Characterization of the Human Burst-Forming Unit-Megakaryocyte

By Robert A. Briddell, John E. Brandt, John E. Straneva, Edward F. Srour, and Ronald Hoffman

Two classes of human marrow megakaryocyte progenitor cells are described. Colony-forming unit-megakaryocyte (CFU-MK)–derived colonies appeared in vitro after 12-day incubation; burst-forming unit-megakaryocyte (BFU-MK)–derived colonies appeared after 21 days. CFU-MK–derived colonies were primarily unifocal and composed of 11.6 ± 1.2 cells/colony; BFU-MK–derived colonies were composed of 2.3 ± 0.4 foci and 108.6 ± 44.4 cells/colony. CFU-MK and BFU-MK were separable by counterflow centrifugal elutriation. CFU-MK colony formation was diminished by exposure to 5-fluorouracil (5-FU); BFU-MK colony formation was unaffected. CFU-MK and BFU-MK were immunologically phenotyped. CFU-MK expressed the human progenitor cell antigen-1 (HPCA-1, CD34, clone My10) and a major histocompatibility class II locus, HLA-DR, and BFU-MK expressed only detectable amounts of CD34. BFU-MK colony formation was entirely dependent on addition of exogeneous hematopoietic growth factors. Recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) possessed such colony-stimulating activity, whereas recombinant erythropoietin (Epo), G-CSF, IL-1α, IL-4, and purified thrombopoiesis-stimulating factor did not. These studies indicate the existence of a human megakaryocyte progenitor cell, the BFU-MK, which has unique properties allowing it to be distinguished from the CFU-MK.

VARIOUS HEMATOPOIETIC progenitor cells have been categorized by in vitro clonal assay systems according to their position in the differentiation schema. The most primitive hematopoietic progenitor cells, as compared with their more differentiated counterparts, have a higher capacity for proliferation and require longer periods of incubation in vitro for their descendant colonies to appear. The burst-forming unit-erythroid (BFU-E) and the high proliferative potential colony-forming cell (HPP-CFC) are two examples of such primitive hematopoietic progenitor cells.

The existence of a more primitive megakaryocyte progenitor cell was first suggested by Long et al., who described a murine megakaryocyte progenitor cell, termed the burst-forming unit-megakaryocyte (BFU-MK), which appeared to have different biologic features than those associated with previously recognized megakaryocyte progenitor cells. BFU-MK–derived colonies required longer incubation periods to develop, were composed of multiple foci of development, and contained larger numbers of megakaryocytic elements than the primarily unifocal colony-forming unit-megakaryocyte (CFU-MK)–derived colonies. The existence of an equivalent primitive megakaryocyte progenitor cell in humans has been assumed, but to date its characteristics have been poorly defined. We previously reported detection of human marrow BFU-MK–derived colonies and described their properties. Using low-density bone marrow (LDBM) cells, BFU-MK–derived colonies were detected in optimum numbers after 21 days of incubation, whereas the greatest numbers of CFU-MK–derived colonies were observed after 12 days of incubation. This report provides a more detailed analysis of the human marrow BFU-MK. The cellular composition and kinetics of appearance of BFU-MK–derived colonies are further clarified, as are the physical properties, sensitivity to 5-fluorouracil (5-FU), antigenic characteristics, and growth factor dependence of the BFU-MK.

MATERIALS AND METHODS

Bone marrow (BM) aspirates were obtained under local anesthesia from the posterior iliac crests of hematologically normal volunteers. Informed consent was obtained from the donors according to guidelines previously established by the Human Investigations Committee of the Indiana University School of Medicine, which adheres to the principles of the Declaration of Helsinki.

Cell separation techniques. BM aspirates were immediately diluted 1:1 with Isscove's modified Dulbecco's media (IMDM, Gibco Laboratories, Grand Island, NY), containing 20 U/mL sodium heparin. This mixture was passed through a 150-μm screen and layered over an equal volume of Ficoll-Paque (sp. g 1.077 g/cm³), containing 20 U/mL human recombinant interleukin-2 (IL-2), 10 U/mL recombinant IL-3, 104 U/mL recombinant GM-CSF, and 105 U/mL recombinant human erythropoietin (Epo). G-CSF, IL-1α, IL-4, and purified thrombopoiesis-stimulating factor did not. These studies indicate the existence of a human megakaryocyte progenitor cell, the BFU-MK, which has unique properties allowing it to be distinguished from the CFU-MK.

