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The Common Myelolymphoid Progenitor: A Key Intermediate Stage in Hemopoiesis Generating T and B Cells

Min Lu, Hiroshi Kawamoto, Yoshihiro Katsube, Tomokatsu Ikawa, and Yoshimoto Katsura

We have previously shown that the common progenitors for myeloid, T, and B cell lineages are enriched in the earliest population of murine fetal liver. However, it remained unclear whether such multipotent progenitors represent the pluriptotent progenitors capable of generating all hemopoietic cells or they also comprise progenitors restricted to myeloid, T, and B cell lineages. To address this issue, we have developed a new clonal assay covering myeloid, erythroid, T, and B cell lineages, and using this assay the developmental potential of individual cells in subpopulations of lineage marker-negative (Lin⁻) c-kit⁺ murine fetal liver cells was investigated. We identified the progenitor generating myeloid, T, and B cells, but not erythroid cells in the Sca-1⁺ subpopulation of Lin⁻c-kit⁺ cells that can thus be designated as the common myelolymphoid progenitor (CMLP). Common myeloiderythroid progenitors were also detected. These findings strongly suggest that the first branching point in fetal hemopoiesis is between the CMLP and common myeloiderythroid progenitors. T and B cell progenitors may be derived from the CMLP through the previously identified myeloid/T and myeloid/B bipotent stages, respectively. The Journal of Immunology, 2002, 169: 3519–3525.

It is well known that all blood cells, including T and B lymphocytes, are generated from the pluripotent hemopoietic stem cells (HSCs). To investigate the mechanism underlying hemopoiesis, it is a necessary prerequisite to outline the exact pathways of differentiation from the hemopoietic stem cell. Early studies on the lineage relationship on myeloid (M)/erythroid (E), T, and B cells have mainly been performed by marking the stem/progenitor cells through chromosome aberrations or retroviral transduction of genes. Recently, trials to more exactly illustrate the developmental process are made by developing clonal assay systems (2–6). In these studies, however, the secondary transfer of cultured cells to the thymus in vivo or to a coculture with a fetal thymic lobe was used for examining the T cell potential. The multilineage progenitor (MLP) assay (7), now designated the MLP(MTB) assay, was the first strategy with which it became theoretically possible seven types, the p-TB type had never been detected in the fetal liver (FL), although the p-TB, popularly called the common lymphoid progenitors (CLP), had been shown to exist in adult bone marrow (4). Our findings strongly suggested that in FL, the lineage restriction of p-Multi to p-T and p-B occurs through the bipotent progenitors p-MT and p-MB, respectively (12).

The problem with the MLP(MTB) assay, however, is that this method is unable to detect the erythroid potential of these progenitors. Therefore, it remained unclear whether the p-Multi represent the HSC themselves or the p-Multi also include progenitors restricted to myeloid, T, and B cell lineages (p-MTB). The earliest progenitors of M/E lineages have been designated as CFU-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEM-Meg), which represent the progenitors showing the broadest developmental capability in a CFU culture (CFU-C) assay. Because it was recently shown that common M/E progenitors, which are distinct from the HSCs, exist in murine bone marrow (13) and FL (14), now it is clear that the CFU-GEM-Meg represents both M/E progenitors and HSCs. In contrast, it remains to be clarified whether myeloid and erythroid potentials are always set together or only myeloid potential accompanies lymphoid branches.

In the present study, we devised new clonal assay systems capable of investigating the developmental potential of a single progenitor for generation of: 1) myeloid, erythroid, and B cell lineages (MLP(MEB) assay) and 2) myeloid, erythroid, T, and B cell lineages (MLP(METB) assay). Using these assays in combination with the CFU-C assay, we investigated the developmental potential of individual cells in various subpopulations of lineage marker-negative (Lin⁻) c-kit⁺ FL cells. The results indicated that the first lineage commitment step in hemopoiesis is the production of common myelolymphoid progenitors (CMLP or p-MT) and common myeloiderythroid progenitors (p-ME). These findings enabled us to illustrate the framework of the early stages of lineage commitment in fetal hemopoiesis.

