Sorting Bone Marrow Mesenchymal Stem Cells with the S3™ Cell Sorter

Xiaoti Guo, PhD
Bio-Rad Laboratories, Inc., 2000 Alfred Nobel Drive, Hercules, CA 94547

Introduction

Bone marrow (BM) mesenchymal stem cells (MSCs) are multipotent adult stem cells with the capacity to differentiate into cells that compose adipose, bone, cartilage, and muscle tissue, presenting a wide potential for cell-based therapies. According to the minimal criteria published by the International Society for Cellular Therapy and multiple studies (Boxall and Jones 2012, Dominici et al. 2006), CD73, CD90, and CD105 are the primary markers to identify MSCs across species, and their expression levels tend to be stable during culture. In contrast, CD106 (VCAM-1) and STRO-1 were reported to represent the most potent and undifferentiated MSCs, and they exhibit reduced expression levels after multiple passages (Boxall and Jones 2012, Halfon et al. 2011, Liu et al. 2008). Although no formal consensus has yet been reached on which markers may be best suited for bone marrow MSC identification, cell sorting by flow cytometry remains the most efficient approach to isolate MSCs from a highly heterogeneous environment like bone marrow. By using flow cytometry, MSC surface markers along with negative selection markers can be used to exclude contaminating hematopoietic cells (for example, CD19, CD34, CD45, or MHC II).

In this study, we used the S3 Cell Sorter to analyze and purify prospective MSCs from passage 1 (P1) and passage 3 (P3) bone marrow cultures. S3 Cell Sorter performance was evaluated and sorting data were compared between P1 and P3. The data presented here show the capability of the S3 Cell Sorter to isolate rare MSCs from a mixed bone marrow culture with satisfying sort efficiency and postsort purity. The finding that most of the P3 cells lose the expression of MSC markers CD106 and CD73 demonstrates the critical need for a better in vitro culture approach that can be implemented for tissue engineering and regenerative medicine strategies.

Results

To identify potential MSCs and to test the capability of the S3 Cell Sorter to purify stem cells from heterogeneous BM culture, P1 and P3 rat BM cells were stained for CD90, CD73 or CD106, and CD45, followed by staining with the viability dye VivaFix™ 498/521. Stained cells were analyzed on the S3 Cell Sorter outfitted with a 100 µm nozzle tip. Viable MSCs and non-MSCs were then sorted on the S3 Cell Sorter at up to 1,500 events per second (eps) and checked for postsort purity. Representative graphs in Figure 1 illustrate the following gating strategy. Since P1 bone marrow displays a relatively high level of heterogeneity, a broad gate is drawn to include

both SSC^{low} and SSC^{high} populations (Figure 1A). Following doublet exclusion (Figure 1B), viable ($VivaFix^{dim}$) and $CD45^{neg}$ populations were gated for surface marker expression (Figure 1C). As shown in Figure 2, about 15% of gated cells stain positive for both CD90 and CD73 (Figure 2B) and 3% coexpress CD90 and CD106 (Figure 2C), as determined by the phycoerythrin fluorescence minus one (PE FMO) control (Figure 2A). The dual positive cells are prospective MSCs and the dual negative ones are likely contaminating hematopoietic and stromal cells. Both prospective MSCs and non-MSCs were then sorted on the S3 Cell Sorter using Purity mode. After sorting, a CD90+ CD73+ population was enriched from 15 to 95% (Figure 2D), and CD90+ CD106+ from 3 to 92% (Figure 2E). Non-MSCs were enriched from 40 to 99% (Figures 2F and 2G).

The sorting performance for each population is summarized in Table 1, which represents results from three independent experiments. Theoretical efficiencies are calculated for a 100 µm nozzle tip at 30 psi sheath pressure and various input rates and target cell frequencies with CytoCalc Software v3.2.

To test whether rat bone marrow MSCs are able to maintain their phenotypic characteristics after in vitro expansion, P1 and P3 bone marrow MSCs were stained and analyzed on the S3 Cell Sorter to compare their expression of MSC-related surface markers (Figure 3 and Table 2). Compared with P1 MSCs, P3 cells revealed less heterogeneity in morphology as determined by microscopy, and SSC^{high} population increased from 50% in P1 to 90% in P3 (data not shown). These more homogeneous P3 cells expressed a greater level of CD90 compared to P1 cells (86 vs. 56%), but the CD73 and CD106 expression within the

CD90+ subset was drastically reduced (23 to 0.9% for CD73+; 6.4 to 0.02% for CD106+). These data suggest that although in vitro expansion may help eliminate contaminating non-MSCs from the culture, it may also compromise stem cell phenotype by downregulating stem cell marker expression.

