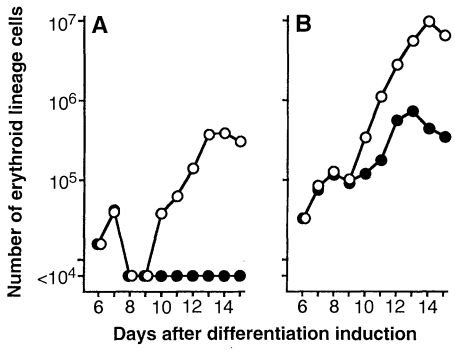

Fig. 1. Development of erythroid lineage cells during differentiation induction of D3 ES cells on OP9 stromal cells, and effects of anti–c-Kit (Ack2) and erythropoietin (EPO) on the development (*24*). Differentiation induction of ES cells was done without (**A**) or with (**B**) the addition of exogenous human recombinant EPO (10 U/ml) (*8*). Data are shown in the absence (○) or presence (●) of Ack2 (10 μg/ml).

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and of α- and β-major–globin mRNA in day 14 erythrocytes was confirmed by reverse transcriptase–polymerase chain reaction (RT-PCR) (Fig. 2G) (*13*). These results show that erythroid lineage cells produced in the first and the second waves of erythropoiesis are EryP and EryD, respectively. During the differentiation induction of ES cells, EryP and EryD first became detectable at day 6 and 10, respectively. The temporal pattern of the appearance of EryP and EryD on the stromal cells is similar to that of erythropoiesis during mouse ontogeny, in which EryP and EryD appear at days 8 and 12, respectively (*1*).

To test whether EryP and EryD require different growth factors for their development and survival, we examined the involvement of c-Kit ligand and erythropoietin (EPO)–induced signaling (Fig. 1). Addition of antibody to c-Kit (anti–c-Kit) (Ack2) (*14*) did not affect EryP development but completely blocked EryD development (Fig. 1A). c-Kit signaling seems to be necessary for the development or maintenance (or both) of multipotential hematopoietic precursors from ES cells in this system because not only EryD but also other myeloid lineage cells did not develop in the presence of Ack2 at day 14 (*15*). Because EPO mRNA was not detectable in the cocultured cells by RT-PCR (*15*), both EryP and EryD developed independently of EPO. This conclusion is consistent with the recent report that EryD precursor formation and impaired EryP production were observed in EPO-targeted and EPO receptor–targeted mice (*16*). In the presence of EPO, the type of erythroid lineage cells as assessed by immunostaining was exclusively EryP before day 9, and the percentage of EryP gradually decreased to ~70% at day 11, ~10% at day 12, and less than 1% after day 13. Even though c-Kit signaling was blocked by Ack2, ~80% of day 14 erythroid lineage cells were EryD in the presence of EPO (Fig. 1B). This impaired but notable production of EryD is comparable to the phenotype of the mice bearing null mutation of c-Kit (*17*).

Further investigation to distinguish precursors for EryP and EryD revealed that EryD precursors could be easily isolated and separated from EryP precursors simply by collecting cells adherent to stroma cells in the EPO-containing culture at day 6 (Table 1). The vast majority of EryD developed from the adherent cell population, whereas EryP were mostly recovered from the nonadherent cell population. Adherent cells grew by day 8 to small round cell clusters consisting of immature hematopoietic cells on OP9 cells (*5*). When 120 individual, well-separated, small round cell clusters were picked at day 7 and transferred separately into methylcellulose cultures containing interleukin-3 and EPO without dispersing the cells (*5*, *18*, *19*), 111 clusters produced blood cell colonies, including 91 erythroid mixed colonies (EryD plus at least one other lineage cell), 7 EryD colonies, 10 nonerythroid colonies, and 3 unidentified cell–type colonies. Immunostaining showed that none of the colonies contained embryonic hemoglobin–positive erythrocytes. Thus, most EryD developed from multipotential hematopoietic progenitors. These data suggest that EryP and EryD are produced by distinct differentiation programs.

