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# Single-platform quality control assay to quantify multipotential stromal cells in bone marrow aspirates prior to bulk manufacture or direct therapeutic use

RICHARD CUTHBERT[1], SALLY A BOXALL[1], HIANG BOON TAN[2], PETER V. GIANNOUDIS[2], DENNIS MCGONAGLE[1] & ELENA JONES[1]

[1]*Academic Unit of Musculoskeletal Disease, Leeds Institute of Molecular Medicine, University of Leeds, UK, and* [2]*Academic Department of Trauma and Orthopaedics, School of Medicine, University of Leeds, UK*

**Abstract**

*Background aims.* The manufacture of multipotential stromal cell (MSC)-based products is costly; therefore, a rapid evaluation of bone marrow (BM) 'quality' with respect to MSC content is desirable. The aim of this study was to develop a rapid single-platform assay to quantify MSC in BM aspirates. *Methods*. Aspirated MSC were enumerated using the $CD45^{-/low}$ $CD271^{bright}$ phenotype and AccuCheck counting beads and compared with a classic colony-forming unit–fibroblast (CFU-F) assay. The phenotype of $CD45^{-/low}$ $CD271^{bright}$ cells was defined using a range of MSC (CD73, CD105, CD90) and non-MSC (CD31, CD33, CD34, CD19) markers. The effect of aspirated BM volume on MSC yield was also determined. *Results*. $CD45^{-/low}$ $CD271^{bright}$ cells had a classic MSC phenotype ($CD73^+$ $CD105^+$ $CD90^+$). Their numbers correlated positively with CFU-F counted manually ($R = 0.81$, $P < 0.001$) or using automatic measurements of surface area occupied by colonies ($R = 0.66$, $P < 0.001$). Simultaneous enumeration of $CD34^+$ cells revealed donor variability ranges compatible with standard International Society of Hematotherapy and Graft Engineering (ISHGE) protocols. Aspirating larger marrow volumes gave a significant several-fold reduction in the frequency of CFU-F and $CD45^{-/low}$ $CD271^{bright}$ cells per milliliter. Therefore aspirated MSC yields can be maximized through a standardized, low-volume harvesting technique. *Conclusions*. Absolute quantification of $CD45^{-/low}$ $CD271^{bright}$ cells was found to be a reliable method of predicting CFU-F yields in BM aspirates. This rapid (< 40 min) procedure could be suitable for intra-operative quality control of BM aspirates prior to volume reduction/direct injection in orthopedics. In the production of culture-expanded MSC, this assay could be used to exclude samples containing low numbers of MSC, resulting in improved consistency and quality of manufactured MSC batches.

**Key Words:** *bone marrow aspirate, enumeration, multipotential stromal cells, single-platform*

## Introduction

Cell therapy with multipotential stromal cells (MSC) is being used increasingly to treat graft-versus-host disease (GvHD) (1), fistulising Crohn disease (2) and to regenerate new bone following injury or as a result of avascular necrosis (3). Most commonly, MSC are manufactured by culture amplification of plastic-adherent cells derived from the bone marrow (BM). BM MSC numbers as typically procured by iliac crest marrow aspiration are low (4) and several rounds of culture passaging are commonly needed to obtain the required cellular yields for therapy applications (5).

It is now well recognized that the quality of manufactured culture-expanded MSC, in respect to both their proliferation and differentiation potentials, is linked directly with their *in vitro* cultivation history, commonly referred to as MSC '*in vitro* age' (6,7). The latter can be measured using telomere length analysis (8); however, the analysis is laborious, time consuming and hard to perform in routine clinical settings (9). Flow cytometry for CD106, CD146 and others molecules (10–12) and quantitative polymerase chain reaction (PCR) for selected potency- or senescence-related markers have been proposed recently (13); however, further cross-center validation is required to establish the potential applicability of these approaches (9).

One critical parameter that logically determines the longevity of manufactured MSC cultures is the number of native MSC present in the seeded sample (9). This is of considerable relevance given the reported large donor variability in the MSC frequency of BM aspirates (4,14,15). A major factor that is thought to

Correspondence: Dr **E. Jones**, Leeds Institute of Molecular Medicine, Clinical Sciences Building, St James's University Hospital, Beckett Street, Leeds LS9 7TF, UK. E-mail: msjej@leeds.ac.uk

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contribute to this variability and potentially very low MSC yields relates to the marrow aspiration procedure that inadvertently collects marrow diluted with peripheral blood from the marrow sinuses (16). The MSC content of BM samples is commonly measured by the classic colony-forming unit–fibroblast (CFU-F) assay (17). As with any other functional progenitor assay, it has many disadvantages, including long duration (2 weeks), dependence on serum lots and plating densities, and considerable subjectivity in defining and scoring colonies. Furthermore, colony sizes are known to differ widely from each other, while colony-scoring criteria remain poorly defined and rarely automated (18). A robust prior knowledge of the number of MSC seeded may therefore have widespread relevance for MSC manufacture processes on an industrial scale, both in terms of quality control and cost savings.

There is also an increased interest in the direct injection of whole BM or BM concentrates in clinical orthopedics (15,19). However, the CFU-F assay cannot be used at the outset to predict the injected quantity of autologous MSC, which is known to be a key factor in fracture repair (15).