Discussion

MSCs are rare in their residing tissues; the frequency of MSCs in bone marrow is 0.001–0.01%, diluted in a heterogeneous mix of hematopoietic cells (Bernardo et al. 2009). When MSCs are isolated, a heterogeneous pool of cells with various proliferation and differentiation potentials can be obtained (Bianco et al. 2001). The rareness and heterogeneity together make it difficult to identify, isolate, and purify MSCs. The adherent culture or panning approach is able to eliminate some of the contaminating hematopoietic cells; however, flow cytometry–based cell sorting remains the most reliable approach to isolate a pure population of MSCs for downstream cellular and molecular characterization.

Using a 100 µm nozzle tip, the S3 Cell Sorter was able to analyze and identify BM-derived MSCs from a mixed bone marrow culture and sort target cells at satisfying purity and efficiency. Cells were sorted at up to 1,500 eps with 30 psi sheath pressure and 41 kHz drop drive frequency, and the sort efficiency ranged from 91 to 98%. Note that the in vitro expanded bone marrow MSCs are relatively large (20–35 µm) for the 100 µm orifice. Therefore, to minimize disturbance of the break-off point and fanning of deflected streams, sort performance at a higher speed was not assessed at current settings. A nozzle tip with a larger diameter will be used to sort MSCs in future studies.

While non-MSCs were sorted at close to 100% purity, dual positive MSCs (CD90+ CD73+ and CD90+ CD106+) achieved only 95 and 92% purity, respectively. There are two possible reasons: 90% of CD90+ cells are larger in size (SSC^{high}) whereas CD90– cells are mainly SSC^{low} population. The large cells (relative to the nozzle tip's orifice diameter) likely have compromised sort purity due to insufficient drop charge and excessive side stream fanning (Arnold and Lannigan 2010, Petersen et al. 2003). Another possible factor contributing to the suboptimal purity is that dual positive cells are much lower in frequency (3% of target population) compared to the dual negative ones (40–50%), which will likely get lower purity and yield with the same instrument setup (Arnold and Lannigan 2010). Presorting rare populations on the S3 Cell Sorter in Enrich mode followed by Purity mode will likely help improve purity and yield.

The P1 cells used in this study were the first passage of Sprague-Dawley (SD) rat bone marrow, in which a significant amount of contaminating hematopoietic cells remained in the culture, mainly contributing to the CD90– population. After two more passages in 5 weeks, most of the contaminating hematopoietic cells were removed from the culture, which greatly increased the frequency of the CD90+ population in P3. But the expression of other stem cell markers, CD73 and CD106, drastically decreased in P3 cells. This finding is consistent with other reports, which showed CD106 downregulated in MSCs by tenfold after 2 weeks of osteogenic differentiation (Liu et al. 2008). Even without induction, the expression of CD106 could also reduce at later passage compared with earlier culture (Halfon et al. 2011). As a surface marker considered to be linked with the stem cell differentiation potential, a reduction in CD106 expression may suggest a compromised stem cell multipotency during in vitro culture (Boxall and Jones 2012, Liu et al. 2008). This could be caused by a serum component in the culture medium, which induces stem cell differentiation, or simply by the fact that cells with less potential outgrow the multipotent MSC subset. As an alternative approach, single cell sorting using Single mode on the S3 Cell Sorter can be used to separate the different stem cell subpopulations. The single colony–derived cells can then be cultured and evaluated separately.

