

MSC isolation and analysis—customer report

Purification of proliferative and multipotential marrow stromal cells (MSCs) from bone marrow aspirate by selection for CD271 (LNGFR) expression

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Introduction

Stromal cells provide a niche and a cytokine support for hematopoietic cell maturation in the bone marrow (BM). At the same time, they form a pool of precursors for BM adipocytes and osteoblasts¹ which may include the most primitive, multipotential stromal cells (MSCs). BM MSCs can be isolated based on their expression of several surface antigens including STRO-1, CD146, fibroblast antigen (D7-FIB), CD73, CD105, and CD271 (LNGFR).^{2–6} In addition, the CD45^{low} phenotype of freshly isolated BM MSCs has been demonstrated in several independent studies.^{3,5,7} In this study we evaluate several MSC surface markers for their selectivity in detecting the CD45^{low} BM MSC population following pre-enrichment by separation with Anti-Fibroblast MicroBeads. Having established that CD271 (LNGFR) provided the best discriminative value, we compared MSC isolation methods based on selection for CD271 (LNGFR) expression to the separation with Anti-Fibroblast MicroBeads in terms of their (1) ability to generate proliferative and multipotential MSC cultures and (2) efficiency in isolating fresh BM MSCs.

Materials and methods

BM aspirates were obtained from the iliac crest of 14 healthy donors. Standard MSC cultures were established from plastic-adherent BM cell fractions.⁸ For positive cell selection using MACS[®] Technology, 5×10⁷ BM mononuclear cells were labeled with either Anti-Fibroblast MicroBeads or MACSelect LNGFR MicroBeads. MSC expansion, tri-lineage differentiation, flow cytometry, and cell sorting were performed as described previously.^{3,9} All antibodies were purchased from Pharmingen, with the exception of CD105-PE (Serotec) and CD45-FITC (DAKO). Cell proliferation rate was assessed in an XTT-based colorimetric assay after MSCs expansion in either MACS[®] NH Expansion Medium or DMEM supplemented with 10% FCS. Differentiation of MSCs to cells of adipocyte, chondrocyte, and osteoblast lineages was performed using MACS[®] NH AdipoDiff Medium, NH ChondroDiff Medium, and NH OsteoDiff Medium.

Results

Comparative analysis of MSC marker selectivity

A good marker for fresh, uncultivated BM MSCs should exhibit a high level of expression on rare MSCs and minimal expression on cells of the predominant hematopoietic lineage cells (HLCs). To compare the selectivity of different markers, MSCs were first selected by using Anti-Fibroblast MicroBeads and then analyzed for the particular marker expression levels on gated CD45^{low} MSCs or CD45^{high} HLCs. Figure 1 demonstrates that CD271 (LNGFR) shows a very high, homogenous level of expression on MSCs as well as minimal labeling of HLCs. CD73 and CD13 were strongly expressed on MSCs, but showed increased staining of HLCs. CD105 and CD146 displayed more heterogeneous and weaker expression on MSCs, and an intermediate expression on HLCs. Based on these data, we concluded that CD271 (LNGFR) could be an excellent marker for the high-purity isolation of MSCs from human BM.

