RAPID COMMUNICATION

High Proliferative Potential Colony-Forming Cell Heterogeneity Identified Using Counterflow Centrifugal Elutriation

By Mervin C. Yoder, Xun Xiang Du, and David A. Williams

Murine high proliferative potential colony-forming cells (HPP-CFC) are known to be heterogenous with respect to proliferative capacity and in vitro responsiveness to hematopoietic growth factors. We have separated HPP-CFC into several subpopulations using counterflow centrifugal elutriation. Although HPP-CFC were identified in all of the elutriated fractions of both C3H/HeJ and C57Bl/6J bone marrow cells, the distribution of HPP-CFC as well as of colony-forming units–granulocyte-macrophage (CFU-GM) in each fraction differed between these two strains of inbred mice. Six subsets of HPP-CFC were resolved that differed in growth factor responsiveness. A low-density HPP-CFC subpopulation was isolated that was distinct from day-12 spleen colony-forming units (CFU-S12), CFU-GM, and bone marrow stromal cells. This unique subpopulation of HPP-CFC is rare (3% to 9% of total HPP-CFC), appears to be lymphocyte-like in morphology, and behaves as the most primitive of the HPP-CFC subsets by requiring multiple hematopoietic growth factors for optimal in vitro cloning. Further characterization of this subpopulation of HPP-CFC will determine the position of these cells in the HPP-CFC hierarchy.

MATERIALS AND METHODS

Mice. Male C57Bl/6J (C57) and C3H/HeJ (C3H) mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 5 to 8 weeks of age.

Elutriation. In each experiment, animals were killed by cervical dislocation and bone marrow cells from six C3H or C57 mice were flushed from the femurs and tibias of each mouse using α-minimal essential medium (α-MEM; Gibco, Grand Island, NY) containing 5% fetal calf serum (FCS; Gibco). Single-cell suspensions were obtained by passing the bone marrow cell suspension through a 25-gauge needle several times before pelleting the cells (50g for 10 minutes) and resuspending the cells in fresh α-MEM medium or elutriation buffer (4.83 g/L NaCl, 0.17 g/L dextrose, 0.05 g/L ethylenediaminetetraacetic acid, and 0.5% bovine serum albumin [BSA]; Sigma, St Louis, MO). A JE-6B elutriation rotor (Beckman Instruments, Palo Alto, CA) installed in a J-6M/E centrifuge (Beckman Instruments) was held at a constant rotor speed of 3,000 rpm (1,260g) and 23°C, whereas 2 to 4 X 10^6 cells were loaded into the standard elutriation chamber at a flow rate of 14 ml/min. A peristaltic pump (Cole-Parmer, Chicago, IL), calibrated with each experiment, was used to maintain the desired flow rate. Cells were collected in 200-ml aliquots as the flow rate (FR) was increased to 22, 24, 26, 30, and 35 ml/min. After the collection of the FR 35 fraction, the rotor was turned off (RO) and a final aliquot of cells was collected. All cells were pelleted, resuspended in α-MEM medium, and counted.

From the Herman B Wells Center for Pediatric Research, Howard Hughes Medical Institute, Indiana University School of Medicine, Indianapolis, IN.

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Address reprint requests to Mervin C. Yoder, MD, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, 702 Barnhill Dr, Indianapolis, IN 46202-5225.

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CFU-GM assay. Myeloid progenitor colony formation was determined as described. Briefly, unfractionated or elutriated marrow cells from each FR were suspended in 10% FCS, 10^5 mol/L 2-mercaptoethanol (Sigma), 2 mmol/L glutamine (GIBCO), 4 U recombinant human erythropoietin (Amgen, Thousand Oaks, CA), 20 U recombinant murine interleukin-3 (IL-3; Genzyme, Cambridge, MA), and 100 ng recombinant rat stem cell factor (SCF; gift of Amgen). The cells were plated in quadruplicate, incubated in a 5% CO^2, humidified incubator at 33°C and fed by demidepopulation (50%) with fresh media added at 7 and 14 days. At 21 days of culture, phase contrast photographs of the adherent layer were obtained with a dissecting microscope.

