Journal of Bone Marrow Research
ISSN: 2329-8820

Milwid et al., J Bone Marrow Res 2013, 1:2
DOI: 10.4172/2329-8820.1000113

Research Article Open Access

Evidence of a Hematopoietic Origin of Human Bone Marrow Stromal Cells

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Abstract

Bone marrow stromal cells (BMSCs) have been considered to be of an ontological lineage distinct from hematopoietic cells in the bone marrow. Numerous studies have shown that BMSCs in culture exhibit an immunophenotype negative for hematopoietic markers such as CD45, CD34 and CD11b. In this study, we discovered that human BMSCs can be isolated by positive selection from CD45+ or CD34+ cells of whole bone marrow aspirates, but not CD11b+ fractions. Adherent cells from the CD34+ and CD45+ fractions displayed growth, morphology, surface markers, and *in vitro* multipotency consistent with BMSCs from whole marrow. These cells were then transplanted subcutaneously in mice and were found to support the formation of hematopoietic tissue in ectopic sites. These results suggest a previously unidentified link between human hematopoietic cells and BMSCs that may point to the existence of a common progenitor.

Keywords: Bone marrow stromal cell; Mesenchymal stem cell; Hematopoietic progenitor cell; Ontology

Abbreviations: BMSC: Bone Marrow Stromal Cell; CFU-F: Colony Forming Unit-Fibroblast

Introduction

Bone marrow stromal cells (BMSCs) are a critical cellular element of the marrow microenvironment [1] and support the production of blood cells from the bone cavity in adults [2,3]. Dysfunction of BMSCs has been directly associated with the promotion of hematological diseases such as multiple myeloma [4] and myelodysplastic syndromes [5]. Clinically, these cells have been increasingly explored in therapeutic trials for regenerative medicine [6,7] and immune-mediated diseases [8-10] based on their pleiotropic functions, many of which remain poorly understood. The ontogeny of BMSCs, although relatively unexplored, may provide insight into the specialized functions of these cells. However, the developmental precursor of BMSCs has been difficult to identify because BMSCs have no distinguishing features to track *in vivo*.

There is evidence that sites of developmental hematopoiesis, including the placenta, aorta-gonad-mesonephros, and fetal liver, are also populated by embryonic BMSCs [11-13]. However, once expanded in culture from human bone marrow samples, these isolated fibroblastoid cells are typically negative for the pan-hematopoietic marker, CD45, and a progenitor cell marker, CD34 [14]. Conflicting reports exist on the variable expression of CD34 in mouse preparations [15,16]. We hypothesized that BMSCs may originate from a hematopoietic cell that ultimately differentiates into a BMSC akin to a hematopoietic-mesenchymal transition. We report here that human BMSCs can be isolated from CD45+ or CD34+ bone marrow cells and display the same, classical *in vitro* and *in vivo* properties of BMSCs derived from conventional whole bone marrow preparations.

Methods

Isolation of BMSCs and cell culture

Fresh human bone marrow or CD34+ cells were purchased from Lonza Biologics. Mononuclear cells were isolated by Ficoll-Paque™ density separation (GE Healthcare). Bone marrow mononuclear cells were either directly plated or sorted for CD45+ or CD34+ cells using magnetic activated cell sorting (MACS) per vendor's instructions (Milltenyi Biotec). Cells were plated at 10,000 cells/cm^2 onto tissue culture flasks in BMSC expansion medium. BMSC expansion medium consisted of α-MEM (Sigma), 20 mg/L gentamycin (Sigma), 10% FBS (Hyclone), 1 ng/mL rhFGF-basic (R&D Systems), 100 U/ml penicillin (Sigma), and 100 µg/ml streptomycin (Sigma). Non-adherent cells were aspirated on day 7 and the adherent population was cultured for another 4-10 days prior to initial passage. Cells were passaged using 0.1% trypsin/0.1 % EDTA, and subcultured at a density of 5×10^3 cells/cm^2. All cultures were used between passages 0-3.

Colony forming unit assays

Limited dilutions of marrow cells were allowed to adhere and proliferate under BMSC expansion for ten days without medium change. The number of adherent colonies was enumerated using a Giemsa stain and visually counted.

In vitro differentiation

The multipotency of BMSCs was assessed *in vitro* by culturing the cells in specified differentiation media as described previously [17] for 3 weeks with medium changes every 3 days. Cells were stained with Alizarin Red or Oil Red O to assess osteogenic or adipogenic differentiation, respectively.

Flow cytometry

Cells were stained with BD Pharmigen™ CD44, CD45, CD29, CD73, CD106 or CD11b antibodies (BD Biosciences) after which flow cytometry was performed (Cell Lab Quanta™ SC, Beckman Coulter).