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from SLC (Shizuoka, Japan), and B6 Ly-5.1 mice were maintained in our animal facility. B6Ly-5.1 fetuses at 13 days postcoitum (dpc) were used as the progenitor source.
**Growth factors**

Recombinant human erythropoietin (Epo; Genzyme, Cambridge, MA), recombinant murine (rm) stem cell factor (SCF) (Genzyme), and rmIL-3 (Genzyme) were used.

**Antibodies**

The following Abs were used: anti-Ly-5.1 (A20-1.7; donated by Y. Saga, Banyu Seiyaku, Tokyo, Japan); anti-c-kit (ACK-2; donated by S.-I. Nishikawa, Kyoto University); anti-IL-7R (A7R34, donated by S.-I. Nishikawa, Kyoto University); TER119 (TER) (15) and anti-FcγRII/III (FcR) (2.4G2; BD Pharmingen, San Diego, CA); FITC anti-Gr-1 (RA3-8C5; Caltag Laboratories, San Francisco, CA); FITC anti-B220, PE anti-B220, and allophycocyanin anti-B220 (RA-682; Caltag Laboratories); FITC anti-Thy-1.2 (5a-8; Caltag Laboratories); PE anti-Sca-1 (E13-161.7; BD Pharmingen); allophycocyanin anti-c-Kit (30F11.1; BD Pharmingen); PE anti-Mac-1 (M1/70; Caltag Laboratories); and Red670-streptavidin (Life Technologies, Grand Island, NY). TER was labeled with FITC. Anti-FcR and anti-IL-7R were biotinylated. Anti-Ly-5.1 and anti-c-kit were labeled with cyanine 5 (Cy5) labeling kit; Biological Detection Systems, Pittsburgh, PA.

**Staining and sorting of progenitors**

Basic methods for surface staining of cells, flow cytometric analysis, and sorting of cells have been previously described (7). FL cells were four-color stained with anti-Lin, anti-Sca-1, anti-FcR, and anti-IL-7R, or with anti-Lin, anti-Sca-1, anti-c-Kit, and anti-FcR plus anti-IL-7R. Cells were sorted using a FACSVantage. Nonviable cells were excluded by forward scatter profiles.

**Stromal cell lines and MLP(MEB) assay**

Stromal cell lines PA6, OP9, and TSt-4 were used to investigate the generation of the MLP(MEB) assay. Stromal cell monolayers were cultured on plastic bags, and the air inside was exchanged with a gas mixture of 5% CO2 and 95% air. The lobes were floated in 0.4% methylcellulose, 1% fraction-V BSA (Sigma-Aldrich, St. Louis, MO), 2-ME (5 × 10⁻⁴ M), D-glucose (1 mM), SCF (10 ng/ml), IL-3 (10 ng/ml), and Epo (2 U/ml). Numbers of colonies were counted on day 2 for CFU erythrocyte and on day 8 for all other colonies.

**RT-PCR**

mRNA was prepared from 10³ cells, and cDNA was synthesized with reverse transcriptase. cDNA equivalent to 500 cells were used for PCR. PCR was conducted as follows: denaturation at 94°C for 1 min, annealing at 53–65°C for 1 min, and elongation at 72°C for 2 min. The sequence of primers, annealing temperature, and cycle numbers were as follows. Primers were for β-actin, 5’-TCTCCTGGGACATCCATAA-3’ and 5’-GGA GCACTTGCGGCTGACAG-3’; for Gata-1, 5’-TCCAAGTCTGCTCTC T3’-3’ and 5’-AAAATGGAATATGATGG-3’; for Gata-2, 5’-AC ACCACCCGATAACCCACT-3’ and 5’-GCCCAGCTGACCCATGC-3’; for EpoR, 5’-GGACACTTACCTGTATTGG-3’ and 5’-GACGTT GAGGCTCGGCTCCT-3’; for c-fms, 5’-CTGGAGAAGAGAATTG TGC-3’ and 5’-TTTGTTGGACGAACATG-3’; for c-myc, 5’-AATATGGTCCG AAGCAGTGG-3’ and 5’-CTACAGGTCTCTGTGTTAT-3’; for G-CSFR, 5’-TACATCTCTGCTCCACTCTT-3’ and 5’-GAGACTACATGACCAGCA AT-3’. Annealing temperature and cycle numbers were 55°C/30 cycles for β-actin; 58°C/35 cycles for Gata-1; 65°C/30 cycles for Gata-2; 53°C/30 cycles for EpoR; 55°C/30 cycles for c-fms; 55°C/30 cycles for G-CSFR; 58°C/35 cycles for c-myc; 55°C/35 cycles for G-CSFR.