CFU-SI assay. As previously described, donor cells from unfractionated and each elutriated FR fraction (except FR 14) were injected via the tail vein into C3H or C57 recipient animals (6 mice per fraction) that had received 11 Gy γ irradiation (96 cGy/min in two doses divided by 4 hours) using a 137Cs irradiator (Nordion, Kanata, Canada).Recipient animals were maintained on acidified (pH 2.4) water before and after the transplant. Twelve days after marrow infusion, the recipient animals were killed by cervical dislocation, spleens were removed and fixed in Bouin’s solution, and the macroscopic surface colonies present in each spleen were counted using a dissecting microscope.

HPP-CFC assay. Double-layer agar cultures were prepared as described by Bradley and Hodgson and McNiece et al. Recombinant hematopoietic growth factors murine macrophage colony-stimulating factor (M-CSF; 1,600 U; gift of Genetics Institute, Boston, MA), IL-3 (200 U), and IL-1α (500 U; Genzyme, Cambridge, MA), and/or rat SCF (100 ng) were added to 10 X 35 mm gridded tissue culture dishes. Triplicate cultures of fractionated and unfractionated cells were plated with each single growth factor or in multiple growth factor combinations and incubated in a 5% O2, 10% CO2, and 85% N2 humidified environment. On day 14 of culture, colonies were stained with 1 mg/mL piodonitrotetrazolium violet-INT (Sigma), and dense, compact, macroscopic colonies greater than 0.5 mm were scored as HPP-CFC.

Morphology. An aliquot of cells from each elutriated fraction and from unfractionated cells was applied to a glass slide using a Cytospin device (Shandon, Pittsburgh, PA). The slides were air-dried and stained with Wright-Giemsa before inspection by light microscopy.

Long-term cultures. Five to 10 million cells from fractions FR 22, 24, 26, 30, 35, and RO and unfractionated cells were added to 25 cm² tissue culture flasks containing 10 mL α-MEM supplemented with 20% horse serum (JRH, Lenexa, KS), 10^-4 mol/L hydrocortisone (Abbott, Chicago, IL), and 10^-3 2-mercaptoethanol, as previously described. Cultures were incubated in a 5% CO2, humidified incubator at 33°C and fed by demidepopulation (50%) with fresh media added at 7 and 14 days. At 21 days of culture, phase contrast photographs of the adherent layer were obtained. Stromal cells were identified using previously published criteria.

Immunofluorescence analysis. Cells (10^6) from FR24 thor RO fractions were incubated with 1 µg of commercially available (Pharminen, San Diego, CA) phycoerythrin (PE)-conjugated rat anti-mouse stem cell antigen 1 (Sca-1); cells from unfractionated bone marrow were also incubated with a PE-conjugated isotype control antibody. After washing, pelleting, and resuspending in phosphate-buffered saline (PBS) with 1% BSA, fluorescence-activated cell analysis (FACS) was performed using a FACSStar Plus instrument (Becton Dickinson, San Jose, CA), as previously described. Background fluorescence was determined using PE-isotype control-stained marrow cells and positive PE-marked cells were recorded as emitting greater fluorescence intensity than did more than 99% of the PE-isotype control cells.

RESULTS

Total cell recovery. The recovered number of nucleated cells in each fraction from 4 elutriations of C3H and 2 elutriations of C57 normal bone marrow is presented on Table 1. The mean ± SD percentage of total recovery of nucleated cells was 84% ± 9% and 81% ± 10% for C3H and C57B1 marrow, respectively.

Recovery of clonogenic cells. The concentration of CFU-GM was very low in fractions FR14 through FR26 for both C3H and C57 marrow (Fig 1, upper panel). For C3H marrow, the highest concentration of CFU-GM reached 1.5 times the un fractionated marrow concentration in fraction FR35, but for C57 marrow, the highest concentration of CFU-GM was in fraction FR30 (twofold more concentrated). Total recovery of CFU-GM was 70% ± 12% for C3H and 67% ± 11% for C57 marrow.

Essentially no CFU-SI were present in elutriated fractions FR14 through FR24 of either C3H or C57 marrow (Fig 1, middle panel). Spleen colonies were rarely detected when C3H or C57 fraction FR26 cells were injected into irradiated recipient mice (Fig 1, middle panel). The highest concentration of CFU-SI was found in fraction FR30, in which a twofold enrichment (compared with the frequency of CFU-SI in unfractionated marrow) was observed for both C3H and C57 marrow cells (Fig 1, middle panel). The overall recovery of CFU-SI was 88% ± 6% and 96% ± 5% for C3H and C57 marrow, respectively.