The principal aim of this study was therefore to develop a rapid, automated single-platform assay for evaluating the MSC content of BM aspirates prior to culture initiation or direct use as therapy. A secondary aim was to 'troubleshoot' the variability of aspirated MSC content and suggest ways to maximize MSC yields. To accomplish these aims, we assessed the utility of a BM MSC enumeration assay based on the $CD45^{-/low}$ $CD271^{bright}$ phenotype (referred to hereafter as $CD271^{bright}$ cells) (11,20–24). CD271-positivity of BM MSC was first suggested by Quirici *et al.* (20), with this MSC identification technique refined further based on the inclusion of CD45 as a negative gating parameter (21). Gating for $CD271^{bright}$ cells was proposed by Buhring *et al.* (22) and later corroborated by Tormin *et al.* (24). CD146 and MSCA-1/W8B2, other candidate BM MSC markers, are also expressed on $CD271^{bright}$ cells (24,22). As a result of this study, we show that MSC can be enumerated easily on a volumetric basis based on the $CD45^{-/low}$ $CD271^{bright}$ phenotype, giving a rapid answer regarding the number of native MSC in a given aspirated sample. Using this approach, we also propose an optimized BM harvesting method for the maximization of the MSC yield in clinical BM aspiration protocols.

## Methods

### *Harvest of BM aspirate*

BM aspirates were obtained from the iliac crest of acute trauma patients or patients undergoing elective orthopedic surgery for metalwork removal; all patients had no other underlying disease ($n = 28$, 16 males, 12 females, median age 37, range 19–85). All samples were collected into $K_3$EDTA vacuettes using a 10-mL syringe and trocar (Stryker, Newbury, UK), both of which were first flushed with 1000 U/mL sodium heparin solution (Leo Pharma, Buckinghamshire, UK) to avoid clotting. Normally, 5 mL BM aspirate were obtained from a single aspiration site; the position of the needle was then altered by removal of the trocar and repositioning, ensuring that the trocar entered the cortical bone at a different angle. This procedure was performed a total of four times to give a final volume of 20 mL.

To investigate the effect of BM dilution with blood on the MSC frequency (dilution studies), the needle site and angle were kept constant and an initial draw of 5 mL was followed by a 15-mL volume draw from the same site, and the two draws were analyzed separately. Once collected, BM specimens were kept at room temperature (RT) and processed within 4 h of collection. The frequency of nucleated cells in BM aspirates was measured manually by dilution with 4% acetic acid and counting on a hemocytometer. Ethical permission for this study was obtained from the Leeds Teaching Hospital National Health Service (NHS) Trust (Leeds, UK).

### *CFU-F assay*

The CFU-F assay was performed as first described by Galotto *et al.* (25) and was used to enumerate MSC volumetrically. In this assay, 200 μL BM aspirate were seeded into duplicate 100-mm diameter tissue culture dishes (Corning, New York, NY, USA) containing 15 mL non-hematopoietic stem cell media (NH media; Miltenyi Biotec, Bisley, UK). The cells were incubated at 37°C, 5% $CO_2$. After 48 h non-adherent cells were removed by washing once with phosphate-buffered saline (PBS; Invitrogen, Paisley, UK) and fresh media were added. Subsequently, half media changes were performed twice a week for 14 days. At the end of the culture period the adherent cells were washed with PBS and fixed with 4% formaldehyde (Fisher Scientific, Loughborough, UK) before staining with 0.5% (v/v) crystal violet (Sigma-Aldrich, Dorset, UK). Fibroblastic colonies were counted macroscopically as described previously (26).

### *Measurements of colony area*

The total area of dishes covered by cells at the end of a CFU-F assay allows indirect evaluation of the sample's CFU-F activity, being a cumulative measure of both CFU-F number and their average proliferative capacity. Because manual counting of colonies and their sizes is considered fairly subjective

(27), digital measurements of total colony area were performed in parallel and correlated with the numbers of $CD271^{bright}$ cells. For this, dishes were scanned using an Epson 3590 digital scanner and digital images were analyzed using NIS elements BR 2.20 imaging software (Nikon, Tokyo, Japan). Using NIS elements, the area occupied by colonies was identified based on the intensity of the crystal violet stain. Dish area was classified into uncolonized and colonized regions and the area taken up by each region was calculated. No determination was made of individual colony size or morphology.

### *Cell staining and data acquisition for enumeration assay*

Enumeration of MSC ($CD45^{-/low}$ $CD271^{bright}$ cells) and hematopoietic progenitors (HP; $CD34^+$) was performed using multiparameter flow cytometry. AccuCheck™ counting beads (Invitrogen) were used to enable enumeration of all populations to be performed on a volumetric basis (i.e. number of cells/mL). To perform the staining, 50 µL BM aspirate were incubated for 15 min at RT with CD45–phycoerythrin (PE)–cyanine dye 7 (Cy7) (BD Pharmingen, Oxford, UK), CD271–allophycocyanin (APC) (Miltenyi Biotec) and CD34–PE (BD Pharmingen) at the manufacturer's recommended concentrations (Table I). Erythrocytes were then lysed with the addition of 2 mL ammonium chloride solution (168 mM $NH_2Cl$, 10 mM $KHCO_3$, 1 mM Ethylenediaminetetraacetic acid (EDTA), pH 8.0) containing 0.5 µg/mL 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) and incubation at RT for 10 min. Immediately following red cell lysis, 50 µL AccuCheck counting beads (Invitrogen) were added and the cell/bead suspension was analyzed immediately using an LSR II flow cytometer (BD Pharmingen). Centrifugation and resuspension of the cell suspension were avoided because this would result in inaccurate reporting of cell frequency because of loss of cells in the centrifuged supernatant.