**Results**

**Fractionation of Lin− c-kit+ FL cells with surface markers**

FL cells from fetuses at 13 dpc were stained with various mAbs for flow cytometric analysis. Approximately 8% of FL cells at this gestational age are Lin− c-kit+ (Fig. 1A), and virtually all Lin− cells are c-kit−. A very small proportion of the Lin− c-kit− cells were c-Kit−/H9252, c-Kit−/H11001, and c-Kit−/H11002. A very small proportion of the Lin− c-kit− cells were CD45−/H11003, CD45−/H9252, and CD45−/H11001. c-Kit is expressed on several cell types, including B cells, monocytes, and mast cells. The c-Kit+/H11001 population consists of 30% FCS, 1% methylcellulose, 1% fraction-V BSA (Sigma-Aldrich, St. Louis, MO), 2-ME (5 × 10⁻⁴ M), D-glucose (1 mM), SCF (10 ng/ml), IL-3 (10 ng/ml), and Epo (2 U/ml). Numbers of colonies were counted on day 2 for CFU erythrocyte and on day 8 for all other colonies.

**CFU-C assay**

FL cells were cultured in MEM (Life Technologies) containing 30% FCS, 1% methylcellulose, 1% fraction-V BSA (Sigma-Aldrich, St. Louis, MO), 2-ME (5 × 10⁻⁴ M), D-glucose (1 mM), SCF (10 ng/ml), IL-3 (10 ng/ml), and Epo (2 U/ml). Numbers of colonies were counted on day 2 for CFU erythrocyte and on day 8 for all other colonies.

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mRNA was prepared from 10³ cells, and cDNA was synthesized with reverse transcriptase. cDNA equivalent to 500 cells were used for PCR. PCR was conducted as follows: denaturation at 94°C for 1 min, annealing at 53–65°C for 1 min, and elongation at 72°C for 2 min. The sequence of primers, annealing temperature, and cycle numbers were as follows. Primers were for β-actin, 5’-TCTCCTGGGACATCCATAA-3’ and 5’-GGA GCACTTGCGGCTGACAG-3’; for Gata-1, 5’-TCCAAGTCTGCTCTC T3’-3’ and 5’-AAAATGGAATATGATGG-3’; for Gata-2, 5’-AC ACCACCCGATAACCCACT-3’ and 5’-GCCCAGCTGACCCATGC-3’; for EpoR, 5’-GGACACTTACCTGTATTGG-3’ and 5’-GACGTT GAGGCTCGGCTCCT-3’; for c-fms, 5’-CTGGAGAAGAGAATTG TGC-3’ and 5’-TTTGTTGGACGAACATG-3’; for c-myc, 5’-AATATGGTCCG AAGCAGTGG-3’ and 5’-CTACAGGTCTCTGTGTTAT-3’; for G-CSFR, 5’-TACATCTCTGCTCCACTCTT-3’ and 5’-GAGACTACATGACCAGCA AT-3’. Annealing temperature and cycle numbers were 55°C/30 cycles for β-actin; 58°C/35 cycles for Gata-1; 65°C/30 cycles for Gata-2; 53°C/30 cycles for EpoR; 55°C/30 cycles for c-fms; 55°C/30 cycles for G-CSFR; 58°C/35 cycles for c-myc; 55°C/35 cycles for G-CSFR.

