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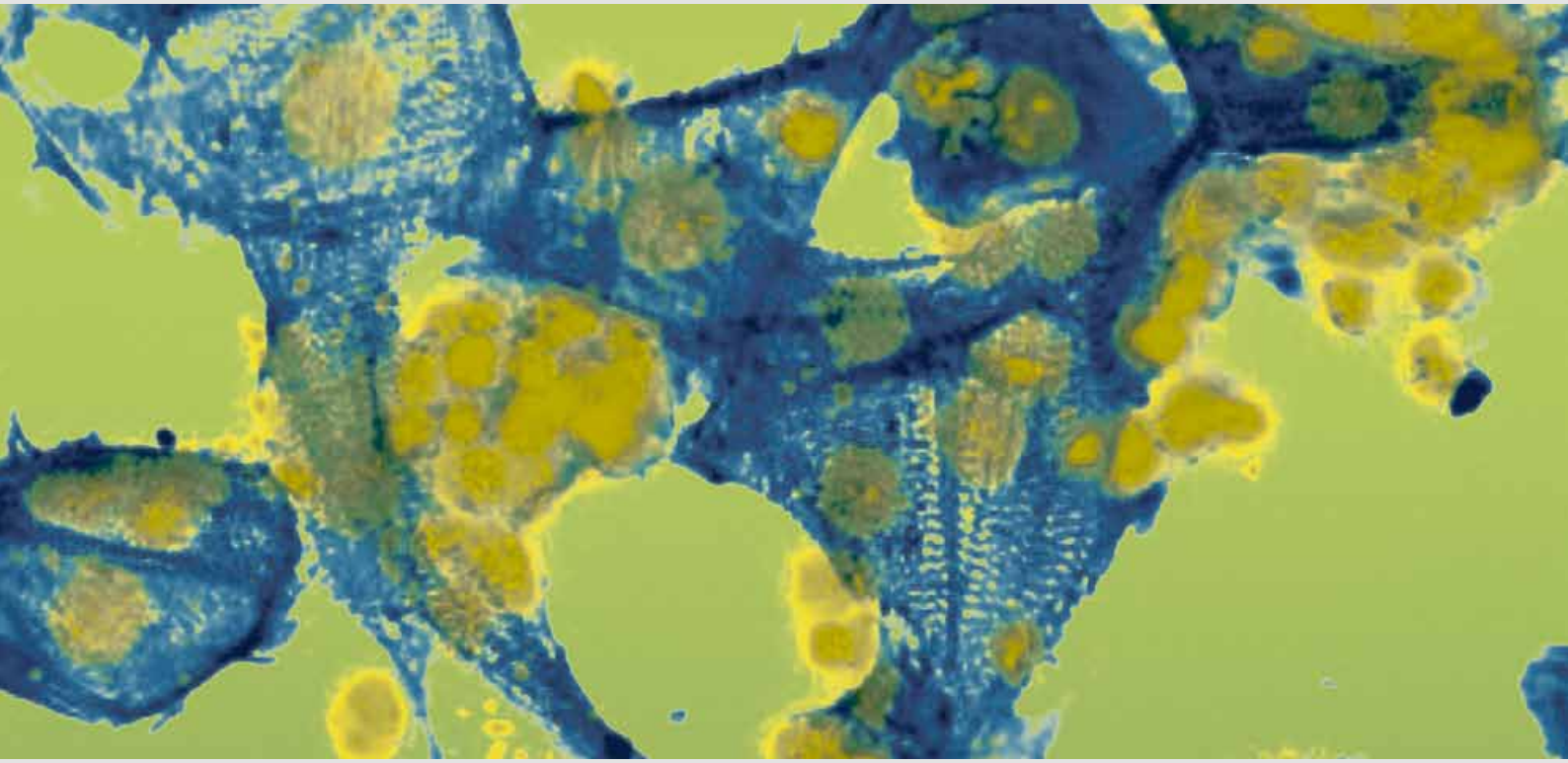

Clinical-scale isolation of mesenchymal stromal cells from bone marrow according to their CD271 expression


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Introduction

Mesenchymal stromal cells (MSCs) have shown their potential for cell therapy in various clinical trials, for example, for the treatment of graft-versus-host disease and tissue regeneration. Studies of several groups have indicated that the clonogenic potential of CD271$^+$ cells magnetically enriched from bone marrow mononuclear cells (BM-MNCs) are about 100-fold higher compared to cells isolated by plastic adherence (PA).[1] The expansion rate of enriched CD271$^+$ cells is one to three orders of magnitude higher than the rate of cells isolated by PA.[2] Expanded CD271$^+$ cells express MSC markers, such as CD73, CD90, and CD105, and retain their multilineage differentiation potential, giving rise to adipocytes, osteoblasts, and chondrocytes. In this study we show the isolation of CD271$^+$ cells from human bone marrow using the CliniMACS® Instrument, an automated cell separation system based on MACS® Technology. The system enables clinical-scale magnetic enrichment of target cells in a closed and sterile system.