Single-antibody stained and unstained controls were used before each experiment to ensure accurate spectral compensation, and isotype controls (Table I) were used to confirm the gating position for all cell populations studied. A minimum of 250 000 events was collected for each sample. The acquisition time was dependent on the cellularity of the sample: on average samples were acquired in 7 min, 23 s (range 3:16–14:42), with the event rate being set as 2000 events/s. If more than 5 min were required to collect a sufficient number of events, the sample was remixed to ensure sample homogeneity. In order to investigate variability between different CD271 antibody clones, three samples were also stained with CD271–PE (Miltenyi Biotec) and NGFR–PE (BD Pharmingen) (Table I).

### *Analysis of flow cytometry data for enumeration assay*

Flow cytometry data were analyzed using FACS-Diva Version 5.02 Becton Dickinson (BD). Beads were distinguished from cells based on their size and fluorescent properties, as described in the Results. Cell debris was excluded based on forward and side scatter, and dead/dying cells were identified by the uptake of DAPI. Cell subsets were identified as $CD271^{bright}$ cells ($CD45^{-/low}$ $CD271^{bright)}$ and HP ($CD45^+$ $CD34^+$). To calculate the absolute count of the cells of interest per milliliter of BM aspirate, the following formula was

Table I. Antibody conjugates used for flow cytometry.

| Target | Flourochrome | Clone | Manufacturer |
|---|---|---|---|
| Enumeration | | | |
| CD271 | Allophycocyanin (APC) | ME20.4-1.H4 | Miltenyi Biotec |
| CD271 | R-phycoerythrin (PE) | ME20.4-1.H4 | Miltenyi Biotec |
| CD271 | R-phycoerythrin (PE) | C40-1457 | BD Pharmingen |
| CD34 | R-phycoerythrin (PE) | 563 | BD Pharmingen |
| CD45 | PECy7 | HI30 | BD Pharmingen |
| Phenotyping | | | |
| CD73 | R-phycoerythrin (PE) | AD2 | BD Pharmingen |
| CD90 | R-phycoerythrin (PE) | MCA90 | Serotec |
| CD105 | R-phycoerythrin (PE) | SN6 | Serotec |
| CD31 | Fluorescein isothiocyanate (FITC) | WM59 | Serotec |
| CD33 | Fluorescein isothiocyanate (FITC) | HIM3-4 | BD Pharmingen |
| CD19 | R-phycoerythrin (PE) | HIB19 | BD Pharmingen |
| Isotype controls | | | |
| IgG1 | Fluorescein isothiocyanate (FITC) | MOPC-31C | BD Pharmingen |
| IgG1 | R-phycoerythrin (PE) | MOPC-21 | BD Pharmingen |
| IgG1 | Allophycocyanin (APC) | IS5-21F5 | Miltenyi Biotec |

used: number of events of interest/total number of bead events × starting concentration of counting beads per milliliter.

### *Extended phenotyping of CD45$^{-/low}$ CD271$^{bright}$ and CD45$^{+}$ CD271$^{low}$ cells*

Extended phenotyping with CD73, CD90 and CD105 (MSC markers) and CD31, CD33 and CD19 lineage markers (endothelial/myelo/megacariocytic, myeloid and B-lymphoid lineages, respectively) was performed to investigate the MSC lineage nature of CD271$^{bright}$ and CD271$^{low}$ (CD45$^{+}$ CD271$^{low}$) cells (Table I). Flow cytometry was performed following mononuclear cell (MNC) isolation using Lymphoprep (Axis Shield, Dundee, UK). The percentage of cells expressing both MSC and lineage markers was determined for each population of interest.

### *Statistics*

The Shapiro–Wilk normality test was used to assess the distribution normality and to determine the appropriate correlation and significance testing. Friedman's two-way analysis of variance by ranks was used to assess differences in cell survival during data acquisition. The Wilcoxon matched-pairs signed rank test was used to detect differences between draw volumes. The Spearman rank correlation test was used to measure statistical dependence as well as to generate a 95% confidence interval of predicted individual values. Statistical significance was defined as $P < 0.05$. All statistics were calculated using SPSS® Version 19. Graphs were generated using GraphPad Prism® Version 5.04.

## Results

### *Reagent selection and gating strategy for the enumeration of CD271$^{bright}$ cells*

The advantages of single-platform, lyse-no-wash protocols include short duration, elimination of the loss of cells through centrifugation and increase in accuracy by avoiding variations inherent with the use of different cell-counting methods (28). The use of fluorescent counting beads provides an absolute count of the cells by measuring the ratio of beads and the cells of interest.

Given these advantages, an AccuCheck bead-based single-platform approach was selected, based on the additional advantage of AccuCheck beads to serve as an internal control for sample mixing (because of the presence of two types of beads) (Figure 1). In our experiments, beads were first discriminated from the cells based on their scatter and fluorescence properties, and enumerated within the 'bead' gate (Figure 1A). Subsequent gating was performed on 'non-beads' (gray) events using scatter thresholds to exclude cell debris and cell aggregates, respectively (Figure 1B). DAPI was used as a viability marker, allowing the exclusion of dead/dying cells based on DAPI-positivity (Figure 1C).