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Subpopulations of FL cells (Fr.1 to Fr.5 in Fig. 1) were cocultured at 50 cells/well on three different stromal cell lines, PA6, OP9, and TSt-4, which efficiently support the generation of myeloid cells, erythroid cells, and B cells, respectively (16–18). T cell potential was investigated by culturing with a dGuo-treated FT lobe. Various days after culture, cells were harvested, counted, and stained with mAb to lineage markers for analysis with a flow cytometer. Because the time course of cell generation differs among lineages (data not shown), the peak cell numbers are shown in Fig. 2. Progenitors in Fr.1 generate cells of all lineages examined, suggesting that this fraction contains the HSC or a mixture of progenitors for all lineages. Progenitors in Fr.2 showed the potential to generate myeloid and erythroid cells, but did not show any T or B cell generation. T and B cell generation was observed when a large number (~10^3/well) of Fr.2 cells were cultured (data not shown), but such T or B cell progenitors may have been diluted out from the triplicate cultures of 50 cells in this experiment. These results indicated that Fr.2 does not contain any HSCs, but contains M/E lineage-restricted progenitors or a mixture of myeloid and erythroid progenitors. Fr.3 was shown to exclusively contain myeloid lineage-restricted progenitors. As has been reported (10), progenitors in Fr.4 are almost completely restricted to the T or B cell lineage, although a marginal level of macrophage potential has also been observed. Fr.5 contains only erythrocyte precursors. The progenitor activity of cells from Fr.1 to Fr.5 was also examined using the CFU-C assay. In Fr.1, the majority of colonies are of the CFU-GEM-Meg or CFU granulocyte-macrophage (CFU-GM) type, although smaller numbers of CFU granulocyte (CFU-G) and CFU macrophage (CFU-M) are also seen (Table I). In Fr.2, the proportions of CFU-GM, CFU-G, and CFU-M are much higher than in Fr.1, and burst-forming unit erythroid appears in this fraction. CFU-GEM-Meg are still seen in this fraction, while progenitors for the T and B cell lineages are undetectable (Fig. 2), demonstrating the existence of M/E progenitors that lack lymphoid potential. A large majority of colonies in Fr.3 were CFU-M or CFU-G, although a small number of CFU-GM were also seen. No CFU-GEM-Meg or erythroid colonies were found in this fraction. Fr.4 contains a small number of CFU-M, conforming to the results shown in Fig. 2. Fr.5 exclusively contains CFU erythrocyte. These results confirmed the findings in Fig. 2 that the lineage restriction progresses in parallel with the phenotypic change.

**Figure 2.** Differences in the developmental potential of progenitors among subpopulations of FL cells. Cells in Fr.1 to Fr.5 (Fig. 1) were cultured at 50 cells/well on a stromal cell monolayer in a 96-well plate. Stromal cell lines PA6 (A), OP9 (B), and TSt-4 (C) were used in the detection of myeloid, erythroid, and B cell potential, respectively. For T cell generation, cells were cocultured with a dGuo-treated FT lobe (D). Recovered cells were counted and stained for various lineage markers for analysis with a flow cytometer. The mean cell number and SD of cells at a peak point in triplicate cultures are shown. Data are representative of three independent experiments.

**Table 1. Progression of the lineage restriction revealed by CFU-C assay**

<table>
<thead>
<tr>
<th>Cells Plated</th>
<th>GEM-Meg</th>
<th>GM*</th>
<th>G*</th>
<th>M*</th>
<th>BFU-E*</th>
<th>CFU-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>No. Per Dish</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fr.1</td>
<td>200</td>
<td>7.3 ± 0.6*</td>
<td>4.6 ± 6.1</td>
<td>3.3 ± 1.5</td>
<td>3.3 ± 1.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Fr.2</td>
<td>200</td>
<td>2.7 ± 1.5</td>
<td>17 ± 3.2</td>
<td>11 ± 1.5</td>
<td>27 ± 4.2</td>
<td>8.7 ± 4.2</td>
</tr>
<tr>
<td>Fr.3</td>
<td>200</td>
<td>0.0</td>
<td>4.7 ± 1.5</td>
<td>24 ± 2.6</td>
<td>21 ± 3.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Fr.4</td>
<td>200</td>
<td>0.0</td>
<td>0.0</td>
<td>7.3 ± 2.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Fr.5</td>
<td>200</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>76 ± 14</td>
</tr>
</tbody>
</table>