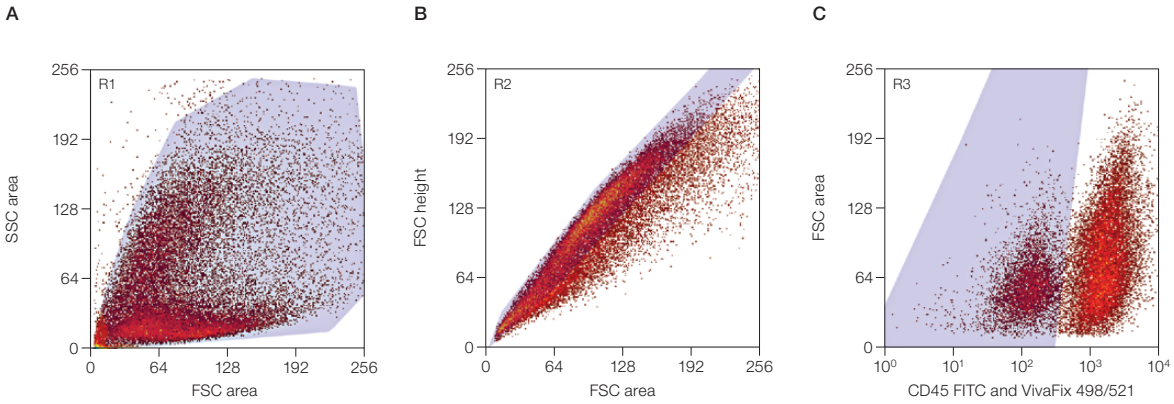

Fig. 1. Rat MSC gating strategy. Rat bone marrow MSCs (P1) were stained with CD90 PerCP-Cy5.5, CD106 PE or CD73 ReadiLink 555/570, and CD45 FITC and VivaFix 498/521, then acquired on the S3 Cell Sorter. After excluding debris and doublets (**A–B**), the fluorescence channel 1 (FL1) dim population was selected for further analysis (**C**). FITC, fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; SSC, side scatter.

