Ann. N.Y. Acad. Sci. ISSN 0077-8923

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Hematopoietic Stem Cells IX*

Isolation and characterization of primary bone marrow mesenchymal stromal cells

Hongzhe Li,[1] Roshanak Ghazanfari,[1] Dimitra Zacharaki,[1] Hooi Ching Lim,[1] and Stefan Scheding[1,2]

[1]Lund Stem Cell Center and Division of Molecular Hematology, Lund University, Lund, Sweden. [2]Department of Hematology, Skåne University Hospital Lund, Lund, Sweden

Address for correspondence: Stefan Scheding, M.D., Lund Stem Cell Center and Division of Molecular Hematology, Lund University, BMC B12, Klinikgatan 26, 22184 Lund, Sweden. stefan.scheding@med.lu.se

Bone marrow (BM) contains a rare population of mesenchymal stromal cells (MSCs), which have been characterized as nonhematopoietic skeletal progenitor cells with central importance for the hematopoietic microenvironment. Classically, MSCs are isolated by plastic adherence and subsequent culture. However, as cultured stromal cells differ from their *in vivo* progenitors, it is important to identify the phenotype of the primary MSCs to study these cells in more detail. In the past years, several surface markers have been reported to be suitable for effective enrichment of BM-MSCs, and recent data indicate that the putative MSC stem/progenitor cell population in human adult BM is highly enriched in Lin$^-$ CD45$^-$ CD271$^+$ CD140a (PDGFRα)$^{low/-}$ cells. Moreover, surface marker combinations have been described for the isolation of MSCs from murine BM. On the basis of these findings, the role of primary MSCs can now be studied in normal and, importantly, diseased BM. Furthermore, genetically engineered mouse models have been developed as powerful tools to investigate well-defined BM stromal cell populations *in vivo*. Our discussion aims to provide a concise overview of the current state of the art in BM-MSC isolation in humans and briefly present murine MSC isolation approaches and genetic models.

Keywords: bone marrow stromal cells; mesenchymal stromal cells; MSC; bone marrow; CFU-F; CD271; PDGFRα

Introduction

The initial findings of Tavassoli and Crosby[1] describing the transplantability of marrow to extramedullary sites and seminal studies by Friedenstein *et al.*[2–4] indicated the existence of a rare population of nonhematopoietic bone marrow (BM) cells with *in vivo* multilineage differentiation capacity toward skeletal lineages.[5] The formation of ectopic BM was assigned to a rare subpopulation of BM cells that could be distinguished from the remaining cells by their plastic adherence properties. Furthermore, these cells were capable of forming single cell–derived fibroblastic colonies, and thus termed colony-forming unit fibroblasts (CFU-Fs), indicating stem/progenitor cell properties.[2–4] Friedenstein and Owen named these cells "osteogenic stem cells" or "bone marrow stromal cells."[6,7] Later, Caplan introduced the term *mesenchymal stem cells* referring to adult BM progenitor cells with similar properties.[8]

The term mesenchymal stem cells has since then mostly and inaccurately been used for culture-derived stromal cell products, which have raised a considerable interest as cell therapy tools for a number of disorders.[9] For example, MSCs have been demonstrated to be therapeutically effective in diseases, such as graft-versus-host disease.[10] However, the widespread clinical use of cultured MSCs has been criticized,[11] and, unfortunately, an increasing number of unproven cell-based therapies outside of approved clinical trials has been made commercially available in recent years, which certainly is a major concern in current MSC therapy.[12,13]

Bona fide primary BM-MSCs have been shown to be important components of the hematopoietic stem cell niche, and they furthermore give

doi: 10.1111/nyas.13102

rise to the hematopoietic microenvironment upon transplantation.[5,14,15] Primary MSCs can be functionally enriched by adherent culture techniques in selected media and propagated further as cultured stromal cells, generating more or less morphologically homogeneous but functionally heterogeneous cell products. Recent progress in identifying the phenotype of the primary MSCs now enables the direct study of this important cell type in its uncultured state, thus providing important information about its physiological role *in vivo*.[16]

Here, we summarize the state-of-the-art BM-MSC isolation in humans, and we also briefly present current murine MSC isolation approaches and genetic models.

Conventional approaches to human MSC isolation: adherence

MSCs can be retrospectively identified on the basis of their ability to form adherent colonies *in vitro*, and these cells were—in analogy to the terminology used for the hematopoietic system—designated as CFU-Fs.[17] MSCs have been described in a number of different species, where they can be isolated from various tissues. Isolation is traditionally based on the typical capacity of MSCs to adhere to plastic surfaces.[18–20] One disadvantage of isolating cells by adherence, however, is the nonspecificity of this approach despite the use of selected sera and media for culture initiation. This is due to the fact that MSCs represent only a very rare fraction of the starting cell population that contains other, more abundant cells types, several of which also adhere to standard cell culture surfaces. Thus, MSC cultures initiated with nonselected cells are heterogeneous, and undesirable interactions of MSCs with non-MSCs might occur.