* Cells in Fr.1 to Fr.5 (Fig. 1) were cultured at 200 cells/dish in a methylcellulose gel containing SCF, IL-3, and Epo. Numbers of colonies were counted on day 2 for CFU-E and on day 8 for all other colonies.

* GM, Granulocyte-macrophage; G, granulocyte; M, macrophage; BFU-E, burst-forming unit erythroid.

* Mean number ± SD of colonies/dish from triplicate cultures are shown.
Distribution of different types of progenitors in FL subpopulations

Progenitor activity of individual cells from Fr.1 (total 100 cells) was examined by culturing them in a 96-well plate with the stromal cell line TsT-4 in the presence of G-CSF and Epo (Fig. 3A). Addition of Epo and G-CSF to cocultures with stromal cell lines barely interfered with the B cell potential of the progenitors (data not shown). These culture conditions support the generation of myeloid, erythroid, and B lineage cells, and this culture system was termed the MLP(MEB) assay. After 7 days of culture, grown cells were harvested for microscopic observation and flow cytometric analysis. Progenitors generating myeloid, erythroid, and B cells (M/E/B type), which can be regarded as the HSCs, are exclusively seen in Fr.1 (Fig. 3B, extreme left panel). Fr.1 contains quite a large number of M/B type progenitors. A large proportion of these M/B type progenitors should be the p-Multi, as previously determined by the MLP(MEB) assay, rather than bipotent p-MB, because p-MB are very rare, but p-Multi are abundant in the Sca-1high population (9) (see also Fig. 4). Distinction of M/B type from M/E/B type may indicate that the p-Multi detected by the MLP(MTB) assay are not necessarily the HSCs, but include the p-MTb, which can be called the CMLP. M/E, M, and B type progenitors were also detected in this population. However, B/E type progenitors were not detected, suggesting that the lineage restriction to a stage generating B cells and erythrocytes does not occur.

Progenitor activity of individual cells from Fr.2, Fr.3, and Fr.4 (a total of 100 cells each) was also examined by the MLP(MEB) assay. A large majority of progenitors in Fr.2 are committed to the myeloid or erythroid lineage, while a small number of M/E type progenitors are also included in this fraction (Fig. 3B). The activity of the M/E type progenitors for production of myeloid and erythroid cells is comparable with that of the M/E/B type progenitors seen in Fr.1 (Fig. 3C). About half of the M/E type clones are comprised of granulocytes, erythroid cells, macrophages, and megakaryocytes, whereas the remaining half tend to lack megakaryocytes. An example of Giemsa staining of the cells in a M/E type clone generated on a TsT-4 monolayer, which corresponds to a CFU-GM cell, is shown in Fig. 3D, compared with cells in an M type clone, which corresponds to a CFU-GM.

Expression of β-globin, mb-1, and c-fms in different types of clones was investigated by RT-PCR as the genes specific for erythroid, B, and myeloid lineages, respectively (Fig. 3E). The results are completely in accordance with the progenitor types, as determined by flow cytometric analysis (Fig. 3B). For example, the absence of lymphoid cells in M/E type colonies from Fr.2 was confirmed by the failure to detect mb-1 mRNA (Fig. 4E, lanes 4–6) that is expressed in B lineage cells beginning at the early stages (20). These results may have confirmed the presence of common progenitors for M/E lineages that have no lymphoid potential.