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form hematopoietic colonies. The optimal dose and time of exposure to 5-FU were defined by criteria established by Brandt et al.9

Cell sorting techniques. Fractions of marrow cells collected at FR of 12 to 14 mL/min by counterflow centrifugal elutriation (FR 12-14) were further characterized by monoclonal antibody (MoAb) labeling and cell sorting, using methods previously reported by our laboratory.9-11 For two-color cell sorting, MoAbs of different sub-classes were selected. The mouse anti-human progenitor cell anti-gen-1 (HPCA-1, CD34, clone My10) and the mouse anti-major histocompatibility class II locus (HLA-DR, clone L243) conjugated with phycoerythrin (PE) are of the IgG1 and IgG2 subclass, respectively (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA). Both MoAbs were dialedyzed to remove sodium azide, titrated, and detectable densities of CD34 and detectable densities of HLA-DR (CD34DR) or no detectable HLA-DR (CD34DR-) were used for analysis. The purity of sorted cells was determined by reanalyzing aliquots of the sorted subsets immediately after the sort was finished. The CD34DR- cell fraction contained >90% CD34 positive cells and >95% HLA-DR- cells, whereas the CD34DR- cell fraction contained >85% CD34+ cells and <5% HLA-DR+ cells.

Complement-dependent cytotoxicity. FR 12-14 cells were first incubated at 4°C for 45 minutes at a concentration of 6 × 10⁶ cells/mL in 50 μL IMDM containing an equal volume of 1% DMSO and an unconjugated anti–HLA-DR antibody (Becton Dickinson Immunocytochemistry Systems) at a plateau killing concentration in sterile microcentrifuge tubes (Tekmar, Cincinnati). Control cells were incubated in a similar fashion with media alone. The tubes were then centrifuged at 500 g for 10 minutes, the supernatant was removed, and the cells were resuspended in 100 μL solution containing 20% rabbit complement, and incubated at 37°C for 60 minutes. Control cells were incubated in a similar fashion with media alone. Cells were next washed three times in IMDM and assayed for megakaryocyte progenitor cell-derived colonies. The number of cells plated was determined by cell counts made before treatment with antibody and complement.

Purified and recombinant human hematopoietic growth factors. Recombinant granulocyte macrophage colony-stimulating factor (rGM-CSF), interleukin-3 (rIL-3), and interleukin-4 (rIL-4) were purchased from Genzyme, Boston. Recombinant human erythropoietin (rEpo) and rG-CSF were purchased from AmGen Biologicals, Thousand Oaks, CA. Recombinant human interleukin-1α (rIL-1α), a gift of Dr Peter Lo Medico, Hoffman La Roche, Nutley, NJ, had a specific activity of 10⁶ U/mg protein, as determined by proliferative effects on D-10 cells. Step III thrombocytopoiesis stimulatory factor (TSF), purified from human embryonic kidney cell conditioned media, was provided by Dr Ted McDonald.12 TSF samples were dissolved in IMDM to form a stock solution of 10⁶ U/mL and stored at −20°C until used.

Assay systems. Various cell fractions were assayed for their ability to produce CFU-MK-derived or BFU-MK-derived colonies in a serum-depleted, fibrin clot culture system as described by Bruno et al.13 or in a serum containing methylcellulose culture system identical to that described by Brandt et al.9 Varying doses of recombinant or purified growth factors were used as a source of megakaryocyte colony-stimulating activity (CSA). Cultures were incubated for 12 to 30 days at 37°C in a 100% humidified atmosphere of 5% CO₂ in air. After incubation, fibrin clots were fixed in situ in methanol/acetic (1:3) for 20 minutes, washed with PBS, and then air-dried. Fixed plates were stored frozen at −20°C until immunofluorescent staining was performed.