For example, the frequency of CFU-Fs in normal adult human BM mononuclear cell preparations is typically about one to two in 100,000 cells. This number refers to cells obtained by aspiration from hematopoietically active iliac crest BM from adult human donors.[15] However, CFU-F frequencies are known to be age dependent (i.e., frequencies decrease with increasing age) and are highest in fetal marrow. Furthermore, differences have been reported between different sites, such as vertebral BM and iliac crest BM.[21]

Prolonged culture is often required to remove the contaminating cells and to obtain a reasonably pure MSC population. Stromal cells in general have a high proliferation capacity and are relatively easy to cultivate, and a number of commercial media formulations have been introduced in the last years. These media save laborious and time-consuming serum testing and also allow better comparisons of results between laboratories, as the products are more standardized compared to investigator-designed media. However, on the other hand, the exact composition of proprietary media might not be known, which is a potential problem when interpreting possible culture-induced MSC changes. Most information about MSCs relies on *in vitro* culture–expanded cells, which are now better referred to as multipotent MSCs[22] or—in our opinion—even better as cultured mesenchymal stromal cells or cultured stromal cells. These cultured stromal cell preparations have to fulfill a number of simple criteria to be classified as MSCs, including plastic adherence in standard media, an MSC-typical surface marker profile (expression of CD105, CD73, and CD90, and lacking expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules) and *in vitro* differentiation potential toward the adipogenic, osteogenic, and chondrogenic lineages.[23]

However, cultured mesenchymal stromal cell preparations are not homogeneous,[24] and, more importantly, they do not necessarily reflect the properties of the native primary MSCs. Native primary MSCs, however, constitute the relevant cell population when studying the physiological role of MSCs *in situ*, for example, as important constituents of the hematopoietic BM microenvironment. Furthermore, cultured MSCs have been demonstrated to gradually lose self-renewal property; they have a decreased multilineage differentiation potential,[25] a poor hematopoiesis-supporting function,[26,27] and they fail to migrate to the BM.[27,28] Cultured MSCs also show a different surface marker profile when compared to the freshly isolated primary cells. For example, a variety of surface markers, such as CD44 and CD318, are highly expressed on cultured cells, but not on primary MSCs,[27,29] while the expression of other markers, such as CD271, is rapidly downregulated in culture.[15] Additionally, there is evidence of marked global gene expression differences between freshly isolated and cultured cells.[26,30] The phenotypical changes from primary to cultured cells are probably due to the biochemical changes in MSCs as they adapt to the *in vitro* culture conditions

Ann. N.Y. Acad. Sci. 1370 (2016) 109–118 © 2016 The Authors. *Annals of the New York Academy of Sciences* published by Wiley Periodicals Inc. on behalf of The New York Academy of Sciences.

and suggest fundamentally altered functional properties of cultured cells. Thus, when aiming to characterize the physiological role of MSC in normal and diseased BM, it appears to be most appropriate to study primary, prospectively isolated MSCs. However, despite its limitations, conventional MSC isolation and propagation in culture is still a valid option when aiming for large-scale stromal cell expansion for clinical applications.

Current approaches to prospectively isolate human MSCs

As mentioned above, most studies conducted thus far have been mainly based on cultured MSCs, which does not allow for proper investigation of many of the important *in vivo* properties and physiological functions of the native bona fide MSCs. Here, recently developed strategies to prospectively isolate primary MSCs enable precise identification and isolation of the target cell population according to a cell-type specific surface marker profile, thus facilitating progress in understanding the biology of this rare cell population.

One of the major hurdles in developing this approach has been the absence of a single marker that specifically defines MSCs. Therefore, it is of utmost importance to establish robust marker combinations and identify novel markers for MSC isolation and, consequently, a better characterization of primary MSCs.

Prospective isolation of human BM-MSCs based on surface marker expression

A variety of candidate markers have been used for isolation of primary MSCs from human adult BM, including positive selection markers, such as Stro-1,[31] CD271,[32,33] CD146,[5,34] CD106,[35] CD73,[36] CD105,[37] FZD9,[38] SUSD2,[39] LEPR, and CD90, and negative selection markers, such as CD44,[27] CD31,[40] CD34,[40] and CD45.[41] The markers CD146, CD105, CD51, and CD140a have been demonstrated to enrich clonogenic stromal cells in human fetal BM.[42,43] However, none of the markers are specific for MSCs, and the precise *in vivo* identity and phenotypic signature of adult BM-MSCs have therefore remained elusive.