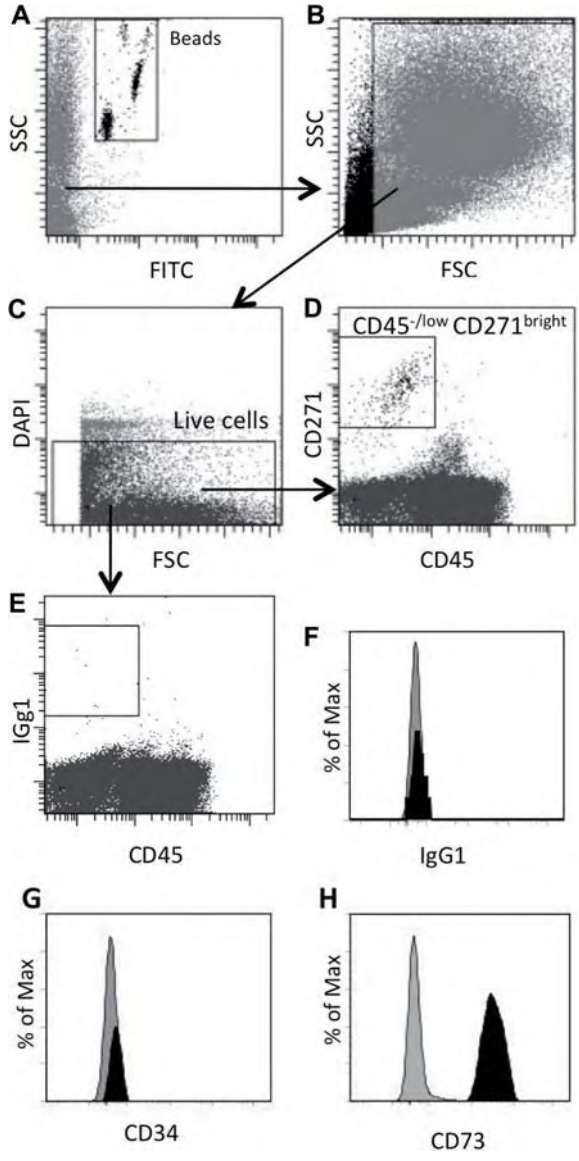


Figure 1. Gating strategy for the enumeration of CD271$^{bright}$ cells. (A) AccuCheck counting beads are quantified and removed from analysis based on emission on the fluorescence (FL1) channel. (B) Cell debris are then removed from the analysis based on forward scatter. (C) Dead cells are gated out based on the uptake of DAPI. (D) CD271$^{bright}$ cells are enumerated on a CD45/CD271 dot-plot within a simple rectangular gate. (E) Minimal non-specific binding is observed when an IgG1 isotype control is used instead of CD271 antibody. (F) Histogram overlay shows no non-specific binding of IgG1 to CD271$^{bright}$ cells (black) in comparison with CD45$^{bright}$ cells (grey). (G, H) Histogram overlays show CD34 and CD73 expression on CD271$^{bright}$ cells (black) in comparison with CD45$^{bright}$ cells (grey).

The accuracy of rare cell enumeration by flow cytometry, including measuring $CD34^+$ cells, is known to increase with the use of multiparameter gating and the inclusion of a low-expressed marker alongside a highly expressed marker. We and others have shown previously that BM MSC have a $CD45^{-/low}$ $CD271^{bright}$ phenotype (21,23,24), confirming that CD45 can serve as a very good 'negative discriminator'. Indeed, using a CD45/CD271 dot-plot data display, $CD271^{bright}$ cells could be easily identified and enumerated (Figure 1D). In the course of this investigation, the proportion of $CD271^{bright}$ cells was determined using CD271–APC and CD45–PE–Cy7, based on a basic rectangular gate (Figure 1D), and the absolute count of $CD271^{bright}$ cells per milliliter of BM aspirate was calculated in relation to the number of events in the bead gate, as described in the Methods. The use of an isotype control antibody instead of CD271–APC showed minimal non-specific binding (Figure 1E).

In the same sample tubes, CD34–PE was added for the simultaneous enumeration of $CD34^+$ cells. Isotype control IgG1–PE demonstrated no non-specific binding (Figure 1F). As shown previously (29), the CD34 expression of gated $CD271^{bright}$ cells was negligible (Figure 1G). On the other hand, $CD271^{bright}$ cells were uniformly $CD73^+$, confirming their MSC identity at a phenotypic level (Figure 1H). The expression of CD73 on $CD271^{bright}$ cells has been shown in several independent investigations (22–24,29).

### *Linearity and precision of absolute cell counting*

Comparison of automated and manual cell counts confirmed a direct linear relationship between these two counting methods ($R = 0.98$, $P < 0.001$; Figure 2A). The high degree of correlation confirmed that AccuCheck counting beads were a reliable means of measuring cell number and also enabled the absolute quantification of cells of interest in BM aspirates. Additionally, close agreement between automated and manual counting techniques confirmed that the chosen gating strategy for removing cell debris was effective at segregating debris from intact cells, thus reducing the risk of experimental error as a result of antibody binding to debris.

### *Correlation between the frequency of $CD271^{bright}$ cells and CFU-F*

Using BM samples from $n = 25$ donors, a close linear relationship was observed between the number of CFU-F colonies counted manually after 14 days of culture and the number of $CD271^{bright}$ cells per milliliter of aspirate ($R = 0.812$, $P < 0.001$; Figure 2B).