Fr.3 exclusively contains myeloid lineage-restricted progenitors (Fig. 3B). A large majority of these myeloid progenitors produce only macrophages or granulocytes. The proportion of the granulocyte/macrophage bipotent type progenitors in Fr.3 was comparable with that of CFU-GM colonies detected by the CFU-C assay (Table 1) in this fraction (data not shown). The absence of B and erythroid lineage cells in the myeloid colonies was confirmed by RT-PCR analysis (Fig. 3E, lanes 7 and 8). These results establish the presence of macrophage/granulocyte type progenitors without lymphoid and erythroid potentials; such progenitors may subsequently be restricted to the macrophage or granulocyte lineages.
Discrimination of CMLP from HSCs with the MLP(METB) assay

The developmental potential of individual progenitors in Fr.1 and Fr.2 was further investigated using the MLP(METB) assay (Fig. 4A), which was also newly devised for the present study. The procedure of this assay is basically the same as the MLP(MTB) assay, which has been designed to examine the developmental potential of a progenitor toward myeloid, T, and B cell lineages. In the MLP(METB) assay, Epo is added to the culture to support erythropoiesis. Because the MLP(METB) assay is able to determine the developmental potential of individual progenitors toward the myeloid, erythroid, T, and B cell lineages, it is formally possible to discriminate 15 different types of progenitors, which are listed in Fig. 4C. Of the 15 types, 9 types were actually detected, which are p-METB, p-MTB, p-ME, p-MB, p-M, p-T, p-B, and p-E. Flow cytometric profiles of cells generated from p-METB, p-MTB, p-ME, and p-E are shown in Fig. 4B. Profiles of cells derived from other types of progenitors have been shown in previous studies (7, 9). Six possible types, p-MET, p-MEB, p-METB, p-ET, p-EB, and p-TB, have never been detected, suggesting that such stages are absent in FL.

The p-Multi, as determined by the previously used MLP(MTB) assay (7, 9), was found to comprise at least two distinct progenitor types, p-METB and p-MTB, with the latter in much larger numbers. The p-METB may represent the HSCs themselves or the immediate progeny of the HSCs retaining the same developmental potential as the HSCs. The p-MTB may represent the earliest stage of differentiation toward T and B cells, and thus can be named as the CMLP. Furthermore, detection of p-ME with the MLP(METB) assay in Fr.2 confirmed the detection of M/E type progenitors in this fraction with the MLP(MEB) assay (Fig. 3B). The inability of the p-MTB to produce erythroid cells was confirmed by the failure to detect the erythroid marker β-globin using RT-PCR in the cells derived from the p-MTB (data not shown).

Expression of lineage-associated genes in FL subpopulations

cDNA samples equivalent to 500 cells from subpopulations of FL cells (indicated in Fig. 1) were used for PCR analysis to examine the expression of transcription factors, which have been shown to play an important role in hemopoiesis or lineage restriction (21, 22). The expression of receptors for lineage-specific growth factors was also examined. As shown in Fig. 5, c-myb, PU.1, Gata-1, Gata-2, and Gata-3 are expressed at the earliest stage (Fr.1), and the expression levels of these genes, except for Gata-3, does not seem to decline until the Fr.2 stage. Expression of Gata-3 was the highest at the Fr.4 stage, in which p-T and p-B are enriched (10). Expression of Gata-1 and PU.1 in Fr.3 to Fr.5 seems reciprocal: Gata-1 is strongly expressed in Fr.5, but hardly detectable in Fr.3.

