# Granulocyte colony-stimulating factor mobilized CFU-F can be found in the peripheral blood but have limited expansion potential

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## ABSTRACT

Bone marrow mesenchymal stem cells are multipotent cells found lining the bone marrow cavity supporting the growth and differentiation of hematologic progenitors. There is growing evidence that these cells can, under the right circumstances, enter the peripheral circulation. We show that granulocyte colony-stimulating factor mobilized peripheral blood contains cells which form colonies and have a similar fibroblastic morphology (termed CFU-F) to bone marrow mesenchymal stem cells. These cells were found at a very low incidence (0.0002%). Mobilized peripheral blood CFU-F were successfully differentiated into osteogenic and adipogenic lineages. FACS analysis showed that the cells had a similar profile to bone marrow mesenchymal stem cells. Importantly, mobilized peripheral blood CFU-F had limited expansion potential and became senescent 20-25 days after isolation. Mobilized peripheral blood CFU-F also did not have any telomerase activity and displayed significant telomere shortening. The rarity of CFU-F in mobilized peripheral blood and the subsequent pressure to divide in cell culture probably contribute to early cellular senescence. Their potential for use in transplant or gene therapy is, therefore, limited.

Key words: granulocyte colony-stimulating factor, mobilized peripheral blood, telomere, mesenchymal stem cell, bone marrow.

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## Introduction

Mesenchymal stem cells were described more than 40 years ago as a subpopulation of cells in the bone marrow that are plastic adherent in a tissue culture environment.[1] They are not a homogenous stem cell population, but contain several multipotent cell types that can differentiate into adipocyte, osteogenic cells, or cartilaginous precursors.[2] Mesenchymal stem cells also have a unique ability to down regulate the immune system with regard to graft versus host disease (GVHD) affecting T, B and NK cell activity and employ soluble as well as direct cell contact mechanisms.[3]

Granulocyte colony stimulating factor (G-CSF) is commonly used to mobilize hematopoietic stem cells from the bone marrow compartment into the peripheral circulation. Whether or not mesenchymal stem cells can migrate or mobilize into the circulation is unclear, but there is growing evidence that this can occur, although at a very low level.[4] Earlier studies in patients receiving G-CSF mobilized stem cell transplants showed that the mesenchymal stem cell population remained of recipient origin post-transplantation, but on a few occasions donor mesenchymal stem cells engrafted.[5-7] Recently, there has been increasing evidence to show that there are multipotent cells in the circulation that have biological traits and morphology that are similar, but not identical, to bone marrow mesenchymal stem cells.[8,9] Two previous studies have successfully used positive selection methods to isolate mesenchymal stem cells from G-CSF mobilized peripheral blood with either CD105 or dense fibrin microbeads.[10,11] Other early attempts to isolate mesenchymal stem cells from peripheral blood using the classical plastic adherence method (with or without G-CSF mobilization) had only limited success.[12-14]

We show the growth characteristics of a plastic adherent cell population which we successfully isolated from mobilized peripheral blood.

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*The online version of this article contains a supplemental appendix.*

## Design and Methods

### Isolation of mobilized peripheral blood CFU-F

Briefly, blood from G-CSF treated healthy volunteers was purchased from AllCells, LLC (Emeryville, CA, USA) (*see Online Supplementary Appendix)*. The use of all tissue was approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation, placed in Iscove's media with 20% fetal calf serum (FCS) (Hyclone), and plated on plastic culture flasks. Cells were left undisturbed for seven days without media changes and colonies of adherent cells were then noted on the flask. Culture flasks were washed with PBS and the adherent cells passaged by traditional means using trypsin digestion. Cell numbers and population doublings were measured throughout the culture period.

To isolate bone marrow derived mesenchymal stem cells, 10 mL of bone marrow aspirate was obtained from healthy volunteers. Mononuclear cells were prepared as above and plated like those of the mobilized peripheral blood CFU-F.

