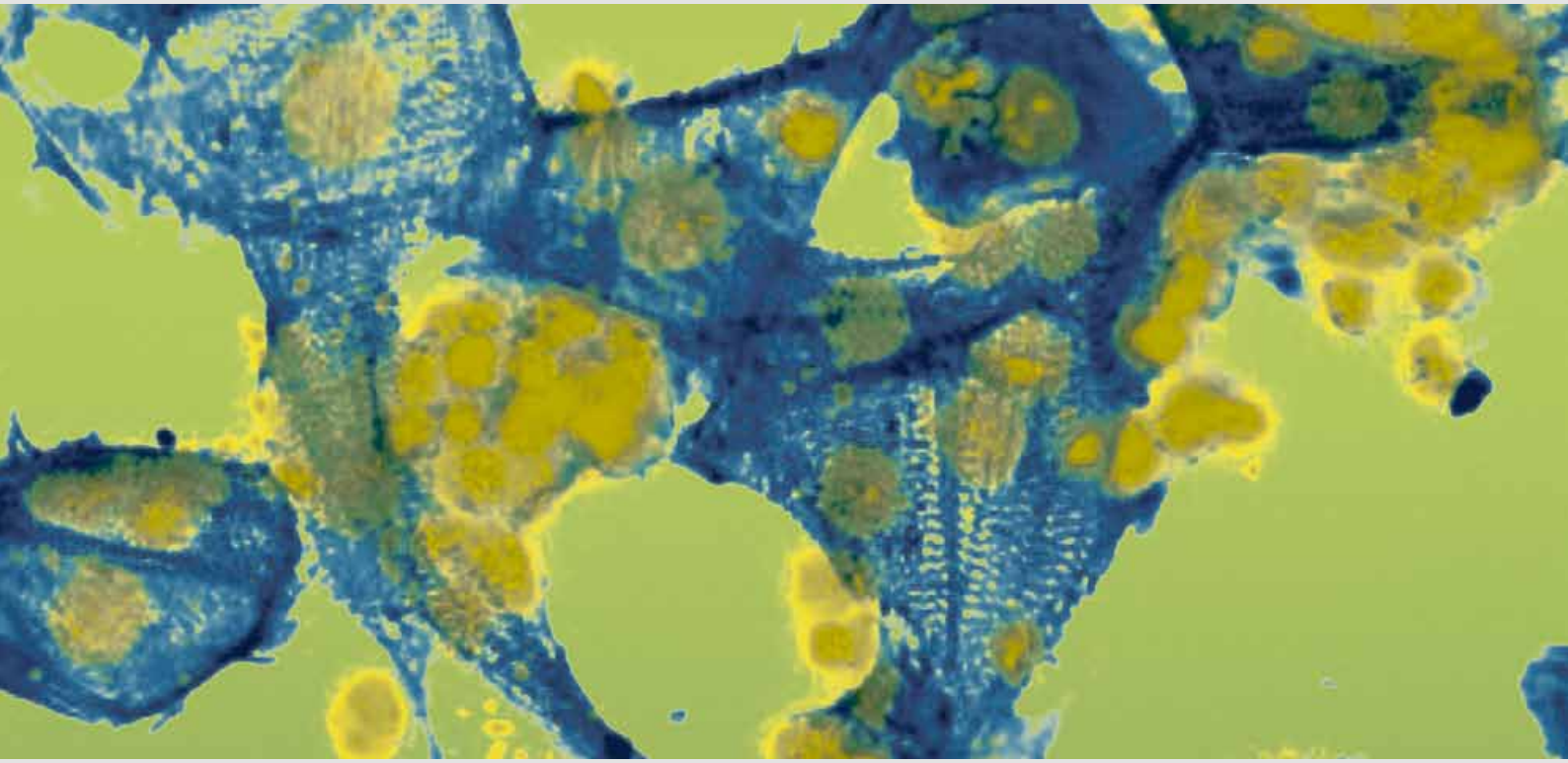
MACS&more

Excerpt from MACS&more Vol 14 – 2/2012

The expression of novel mesenchymal/stromal lineage transcripts in uncultured CD271-positive MSCs: differences from culture-expanded MSCs