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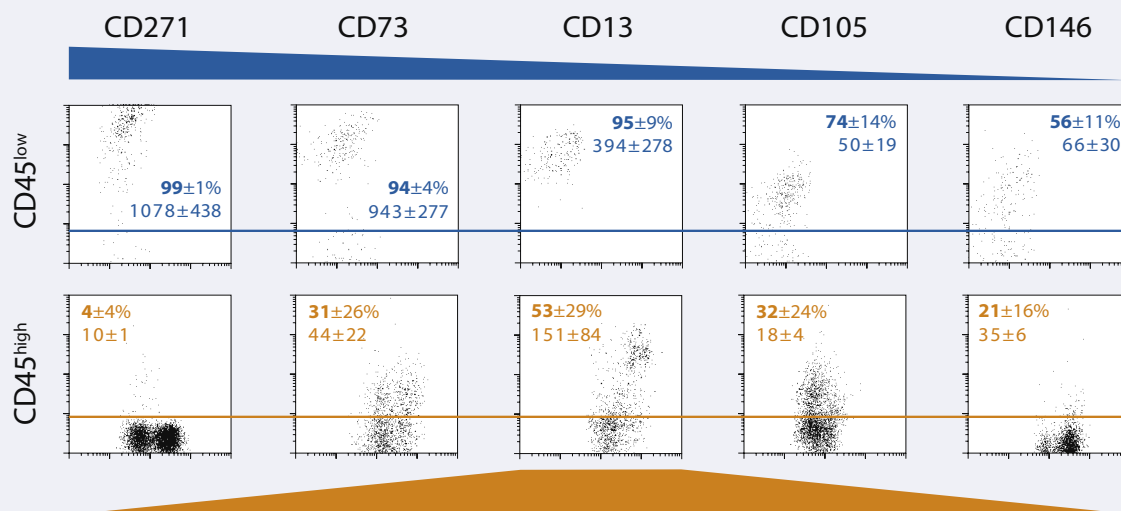
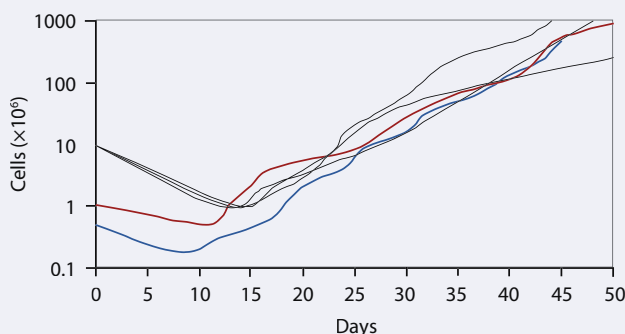
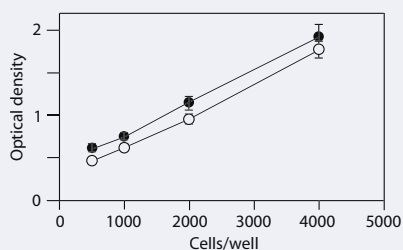


Figure 1 Comparative analysis of MSC marker selectivity. MSCs were pre-enriched with Anti-Fibroblast MicroBeads; marker expression levels were analyzed on gated CD45^{low} MSCs (top panel) and CD45^{high} hematopoietic lineage cells (HLCs) (bottom panel). Percentage and mean fluorescence intensity of marker-positive cells for MSCs (shown in blue) and HLCs (shown in orange) represent a mean ± SD for a minimum of 5 donors.

Growth kinetics of PA-selected and CD271⁺ BM-MSCs



PA-selected BM-MSCs



CD271⁺ BM-MSCs

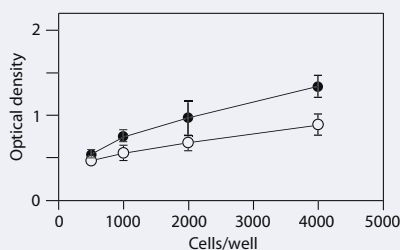


Figure 2 Growth characteristics of cells isolated using MACS[®] Technology vs. plastic adhesion-enriched cells (PA). (A) Expansion kinetics of BM cells isolated for CD271 (LNGFR) expression (blue line) compared with cells isolated with Anti-Fibroblast MicroBeads (red line) and three control cultures expanded from plastic-adherent BM cells (black lines). (B) Cell yields (measured as optical densities in the duration of a four-day XTT proliferation assay) in control cultures derived from BM plastic-adherent fraction (left panel) and cultures from CD271 (LNGFR)-selected cells (right panel). MACS[®] NH Expansion Medium (dark circles) produced higher cell yields than conventional DMEM/10% FCS (empty circles).

