Standardized quantification of human bone marrow mesenchymal stromal cells based on a flow cytometry assay

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Introduction

The mesenchymal stromal cell (MSC) content is a major quality determinant of human bone marrow aspirates (BMA). It depends on the aspiration site, contamination with peripheral blood, as well as the aspiration procedure, and it negatively correlates with donor age. The common way of quantifying MSCs is based on the cultivation of cells using the colony forming unit–fibroblast (CFU-F) assay, which is time consuming and highly variable. Cuthbert *et al.* described a close linear relationship between the number of CFU-F colonies counted manually after 14 days of culture and the number of CD271^{bright} cells per mL of BMA[1]. The aim of this work was the optimization of a fast and reproducible flow cytometry–based MSC quantification method enabling prospective quality assessment of BMA.

Results

1 Clonogenic potential of CD271$^+$MSCA-1$^+$ human bone marrow cells

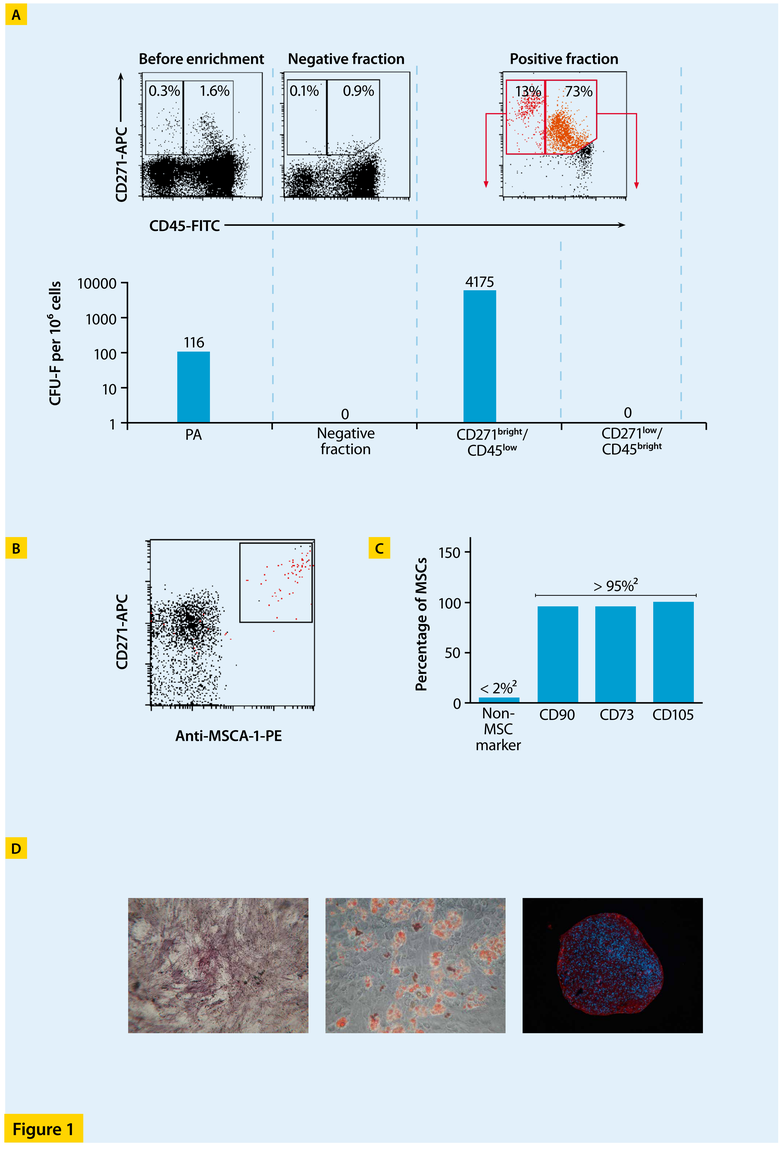

Figure 1

In previous experiments we have shown that only CD271^{bright} cells, which also express MSCA-1, give rise to CFU-F. MSCs were isolated from 2×10^7 bone marrow mononuclear cells (BM MNC) using CD271 (LNGFR)-APC and Anti-APC MicroBeads. Samples of all fractions were labeled with CD45-FITC. PI fluorescence and light scatter signals were used for gating live cells. The positive fraction contained CD271$^+$ cells with a purity of about 86% (Mean: 83±4.1%; n = 4). This population contained about 73% CD271^{low}CD45^{bright} cells (A, orange fraction) and about 13% CD271^{bright}CD45^{low} cells (A, red fraction). Further separation of CD271^{bright} and CD271^{low} cells was achieved by flow sorting. The different fractions were cultivated and CFU-F numbers were