Sarah Churchman and Elena Jones

Leeds Musculoskeletal Biomedical Research Unit, University of Leeds, Leeds, United Kingdom



The expression of novel mesenchymal/stromal lineage transcripts in uncultured CD271-positive MSCs: differences from culture-expanded MSCs


Sarah Churchman and Elena Jones
Leeds Musculoskeletal Biomedical Research Unit, University of Leeds, Leeds, United Kingdom

Introduction

The expression of low-affinity nerve growth factor receptor (LNGFR, now designated as CD271) on bone marrow (BM) non-hematopoietic reticular stromal cells was shown in the early 1990s[1]. Nearly a decade later, two independent studies have documented the exclusive residence of colony-forming unit fibroblasts (CFU-Fs) in the CD271$^+$ cell fraction[2,3]. Subsequent phenotypic analysis using multiparameter flow cytometry revealed the expression of common mesenchymal stem/stromal cell (MSC) markers (CD73, CD105) on cells that had the strongest levels of CD271 (CD271^{bright}) and negligible levels of CD45[4]. Recent studies have confirmed the superiority of CD271 over other candidate markers for the isolation of native BM-MSCs[5].

Only limited data yet exist on the transcriptional profile of BM CD271^{bright} cells. Notably and perhaps not surprisingly, these cells were found to express transcripts indicative of adipogenic and osteogenic fates[5,6]. At the same time, strong expression of stromal-derived factor-1 (SDF-1/CXCL12) was observed in CD271^{bright} cells[6], consistent with findings obtained using Stro-1-based MSC selection[7]. Larger-scale transcriptional profiling of CD271^{bright} MSCs should provide more insights on their *in vivo* functions and cellular interactions. It is additionally interesting to investigate how a native MSC fingerprint differs from that of MSCs cultured in standard conditions.

Materials and methods

CD271^{bright} cells and donor-matched HLCs were purified from iliac crest BM aspirates (8 donors) using a 3-stage procedure. Firstly, BM mononuclear cells were isolated by density gradient centrifugation using Lymphoprep™. Secondly, MSCs were pre-enriched using positive selection with Anti-Fibroblast MicroBeads (Miltenyi Biotec), as described previously[3]. In a third stage, cells were isolated by flow sorting. MSC cultures (n=3) were grown in non-hematopoietic (NH) culture medium optimized for MSC growth (Miltenyi Biotec) as previously described[6]. Following RNA isolation (Norgen Biotek RNA/DNA/Protein Purification Kit, Geneflow) and reverse transcription (High Capacity cDNA reverse transcription kit, Life Technologies), quantitative real-time (q) PCR was performed using Custom TaqMan® Array (Life Technologies) on sorted cells and cultured MSCs (cMSCs). Data were analyzed using the $2^{(-\Delta Ct)}$ method, normalizing to the reference gene *HPRT*.

For fluorescent microscopy, cytopreparations were fixed with 4% paraformaldehyde/0.1% bovine serum albumin (BSA), treated with 10% donkey serum (Dako) and permeabilized with 0.1% Triton® X-100/1% BSA. SDF-1 and FABP4 antibody binding (both from R&D Systems) was detected using biotinylated secondary antibodies (Dako) followed by Streptavidin-FITC (Becton Dickinson/Pharmingen) and DAPI (Sigma). Images were taken using a Carl Zeiss Axio Imager microscope equipped with AxioVision® Software.

Results

CD271^{bright} cells were obtained following a 3-stage procedure as described in materials and methods. The pre-enrichment using Anti-Fibroblast MicroBeads resulted in an approximately 60-fold enrichment of CD271^{bright} cells (from a frequency of 0.03% to 1.8%) allowing subsequent cell sorting to be performed much faster and more accurately. Sorting gates for CD271^{bright} cells and control HLCs are shown on figure 1, left panel. The purity of the sorted fractions was confirmed by qPCR for *NGFR*/CD271 showing ~170-fold higher levels in the sorted CD271^{bright} fraction compared to the HLC fraction (fig. 1, right panel). The cMSCs were obtained by a standard plastic adherence/culture expansion procedure (fig. 1, middle panel) and trypsinized at early passage[6]. The down-regulation of CD271 in cMSCs, first documented at the protein level[3], was

confirmed for the NGFR transcript (9-fold, fig. 1, right panel).