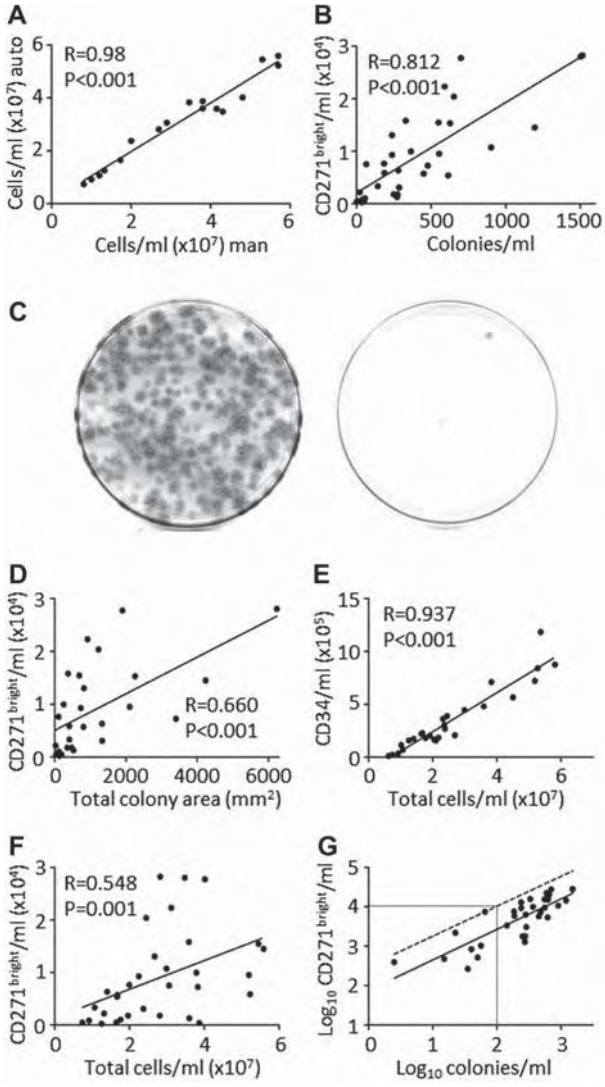


Figure 2. Quantification of $CD271^{bright}$ and $CD34^+$ cells by flow cytometry and correlations with CFU-F assay and sample cellularity. (A) Correlation between cell counts obtained using a hemocytometer (manual, man) and using AccuCheck counting beads (automatic, auto). (B) Correlation between the frequency of day-14 CFU-F and the frequency of $CD271^{bright}$ cells per milliliter of BM aspirate. (C) Representative CFU-F dishes showing a specimen with high (left) and low (right) MSC frequency. (D) Correlation between the total CFU-F colony area and frequency of $CD271^{bright}$ cells. (E) Correlation between the cellularity of aspirate and the frequency of $CD34^+$ cells. (F) Correlation between the cellularity of aspirate and frequency of $CD271^{bright}$ cells. (G) Predicting a sample's CFU-frequency based on $CD271^{bright}$ cell enumeration.

The median values of CFU-F/mL and $CD271^{bright}$ cells/mL for the studied cohort were 280 and 7230 (range 3–1503 and 398–28210), respectively. The line of best fit suggested that approximately 1 in 17 $CD271^{bright}$ cells formed a CFU-F colony in our experimental conditions. CFU-F colony assay dishes representing specimens with very high and very low MSC contents are shown on Figure 2C.

The numbers of CD271$^{bright}$ cells also correlated strongly with the total area occupied by CFU-F colonies ($R = 0.66$, $P < 0.001$; Figure 2D). The total area occupied by CFU-F colonies and CFU-F colony number correlated very well with each other ($R = 0.86$, $P < 0.001$).

### *Correlation between sample cellularity and the frequency of CD34$^+$ cells and CD271$^{bright}$ cells*

The enumeration of CD34$^+$ cells was included in our experimental protocol for two reasons: first, as a quality control for the CD271$^{bright}$ cell population (Figure 1), and second, to assess the feasibility of the enumeration of these two important cells subsets (MSC and HP) in a single tube. Our data on the numbers of CD34$^+$ cells/mL showed very good compatibility with the known ranges obtained using International Society of Hematotherapy and Graft Engineering (ISHGE) CD34 enumeration protocols (median 221/μL, range 22–749) (30). The CD34$^+$ cell counts correlated very closely with the cellularity of the aspirate ($R = 0.937$, $P = 0.001$; Figure 2E). A significant, but much weaker, correlation was observed between sample cellularity and the number of CD271$^{bright}$ cells ($R = 0.548$, $P = 0.001$; Figure 2F).

### *The ability of CD271$^{bright}$ cell enumeration to predict a sample's CFU-F content*

We next explored the ability of the CD271-based enumeration assay to predict a specimen's CFU-F content (Figure 2G). The logarithmic data transformation was used to display better the whole range of values (particularly the bottom range) and a 95% confidence interval of predicted individual values was calculated to establish a cut-off point for the exclusion of specimens with potentially poor CFU-F content (< 100 CFU-F/mL). As seen in Figure 2G, samples with CD271$^{bright}$ cell values of greater than $10^4$ cells/mL (or 10 cells/μL) were 95% likely to produce good yields of CFU-F colonies (>100 CFU-F/mL).