### Differentiation cultures

Approximately two weeks after isolation, mobilized peripheral blood CFU-F were in sufficient number to differentiate and 50,000 cells per well were placed in a 6-well plate with the appropriate media for osteogenic or adipogenic differentiation (*see Online Supplementary Appendix*). The culture period was 21 days, after which cells were washed with PBS, fixed for 10 mins. in 10% buffered formalin at room temperature and stained with Alizarin Red (for osteogenic cells) or Oil Red-O (for adipogenic cells). Undifferentiated cells were washed with PBS and stained with crystal violet.

### Telomere analysis

DNA was extracted from 2 million cells by standard methods and 1 microgram digested with the restriction enzymes HinfIII and RsaI (Invitrogen, Carlsbad, CA, USA). Fragments were separated on an 0.5% agarose gel and transferred to positively charged nylon membrane by vacuum. Standard Southern analysis was performed using a digoxigenin (Dig) 5' and 3' end-labeled telomere probe (TTAGGG). Detection was by a horseradish peroxidase labeled anti-Dig antibody (Roche) and chemiluminescence using CDP-STAR® (Roche). Average telomere lengths were determined by scanning the resulting Southern blot. The Telometric software program was used for the analysis.[15]

## Results and Discussion

### Isolation of mobilized peripheral blood CFU-F

The study shows that colony forming cells can be isolated from G-CSF mobilized peripheral blood by standard plastic adherence. The morphological appearance was identical to that of bone marrow derived mesenchymal stem cells (Figure 1A). On the basis of their appearance and phenotypic analysis these cells were termed mobilized peripheral blood colony forming unit-fibroblasts (mobilized peripheral blood CFU-F). The success of this method was limited because cultures could only be established in 50% of six attempts. The growth curves of three cultures that grew to three weeks are shown in Figure 2. An additional culture appeared as CFU-F, but did not proliferate.

Mobilized peripheral blood CFU-F were shown to have both osteogenic and adipogenic potential (Figure 1B). Attempts at chondrogenic differentiation were only minimally successful indicating that these cells are more limited in ability than typical bone marrow mesenchymal stem cells (*Online Supplementary Figure 1*).

Although there is no single cell surface antigen specific for mesenchymal stem cells, a panel of commonly positive CD markers on bone marrow mesenchymal stem cells was used to evaluate mobilized periph-

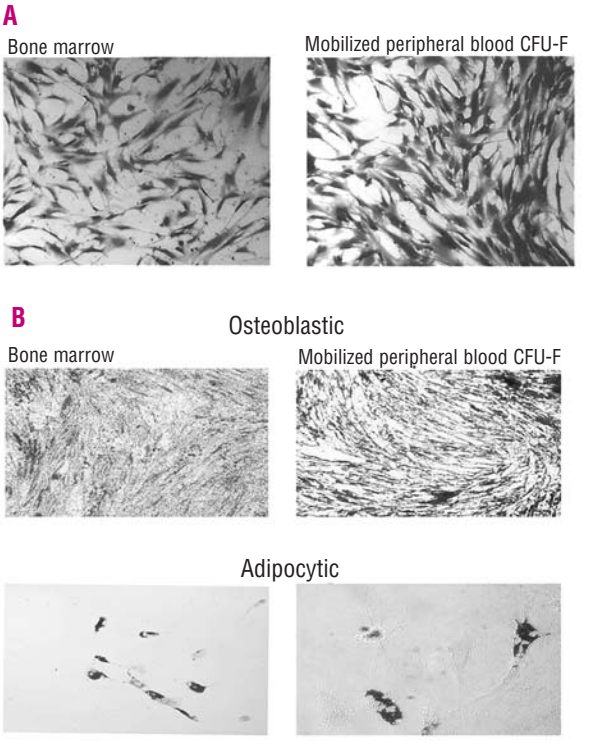
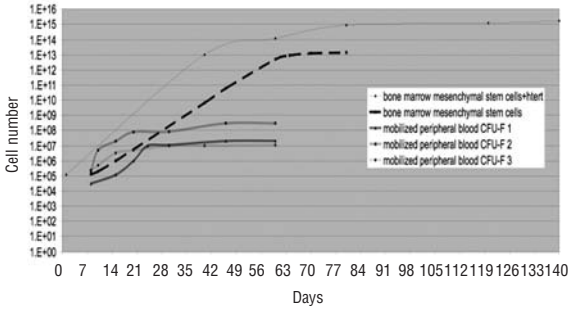