A small number of HPP-CFC from each murine strain were elutriated during the loading (FR14) of the elutriation chamber (<0.01% total HPP-CFC), but no HPP-CFC were isolated from fraction FR22 cells (Fig 1, lower panel). HPP-CFC from each mouse strain were isolated in all remaining FR fractions. In contrast to CFU-GM and CFU-SI, which were not present or rare in fractions FR24 and FR26, significant numbers of HPP-CFC were isolated from both C3H and C57 mice in fractions FR24 and FR26 (Fig 1, lower panel). The overall recovery of HPP-CFC from all fractions was 72% ± 9% for C3H and 79% ± 11% for C57 marrow.

Distribution pattern of all colony-forming cells (CFC). When all CFC from each FR fraction were analyzed as a percent of total CFC, differences in the distribution of elutriated CFC between C3H and C57 mice became even more apparent (Fig 2). Significantly, 9% of HPP-CFC elutriated from C3H marrow were present in fractions FR24 and FR26, with a graded increase in subsequent fractions to the peak number of cells (31%) in the RO fraction (Fig 2, bottom panel). In contrast, fewer than 1% of total C3H CFU-SI and CFU-GM were elutriated in fractions FR14 through FR26. The peak number of C3H CFU-SI (43%) and CFU-GM (49%) were elutriated in fraction FR35 (Fig 2, bottom panel). Thus, a distinct difference in the distribution profile for HPP-CFC as compared with CFU-SI and CFU-GM allows for enrichment of a portion of one population over another.

The distribution profile of elutriated CFC from C57 mice was different from the C3H profile (Fig 2). Rare CFU-SI (<0.1%), CFU-GM (<0.1%), and few HPP-CFC (0.6%) were elutriated in fractions FR14 through FR24. Fraction FR26 also contained few CFU-SI (0.8%) but more CFU-
Table 1. Nucleated Cell Counts in the Elutriated and Pre-Elutriation Unfractionated Samples

<table>
<thead>
<tr>
<th></th>
<th>UF</th>
<th>14</th>
<th>22</th>
<th>24</th>
<th>26</th>
<th>30</th>
<th>35</th>
<th>RO</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H</td>
<td>280</td>
<td>±</td>
<td>1</td>
<td>±</td>
<td>6</td>
<td>±</td>
<td>21</td>
<td>±</td>
</tr>
<tr>
<td>C57</td>
<td>366</td>
<td>±</td>
<td>1</td>
<td>±</td>
<td>11</td>
<td>±</td>
<td>29</td>
<td>±</td>
</tr>
</tbody>
</table>

The data represent the mean ± SD of four experiments with C3H (24 animals) and two experiments with C57 (12 animals) inbred mouse strains.

GM (3.2%) and HPP-CFC (3.0%) (Fig 2, top panel). Whereas the number of CFU-S12 peaked (43%) in fraction FR35 and then declined in the RO fraction, both CFU-GM and HPP-CFC were elutriated in peak number (36% to 39%) in fraction RO (Fig 2, top panel). Thus, HPP-CFC from C57 mice, like C3H mice, were elutriated essentially free of CFU-GM and CFU-S12 in fraction FR24; however, in fraction FR26, the percentage of total C57 CFU-GM and HPP-CFC elutriated was similar.

Hematopoietic growth factor requirements for HPP-CFC cloning in agar cultures. Table 2 lists the number of HPP-CFC identified in each fraction of elutriated or unfractionated C3H marrow cells responsive to single or multiple growth factors. No HPP-CFC were identified in any FR fraction in plates in which IL-1α or SCF were used as single growth factors. Few HPP-CFC were identified from fraction FR24 and FR26 cells grown in the presence of only IL-3 or CSF-1. However, a significant number of HPP-CFC responsive to these single agents were present in higher flow rate fractions (Table 2). Only 10% to 11.5% of the total HPP-CFC (colonies responsive to all four growth factors) present in fractions FR24 and FR26 were responsive to a single factor, whereas greater than 35% of the HPP-CFC cloned from the remaining elutriated fractions (and the unfractionated sample) were responsive to a single hematopoietic growth factor (Table 2). Approximately 50% of HPP-CFC from fraction FR24 and FR26 cells (cloned in the presence of IL-1α + SCF + IL-3 + CSF-1) were responsive to the combination of IL-3 and CSF-1 (Table 2). More dual factor...
(IL-3 + CSF-1)-responsive HPP-CFC were present among the cells from higher flow rate fractions and unfractonated cells (Table 2).