The time course of the appearance of precursors of EryP and hematopoietic clusters, from which EryD were derived as discussed above, was examined by limiting dilution analysis (Table 2). Substantial numbers of precursors of EryP and hematopoietic clusters appeared at days 3 and 4 of the induction, respectively, and the number of precursors increased with time. We then tested whether common precursors for EryP and EryD existed in cultured ES cells at early stages of differentiation induction, that is, day 3 and day 4. Most of the hema-

Table 1. Development of EryP and EryD from day 6 adherent or nonadherent cells in the culture containing EPO. Cells were examined at day 8 and day 13 in the culture containing EPO. Cocultured D3 ES cells were trypsinized and transferred onto fresh OP9 cells at day 5. On the next day, cells nonadherent to stromal cells were collected by washing gently three times with culture medium and transferred onto fresh OP9 cells as nonadherent population. Culture medium was added to the remaining adherent cell layers, and cocultures were continued as adherent population. A control unseparated cell population received no treatment except that total cells were transferred to fresh OP9 cell layers at day 10. Differentiation induction was carried out in the presence of human recombinant EPO (10 U/ml). Erythroid lineage cells were identified by May-Giemsa staining, and the data are shown as the mean ± SE. Day 5 cells (10^5) were used for separated and unseparated cell populations.

Cell fractions at day 6	Erythroid cells ($\times 10^{-4}$)		
	Day 8 (EryP)	Day 13 (EryD)	EryP/EryD
Unseparated	12.2 ± 0.4	271.1 ± 13.3	0.045
Nonadherent	7.0 ± 0.6	21.5 ± 2.8	0.326
Adherent	1.6 ± 0.2	225.4 ± 16.9	0.007

Table 2. Time course of increase in the number of EryP precursors and hematopoietic cell cluster precursors generated from 10^4 D3 ES cells (*25*). Cocultured D3 ES cells were harvested by trypsinization 3, 4, and 5 days after the initiation of the differentiation induction in the presence of EPO. Sixty, 150, 300, and 600 induced cells per well were transferred onto OP9 cell layers of four 24-well plates in the presence of EPO. Three days after the transfer, dianisidine solution was added to the media (26), and dianisidine-positive nonadherent cells were counted as EryP. Thereafter, culture media were carefully removed and adherent small round cell clusters were counted.

Experiment	Precursors of	Number of precursors at		
		Day 3	Day 4	Day 5
1	Primitive erythrocytes	270	2000	8500
	Hematopoietic cell clusters	<10	1100	4800
2	Primitive erythrocytes	250	1100	12,500
	Hematopoietic cell clusters	<10	1100	4300

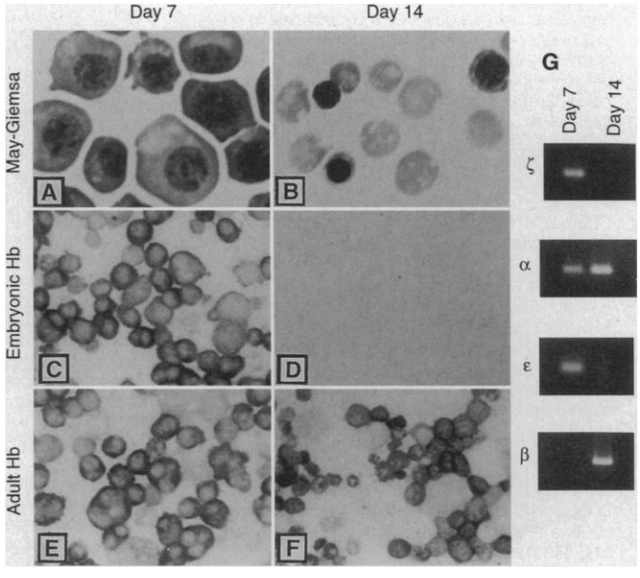

Fig. 2. Morphology of erythroid lineage cells and globin gene expression at days 7 and 14 of the differentiation induction in the absence of EPO. Cytospin preparations of the cells harvested from day 7 (**A**, **C**, and **E**) or day 14 (**B**, **D**, and **F**) were stained either with May-Giemsa (A and B) or by the PAP immunoenzymatic method (C to F). Polyclonal anti-mouse embryonic hemoglobin (C and D) and polyclonal anti-mouse adult hemoglobin (E and F) were used as primary antibodies (*11*). Expression of embryonic globin genes (ζ and ε) and adult globin genes (α- and β-major) was examined by RT-PCR (**G**) (*13*).

Table 3. Low frequency for generation of both EryP and hematopoietic cluster precursors from day 3 or day 4 cultured ES cells. Experiments were done essentially as described in Table 2. Numbers of the wells positive for EryP precursor, for hematopoietic cluster precursors, or for both were counted after either 60 or 150 induced cells per well were seeded in 192 wells (*27*).