Subcutaneous transplantation and ectopic marrow formation

This experimental procedure was approved by Subcommittee on Research Animal Care at Massachusetts General Hospital. Adherent

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Received April 05, 2013; **Accepted** April 17, 2013; **Published** April 19, 2013

Citation: Milwid JM, Li M, Lee J, Yarmush ML, Parekkadan B (2013) Evidence of a Hematopoietic Origin of Human Bone Marrow Stromal Cells. J Bone Marrow Res 1: 113. doi:10.4172/2329-8820.1000113

cells were suspended in PBS and mixed 1:2 v/v with hydroxyapatite (HA) and tricalcium phosphate (TCP) powders (65:35, HA:TCP w/w; Sigma Aldrich) to establish HA/TCP slurries for each cell population. 150 uL of the slurry was implanted subcutaneously in four sites using an 18G needle in female nih/nu/xid/bg mice (Harlan). Eight weeks following transplantation, the implants were harvested and prepared for conventional hematoxylin & eosin (H&E) histology. Images were captured on a Nikon Eclipse E800 Upright Microscope.

Results and Discussion

BMSCs are typically isolated from whole bone marrow mononuclear cells based on differential adhesion. Upon isolation, these cells can be qualitatively described as fibroblastic, nonphagocytic, and able to give rise to colony forming units-fibroblastic (CFU-F) in a clonogenic manner [18]. To initially evaluate the hypothesis that hematopoietic marrow cells give rise to BMSCs, we enumerated CFU-F number from whole bone marrow compared to $CD34^+$ or $CD45^+$ sorted marrow cells. Using a limited dilution assay, we observed no statistical difference in the number of CFU-F from the three marrow preparations (Figure 1A). Morphologically, the cells derived from CFU-F displayed a similar fibroblastic morphology consistent with BMSCs (Figure 1B). Fibrocytes are circulating bone marrow-derived cells that are phenotypically a mixture of monocytes and fibroblasts expressing type I collagen and surface markers that include CD45, CD34, and CD11b [19]. To ensure that these data were not a result of fibrocyte contaminants or other impurities of our sorting methods, we measured CFU-F outgrowths from marrow cells that were purified for CD11b. No CFU-F were observed after plating CD11b+ cells in culture, which validated that the isolated cells were distinct from fibrocytes and our sorting method had insignificant impurities (data not shown). In addition, we also isolated CFU-F from CD34+ bone marrow cells that were provided by a commercial vendor, which assured ≥ 95% purity based on their manufacturing processes (data not shown). Taken together, these data suggested that $CD34^+$ and $CD45^+$ fractions contain the same number of clonogenic, fibroblastoid cells as whole bone marrow.

We next evaluated the immunophenotype of adherent cells that were culture expanded from whole bone marrow or sorted marrow populations. The expression of a classical panel of BMSC surface markers verified that the isolated fibroblastoid cells expressed CD44+, CD45-, CD11b-, and CD73+ (Figure 1C). Subtle variations in the mean fluorescent intensity of CD29 and CD106 expression may indicate phenotypic heterogeneity due to selection criteria during the initial sample fractionation. Alternatively, the data may suggest the gain of these markers as a cell transitions from a CD34+ cell towards a differentiated BMSC after culture expansion.

Under certain mechanochemical stimuli, it is reported that CFU-F can give rise to connective tissue cells such as osteoblasts and adipocytes [20,21]. We measured the capacity for CFU-F cells to differentiate into osteogenic and adipogenic lineages by using lineage specifying differentiation medium. As seen in figure 1D, all three fractions stain positive for transformation into osseous or adipose cells, respectively.

In vivo transplantation of expanded BMSCs in subcutaneous spaces has been reported to result in the ectopic formation of hematopoietic tissue [20,22]. We coated HA/TCP particles with human BMSCs derived from whole bone marrow, CD34+, or CD45+ cultures and subcutaneously implanted these constructs into immunocompromised mice. Eight weeks after implantation, ectopic bone marrow-like tissue