Overall, these data showed that although the cellularity of an aspirate weakly correlated with its MSC content, a more accurate quantification, with a high degree of confidence across the whole spectrum of values, was achieved by CD271$^{bright}$ cell enumeration.

### *Method validation*

As the CD271$^{bright}$ population is extremely rare (median 0.026%, range 0.001–0.100) and the event rate was kept to 2000 events/s to avoid blockage of the cytometer flow cell, up to 10 min were normally required to collect a sufficient number of events for analysis (a minimum of 100 cells in the CD45$^{-/low}$ CD271$^{bright}$ gate). To investigate any potential loss of cells other than erythrocytes during prolonged data acquisition in ammonium chloride-containing buffer, the number of cells that had not taken up DAPI was next analyzed step-wise over a 10-min period following the initial 10-min incubation to remove erythrocytes. The 10-min data collection acquisition period was divided into five 2-min intervals and the number of cells detected was expressed as a proportion of the total (Figure 3A,B).

As seen in Figure 3A, there were no significant differences in the proportions of total live cells detected during earlier or later periods of data acquisition. This indicated that ammonium chloride did not have any detrimental effect on total cell viability. The lack of a decline in cell acquisition rate at the later time points also suggested that cell sedimentation at the bottom of a tube during later stages of acquisition was unlikely. Similarly, the proportions of CD271$^{bright}$ cells were analyzed, and no significant differences between any time-points were observed (Figure 3B). This indicated that the detection of CD271$^{bright}$ cells was

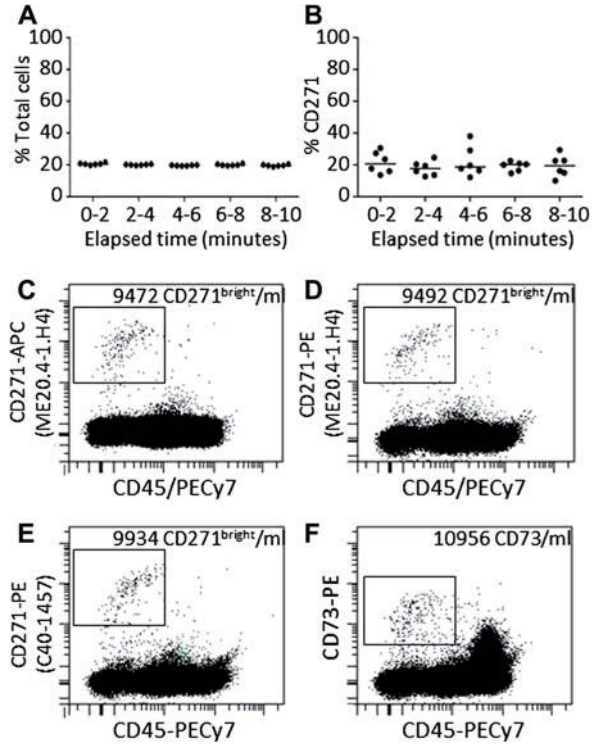


Figure 3. Method validation: sample viability during acquisition (A, B) and the use of different antibodies (C–F). Total cells (A) or CD271$^{bright}$ cells (B) detected over five continuous 2-min intervals as a proportion of the total respective cells acquired during the whole 10-min interval. Similar proportions indicate that cells remain viable. Representative flow cytometry plots showing enumeration of CD271$^{bright}$ cells on the same sample using anti-CD271 clone ME20.4-1.H4 APC (C) or PE conjugate (D) and anti-CD271 clone C40-1457 PE conjugate (E). Enumeration of CD45$^{-/low}$ CD73$^{bright}$ population (F).

not affected by prolonged incubation in ammonium chloride. To characterize the intra-assay variability of this technique, a single aspirate was split into six tubes, which were analyzed consecutively. The coefficient of variation for the proportion of CD271$^{bright}$ cells was measured at 0.105, indicating a signal to noise ratio of approximately 10:1.

To assess the effect of different anti-CD271 antibody conjugates or clones on the detection of CD271$^{bright}$ cells, enumeration data obtained using CD271–APC and CD271–PE (both from Miltenyi Biotec; clone ME20.4-1.H4) were compared with data obtained with BD CD271–PE (clone C40-1457) ($n = 3$). Additionally, CD73–PE was evaluated alongside CD271–APC ($n = 7$ donors). The representative experiment shown in Figure 3C illustrates the very similar results obtained with all three CD271 antibodies, and no statistical significance was found (average values of 4180, 3954 and 4198 cells/mL for CD271–APC, CD271–PE clone ME20.4-1.H4 and CD271–PE clone C40-1457, respectively). Data obtained on a subset of samples using CD73 in combination with CD271 showed slightly higher frequencies of CD73$^{bright}$ cells (median 9933, range 2711–36193 cells/mL) compared with CD271$^{bright}$ cells (median 7637, range 1778–28210, $P = 0.018$). The strong correlation between the frequencies of CD73$^{bright}$ and CD271$^{bright}$ cells ($R = 0.964$) indicated that CD73 could also be used for MSC enumeration in BM aspirates (16), but in our hands the CD271-based method provided better discrimination and less 'noise'.