**Figure 1. Morphology and differentiation of adherent cells isolated from mobilized peripheral blood. A. Cells were isolated by plastic adherence as described in the Methods section. Adherent cells from bone marrow mononuclear cells were also isolated in an identical fashion and used for comparison. The isolated cells have a triangular and fibroblastoid appearance typical of mesenchymal stem cells. B. Cells at population doubling 5 were placed in a 6-well plate and differentiated for 21 days along osteogenic or adipogenic lines in the appropriate media. At the end of the culture period, cells were washed, fixed, and stained with alizarin red for calcium indicative of osteoblasts or Oil-Red-O, a lipid stain characteristic of adipogenic cells.**




| | *CFU-F* | *% of starting PBMC* | *Day 8 cell count* |
|---|---|---|---|
| Mobilized peripheral blood CFU-F1 | 300/$10^9$ | 0.00003 | 31,500 |
| Mobilized peripheral blood CFU-F2 | 200/$10^9$ | 0.00002 | 200,000 |
| Mobilized peripheral blood CFU-F3 | 1900/$10^9$ | 0.00019 | 245,000 |
| Bone marrow mesenchymal stem cells | 781/$10^7$ | 0.008 | 130,000 |

**Figure 2. Mobilized peripheral blood CFU-F have limited expansion potential in culture. Cells were isolated from bone marrow or from G-CSF mobilized peripheral blood from healthy donors. Culture initiation was performed as described in the *Design and Methods* section. The graph represents the cumulative number of cells during the culture period. Senescence occurred when the cells undertook no further divisions (curve flattens). A control cell line, (+htert), was taken from a separate experiment with bone marrow mesenchymal stem cells at population doubling 5 via infection with the human telomerase gene *htert* encoded via a retrovirus containing a bicistronic eGFP. Green cells were FACS sorted for eGFP expression and 100,000 cells were used to start a new culture on Day 1. The number of colonies with 30 or more cells counted at day 8 in culture is given in the table below as well as the percentage of the starting cell number.**