We have previously shown very high levels of SDF-1/*CXCL12* expression in sorted CD271^{bright} cells that were diminished in cMSCs grown in a clinical-grade defined medium[6]. The SDF-1 protein was present in the cytoplasm of CD271^{bright} cells and absent in control donor-matched HLCs (fig. 2A). A similar down-regulation of *CXCL12* mRNA was observed when CD271^{bright} cells were compared with cMSCs grown in a research-grade NH medium (fig. 2A). This suggested that lower levels of *CXCL12* in cMSCs reflected common processes of culture adaptation and MSC expansion rather than the media used.

BM is known to contain large numbers of adipocytes, which have important metabolic and hematopoiesis-supporting functions, as well as acting as mere space-fillers. The transcript expression for *FABP4*, a mature fat-lineage protein involved in binding fatty acids and transporting them to other parts of the cell, displayed a pattern similar to SDF-1 (fig. 2B). It was significantly over-expressed in CD271^{bright} cells compared to HLCs (~50-fold) and reduced in expression in cMSCs (~120-fold). The specificity of FABP4 expression at the protein level was confirmed by fluorescence microscopy (fig. 2B), indicating that CD271^{bright} cells were most likely precursors of BM fat cells.

We next analyzed the expression of transcripts traditionally linked to the differentiation of MSCs towards bone. Amongst the markers shown in figure 3A, the highest levels of expression were observed for the inhibitor of BMP signalling noggin/*NOG*. Approximately similar levels of expression were observed for *BMP2*, *RUNX2,* and osteomodulin/*OMD*. Whereas a role of BMP2 and RUNX2 in osteoblast differentiation is well known, the function of OMD as an osteoblast maturation marker is less documented[8]. The expression of all four molecules was reduced in cMSCs (range 11–47-fold).

The described approach can be additionally used to identify new receptors and potential cellular interactions of BM-MSCs *in vivo*. Membrane receptor molecules: *GHR*, *S1PR1*, *ACVR1B,* and *BMPR1A* were all proven to be CD271^{bright} cell–specific at the transcript level, showing noticeably lower levels in donor-matched HLCs (fig. 3B). Whereas the expression of genes encoding the TGFB/BMP pathway receptors BMPR1A and ACVR1B reflects a capacity of CD271^{bright} cells to respond to BMPs and activins, the presence of mRNA for growth hormone receptor/GHR suggests potential interactions with GH, which can possibly exert direct anabolic effects on MSCs *in vivo*[9]. G-protein–coupled receptor S1PR1 has been previously shown to be expressed on osteoclast precursors[10] and B cells in the marrow[11]; interestingly, our transcript data indicate that its expression is even higher on uncultured MSCs (fig. 3B).

The observed high-level Jagged 1/*JAG1* expression (fig. 4) in CD271^{bright} cells is consistent with earlier publications[12,13] and indicative of hematopoiesis-supporting and