Growth characteristics of cells selected for CD271 (LNGFR) or fibroblast antigen expression

Cells isolated by MACS[®] Technology displayed similar growth characteristics to control cultures that had been expanded from plastic-adherent BM cell fractions, however they entered an exponential growth phase several days earlier (fig. 2A). Similarly to controls, cultures derived from the cells, isolated with MACSelect LNGFR MicroBeads, were formed by spindle-shaped cells and showed surface marker profiles consistent with cultured BM MSCs^{3,8} (table 1). Cultures grown in MACS[®] NH Expansion Medium proliferated faster than cultures grown in conventional DMEM/10% FCS, regardless of whether or not the cell isolation step was used before culturing (fig. 2B).

Differentiation potentials of cultures derived from isolated cells

Similarly to cultures, isolated by using Anti-Fibroblast MicroBeads³, MSC cultures established from cells selected for CD271 (LNGFR) expression retained full tri-lineage differentiation potential (fig. 3). In quantitative *in vitro* differentiation assays⁹, levels of differentiation (measured by production of adipocytes, Ca²⁺, or sulfated glycosaminoglycans) in cultures maintained in MACS[®] NH AdipoDiff,

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	CD45	CD34	CD133	CD105	CD73	CD13	CD106	CD146	CD166	FA*
Standard MSC culture	0	0	0	98	99	98	52	81	99	95
Fibroblast antigen–selected cells	0	0	0	97	98	98	78	63	98	81
CD271 (LNGFR)–selected cells	0	0	0	91	100	99	37	69	100	82

All values are % positive cells

* Fibroblast antigen

Table 1 Cell surface marker profiles of growing MSC cultures.

NH OsteoDiff, and NH ChondroDiff Media were 2-fold to 5-fold higher than those in cultures grown in respective home-made differentiation media.

Purification of adherent stromal cells in fractions isolated with MACS® Technology

One day after magnetic selection for either CD271 (LNGFR) or fibroblast antigen expression, the same

morphologically distinct adherent cell type could be observed in the positive fractions (fig. 4A, left panel), but not in the negative fractions (right panel). These cells had a stromal (spindle-shape or stellate) morphology and cytoplasmic projections. Seeded on grid coverslips, the fate of individual adherent cells could be followed for several days³, and their exponential growth was observed from as early as day three of culture (fig. 4B), whereas small round cells did not amplify.

As the cells, selected Anti-Fibroblast MicroBeads, cell fractions isolated for CD271 (LNGFR) expression contained a population of CD45^{low}CD73⁺ cells (fig. 4C). This population was present at a much lower proportion before MACS® Separation and absent in the negative cell fraction. Cells sorted for the CD45^{low}CD73⁺ phenotype yielded a pure population of stromal cells (fig. 4D) and contamination with small HLCs was no longer observed.

Discussion

This study demonstrated that magnetic enrichment of cells expressing CD271 (LNGFR) could be used for the purification of fresh BM MSCs and for the establishment of highly proliferative and multipotential MSC cultures. The effectiveness of this isolation method could be explained by the high degree of specificity and selectivity of the CD271 (LNGFR) marker for human *in vivo* BM MSCs. Similar to the selection with Anti-Fibroblast MicroBeads, isolation of MSCs by selecting for CD271 (LNGFR) expression did not inhibit MSC growth kinetics. In addition, it proved critical for the morphological identification and purification of culture-initiating cells (fresh MSCs). A similar morphology in freshly purified BM MSCs was previously described after selection with Anti-Fibroblast MicroBeads³ or cell sorting using a combination of STRO-1/VCAM-1¹⁰. In terms of cell growth kinetics, the choice of expansion medium was more important than the initial MSC isolation step. Similarly, the extent of tri-lineage differentiation from the same