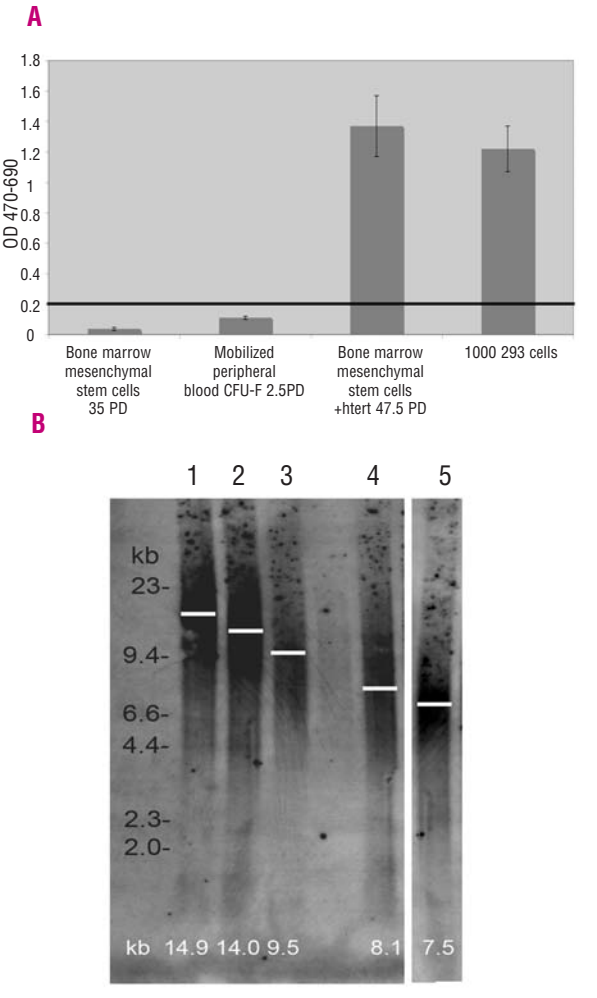



**Figure 3. Mobilized peripheral blood CFU-F lack telomerase activity and have shortened telomeres at early onset in culture. A. Protein was prepared from 250,000 cells and normalized by Bradford assay (Pierce). Telomerase activity was evaluated by the TRAPEZE® ELISA kit from Chemicon (Temecula, CA, USA). Units are expressed as OD 470–OD 690 nm absorbance. Background was determined from heat-inactivated extracts and subtracted from total activity. Samples with OD 470-690 >0.2 are considered to possess telomerase activity (red line) (n=4). Extracts from the 293 cell line and bone marrow mesenchymal stem cells+htert (bone marrow mesenchymal stem cells harboring the human telomerase gene) cell line were used as positive controls. B. Cells were harvested at the population doubling (PD) as noted and DNA prepared by standard methods. A Southern blot was performed using a labeled telomere probe: (TTAGGG)3. The white bar indicates the mean telomere length determined by unidirectional signal strength averaging using Telometric software (actual kb values are listed below each lane). Lane 1 is bone marrow mesenchymal stem cells+htert, lanes 2 and 3 are bone marrow mesenchymal stem cells PD 4 and 35 respectively, lane 4 is mobilized peripheral blood CFU-F1 at PD 5, and lane 5 is mobilized peripheral blood CFU-F2 at PD 5.**

eral blood CFU-F. The cells were consistently positive for CD13, CD29, CD105 and CD166. Cells were negative for CD14, CD34, CD45 and CD133. This pattern of expression was maintained throughout culture. An identical pattern of expression was seen in our bone marrow derived mesenchymal stem cells and is consistent with the published literature on mesenchymal stem cells[16] (*Online Supplementary Figure 2*).

### *Growth characteristics of mobilized peripheral blood CFU-F*

Enumeration and calculation of frequency show the cells to be at least 50 times less frequent than bone marrow mesenchymal stem cells existing at 0.00003%-0.0002% (Figure 2). The mobilized peripheral blood CFU-F expanded after some initial lag time in culture. After reaching approximately 6,500 cells/cm$^2$, mobilized peripheral blood CFU-F entered a more robust state of growth dividing roughly every 48 hrs. for about three weeks until they reached a point of senescence. Bone marrow derived mesenchymal stem cells were also isolated and grown in comparison to the mobilized peripheral blood CFU-F. Bone marrow mesenchymal stem cells continued in culture for a longer duration until reaching senescence at about 35-50 population doublings. There are several other reports of isolating mesenchymal stem cells from human peripheral blood by a variety of methods.[10,11,17] These reports have shown an early exponential expansion of the peripheral blood mesenchymal stem cells, but few comment on longetivity. Work by Zvaifler *et al.* has shown that cells with mesenchymal stem cell morphology and phenotype can be found in the peripheral blood. [18] It was estimated that several thousand mesenchymal stem cells were found in 0.5 liter of peripheral blood which is certainly less than <0.1% of PBMCs. Growth curve data showed evi-

dence of early exponential growth in culture, but was only reported to 17 days and achieved 7 population doublings. It is worth noting that in the work of Kassis *et al.*, mesenchymal stem cells were isolated from G-CSF mobilized peripheral blood, but could not grow past 18 days in culture.[10] Our growth curve data agree with both these observations. Lazarus *et al.* attempted to isolate mesenchymal stem cells from G-CSF mobilized peripheral blood but was unsuccessful. This was possibly due to the small volumes used (only 10 mL of blood).[14]