![FIGURE 4. Discrimination of p-MTB (CMLP) from p-METB (HSC). A, Procedure of the MLP(METB) assay. Individual cells in Fr.1 or Fr.2 from 13 dpc FL were cultured with a dGuo-treated lobe supplemented with SCF, IL-3, IL-7, and Epo. Generation of myeloid and erythroid cells was assessed on day 6 of culture, whereas the generation of T and B cells was assayed on day 11. B, Representative flow cytometric profiles of cells judged to be derived from p-METB, p-MTB, p-ME, and p-E. Cell numbers from clones of p-METB, p-MTB, p-ME, and p-E were 6.8 × 10^4, 6.2 × 10^4, 3.5 × 10^4, and 0.8 × 10^4, respectively. C, Distribution of different types of progenitors determined with the MLP(METB) assay. A total of 390 cells from Fr.1 and 100 cells from Fr.2 was examined. Cumulative results from five experiments are shown.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.150.8.3523)
and Fr.4, whereas the reverse is true for PU.1. B cell lineage-specific Pax-5 and erythroid lineage-specific EpoR are exclusively expressed in Fr.4 and Fr.5, respectively. c-fms and G-CSFR are expressed from the earliest stage, and the expression continued onto the stage of Fr.4. Because Fr.4 almost exclusively contains T and B cell progenitors (4) (see also Fig. 2), and the cells in this fraction barely respond to M-CSF or G-CSF (data not shown), the expression of these genes could be related to remnant myeloid potential.

**Discussion**

Development of T, B, and myeloerythroid cells has traditionally been investigated separately; thus, the relationship among these lineages has not been well understood. It was only after the development of the MLP(MTB) assay that it became possible to examine the existence of progenitors having a developmental potential across these lineages. In addition to p-M, p-T, and p-B, the bipotent progenitors p-MT and p-MB, as well as the multipotent progenitors (p-Multi) were identified (7, 9). With the MLP(MTB) assay, however, the heterogeneity among the p-Multi could not be determined. In the present study, by developing the MLP(METB) assay, p-Multi was subdivided into p-METB (HSCs) and p-MTB (CMLP). A difference between the HSC and CMLP was also observed in experiments using the MLP(MEB) assay, which discriminates the M/B type from the M/E/B type (Fig. 3).

The p-METB is thought to be the HSC, because it is able to generate all major lineage cells, and both p-METB and HSCs with long-term reconstitution ability are exclusively found in Fr.1 (Fig. 4, and our unpublished data). In contrast, in any in vitro clonal assay systems, the detection of progenitors showing restricted potential may face the problem as to whether the developmental potential of the progenitor is fully induced in the culture condition used. The existence of p-ME may be evident, because this type of progenitor was found in the population in which no multipotent progenitors reside (Fr.2, Figs. 3B and 4C). As for the discrimination of p-MTB from p-METB, however, the possibility could not completely be ruled out that some of p-METB are judged as p-MTB. Nevertheless, we regarded that most of the progenitors that have expressed myeloid, T, and B cell potentials, but not erythroid potential are really the p-MTB. This can be said because the number of MEB type progenitors detected by using Epo-supplemented OP9 stromal cells (data not shown), which is known to be the most efficient culture system for induction of erythroid potential (16), was comparable with the number of p-METB or MEB type progenitor determined in the present study. The finding that the number of HSCs (120 repopulating U/13 dpc FL) estimated by Ema and Nakauchi (23) is comparable with that of p-METB (350/13 dpc FL, see Fig. 4C), but not to the sum of p-METB and p-MTB, may also support the discrimination of p-MTB from p-METB.

The existence of p-MTB was elucidated for the first time in the present study, which can be termed the CMLP. It has recently been proposed that the first major forking point in hemopoiesis in the bone marrow is the production of M/E progenitors and CLP (4, 13). Because our present findings in FL indicated that the CLP is not p-TB but p-MTB, p-MTB rather than p-TB may be the key stage in branching toward the T and B cell lineages in FL. During the past several years of investigation on FL progenitors, we have repeatedly shown that the p-TB type does not exist in FL. The present investigation with the MLP(METB) assay strongly suggests that the absence of some possible types of progenitor such as the p-TB is not exceptional. As seen in Fig. 4C, p-MET, p-MEB, p-ETB, p-ET, and p-EB types are also undetected, implying that such stages do not exist. These results further suggested that the erythroid potential is not maintained in the lymphoid pathway, thus proposing that the first branch in hemopoiesis may be between p-MTB and p-ME (Fig. 6). A large proportion of p-MTB identified in our study was able to produce both macrophages and granulocytes (data not shown). However, it is probable that the granulocyte/macrophage double producer gives rise to a macrophage producer along with the progress of differentiation, because T/B macrophage type progenitors have been reported (6). The CMLP or p-MTB is exclusively found in Fr.1, in which the HSCs belong, but not in Fr.4, in which p-T and p-B are enriched (10). The phenotypic similarity between HSCs and CMLP suggests that the CMLP is an immediate progeny of the HSCs.