Materials and methods

Enrichment of CD271$^+$ cells from bone marrow

50 mL human bone marrow was harvested and CD271$^+$ cells were purified using CD271 antibody–conjugated MicroBeads and the CliniMACS Plus Instrument (Miltenyi Biotec). Bone marrow samples before and after enrichment were labeled with CD271-PE (Miltenyi Biotec) and analyzed by flow cytometry. PI immunofluorescence and light scatter signals were used to gate live cells.

Colony-forming unit fibroblast (CFU-F) assay

Cells were Giemsa-stained after 14 days of culture using NH CFU-F Medium (Miltenyi Biotec) and CFU-F numbers were determined microscopically.

MSC expansion

Cells isolated by magnetic enrichment or PA were cultured in NH Expansion Medium (Miltenyi Biotec) for 41 days. Population doubling (PD) and cumulative population doubling (CPD) were determined using the following equations:

PD for each subculturing =
$(\log_{10}(N_H) - \log_{10}(N_I))/\log_{10}(2)$

N_I = number of inoculated cells; N_H = number of harvested cells

CPD for 41 days of culturing = Σ (PD)

Phenotyping of *in vitro* expanded MSCs

After 41 days of culture in NH Expansion Medium, MSCs were trypsinized, stained for CD73, CD90, CD105, CD14, CD20, CD34, and CD45 (MSC Phenotyping Kit, Miltenyi Biotec), and analyzed by flow cytometry using the MACSQuant® Analyzer.

Differentiation assays

Isolated CD271$^+$ cells cultured in NH OsteoDiff Medium (Miltenyi Biotec) for 10 days were analyzed for their alkaline phosphatase (AP) activity to assess differentiation into osteoblasts. AP activity was evaluated using SigmaFast™ BCIP/NBT (Sigma-Aldrich) as a substrate.

Isolated CD271$^+$ cells cultured in NH AdipoDiff Medium (Miltenyi Biotec) for 18 days were analyzed for the presence of intracellular lipid vacuoles by Oil red O staining to evaluate differentiation into adipocytes.

Isolated CD271$^+$ cells cultured in NH Expansion Medium (Miltenyi Biotec) served as negative controls.

Suppression of T cell proliferation by CD271$^+$ cells

Human MSCs isolated by PA were expanded for 2 passages (P2) in NH Expansion Medium. These MSCs are further referred to as PA-MSCs. Responder T (Tresp) cells were

magnetically isolated from buffy coat by positive selection of CD4$^+$ cells and depletion of CD25$^+$ regulatory T cells. CD4$^+$CD25$^-$ Tresp cells were labeled with carboxyfluorescein succinimidyl ester (CFSE). CD4$^+$CD25$^-$ Tresp cells (1×10^5) were either cocultured with 1×10^5 CD271$^+$ cells directly after magnetic isolation or with 1×10^5 PA-MSCs. Tresp cells were stimulated with MSC Suppression Inspector, human (Miltenyi Biotec), which contains Anti-Biotin MACSiBead™ Particles pre-loaded with biotinylated CD2, CD3, and CD28 antibodies. Cells were harvested on day 5 and Tresp proliferation was flow cytometrically measured by determining CFSE fluorescence.

Results

Isolation of CD271$^+$ cells from bone marrow

CD271$^+$ cells were isolated by MACS Technology at purities of about 96% (median 93% ± 6.0%, n=4) and recoveries of about 94% (median 87%, n=2). A representative example is shown in figure 1.

Clonogenic potential of MSCs isolated by MACS Technology and PA-MSCs

The clonogenic (CFU-F) potentials of isolated CD271$^+$ cells, CD271$^-$ cells, and MSCs obtained by PA were compared. CFU-F numbers of CD271$^+$ cells amounted to 2952 ± 431 (median; n=3). The numbers of CFU-F increased by more than 200-fold when MSCs were isolated by MACS Technology as compared to PA. No CFU-Fs were detected in the CD271$^-$ cell fraction (fig. 2).

Expansion of CD271$^+$ MSCs and PA-MSCs

PA-MSCs and the CD271$^+$ cell fraction isolated by MACS Technology were cultured for 41 days in order to assess their proliferative capacity. CPD level nearly doubles with enriched CD271$^+$ cells compared to MSCs isolated by PA (fig. 3).