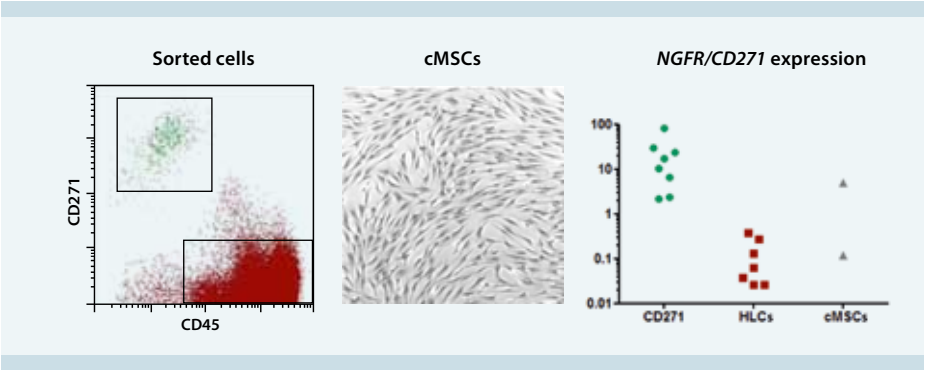


Figure 1 Generation of cell fractions for transcript expression study and their purity control based on *NGFR/CD271* transcript expression. Left panel: purification of CD271^{bright} cells (CD45$^{-/low}$CD271^{bright}) and HLCs (CD45^{+}CD271^{-}) by flow sorting following pre-enrichment with Anti-Fibroblast MicroBeads. Sorting gates are shown as rectangles. Middle panel: cMSCs grown in NH media display typical MSC morphology. Right panel: expression (relative to *HPRT*) of *NGFR/CD271* confirming purity of analyzed fractions. Single points represent individual donors (cMSC value for one donor was below detection).

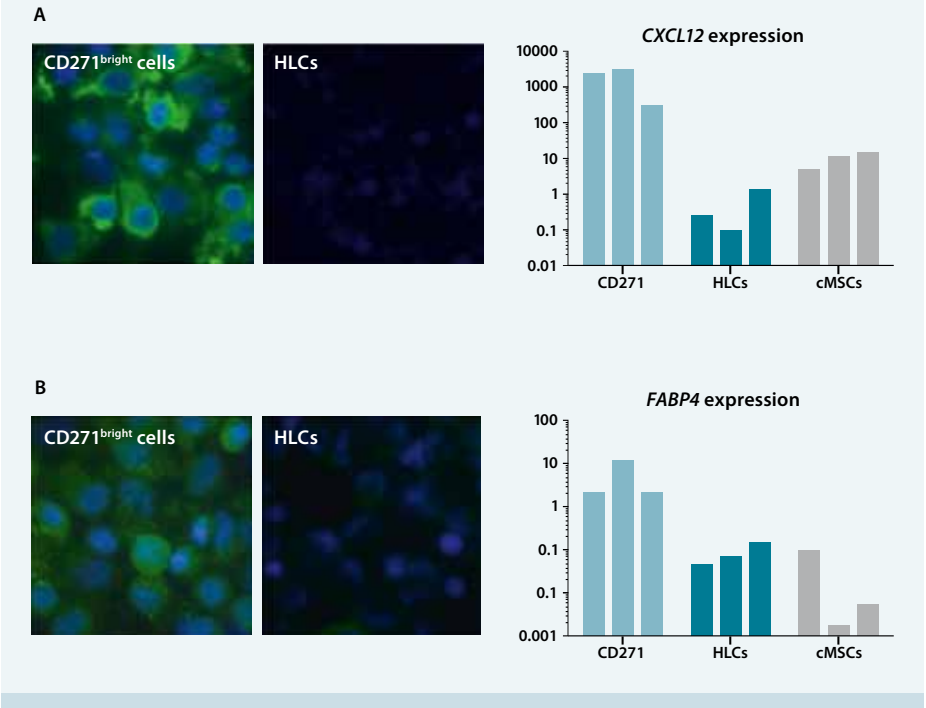


Figure 2 Expression of *CXCL12* (A) and *FABP4* (B) mRNA and corresponding proteins in analyzed fractions. Left panels: protein expression, green; cell nuclei, blue; original magnification 400×. Right panels: transcript expression levels (relative to *HPRT*), three age-matched donors (22, 35, and 72 years old).