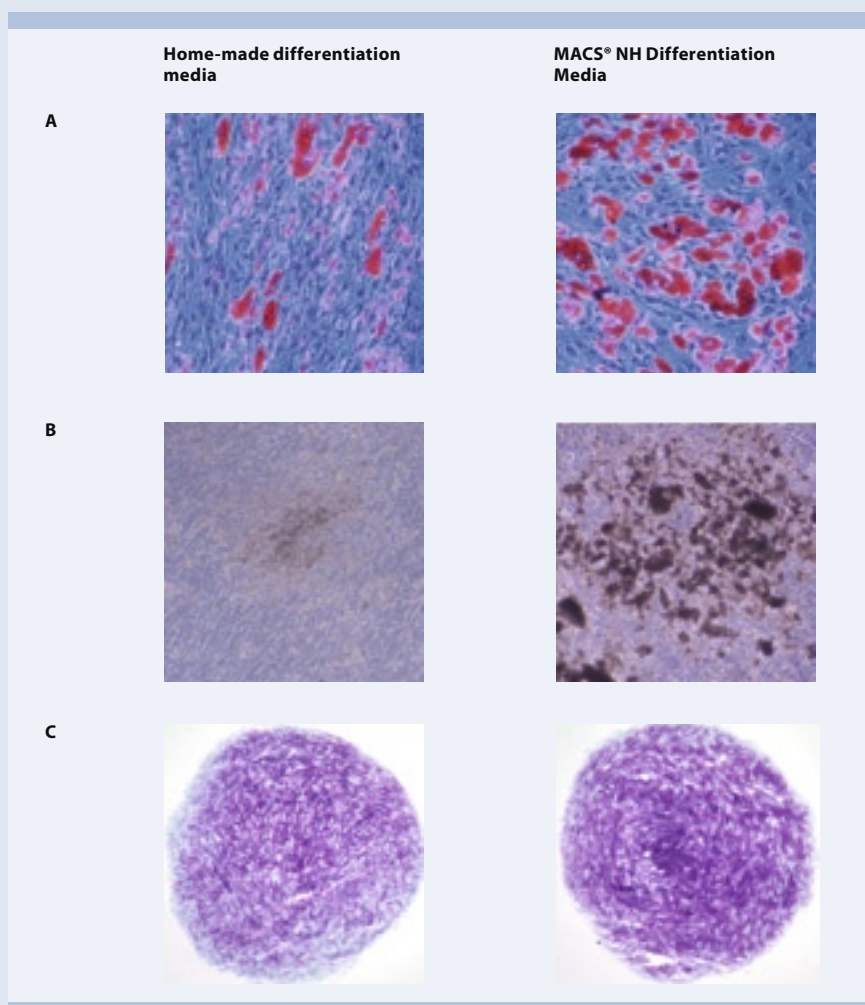


Figure 3 The effect of differentiation media on tri-lineage differentiation potential of CD271 (LNGFR)–isolated cells.

The same "passage 2" cell culture, derived from CD271 (LNGFR)–isolated cells, was used in all differentiation assays. The top panels (A) show adipogenesis (oil red O staining); the middle panels (B) show osteogenesis (calcium deposits); the bottom panels (C) show chondrogenesis (toluidine blue staining). All microphotographs were taken on day 21 of culture.