Phenotyping of *in vitro* expanded PA-MSCs and CD271$^+$ MSCs

After 41 days of culture, MSCs were trypsinized and analyzed for various markers by using the MSC Phenotyping Kit. Expanded MSCs were CD73$^+$, CD90$^+$, CD105$^+$, CD14$^-$, CD20$^-$, CD34$^-$, and CD45$^-$ regardless of whether MSCs were isolated by PA or by MACS Technology according to CD271 expression (fig. 4A). The histograms (fig. 4B) exemplify marker expression of CD271$^+$ MSCs.

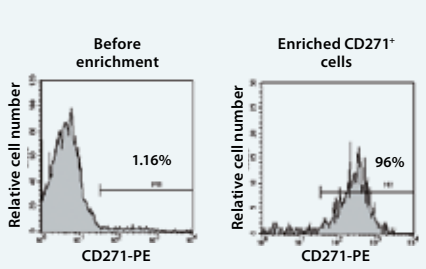

Figure 1 Isolation of CD271$^+$ cells from bone marrow. Cells were labeled with CD271-PE before or after enrichment and analyzed by flow cytometry.

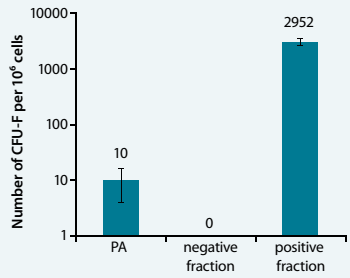

Figure 2 Clonogenic potential of PA-MSCs, CD271$^-$ cells, and CD271$^+$ cells. For details see the materials and methods section.

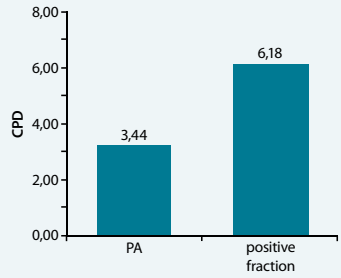

Figure 3 Expansion of PA-MSCs and CD271$^+$ MSCs. CPD defines the cumulative population doubling over 41 days.

A

	CD73	CD90	CD105	CD14	CD20	CD34	CD45
CD271$^+$ MSCs	+	+	+	–	–	–	–
PA-MSCs	+	+	+	–	–	–	–

B

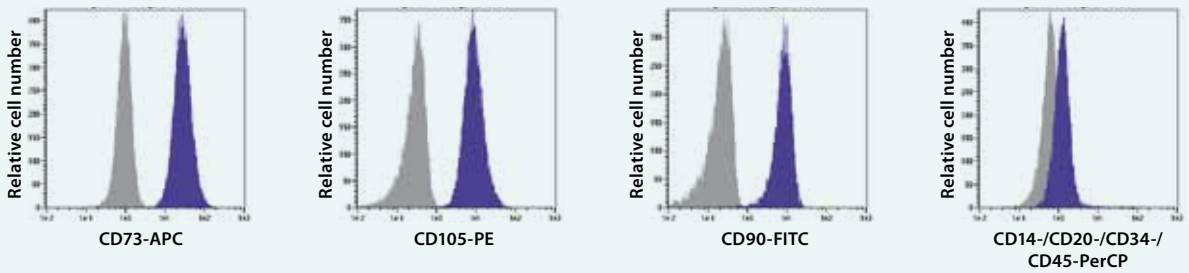

Figure 4 Phenotyping of *in vitro* expanded CD271$^+$ MSCs and PA-MSCs. Cells were stained by using the MSC Phenotyping Kit and analyzed by flow cytometry. Figure 4B shows the analysis of CD271$^+$ MSCs. Purple peaks indicate staining with marker antibodies, whereas isotype controls are indicated as gray peaks.

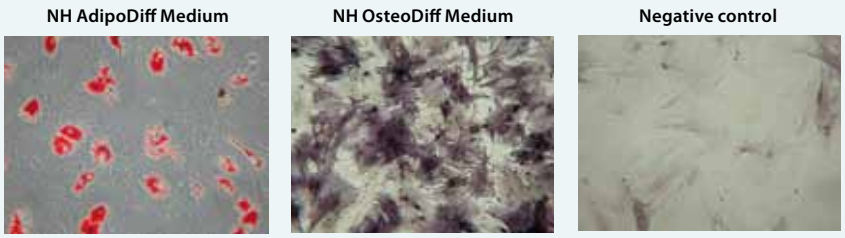

Figure 5 Multipotent differentiation potential of isolated CD271⁺ MSCs. Cells were cultured in media that induce differentiation into either adipocytes (left) or osteoblasts (middle). The negative control (right) was cultured in NH Expansion Medium.