**Presence of stromal cells in elutriated fractions.** In each experiment, fractions FR24 through RO (fractions FR14 and FR22 were excluded due to an insufficient number of cells) were assayed for the presence of cells capable of forming an adherent stromal layer in long-term bone marrow cultures. Figure 3 shows the typical results obtained. No stromal layer formed using cells from fraction FR24 or FR26 from either strain (Fig 3A and B). Fraction FR30 cells produced an adherent layer of macrophages (Fig 3C), whereas fractions FR35 and RO and the unfractionated sample cultures had complex stromal adherent layers (Fig 3D through F). Cobblestone areas of hematopoiesis were apparent in only the cultures derived from fractions FR35 and RO and unfractionated bone marrow cells.

**Morphology of cells from elutriated fractions.** FR14 and FR22 fraction cells were composed primarily of nucleated or mature red blood cells and rare lymphocytes or myeloid cells. FR24 and FR26 fraction cells resembled mature lymphocytes with few nucleated red blood cells and rare immature or mature myeloid cells. In contrast, FR30, FR35, and RO fractions contained few cells with a lymphocyte morphology and were composed primarily of mature monocytes, macrophages, neutrophils, and many immature “blast” forms, as well as nonhematopoietic lineage stromal cells (data not shown).

**FACS analysis.** Data from three experiments showed that the percentage of positively PE-labeled Sca-1⁺ cells in FR24 was 5.6 ± 1.5, in FR26 was 1.7 ± 0.9, in FR30 was 1.3 ± 1.0, in FR35 was 1.3 ± 0.7, and FRRO was 0.8 ± 0.4, and in unfractionated marrow was 0.2 ± 0.1.

**DISCUSSION**

Subpopulations of HPP-CFC have generally been ordered by their in vitro growth factor requirements. Those HPP-CFC requiring three or more factors including IL-1α, CSF-1, IL-3, and/or SCF for in vitro growth are thought to be the most primitive HPP-CFC, whereas those cells responding to two factors or single agents are considered less primitive.²⁻³ Based on this hierarchy, subpopulations of HPP-CFC have been further characterized according to the rate and extent of bone marrow reconstitution after myeloablation with 5-FU, retention of the fluorescent dye rhodamine 123, and recloning experiments after hematopoietic growth factor stimulation in liquid culture.⁴⁻¹² We report that HPP-CFC subpopulations can also be resolved using CCE and that the HPP-CFC elutriated at the lower flow rates differ in morphologic appearance and as a population require more hematopoietic growth factors for cloning than HPP-CFC elutriated at higher flow rates.

The elutriation protocol used in the present experiments is similar to several recently published methods. Jones et al.¹³ using B6D2F1 mice and the same elutriation rotor (JE-6B) and rotor speed (1,260g), elutriated normal marrow cells into four fractions and recovered 78% of the input cells. They reported that fraction FR25 cells were enriched for long-term reconstituting PHSC and were essentially devoid of CFU-S₁₂ and CFU-GM, whereas the RO fraction was enriched for CFU-GM and was depleted of long-term reconstituting PHSC. Intermediate FR29 and FR33 fractions were not tested for PHSC content, but were enriched for CFU-S₁₂ and CFU-GM, respectively. Orlic and Bodine,¹⁴ using C57Bl/6J mice and the same elutriation rotor and rotor speed, reported that PHSC were present in FR25, FR29/30, FR35, and RO fractions, but CFU-S₁₂ were not found in the FR25 fraction. They found threelfold PHSC enrichment in the FR29/30 fraction and twofold CFU-S₁₂ enrichment in the FR35 fraction and hypothesized that overall population differences in density resulted in the ability to isolate a fraction (FR25) of cells containing PHSC but no CFU-S₁₂.