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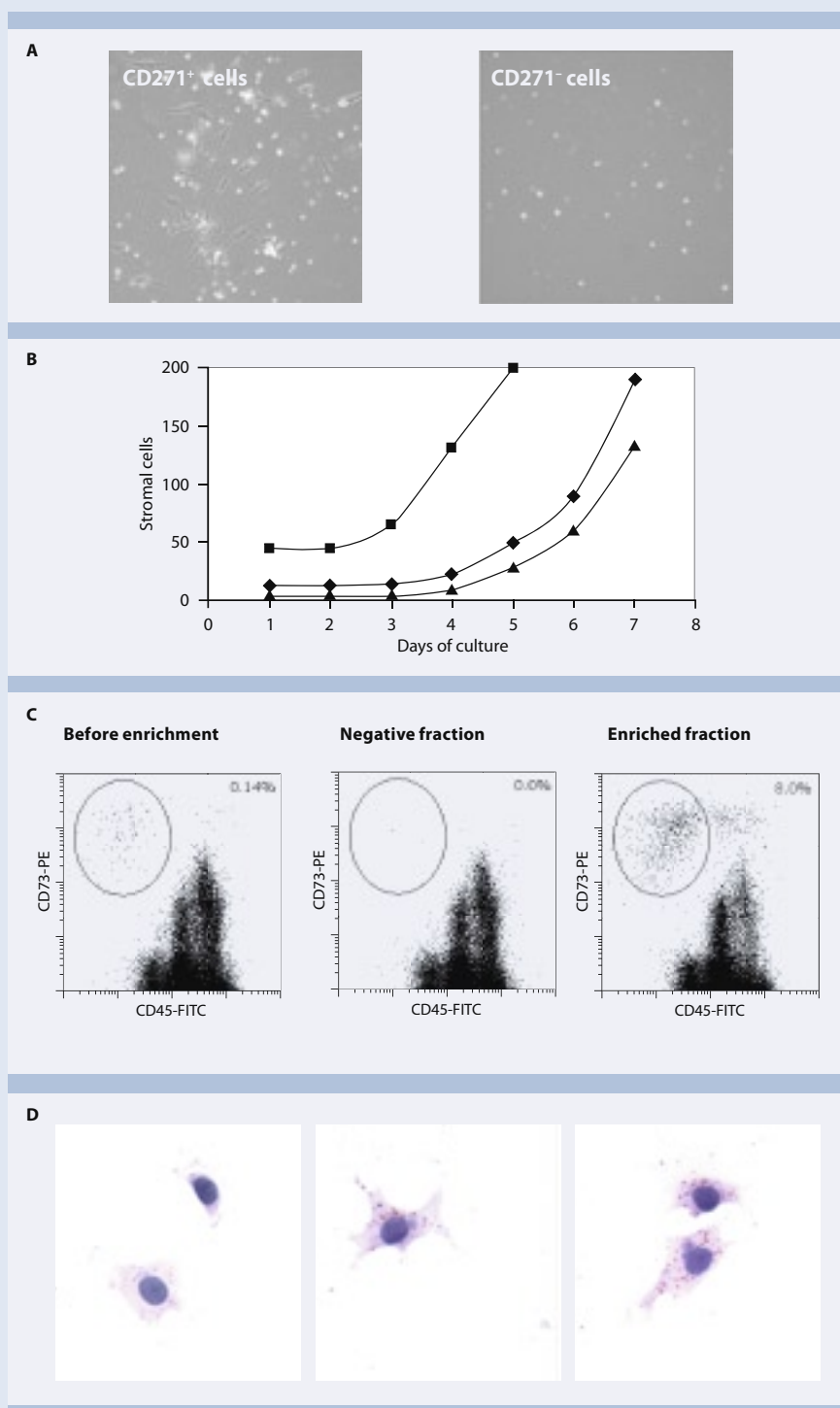


Figure 4 Purification and expansion of marrow stromal cells from the CD271 (LNGFR)-selected cells. (A) Microphotographs of adherent cells from the CD271 (LNGFR)-positive cell fraction (left) and the CD271 (LNGFR)-negative cell fractions (right). Small round cells are present in both fractions, whereas dark spindle- and stellate-shaped cells are present exclusively in the positive fraction. (B) Proliferation kinetics of CD271 (LNGFR)-isolated stromal cells grown in DMEM/10% FCS showing their exponential growth as early as day three of culture (n=3 donors). (C) Enrichment of CD45^{low}CD73⁺ MSCs (circled) in the CD271 (LNGFR)-positive fraction. (D) Stromal morphology of sorted CD45^{low}CD73⁺ cells following overnight adherence to plastic (oil red O staining). Tiny droplets of fat in the cytoplasm of many cells could indicate their pre-adipocyte nature.

MSC culture clearly depended on the potency of the differentiation medium. In the area of MSC research, the standardization of isolation, expansion, and differentiation protocols are needed. This study shows that with respect to BM MSC isolation from humans, CD271 (LNGFR)-based methods hold great promise. In addition, these methods can be used for the isolation of stromal reticular cells in BM diseases (such as aplastic anemia and myeloma), in which stromal cell function is compromised.^{11,12} Further work is needed to test the utility of CD271 (LNGFR) as a marker of MSCs in other tissues in both humans and in other species, but our data clearly display the potential of CD271 (LNGFR) as a selection marker of proliferative and multipotential MSCs from BM.

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