Days after differentiation induction	No. of cells seeded per well	No. of positive wells containing precursors of			Estimated coincidence of two independent precursors
		Primitive erythrocytes	Hematopoietic cell clusters	Both	
Day 3	60	35	1	0	0
	150	76	4	2	1
Day 4	60	30	23	5	4
	150	54	49	16	14

topoiesis-positive wells contained either EryP or adherent small round cell clusters (Table 3). The frequency of the wells containing both types of precursors was low and close to the expected value calculated on the assumption that the two types of precursors developed independently. Two types of erythroid precursors for EryP and EryD had been reported, but whether these two types of precursors developed from common committed precursors or not had not been clear (*4*). Our data suggest that the two types of precursors belong to different cohorts, although we cannot exclude the existence of infrequent or transient common precursor cells to both EryP and EryD.

Differentiation of EryD took longer than that of EryP, presumably because EryP precursors emerged directly from committed precursor cells but EryD emerged by way of noncommitted multipotential hematopoietic precursors. The cell fate determination to EryP or EryD is most likely governed by the intrinsic differentiation program per se rather than by the microenvironment, because this system provided the same hematopoietic microenvironment for both erythroid lineage precursors on cloned OP9 stromal cells. Recently, Dzierzak and colleagues (*20*) reported new insights into hematopoietic cell development during mouse ontogeny: (i) There are two temporal waves of the hematopoietic cell production that could produce EryD; (ii) nonself-renewing hematopoietic progenitors appear first, followed by self-renewing hematopoietic stem cells; and (iii) these two progenitors develop from different anatomical sites. Taken together with the present data, these results indicate that erythroid lineage cells develop in three waves from three distinct types of the cells during mouse ontogeny, that is, (i) EryP from EryP precursors, (ii) EryD from nonself-renewing multipotential precursors, and (iii) EryD from self-renewing stem cells. Considering that the OP9 differentiation induction system could not give rise to self-renewing hematopoietic stem cells (*15*), this system seems to represent the first two waves of erythroid cell diffentiation in mouse embryos. The transition from embryonic to adult hemoglobin in mouse as well as that from embryonic to fetal hemoglobin in human (*21*) may thus reflect a switching of the cell lineage rather than a switching of the globin gene transcription within an erythroid committed cell (*22*).

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8. Differentiation induction of ES cells was carried out as described (*5*). D3 ES cells that had been maintained by standard methods (*6, 23*) in the presence of leukemia inhibitory factor and feeder cells were transferred onto confluent OP9 stromal cells in six-well plates at a density of 10^4 cells per well. Culture media for the maintenance of OP9 cells and for the differentiation induction were α-MEM (Life Technologies, Gaithersburg, MD) supplemented with 20% fetal bovine serum (Summit, Australia) and standard antibiotics. The induced cells were trypsinized at day 5, and 10^5 cells per well were transferred onto fresh OP9 cells. Total cells of individual wells were harvested by vigorous pipetting and transferred to a fresh OP9 cell layer on individual wells again at day 10. Erythroid lineage cells were counted by 2,7-diaminofluorene or May-Giemsa staining or both, and the two counts were in general agreement (*11*).
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19. Day 4–induced ES cells were trypsinized and transferred onto an OP9 cell layer. Three days after the transfer, individual, well-separated, adherent small round cell clusters were picked and put en bloc into methylcellulose media containing EPO and interleukin-3 (*18*). Five to 7 days later, emerged colonies were picked, washed, and divided into three portions. Cytospin specimens were produced from the three portions. One portion was stained with May-Grünwald Giemsa, and the two other portions were stained with the PAP method by use of rabbit anti-mouse embryonic hemoglobin or rabbit anti-mouse adult hemoglobin as the primary antibodies (*10*).
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27. Culture plates that were seeded with day 4–induced cells were examined 4 days after the transfer as described in Table 2. However, appearance of EryP in culture plates seeded with day 3–induced cells was examined microscopically 3 days after the transfer without dianisidine staining. This microscopic assay was relatively easy to perform because more than 90% of nonadherent cells were EryP (*15*). Day 3 cell cultures were continued for another 2 days to examine subsequent appearance of adherent hematopoietic cell clusters microscopically. This step was necessary because a large number of EryP appeared that originated from secondarily differentiated nonhematopoietic colonies when we waited until 5 days after the transfer (*15*).

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