The presence of p-MT and p-MB stages has been elucidated using the MLP(MTB) assay (7–10). Now it is clear that the p-MTB is the immediate progenitor generating p-MT and p-MB. Because p-TB type progenitors are undetectable and both p-MT and p-MB are routinely detected in FL as well as in the aorta-gonad-mesonephros region (11, 18), lineage commitment of p-MTB seems to progress by losing B and T cell potentials to give rise to p-MT and p-MB, respectively. Although p-T is unable to generate granulocytes or macrophages, it retains the potential to generate dendritic cells (DC) in addition to NK cells (24, 25). Thus, in the process of restriction of p-MT to p-T, some major molecular system participating in the myeloid lineage may remain active, as is the case of PU.1, c-fms, or G-CSFR, which is found to be expressed at high levels in Fr.4 (Fig. 5), in which myeloid potential is only marginally present (10). Similarly, p-B may be the immediate progeny of p-MB. Although p-B scarcely generate DC (26, and our unpublished data), B cells retain an Ag-presenting function, and they sometimes show a morphology indistinguishable from DC (27).

The findings that the myeloid potential accompanies all erythrocyte, T cell, and B cell branches may be compatible with the idea that the myeloid lineage represents the prototype of hemopoietic cells. The process of lineage commitment revealed in the present study could be the recapitulation of the phylogenetic development of hemopoietic cells, in that all blood cells are thought to have evolved from macrophage-like phagocytic cells (28). It is likely that genes for molecules constructing erythrocytes, T cells, and B cells are controlled to be expressed after the expression of a basic program, which may lead to the macrophage or myeloid lineage. It remains unclear, however, whether myeloid differentiation is always accompanied with differentiation of erythrocytes, T cells, and B cells, or whether a myeloid-specific pathway independent of the other lineages exists.

It is highly probable that transcription factors are involved in lineage restriction, although the key molecules, except for Pax5 in
the B cell lineage (29), have not yet been identified. The results of RT-PCR analysis indicate that the expression of many of the lineage-specific transcription factors begins at the earliest stage, conforming to the previously proposed idea that some of the lineage-specific genes are initiated to be expressed at an uncommitted progenitor stage (30). Reciprocal expression of PU.1 and Gata-1 between Fr.3 and Fr.5, which represent P-M and P-E stages, respectively, seems to be related to the shutting down of erythroid potential and myeloid potential (Fig. 5). These observations are in complete agreement with the previous findings that predominant expression of PU.1 or Gata-1 promoted the commitment toward myeloid or erythroid lineage, respectively (31, 32). To date, no candidate genes have been found that could play a part in controlling the lineage commitment between lymphoid and erythroid lineages.

The present findings indicate that hemopoiesis progresses through an ordered restriction process rather than random processes. The ordered restriction process has previously been proposed (33), and recent molecular studies on hemopoiesis have suggested a hierarchy in the lineage restriction process (21, 22). Although the models proposed in these studies are different from each other and from the model we propose (Fig. 6), they are based on the common concept that hemopoiesis progresses according to a set program. Nevertheless, lineage restriction in hemopoiesis is frequently referred to, without any firm evidence, as a random process. This is usually called the stochastic model rather than the random restriction model, the latter more exactly expressing the meaning of this concept (34, 35). If hemopoiesis progresses through a random process, all 15 possible types of progenitors should be constantly detected with the ML/P(METB) assay. In the present study, however, of the 15 theoretical types of progenitors, 6 are thought to be defective (Fig. 4), strongly suggesting that the hemopoiesis progresses through an ordered restriction process.

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References