Whether CFU-F truly migrate from the bone marrow is difficult to prove. Several groups have shown that there are cells circulating which have mesenchymal stem cell-like properties and can contribute to organ repair when evaluated under injury or transplant conditions.[19] The native migration of PB CFU-F is unknown, but if they are present in the peripheral circulation they are likely to be very rare indeed. We did not find any CFU-F in non-G-CSF treated peripheral blood. While G-CSF allowed CFU-F identification to be made, it is possible that other cytokines such as interleukin-8 may be more effective for their mobilization.[20]

### Mobilized peripheral blood CFU-F lack telomerase and have short telomeres

Typically, bone marrow mesenchymal stem cells lack telomerase and have a defined lifespan of 30-50 population doublings. Neither our bone marrow mesenchymal stem cells or mobilized peripheral blood CFU-F showed any detectable telomerase activity. We also aimed to determine if there was expected telomere erosion *in vitro*. In evaluating telomere length, bone marrow mesenchymal stem cells at an early population doubling have relatively long telomeres at 14 kb, while those at higher population doublings show significant shortening (Figure 3B). Mobilized peripheral blood CFU-F from two samples were evaluated at an early population doubling and have approximately 50% shorter telomeres than their bone marrow counterparts.

It has been reported that bone marrow stroma cells isolated from older individuals have a decreased life span and undergo senescence earlier than those from younger people.[21] In addition, bone marrow mesenchymal stem cells do not express telomerase de novo, but ectopic telomerase expression can be induced to extend the lifespan of these cells while retaining their differentiation potential.[22] When evaluating the telomere length in peripheral blood mononuclear cells, some correlation to donor age has been shown.[23] We do not have that information for our cells (only that donors were over 18 years of age and in good health). Our culture outgrowths underwent significant cell divisions and subsequently lost telomeres for two main reasons. First, the cells were placed into optimal culture conditions, and second and more importantly, they are very rare in number and therefore under tremendous pressure to divide. Further population doublings of mobilized peripheral blood CFU-F could not be analyzed as cell quantities were limited. The significant telomere shorting probably contributes to mobilized peripheral blood CFU-F early cell senescence, although this could also be influenced by donor age. An ideal experiment would involve isolating both marrow derived mesenchymal stem cells and mobilized peripheral blood CFU-F from the same donor, but availability of this type of donation would be unlikely.

Despite limited ability to expand *in vitro*, one may theorize that the presence of mesenchymal stem cells in the peripheral circulation in times of organ damage/injury serves a specific purpose. Cells could be actively recruited to damaged tissues (via appropriate cytokines) and promote healing/repair as well as the recruitment of other cell types. Because mesenchymal stem cells are potent secretors of many cytokines, they may have a significant biological effect even in small cell numbers.[16,24]

While our isolation of CFU-F from mobilized peripheral blood is not necessarily novel, it does underlie the difficulty of isolating mobilized peripheral blood CFU-F using the current standard method of plastic adherence (our success rate was only 50%). This may be quite a significant limitation if these types of cells are ever to be considered for clinical studies. Additionally, their lack of expansion and limited differentiation show they have restricted potential, and although they share surface markers with bone marrow mesenchymal stem cells, they may, in fact, be different cells altogether.

In conclusion, we have shown that G-CSF mobilized PBMCs contain a population of cells with classical phenotypic markers of mesenchymal stem cells and limited differentiation properties. These cells are properly termed mobilized peripheral blood CFU-F. They are very rare in G-CSF mobilized peripheral blood and display limited expansion *in vitro*, most likely due to early cell senescence secondary to significant telomere erosion which will limit their potential for future use in the clinical arena.

## Authorship and Disclosures

TL: performed all experiments and wrote manuscript; JT: key experimental design and critical reading; PO: laboratory PI. The authors reported no potential conflicts of interest.

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