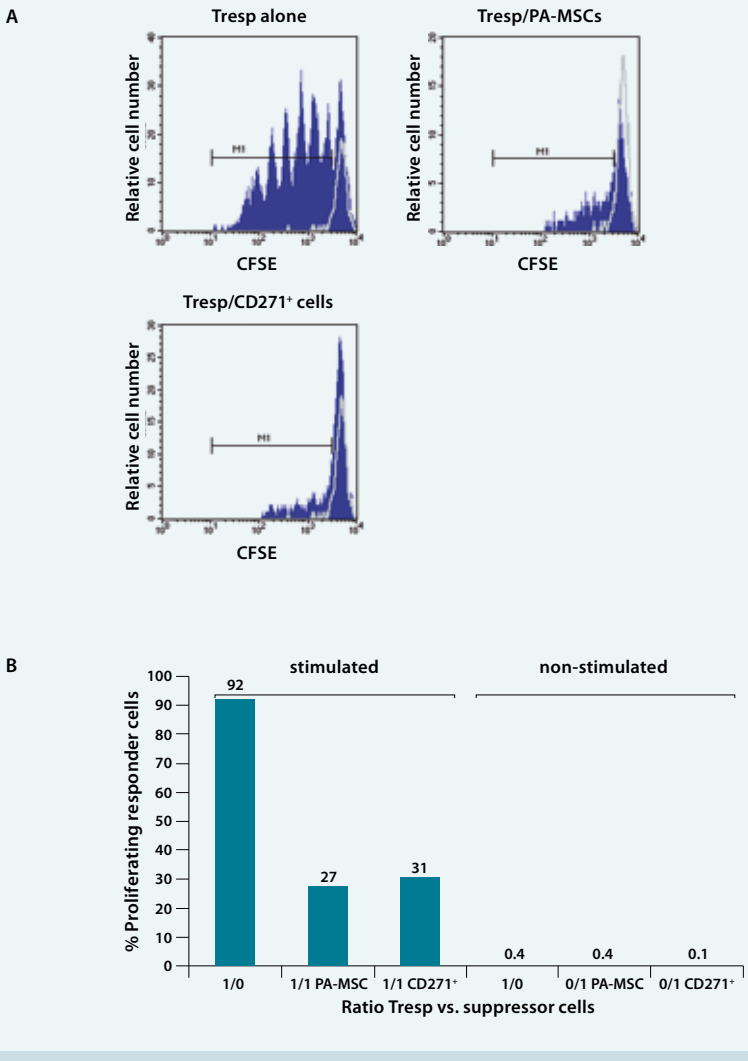

Figure 6 Suppression of T cell proliferation by PA-MSCs or CD271⁺ MSCs isolated by MACS Technology. Tresp cells labeled with CFSE were cocultured with either CD271⁺ cells or with PA-MSCs. Cells were stimulated by using the MSC Suppression Inspector or left untreated. (A) Tresp cell proliferation was flow cytometrically measured as CFSE dilution (indicated by M1). (B) Data are presented as percentage of proliferating Tresp cells cultured in the presence of PA-MSCs or CD271⁺ cells with respect to responder cells cultured alone.

Multipotent differentiation potential of CD271⁺ MSCs

Isolated CD271⁺ cells were cultured in specific differentiation media and subsequently analyzed regarding their potential to differentiate into the respective cell types. After 18 days of culturing in NH AdipoDiff Medium, all cells showed an increased accumulation of intracellular lipid vacuoles, indicating differentiation into adipocytes. After 10 days of culturing in NH OsteoDiff Medium all cells showed a high alkaline phosphatase activity, indicating differentiation into osteoblasts. Negative controls showed no differentiation.

Suppression of T cell proliferation by PA-MSCs or CD271⁺ cells

Suppression of T cell proliferation was measured as described in materials and methods. Tresp cells proliferated in the presence of the MSC Suppression Inspector (fig. 6A, top left). Suppression of T cell proliferation by isolated CD271⁺ cells prior to culturing was as robust as by PA-MSCs (fig. 6A, top right and bottom, and 6B).

Conclusion

We developed a protocol for the isolation of CD271⁺ MSCs with high purity and recovery, in a closed and sterile system. With their increased expansion potential, CD271⁺ cells represent an optimal, homogeneous starting population for the time-efficient clinical-scale MSC expansion. The isolated CD271⁺ cells showed the typical MSC phenotype expressing the MSC markers CD73, CD90, and CD105. They also showed multipotent differentiation potential and effectively suppressed T cell proliferation.

References

1. Quirici, N. *et al.* (2002) Exp. Hematol. 30: 783–791.
2. Poloni, A. *et al.* (2009) Cytotherapy 11: 153–162.

Acknowledgment

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Relevant products	Order no.
CD271 (LNGFR) MicroBead Kit (PE), human	130-092-819
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MSC Phenotyping Kit, human	130-095-198
NH Expansion Medium, human	130-091-680
NH AdipoDiff Medium, human	130-091-677
NH ChondroDiff Medium, human	130-091-679
MSC Suppression Inspector, human	130-096-207

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