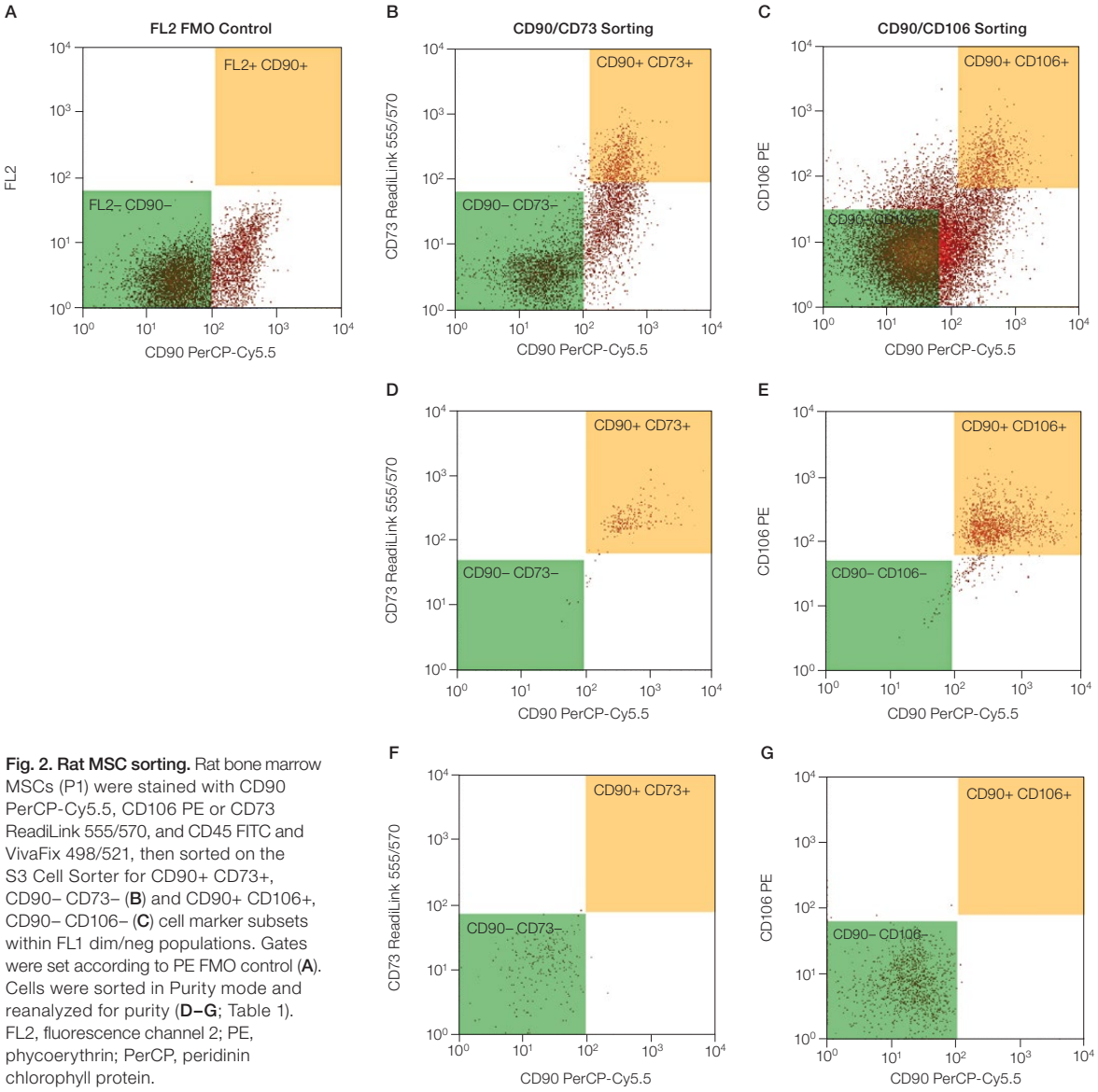

Fig. 2. Rat MSC sorting. Rat bone marrow MSCs (P1) were stained with CD90 PerCP-Cy5.5, CD106 PE or CD73 ReadiLink 555/570, and CD45 FITC and VivaFix 498/521, then sorted on the S3 Cell Sorter for CD90+ CD73+, CD90– CD73– (**B**) and CD90+ CD106+, CD90– CD106– (**C**) cell marker subsets within FL1 dim/neg populations. Gates were set according to PE FMO control (**A**). Cells were sorted in Purity mode and reanalyzed for purity (**D–G**; Table 1). FL2, fluorescence channel 2; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

Table 1. Sort performance.*

Cell Marker Subset	Presort Frequency (% of parent)	Postsort Purity (% of parent)	Sort Efficiency, %	Theoretical Efficiency, %**
CD90+	40	99	98	98.2
CD90+ CD73+	15	95	91	97
CD90– CD73–	40	97	94	97.8
CD90+ CD106+	3	92	96.2	96.6
CD90– CD106–	50	99	92	98.4

* The size of rat MSCs range from 20 to 35 µm. P1 MSCs were sorted in Purity mode at 1,000–1,500 eps with a 100 µm nozzle tip, 30 psi sheath pressure, and 41 kHz drop drive frequency. Data represent three independent experiments.

** Theoretical efficiencies are calculated with CytoCalc Software v3.2 for a 100 µm nozzle tip at 30 psi sheath pressure at various input rates and target cell frequencies.