counted after 14 days. The numbers of CFU-F were about 40-fold higher when MSCs were cultivated from the CD271^{bright}/CD45^{low} fraction compared to MSCs obtained by plastic adherence (PA). No CFU-Fs were detected in the CD271$^-$ and CD271^{low}CD45^{bright} cell fractions (A). Counterstaining of isolated CD271$^+$ cells revealed a 100% co-expression of MSCA-1 in CD271^{bright}CD45^{low} cells (B). CD271^{bright}CD45^{low} MSCs met ISCT criteria[2] after culture expansion (3 passages) with respect to MSC-specific marker expression (C) and their differentiation potential towards osteocytes, adipocytes, and chondrocytes (D, left to right)

2 Flow cytometric detection of CD271$^+$MSCA-1$^+$ MSCs

To assess the MSC frequency in human BMA, two samples (MSC sample and control sample) were used. The MSC sample was stained with MSC Staining Cocktail (CD45-FITC, CD235a-VioBlue®, CD271-PE) and an Anti-MSCA-1 (W8B2)-APC antibody. To assess MSCA-1 specificity, the control sample was stained with MSC Control Cocktail (CD45-FITC, CD235a-VioBlue, CD271-PE, and the Anti-MSCA-1 isotype control, i.e., Mouse IgG1-APC). For details on the incubation steps see figure 2. Both samples were analyzed using the same gating strategy (fig. 3):

1: Debris was excluded by gating of leukocytes according to light scatter signals (P1).

2+3: Dead cells were excluded according to 7-AAD fluorescence (P2), erythrocytes according to CD235a expression (P3).

4: All CD271$^+$ cells were defined in P4 gated on P1, P2, and P3.

5: Shown is the control sample gated on P1, P2, P3 and P4. P5 was set as close as possible to MSCA-1$^-$ events (a) to include all MSCA-1$^+$ and MSCA-1^{dim} events in the MSC sample (b).

6: Median number of CD271$^+$MSCA-1$^+$ cells per mL of BMA amounted to 2200 (range: 330–5368) using bone marrow samples from 19 male and female donors (age: 21–80).