The overall nucleated cell count and total CFC recovery

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**Table 2. Hematopoietic Growth Factor Requirements of Elutriated and Pre-Elutriation Unfractionated HPP-CFC in Agar Culture**

<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>24</th>
<th>26</th>
<th>30</th>
<th>35</th>
<th>RO</th>
<th>Unfractionated</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SCF</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-3</td>
<td>0.6 ± 0.5</td>
<td>1.5 ± 0.5</td>
<td>2.5 ± 2.0</td>
<td>15.1 ± 2.8</td>
<td>15.5 ± 12.0</td>
<td>16 ± 11.3</td>
</tr>
<tr>
<td>CSF-1</td>
<td>0.5 ± 0.2</td>
<td>1.5 ± 2.0</td>
<td>16.2 ± 15.5</td>
<td>12.5 ± 8.9</td>
<td>5.5 ± 3.5</td>
<td>6.5 ± 3.5</td>
</tr>
<tr>
<td>IL-3 + CSF-1</td>
<td>5.2 ± 1.9</td>
<td>12.0 ± 1.0</td>
<td>39.3 ± 15.2</td>
<td>42.6 ± 11.8</td>
<td>46.5 ± 5.3</td>
<td>41.6 ± 15.9</td>
</tr>
<tr>
<td>IL-1α + SCF + IL-3 + CSF-1</td>
<td>10.6 ± 0.5</td>
<td>26.0 ± 5.6</td>
<td>53.0 ± 2.8</td>
<td>64.5 ± 4.9</td>
<td>49.0 ± 16.9</td>
<td>51.1 ± 14.0</td>
</tr>
<tr>
<td>IL-1α or SCF or IL-3 or CSF (IL-3 + CSF-1) (%)</td>
<td>10.4</td>
<td>11.5</td>
<td>35.2</td>
<td>42.8</td>
<td>42.8</td>
<td>44.0</td>
</tr>
<tr>
<td>IL-3 + CSF-1 (%)</td>
<td>49.0</td>
<td>46.1</td>
<td>73.5</td>
<td>65.1</td>
<td>93.8</td>
<td>81.4</td>
</tr>
</tbody>
</table>

These results represent the mean ± SD of HPP-CFC/20,000 cells from each elutriated fraction or the pre-elutriation unfractionated sample cloned in the presence of single growth factors and in two or four factor combinations. The percentage of HPP-CFC responsive to any single factor (IL-1α or SCF or IL-3 or CSF-1) was calculated for each fraction using the response to four factors as the total number of HPP-CFC. The percentage of HPP-CFC responsive to the combination of IL-3 and CSF-1 was also calculated for each fraction. HPP-CFC in the fractions FR24 and FR26 were less responsive to single growth factors or the combination of IL-3 + CSF-1 than HPP-CFC in higher flow rate fractions or the unfractionated sample.
in our experiments was similar to the above published results. However, we have made several novel observations regarding the use of CCE to separate bone marrow populations by assaying elutriated fractions for cell types not previously studied or by modifying the elutriation protocol to further resolve previously studied hematopoietic CFC subpopulations. For example, our observations that stromal cell precursors can be depleted from clonogenic hematopoietic cells at low flow rates using CCE may be a useful technique for investigators examining hematopoietic stromal cell-stem cell interactions.

Our modified elutriation protocol included keeping the elutriation rotor, elutriation chamber, and rotor speed the same as the above protocols, but we changed the lowest selected FR fraction to 22, 24, and 26 mL/min fractions, respectively. We hypothesized that the FR22 fraction would not contain significant numbers of HPP-CFC and would remove most nucleated red blood cells. We found no HPP-CFC in this nucleated red blood cell-laden fraction. We also hypothesized that collecting fractions at FR24 and FR26 rather than FR25 would be useful in identifying subpopulations of HPP-CFC. Strain differences in HPP-CFC recovery from these fractions were observed. Despite the changes in elutriation FR selected, many similarities exist in our results with those of Jones et al. and Orlic and Bodine. FR24 and FR26 fractions in our experiments were depleted of CFU-S12, similar to the CFU-S12-depleted FR25 fraction in the previous studies. Likewise, the peak concentrations of CFU-S12 and CFU-GM were present in fractions FR30 and FR35 in our experiments and in the previous studies. Therefore, our protocol modifications did not influence the expected results for CFU-S12 and CFU-GM elutriation.

In the present experiments, 9% of the total HPP-CFC recovered from C3H marrow and 3% from C57 marrow were found in the FR24 and FR26 fractions. Schwartz et al. recovered 48% of all HPP-CFC in fraction FR25, but used elutriation conditions that varied from ours with respect to rotor type (JE-6), rotor speed (867g), and number of FR fractions selected. Most HPP-CFC (50%) from the B6D2F1 mice used in their study remained in the FR35 fraction, in contrast to the peak concentration residing in fraction FR30 from both C3H and C57 marrow in our study. Furthermore, HPP-CFC were not separable from CFU-S in the study of Schwartz et al. We propose that the differences in our results arise primarily from the methodological differences noted between their and our protocols; however, we cannot exclude that some differences may be related to choice of donor murine strain. Nevertheless, by increasing the number of FR fractions, using a standard elutriation chamber, and using a higher rotor speed, we have been able to identify subpopulations of HPP-CFC (from two different inbred strains) that were not apparent in the previous study.