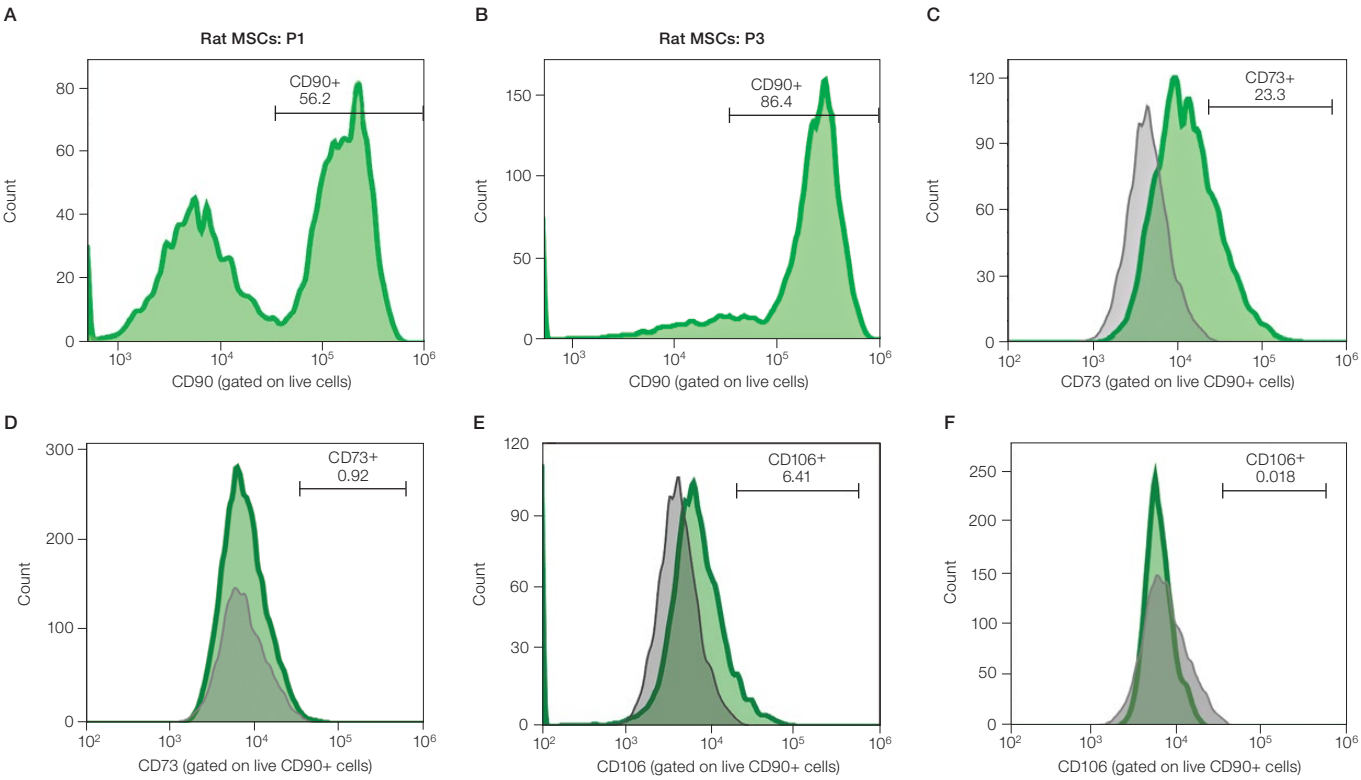

Fig. 3. Characterization of P1 vs. P3 rat bone marrow MSCs. Rat bone marrow MSCs were stained with anti-CD90 PE-Cy7, CD106 PE or CD73 ReadiLink 555/570, and CD45 FITC and VivaFix 498/521, then acquired on the S3 Cell Sorter. Live CD90+ (**A–B**), CD90+ CD73+ (**C–D**), and CD90+ CD106+ (**E–F**) subsets were compared between P1 and P3 MSCs. Gray traces indicate FMO control for the PE channel. The frequency of each cell marker subset is shown in Table 2.

Table 2. Frequency of P1 vs. P3 rat bone marrow MSCs.

Cell Marker Subset	P1 Frequency, %	P3 Frequency, %
CD90+	56	86
CD90+ CD73+	23	0.9
CD90+ CD106+	6.4	0.02

Conclusions

By using appropriate surface markers, the S3 Cell Sorter is capable of identifying bone marrow MSCs from a mixed BM culture and sorting target cells at satisfying purity and efficiency. Compared to P1 cells, P3 rat MSCs display a more homogeneous phenotype with an increased level of CD90 but decreased levels of CD90+ CD106+ and CD90+ CD73+ stem cell subsets, suggesting that prolonged culture may help remove contaminating cells but also could compromise stem cell phenotype and possibly affect their pluripotency. An improved long-term cell culture strategy should be investigated to minimize stem cell differentiation before therapeutic applications of MSCs can be realized.

Materials and Methods

Culture-expanded P1 and P3 rat bone marrow MSCs were kindly provided by Drs. Haixiang Liang and Daniel Grande at the Feinstein Institute for Medical Research, Manhasset, NY. Cells (1 x 10^7) were stained with the following mouse anti-rat monoclonal antibody panel: CD45 FITC (AbD Serotec®, catalog #MCA43FA), CD90 PE-Cy7 (BioLegend, Inc., #202517) or CD90 PerCP-Cy5.5 (BioLegend, #202515), CD106 PE (BioLegend, #200403), or CD73 ReadiLink 555/570 (Bio-Rad, #1351003). Purified anti-rat CD73 (BD Biosciences) was labeled with ReadiLink 555/570 according to the instructions in the ReadiLink Antibody Labeling Kit Product Insert (#10033055). Flow cytometry analysis and cell sorting were performed on the S3 Cell Sorter using ProSort™ Software (Bio-Rad) for acquisition and sorting. Dead cells were excluded with VivaFix 498/521 Dye (Bio-Rad, #1351115). Using the Purity mode, CD90/CD73 or CD90/CD106 dual positive (prospective MSCs) and dual negative (non-MSCs) subsets were sorted on the S3 Cell Sorter and separated from CD45+ hematopoietic cells and evaluated, and the frequencies of prospective MSCs in P1 and P3 were compared. Sorted cells were maintained in culture for downstream applications, including immunophenotyping and gene expression analysis.

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