Morphologic identification. Colonies composed of either undifferentiated cellular elements or megakaryocytes were plucked from the methylcellulose assays under direct microscopic visualization with sterile 100-μL glass micropipettes (Drummond Scientific, Broomall, PA) and suspended in 200 μL IMDM in sterile 1.5-mL microcentrifuge tubes (Tekmar). The cell suspension was transferred to slides on a Cytospin 2 (Shandon Southern Instruments, Sewickly, PA) at 750 rpm for 5 minutes and subsequently stained with either Wright’s-Giemsa, benzidine, butyrate esterase, or peroxidase, and was microscopically examined.

Immunofluorescent identification. Rabbit antiserum to human platelet glycoproteins (GPs), previously established as an immunologic probe for identifying human megakaryocytes, were diluted in PBS (1:200), layered over fixed fibrin clot cultures, and incubated for 60 minutes at room temperature in 100% humidified air.14 After being washed with PBS, the specimens were reincubated for an additional 60 minutes at room temperature with fluorescein conjugated, goat F(ab)² specific anti-rabbit IgG, (Tago, Burlingame, CA) diluted in PBS at a final concentration of 0.76 mg/mL protein. Specimens were washed with PBS, counterstained with 0.125% wt/vol Evans blue for 1.5 minutes, washed with distilled water, and mounted in isotonic barbital buffer, pH 8.6, in glycerol (1:3). Nonspecific immunofluorescent staining of eosinophils was quenched by the counterstaining procedure, allowing specific identification of megakaryocytes.

Cultures were scored in situ to enumerate fluorescein-positive colonies. The 35-mm Petri dishes were inverted, and the base area was completely scanned with a fluorescent microscope at ×100 (Zeiss standard microscope 18 with IV FL vertical fluorescent illuminator; Carl Zeiss, New York). A CFU-MK–derived megakaryocyte colony was defined as a cluster of three or more fluorescent cells. BFU-MK–derived colonies were identified by criteria established by Long et al.13 These colonies appeared as clusters of cells distributed at least 80 microns apart. Based on our determination of the optimum in vitro time of appearance of BFU-MK–derived and CFU-MK–derived colonies, previously reported by our laboratory, CFU-MK–derived colonies were enumerated after 12 days of incubation and BFU-MK–derived colonies were scored after 21 days of incubation. Each experimental group was cultured in duplicate or quadruplicate.

Statistical analysis. Results are the mean ± SEM of two or more points for each experiment. Statistical significance was determined with Student’s t test.

RESULTS

Colony morphology. The morphologic characteristics of BFU-MK–derived colonies were initially examined and compared with those of CFU-MK–derived colonies. BFU-MK–derived colonies were composed of almost ten times the number of cells (108.6 ± 4.4) as CFU-MK–derived colonies (11.2 ± 1.2). The cells composing BFU-MK–derived colonies were distributed in almost twice the number of foci (2.3 ± 0.4) as observed with CFU-MK–derived colonies (1.2 ± 0.1). A photomicrograph of a representative BFU-MK–derived colony is shown in Fig 1A. The characteristic appearance of a BFU-MK–derived colony differs from that of a CFU-MK–derived colony shown in Fig 1B. These colonies were labeled with a platelet GP antiserum to facili-
10 11 12 14 16 18 20 22 24 28

HUMAN BURST-FORMING UNIT MEGAKARYOCYTE

Fig 1. (A) Typical BFU-MK-derived colony cloned in the serum-depleted assay system after 21-day incubation. Cells were stained in situ with antiplatelet glycoprotein antibodies and F(ab')² specific fluorescent-conjugated IgG antibodies. (Original magnification ×270.) (B) Typical CFU-MK-derived colonies cloned in the serum depleted system after 12-day incubation. Cells were stained in the identical fashion as described above. (Original magnification ×540.)

The possibility that BFU-MK and CFU-MK have different physical properties that might facilitate separation of these two progenitor cells was then determined. By counterflow centrifugal elutriation, almost 80% of the BFU-MK-derived colonies were present in marrow fractions obtained at flow rates (FR) of 12 to 14 mL/min, whereas nearly 70% of the CFU-MK-derived colonies resided in marrow fractions obtained at FR of 18 to 20 mL/min (Fig 2). When cloned in the presence of rIL-3, FR 12-14 gave rise to 4.5 ± 1.0 CFU-MK-derived colonies/1 × 10⁶ cells plated and 18.5 ± 1.1 BFU-MK-derived colonies/1 × 10⁶ cells plated; FR 18-20 gave rise to 34.0 ± 3.0 CFU-MK-derived colonies/1 × 10⁶ cells plated and 0.0 ± 0.0 BFU-MK-derived colonies/1 × 10⁶ cells plated. These studies indicate that BFU-MK and CFU-MK are readily separable according to characteristic differences in size and density.