Applying the rationale that those HPP-CFC requiring multiple hematopoietic growth factors for optimal in vitro
cloning are the most primitive HPP-CFC.\textsuperscript{2,3} we found that HPP-CFC isolated in FR24 and FR26 fractions appeared more primitive than any of the HPP-CFC subpopulations in the higher flow rate fractions. A previous report of a shift of the majority of elutriated murine HPP-CFC from lower to higher FR fractions in association with an increase in cell cycling (4 days post-5-FU treatment) supports our speculation that elutriated low-density HPP-CFC are more primitive than higher-density elutriated HPP-CFC.\textsuperscript{15} Experiments using additional hematopoietic growth factors that enhance detection of primitive HPP-CFC, such as IL-6,\textsuperscript{15} may further resolve the cycling status of the low-density HPP-CFC. Furthermore, Sca-1 is a monoclonal antibody that has been extensively used as a positive marker for murine hematopoietic stem and progenitor cell isolation using FACS analysis and cell sorting.\textsuperscript{2,3} The increased percentage of Sca-1\textsuperscript{+} cells in FR24 compared with other FR fractions and unfractionated marrow provides additional support for FR24 eluted cells being composed of primitive hematopoietic cells. Several reports on the use of rhodamine 123 dye retention to identify the most quiescent population of HPP-CFC, pre-CFU-S, or PHSCs\textsuperscript{6,12,16} suggest an additional method that could be applied to characterize the hierarchical relationship between the low-density and higher-density HPP-CFC described in these experiments.

Differences in the distribution of elutriated HPP-CFC in the various FR fractions were apparent when comparing bone marrow cells isolated from C3H and C57 mice. The graded increment in the percent of total C3H HPP-CFC isolated at each increase in FR, compared with the more abrupt changes in CFU-S\textsubscript{12} and CFU-GM distributions, suggests differences in cell size or density in the C3H HPP-CFC compartment that are distinctly associated with HPP-CFC proliferative status, maturation, and/or commitment. For CFU-S cells, differences in cell size appear to be a major determinant at which elutriation fraction the cells are extracted, although increasing cell density associated with a higher proliferative state is also involved in determining the distribution of CFU-S in elutriation fractions.\textsuperscript{15,17,19} There is little apparent overall difference in the distribution profile of elutriated HPP-CFC compared with that of CFU-S\textsubscript{12} and CFU-GM in marrow isolated from C57 mice, suggesting less differences in cell size and/or density among HPP-CFC, CFU-S\textsubscript{12}, and CFU-GM populations in this mouse strain. The significance of the differences in the physical characteristics of HPP-CFC from C3H and C57 mice that give rise to different elutriation profiles is unclear. However, if similar differences in PHSC characteristics exist between these strains, methods of enriching a population for PHSC using CCE may not be directly applicable from strain to strain. Some mouse strain differences in PHSC characteristics, such as the percentage of hematopoietic stem cells in cycle, have been reported for DBA, C3H, and C57 animals\textsuperscript{20} and different elutriation profiles of PHSC from normal marrow of B6D2F\textsubscript{1} and C57 mice have already been reported.\textsuperscript{13,14}

Defining the relationship between HPP-CFC and PHSC continues to be an active area of investigation. The unique subpopulation of HPP-CFC isolated by CCE at low flow rates will provide us with primitive cells that can be extensively characterized in the absence of more committed cells (CFU-S\textsubscript{12} and CFU-GM) and bone marrow stromal cell precursors. Combining monoclonal antibody identification and depletion of mature lineage-committed cells and positive selection of PHSC using monoclonal antibodies to stem cell antigens to the FR fractions containing low-density HPP-CFC will determine what proportion, if any, of these low-density HPP-CFC are PHSCs.\textsuperscript{5,7,12,13}

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High proliferative potential colony-forming cell heterogeneity identified using counterflow centrifugal elutriation

MC Yoder, XX Du and DA Williams