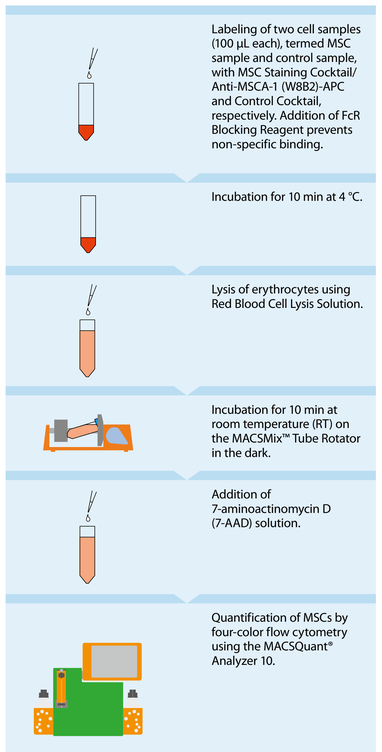

Figure 2

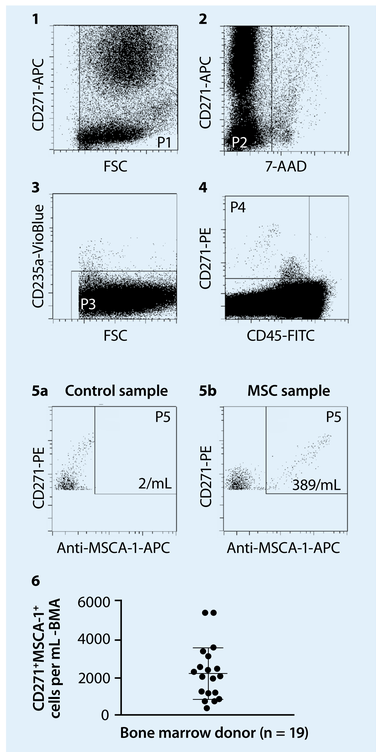

Figure 3

3 Assay accuracy: Detection of MSC-like cell line spiked into whole blood

Reproducibility and variability of the assay was assessed by spiking experiments. To this end, the indicated numbers of cells from the CD271$^+$MSCA-1$^+$ osteosarcoma cell line, SAOS-2, were titrated (triplicate) into 100 µL of human whole blood and then enumerated by flow cytometry using the MSC Enumeration Kit. An excellent correlation between input and recovered cells was observed (R2 = 0.997; P<0.0001) with low cell numbers.

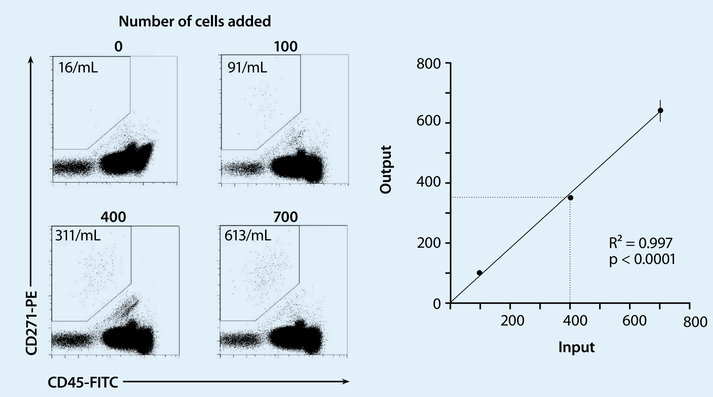

Figure 4

4 Intra-experimental validation

To evaluate intra-experimental variation, the numbers of CD271$^+$MSCA-1$^+$ cells in the MSC sample (stained with MSC Staining Cocktail) and control sample (stained with MSC Control Cocktail) were calculated for one milliliter of BMA in six parallel experiments with a sample from a single donor. The median number of MSCs detected in the MSC sample was 2882 (range: 2552–3366; SD = 348; CV = 11.9%). Non-specific staining of CD271$^+$MSCA-1$^+$ cells in the control sample was below 1% (median = 22; range: 0–88; SD = 30).

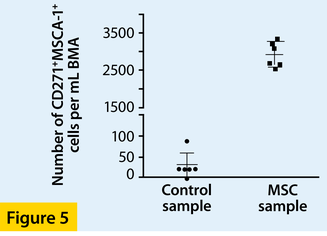

Figure 5

5 Correlation between CFU-F number and CD271$^+$MSCA-1$^+$ cells

The MSC frequency was determined by CFU-F assay and flow cytometry in parallel from 12 bone marrow samples. For this purpose, bone marrow cells were cultivated in a 6-well plate (4×10^5 cells per well) using StemMACS™ MSC Expansion Media Kit XF. Media was replaced with fresh media after 48 h and colonies were counted after 11 days. Furthermore, CD271$^+$MSCA-1$^+$ cells were quantified using the MSC Enumeration Kit. A linear relationship was observed between the number of colonies and the number of CD271$^+$MSCA-1$^+$ cells per mL of BMA (R2 = 0.753, P = 0.0003, n = 12 donors). The median values of CFU-F/mL and CD271$^+$MSCA-1$^+$ cells/mL were 485 and 2112 (range: 45–1202 and 330–5368) respectively.

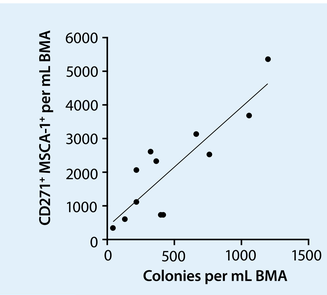

Figure 6

Conclusion

Based on an optimized cocktail of fluorochrome-conjugated antibodies and a four-color gating strategy we developed a reliable and quick procedure for absolute quantification of MSCs. The method allows prospective assessment of CFU-F yields in BMA in less than 30 minutes.

References
1. Cuthbert, R. *et al.* (2012) Cytotherapy 14: 431–440.
2. Dominici, M. *et al.* (2006) Cytotherapy 8: 315–317.

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 305436.