5-FU sensitivity. The two classes of megakaryocyte progenitor cells also had markedly different sensitivities to in vitro treatment with the chemotherapeutic agent, 5-FU. Table 1 shows that when LDBM cells were exposed to 5-FU, CFU-MK cloning efficiency was markedly reduced whereas BFU-MK colony formation was unaltered.

Immunologic phenotyping. To phenotype the BFU-MK immunologically, FR 12-14 cells were incubated with FITC-labeled CD34 antibody and HLA-DR antibody conjugated with PE and analyzed for subsequent separation of marrow populations by FACS. The distribution of HLA-DR and CD34 antigens on these cells was similar to that previously described.
reported by our laboratory. The two populations of interest both expressed high densities of the CD34 antigen, whereas only one expressed significant levels of HLA-DR. The viability of cells after sorting was ≥99% by trypan blue exclusion. BFU-MK–derived colony formation by these two populations of cells is shown in Fig 3. Assayable BFU-MK resided only in the CD34DR cell population. FR 12-14 cells constituted 10.2% ± 1.6% of LDBM and provided an eightfold enrichment of BFU-MK–derived colonies as compared with that assayed from LDBM cells (Table 2, Figs 2 through 4). The BFU-MK–derived colonies enumerated is shown in Fig 4. These data indicate that increasing numbers of BFU-MK–derived colonies were observed with the addition of increasing numbers of CD34DR cells plated.

Complement-dependent cytotoxicity was also used as an alternative method to eliminate HLA-DR–containing cells. Only BFU-MK–derived colony formation was affected by such treatment, confirming that BFU-MK are HLA-DR~ (Table 3).

Cellular morphology. Our group previously reported that the colony forming unit-blast (CFU-BI) can also be assayed from this same CD34DR cell population. To establish the relationship between the colonies derived from CFU-BI and BFU-MK, representative colonies were examined in vitro, plucked, and morphologically examined. The CFU-BI–derived colonies appeared in vitro as dispersed clusters of round, agranular, nonpigmented cells with no evidence of morphologic characteristics of myeloid or erythroid colonies. The cells comprising these colonies were predominantly agranular blasts with basophilic cytoplasm and multiple, large nucleoli, as has been reported by our laboratory. No megakaryocytic elements were observed in such colonies. The BFU-MK–derived colonies, like those of the CFU-BI–derived colonies, were also composed of agranu-

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**Table 1. Effect of Pretreatment of Marrow Cells With 5-FU on Megakaryocyte Colony Formation**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>CFU-MK/2 x 10^5 Cells Plated</th>
<th>BFU-MK/2 x 10^5 Cells Plated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>31.3 ± 0.8*</td>
<td>6.1 ± 0.4</td>
</tr>
<tr>
<td>5-FU</td>
<td>1.9 ± 0.6†</td>
<td>5.3 ± 0.7†</td>
</tr>
</tbody>
</table>

Low-density marrow cells were pretreated with or without 25 µg/mL 5-FU for 24 hours and then assayed with human rIL-3 in a serum-depleted assay system. CFU-MK–derived colonies were enumerated on day 12, and BFU-MK–derived colonies were enumerated on day 21. *Each point is the mean ± SEM of multiple points from three studies. †P < .001.