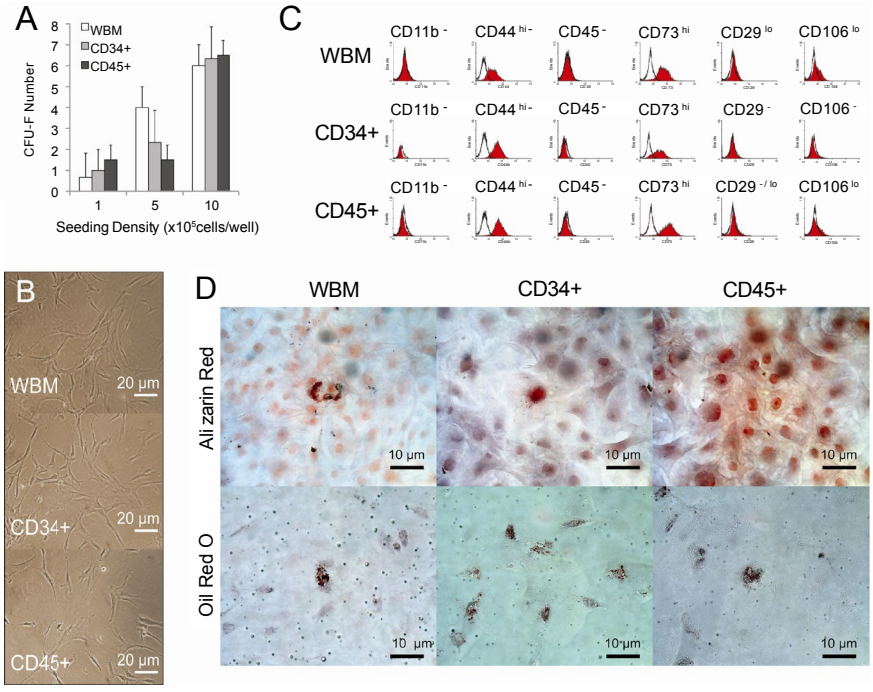

Figure 1: Adherent populations of $CD34^+$ and $CD45^+$ cells from whole human bone marrow exhibit BMSC phenotypes *in vitro*. (A) CFU-F for different seeding densities of CD34+, CD45+ and whole bone marrow fractions. (B) Morphology, (C) immunophenotype, and (D) osteogenic/adipogenic potential of the adherent cells in culture from the three fractions.

Micrographs were taken with 10x magnification. Results are representative of two independent trials.

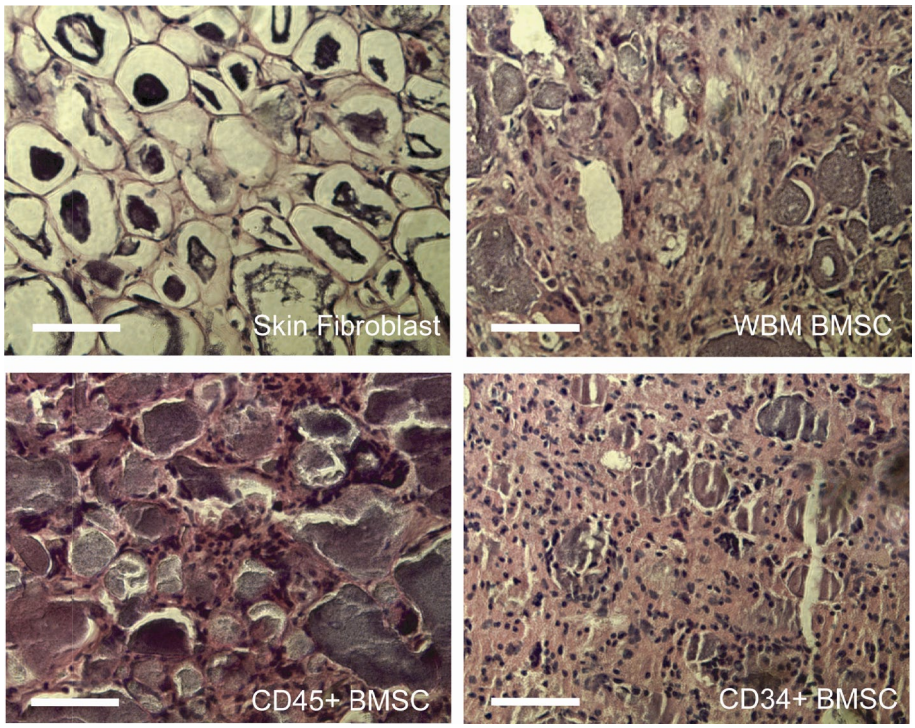

Figure 2: BMSCs derived from CD34$^+$ and CD45$^+$ populations possess the same capacity for ectopic marrow formation as whole bone marrow BMSCs. HA/TCP mixture with BMSCs from one of the three fractions were subcutaneously implanted into immunocompromised mice. Human skin dermal fibroblasts were used as control cells. Representative images of implants after 8 weeks. N=3 per group.

formed using all three BMSC preparations (Figure 2). Similar tissue formation and structure was observed in the whole bone marrow, CD34+, and CD45+, but could not be reproduced using human skin fibroblasts as a mock cell control.

In conclusion, we demonstrate, by multiple comparators, that human BMSCs can be isolated from hematopoietic cells by positive selection. These data may enable new purification methods of BMSCs and provide a new insight into a hematopoietic ontology of BMSCs.

Author Contributions

J.M.M. conceived idea, designed and executed experiments, interpreted data, and prepared manuscript; M.L. carried out histological techniques and prepared manuscript; J. L., created carrier particles and prepared manuscript; M.L.Y. designed experiments, prepared manuscript, and provided financial support; B.P. conceived idea, designed experiments, interpreted data, prepared manuscript, and provided financial support.

Acknowledgement

This work was partially supported by grants from the National Institutes of Health: K01DK087770 (BP), R01EB012521 (BP) and the Shriners Hospitals for Children (MLY, BP).

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