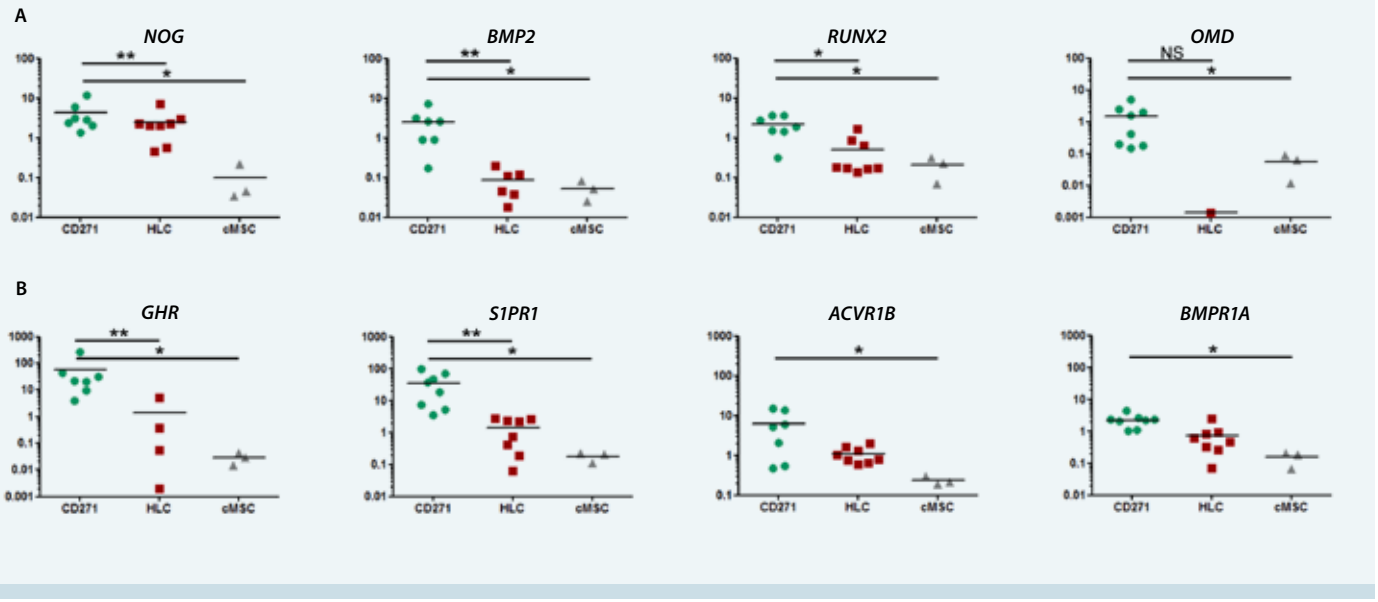


Figure 3 **Transcripts implicated in MSC osteogenesis (A) and novel MSC surface receptors (B).** *$P<0.05$, **$P<0.01$, Kruskal-Wallis analysis, corrected with the Bonferroni-Dunn multiple group comparison test. NS=no statistics due to low detection in HLCs in some donors.

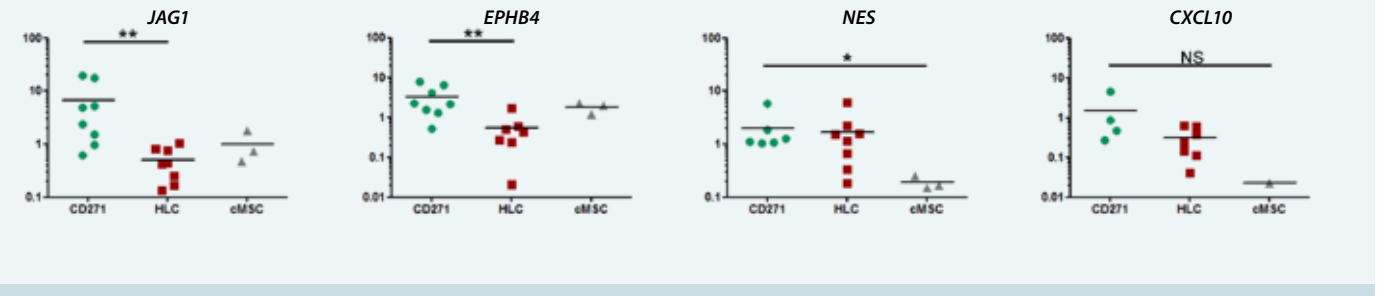


Figure 4 **Transcripts implicated in MSC hematopoiesis–supporting and immunomodulatory functions.** *$P<0.05$, **$P<0.01$, Kruskal-Wallis analysis, corrected with the Bonferroni-Dunn multiple group comparison test. NS=no statistics due to low detection in HLCs in some donors.

immunomodulatory functions of MSCs, whereas *EPHB4* could be involved in MSC interactions with osteoblasts and osteoclasts[14]. Nestin/*NES* has been previously described as highly MSC-specific in a mouse system[15]. In our dataset, the differences between human CD271^{bright} MSCs and HLCs were insignificant due to large donor variation observed for HLCs (fig. 4). *CXCL10* (chemokine (C-X-C motif ligand 10) is induced by Toll-like receptor signaling[13] and may be involved in the immunosuppressive activity of MSCs *in vivo*.