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**Table 2. Effect of Complement-Dependent Cytotoxic Depletion of HLA-DR+ Marrow Cells on Megakaryocyte Colony Formation**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>CFU-MK/10^5 Cells Plated</th>
<th>BFU-MK/10^5 Cells Plated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>18.5 ± 1.1*</td>
<td>21.5 ± 1.1</td>
</tr>
<tr>
<td>Anti-HLA-DR</td>
<td>18.6 ± 1.0</td>
<td>21.9 ± 1.2</td>
</tr>
<tr>
<td>Rabbit C'</td>
<td>20.0 ± 1.8</td>
<td>20.7 ± 0.9</td>
</tr>
<tr>
<td>Anti-HLA-DR + rabbit C'</td>
<td>3.9 ± 0.5†</td>
<td>18.6 ± 1.1†</td>
</tr>
</tbody>
</table>

Each point represents colonies assayed from 10^5 marrow cells elutriated at FR of 12 to 14 mL/min with human rIL-3. CFU-MK–derived colonies were enumerated on day 12, and BFU-MK–derived colonies were scored on day 21. *Each point is the mean ± SEM of multiple points from three studies. †P < .001.

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**Fig 3. Time of appearance (days) of BFU-MK–derived colonies assayed from CD34DR (My10)DR+ and CD34DR (My10)DR- cells obtained by MoAb labeling and FACS. BFU-MK–derived colonies were observed only in the CD34DR (My10)DR+ cell population and appeared in optimal numbers after 21 days of incubation. Data points are the mean ± SEM of duplicate assays performed on two separate donors.**
lar, refractile cellular elements arranged in multiple foci; however, the cells composing these colonies were larger and had irregular cytoplasm. The cells comprising the BFU-MK-derived colonies, when plucked from culture and stained with Wright's-Giemsa, could be readily identified as being of megakaryocytic origin by morphologic criteria. The megakaryocytic origin of these cells was confirmed by their lack of reactivity to staining with either benzidine, butyrate esterase, or peroxidase.

Cytokine response. The effect of addition of exogenous growth factors on BFU-MK-derived colony formation is shown in Figs 5 and 6. No BFU-MK-derived colonies appeared in the absence of an exogenous growth factor. In Fig 5, the effect of addition of increasing concentrations of rGM-CSF on the number of BFU-MK-derived colonies assayed is shown. A dose-response relationship was observed with optimal cloning efficiency occurring at a concentration of 10 U rGM-CSF/mL. A similar dose-response relationship between the number of BFU-MK-derived colonies assayed and the amount of rIL-3 added to culture is shown in Fig 6.

The concentration of rIL-3 needed to promote formation of optimal numbers of BFU-MK colonies was 100 U/mL. The optimal dose of rIL-3 promoted the appearance of more than twice the numbers of BFU-MK than the optimal concentration of rGM-CSF (16.7 ± 1.8/1 × 10^5 FR 12-14 cells plated v 7.0 ± 0.6/1 × 10^5 FR 12-14 cells plated). The effect of suboptimal and optimal concentrations of rGM-CSF and rIL-3, alone or in combination, on BFU-MK-derived colony formation by CD34^+ DR ^- cells is shown in Table 3. The effects of rGM-CSF and rIL-3 added to culture were additive. In contrast, varying concentrations of rEpo (0.25 to 2.0 U/mL), rGCSF (5 to 50 U/mL), rIL-1α (25 to 200 U/mL), rIL-4 (5 to 100 U/mL), and purified TGF (10 to 100 mg/mL) alone did not promote any BFU-MK-derived colony formation. BFU-MK growth factor responsiveness was identical whether LDBM, FR 12-14 cells, or CD34^+ DR ^- cells were used as target cells. When CD34^+ DR ^- cells were cloned in the methylcellulose system with 100 U rIL-3/mL, similar cloning efficiency was observed (1.0 ± 1.0/1.0 × 10^5 cells plated). Colonies cloned with rEpo and rIL-3 were analyzed by morphologic criteria and benzidine reactivity and had properties identical to those assayed with rIL-3 alone.

**DISCUSSION**

We provide evidence of the existence of a primitive human megakaryocyte progenitor cell, the BFU-MK. The delayed time of appearance required for its descendant colonies to appear in vitro, the large number of megakaryocytic elements composing such colonies, and the multifocal nature of such colonies allows one to distinguish BFU-MK-derived colonies from megakaryocyte colonies which originate from CFU-MK. The distinctiveness of these two cell types is further confirmed by the observation that human BFU-MK and CFU-MK can be physically separated by counterflow...
Fig 5. Effect of the addition of varying amounts of human GM-CSF on BFU-MK-derived colony formation by enriched fractions of human marrow cells obtained by counterflow centrifugal elutriation. Target cells were obtained at a flow rate of 12 to 14 mL/min. Each point is the mean ± SEM of quadruplicate points performed on two separate donors.