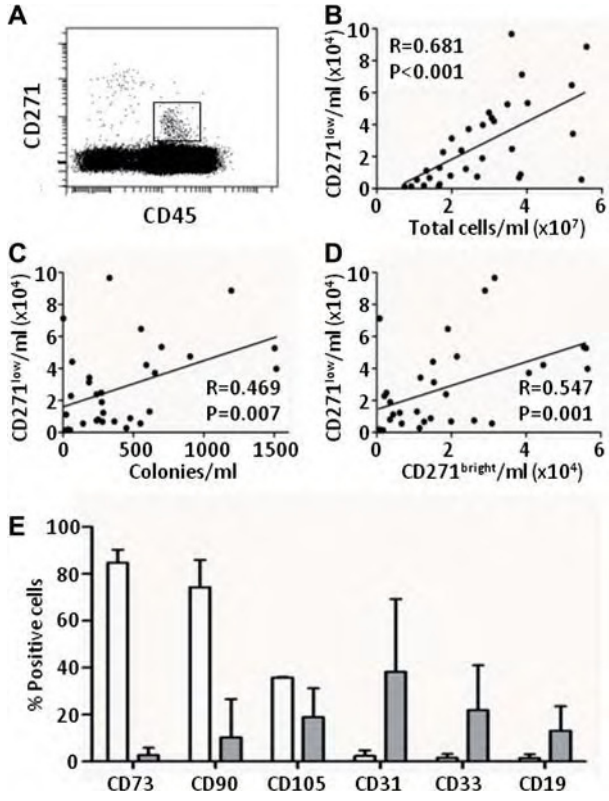


Figure 4. Investigation of CD45$^+$ CD271$^{low}$ cell population. (A) Representative flow cytometry plot showing gating for the CD45$^+$ CD271$^{low}$ cell population. (B) Correlation between the sample cellularity and the proportion of CD271$^{low}$ cells. (C) Correlation between the proportions of CFU-F and CD271$^{low}$ cells. (D) Correlation between CD271$^{bright}$ and CD271$^{low}$ cells. (E) Expression of MSC and non-MSC specific markers on CD271$^{bright}$ cells (white) and CD271$^{low}$ cells (grey). Values are expressed as a percentage of the positive cells above isotype control levels. All values are significant at $P = 0.05$ except for CD19.

### *Investigation of the CD271$^{low}$ cell population*

Apart from CD271$^{bright}$ cells, BM aspirates consistently contained a population of CD45$^+$ CD271$^{low}$ cells (CD271$^{low}$) (Figure 4). In fact, this population was more numerous than CD271$^{bright}$ cells (median frequencies of 0.073 and 0.026%, respectively) (Figure 4A). The proportions of CD271$^{low}$ cells positively correlated with total sample cellularity ($R = 0.681$, $P < 0.001$; Figure 4B), suggesting that these cells were unlikely to originate from blood.

When the frequency of CD271$^{low}$ cells was compared with CFU-F data from the same donors, only a weak relationship was found (Figure 4C) and the same was observed for the correlation between CD271$^{low}$ cells and CD271$^{bright}$ cells (Figure 4D). The presence of CD45 and the lack of a strong correlation with CFU-F indicated that CD271$^{low}$ cells were most likely to be hematopoietic in origin. Indeed, when extended phenotyping of CD271$^{low}$ cells and CD271$^{bright}$ cells was performed in parallel, the non-MSC nature of CD45$^+$ CD271$^{low}$ cells was confirmed (Figure 4E). This was consistent with data reported by Tormin *et al.* (24), who documented that CD45$^+$ CD271$^{low}$ cells were enriched for HP. In contrast, CD271$^{bright}$ cells had a clear MSC phenotype (Figure 4E).

### *Quality control of BM aspiration*

A highly reproducible quality-controlled marrow aspiration technique is essential for investigation of the effect of age and disease on MSC dynamics in the marrow. As reported previously, hemodilution of BM may be the prime reason for the huge (up to 500-fold) variability in MSC frequency in aspirated marrows (16). In order to examine marrow dilution effects further and to optimize the collection of MSC in BM aspirates, the frequency of CD271$^{bright}$ cells in the first 5 mL of the aspirate was compared with that in the following 15 mL aspirated from the same site (Figure 5A). The results demonstrated a significant 7-fold drop in the frequency of CD271$^{bright}$ cells in the second 15-mL draw fraction (average frequency 2197 cell/mL, $n = 7$) compared with the first 5-mL

draw (average frequency 14450 cell/mL, $P = 0.018$). The same trends were observed for CFU-F (9-fold, $P = 0.028$; Figure 5B). Representative dot-plots are shown on Figure 5C,D.

Altogether these data have provided evidence that prolonged marrow aspiration from the same site yields marrow specimens considerably diluted with blood. Collection of the same volume of aspirate is therefore essential for investigation of MSC frequency in ageing and disease. Finally, in order to maximize the number of MSC aspirated from the iliac crest for the purpose of MSC expansion or direct one-stage therapy, our data suggest that each draw should be limited in volume, ideally to no more than 5 mL, at a single site.

## Discussion

In this work we have shown that a cytometric assay can be used reliably to evaluate and potentially predict the frequency of MSC in a BM aspirate. This assay is therefore of considerable relevance to MSC manufacturing protocols and could be used to exclude those BM specimens that have a very low initial MSC content and for which industrial-scale cultivation would result in pre-senescent, poor-quality MSC. The exclusion of poor-quality BM specimens before cultivation in good manufacturing practice (GMP) conditions would reduce considerably the variability and improve the quality of MSC-based therapies. This approach could also potentially be relevant for cost savings in MSC manufacture, as only quantitatively good batches of MSC aspirates would be taken forward in GMP settings, thus avoiding waste.