Discussion

Several lines of evidence have now converged to indicate that CD271^{bright} cells represent the *in vivo* counterpart of BM-MSCs. Firstly, all assayed CFU-Fs were shown to be confined to this fraction[5] and the number of CD271^{bright} cells per milliliter of aspirate predicted the number of CFU-Fs[16]. Secondly, CD271^{bright} cells expressed standard ISCT-approved MSC markers as well as additional MSC- and pericyte-linked markers such as CD146 and MSCA-1[4]. Finally, their transcriptional signature as illustrated here and elsewhere[5,6], suggests their belonging to the mesenchymal lineage, with a potential strong engagement in BMP-, Wnt-, and Notch-pathway signaling processes. The expression of immunoregulatory molecules in CD271^{bright} cells is particularly noteworthy given their potent allosuppressive properties[17].

It remains unresolved as to whether every single CD271^{bright} cell is highly proliferative and multipotential. Recent enumeration and sorting experiments showed that only ~3–15% of CD271^{bright} cells are colony-forming following plastic adherence[5,16]. This could imply that CD271^{bright} cells would be further subdivided into 'true MSCs' and other 'contaminating' cells. Our transcript expression data however, challenge this concept. For the overwhelming majority of transcripts studied, and irrespective of the

expansion media and/or prior pre-selection step used[6], the transcript expression in CD271^{bright} cells was never lower than in cMSCs. On the contrary, the fact that it was commonly above cMSC levels (figs. 3 and 4) suggests that a plastic adherence–based cultivation may in fact lead to the loss of some poorly adherent, but highly potent MSCs. QPCR experiments using single sorted CD271^{bright} cells will be required to address this question.

Conclusion

Overall, the data presented in this report suggest that freshly purified CD271^{bright} cells are transcriptionally active and multipotential and may have a significant value as cell therapeutics for multiple indications, including immunomodulation, and without the need for adherence-based culture expansion. Further work on maximizing their recovery from the marrow niche, and functional tests in animal models will be required to prove their potential in regenerative medicine applications.

References:

1. Cattoretti, G. *et al.* (1993) Blood 81: 1726–1738.
2. Quirici, N. *et al.* (2002) Exp. Hematol. 30: 783–791.
3. Jones, E.A. *et al.* (2002) Arthritis Rheum. 46: 3349–3360.
4. Buhring, H.J. *et al.* (2009) Phenotypic characterization of distinct human bone marrow-derived MSC subsets; in Kanz, Weisel, Dick, and Fibbe (eds.): Hematopoietic stem cells VII, p. 124–134. Oxford, Blackwell Publishing.
5. Tormin, A. *et al.* (2011) Blood 117: 5067–5077.
6. Churchman, S. *et al.* (2012) Arthritis Rheum.: Epub ahead of print, Feb. 29.
7. Kortesidis, A. *et al.* (2005) Blood 105: 3793–3801.
8. Ninomiya. K. *et al.* (2007) Biochem. Biophys. Res. Commun. 362: 460–466.
9. Kassem, M. *et al.* (1993) Calcif. Tissue Int. 52: 222–226.
10. Ishii, T. *et al.* (2011) Mol. Cells 31: 399–403.
11. Allende, M.L. *et al.* (2010) J. Exp. Med. 207: 1113–1124.
12. Jones, P. *et al.* (1998) Blood 92: 1505–1511.
13. Liotta, F. *et al.* (2008) Stem Cells 26: 279–289.
14. Zhao, C. *et al.* (2006) Cell Metab. 4: 111–121.
15. Mendez-Ferrer, S. *et al.* (2010) Nature 466: 829–834.
16. Cuthbert, R. *et al.* (2012) Cytotherapy 14: 431–440.
17. Kuci, Z. *et al.* (2011) Cytotherapy 13: 1193–1204.

Relevant products	Order no.
Anti-Fibroblast MicroBeads, human	130-050-601
NH Expansion Medium, human	130-091-680