Fig 6. Effect of addition of varying amounts of rIL-3 on BFU-MK-derived colony formation by enriched fractions of human marrow cells obtained by counterflow centrifugal elutriation. Target cells were obtained at a flow rate of 12 to 14 mL/min. Each point is the mean ± SEM of quadruplicate points performed on two separate donors.

centrifugal elutriation. Each of these observations is remarkably similar to the findings reported by Long et al, who initially identified BFU-MK in murine marrow cells.5

Our studies indicate that the antigenic phenotypes of BFU-MK and CFU-MK also differ. Many investigators have shown that most human marrow CFU-granulocyte macrophage (GM), BFU-E, and CFU-granulocyte erythroid macrophage megakaryocytes (GEMM) express both CD34 and HLA-DR.10,15-17 Watt et al and Lu et al recently reported that most normal human CFU-MK also express both CD34 and HLA-DR determinants.9,11,13-17 Such findings are also in agreement with those of Long and Shapiro who showed that murine CFU-MK are Ia antigen positive.18 With combinations of CD34 and HLA-DR antibodies and two-color FACS, we isolated hematopoietic progenitor cells differing in density expression of HLA-DR and CD34 antigens. Our studies indicate that human BFU-MK express the CD34 antigen but possess nondetectable amounts of HLA-DR. The lack of detectable expression of HLA-DR by BFU-MK was confirmed with antibody-directed, complement-mediated cytotoxicity. Localization of the BFU-MK to an HLA-DR population of cells might initially be considered surprising. Recently, however, our laboratory studied a primitive human hematopoietic cell, CFU-B1, which in vitro forms colonies composed of blast cells which have both extensive self-renewal capacity and the ability to differentiate along multiple lineages. These studies indicate that CFU-B1 also express CD34 but not HLA-DR determinants.9 The cellular composition of these two hematopoietic colonies allows them to be easily distinguished.

In our studies, human BFU-MK and CFU-MK differed with regard to their sensitivity to pretreatment with 5-FU. Cloning efficiency of the CFU-MK was markedly reduced by such pretreatment, whereas the proliferative capacity of BFU-MK was unaltered. In a similar fashion, human CFU-B1 have also been shown to be resistant to 5-FU pretreatment, whereas more differentiated progenitor cells were destroyed by exposure to 5-FU.9 Ability to survive such in vitro chemotherapeutic exposures therefore appears to be characteristic of the most primitive hematopoietic progenitor cells.

Formation of BFU-MK-derived colonies was entirely dependent on the presence of either of two different recomb-
nant growth factors which affect multiple hematopoietic lineages; rGM-CSF and rIL-3. Other growth factors, such as rEpo, rG-CSF, rIL-1α, rIL-4, or TSF previously reported by several groups of researchers to affect CFU-MK proliferation alone or in combination, were shown not to affect BFU-MK colony formation individually. Based on the present studies, rIL-3 appears to be more effective in supporting BFU-MK–derived colony formation, leading to appearance of more than twice the numbers of BFU-MK–derived colonies than result with optimal concentrations of rGM-CSF (16.7 ± 1.8/1 × 10^5 cells plated × 7.0 ± 0.6/1 × 10^5 cells plated. In addition, the effects of various concentrations of rGM-CSF and rIL-3 when added in combination to culture are additive with regard to their ability to promote BFU-MK–derived colony formation. Such additive effects of cytokines on megakaryocytopoiesis have previously been described at the level of CFU-MK. We have provided substantial evidence for the existence of a primitive megakaryocyte progenitor cell in normal human BM. The delayed time of appearance, higher proliferative capacity, relative resistance to 5-FU pretreatment, and lack of expression of the HLA-DR antigen by BFU-MK suggest that this megakaryocyte progenitor cell is more primitive than CFU-MK. BFU-MK appear to be the immediate ancestor of CFU-MK, but further studies will be required to define this relationship. Such an organizational structure is consistent with an age-structured model of cell proliferation. Continued investigation of BFU-MK will surely clarify such issues and shed further light on this important cellular stage of megakaryocyte development.

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