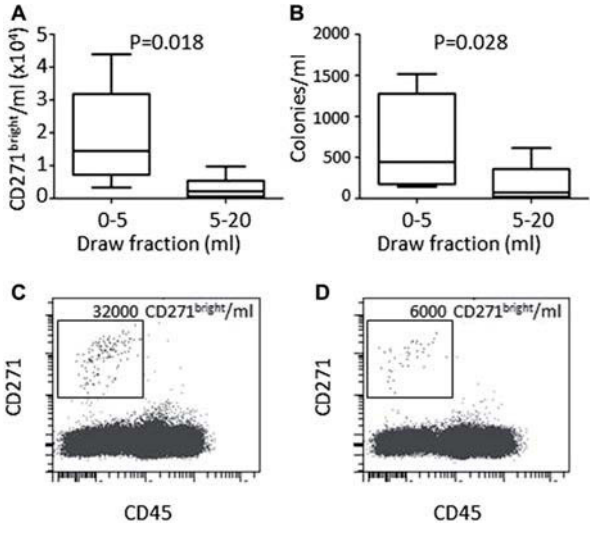


Figure 5. Quality control of BM aspiration. A higher number of $CD271^{bright}$ cells/mL (A) or CFU-F/mL (B) was observed in the first 5 mL of aspirate compared with the following 15-mL draw. Representative flow cytometry plots showing the population of $CD271^{bright}$ cells in the first 5-mL draw (C) and the following 15-mL draw (D).

A second major implication of this work is its relevance to the use of 'minimally manipulated MSC' for therapy development. The rationale for this statement comes from the observations that aspirated CFU-F yields as measured in retrospect were found to correlate with the healing potential of BM concentrates injected directly into fracture sites (15). However, the dose of injected MSC could not be controlled at the outset as the CFU-F assay takes 14 days to complete. In contrast, the rapid evaluation of the number of MSC in the harvested BM sample using the proposed 40-min assay would permit the surgeon to determine the yield range and estimate the dose of MSC prior to injection. This has significant implications for clinical orthopedics trials utilizing uncultured MSC, which could be further concentrated by size-filtering (31) or using CD271-based immunomagnetic selection. One-stage therapy with uncultured MSC has an additional advantage of using freshly procured cells that have not been artificially 'aged' in culture by multiple rounds of *in vitro* passaging (9,11).

Single-platform approaches utilizing lyse-no-wash protocols are commonly used in hematology practice to enumerate CD34 cells and CD4/CD8 cells (28,30,32,33). The correlation between the number of HP colonies and the proportions of $CD34^+$ cells was first published in 1993 (34). Subsequently, the flow cytometry-based assay for $CD34^+$ cells was refined to take into account sample viability during extended storage (32) and the provision of counting standards (counting beads or tubes) (28,30,32,33). The MSC assay reported in this study takes these variables into consideration and, similarly to single-platform CD34 protocols, allows a direct enumeration of MSC per milliliter or microliter of marrow. Of note, the results of this study have confirmed fully that BM MSC are considerably less numerous than $CD34^+$ HP (16).

Using the current experimental system, one in 17 $CD271^{bright}$ cells formed a colony in the CFU-F assay. This is *c.* 2-fold lower compared with observations using sorted $CD271^{bright}$ cells obtained by us (11,23) but is *c.* 2-fold higher compared with data obtained by Tormin *et al.* (24). The reason for this small discrepancy may be explained by different media/sera and cell seeding densities used. We additionally acknowledge that performing the CFU-F assay volumetrically by direct plating (25,26) rather than by cell density from BM MNC (16,17) could have affected MSC clonogenicity *in vitro*, because of the presence of large numbers of red cells. On the other hand, the CFU-F assay based on MNC

that are prepared using multiple centrifugation steps was deemed inappropriate for this study as a direct comparison with the volumetric flow cytometry enumeration assay was sought.

Having developed the flow cytometry-based MSC enumeration assay, we next addressed the issues pertaining to marrow harvesting and repeated marrow aspiration. The collection of large volumes of marrow (up to 300 mL) is commonly employed in orthopedic practice prior to volume reduction using marrow concentrators and their direct injection (35). Of course, low-volume aspiration from multiple sites has been recognized in the hematology field, being a standard marrow harvesting technique for transplantation purposes for decades. The novelty of our study in this context lies in the direct demonstration of a significant reduction in MSC abundance following the prolonged specimen draw from the same site. MSC form an integrated reticular network *in vivo* and it is also likely that prolonged aspiration primarily extricates hematopoietic cells from the marrow cavity leaving interconnected MSC behind (36). This also shows that the precise enumeration of marrow MSC in ageing and disease cannot be performed without prior standardization of the marrow harvesting technique and clear reporting of the volumes of marrow drawn.

In conclusion, this study reports methodologies that will introduce an important quality control for current MSC manufacture. The incorporation of a prospective MSC enumeration assay in MSC-expansion protocols and the establishment of the dose of seeded MSC prior to the commencement of culture, should undoubtedly lead to the manufacture of MSC batches with more consistent and predictable biologic behaviors. This study also confirms the critical importance of clinical techniques for harvesting MSC and for obtaining maximal yields of MSC for the 'instant' therapy of fractures. Finally, this study calls for standardization in the marrow-harvesting technique to allow more precise evaluation of the marrow MSC content in healthy ageing and its possible disturbances in diseases such as osteoporosis and myeloma.

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