On the basis of our previous data showing that all the BM colony-forming cells (CFU-Fs) were exclusively enriched in Lin^- $CD45^-$ $CD271^+$ cells, but not in the $CD271^-$ subset,[15] an array-based gene expression profiling analysis was conducted comparing colony-initiating cells ($CD271^+$) versus non-colony-initiating cells ($CD271^-$).[44] The aim of this comparison was to identify potential novel MSC surface markers that could be used to improve current MSC isolation approaches.

Using this approach, 28 surface-expressed molecules could be identified that were highly expressed in primary MSCs (Table 1). These markers were further divided into two groups according to their expression patterns in relation to CD271. Group I markers showed a staining pattern that paralleled CD271 expression, which means that the expression of the marker proportionally increased with increasing CD271 expression. Therefore, these markers could not be used to further enrich the clonogenic cell population in Lin^- $CD45^-$ BM cells, but are candidates to be used in combination with CD271 to isolate BM-MSCs that show all typical MSC properties *in vitro* and *in vivo*.[44]

In contrast to group I markers, group II markers (CD140a and FGFR3) showed a more orthogonal distribution in the fluorescence-activated cell sorting (FACS) plot, allowing the separation of the $CD271^+$ cell population into subpopulations of $CD271^+$ $FGFR3^+$, $FGFR3^-$ and $CD271^+$ $CD140a^+$, and $CD140a^{low/-}$ cells. Sorting based on FGFR3 expression did not improve CFU-F enrichment rates compared to sorting based on CD271 alone (i.e., CFU-F frequencies were about one in 20 cells). On the other hand, a very high enrichment of CFU-F was achieved by sorting $CD271^+$ cells in combination with CD140a (PDGFR-α) (Fig. 1). Here, the mean CFU-F frequency in $CD45^-$ $CD271^+$ $CD140a^{low/-}$ cells from bulk sorting was 20.8 ± 9.6 CFU-Fs per 100 cells, compared with 0.8 ± 1.2 CFU-Fs per 100 $CD140a^+$ cells, which indicates that the majority of the colony-initiating cells reside in the $CD140a^{low/-}$ subpopulation. The combination of CD271 with $CD140^{low/-}$ staining thus considerably enriched the frequency of colony-forming cells more than 20-fold compared with sorting on CD271 alone. CFU-F frequencies were additionally confirmed in single-cell and limiting-dilution assays.[44]

Recently, Zhou *et al.*[45] reported that leptin receptor (LepR) was a marker that highly enriched BM CFU-Fs in mice, and that approximately 94% of BM CFU-Fs were $LepR^+$. Interestingly, the LepR gene was also one of the genes found to be upregulated in human $CD271^+$ cells, compared with $CD271^-$ cells,

Table 1. Upregulated surface-expressed molecules in Lin$^-$ CD45$^-$ CD271$^+$ cells

Group I molecules				
Name	Description	Fold change	Previously reported	Reference
VCAM1/CD106	Vascular cell adhesion molecule 1	52.23	Y	35
ITGB5	Integrin β5	41.91	N	
IL11RA	Interleukin 11 receptor α	27.62	N	
GHR	Growth hormone receptor	16.08	N	
LEPR/CD295	Leptin receptor	15.40	Y	45
PDGFRB/CD140b	β-Type platelet-derived growth factor receptor	14.60	Y	38
CDH11	Cadherin-11	14.45	N	
CD81	Tetraspanin-28 (Tspan-28)	13.66	N	
TNFRSF19	Tumor necrosis factor receptor superfamily, member 19	8.74	N	
TGFBR2	Transforming growth factor β receptor II	7.48	N	
TNFRSF1A	Tumor necrosis factor receptor superfamily member 1A	6.95	N	
TMEM98	Transmembrane protein 98	6.77	N	
TMEM119	Transmembrane protein 119	6.22	N	
ITGB2/CD18	Integrin β2	6.10	N	
IFNGR2	Interferon γ receptor 2	5.90	N	
CNTNAP2	Contactin-associated protein–like 2	5.74	N	
ABCA8	ATP-binding cassette subfamily A member 8	5.57	N	
IL1R1	Interleukin 1 receptor, type I	4.84	N	
TGFBR3	Transforming growth factor β receptor III	4.61	N	
CD151	A member of the tetraspanin family	4.52	N	
MME/CD10	Membrane metalloendopeptidase, neprilysin	3.97	Y	29
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	3.92	N	
TMEM2	Transmembrane protein 2	3.75	N	
BST2/CD317	Tetherin, bone marrow stromal antigen 2	3.61	N	
FCRLA	Fc receptor–like A	3.13	N	
EFNA1	Ephrin-A1	3.10	N	
Group II molecules				
FGFR3/CD333	Fibroblast growth factor receptor 3	11.02	N	
PDGFRA/CD140a	α-Type platelet-derived growth factor receptor	10.93	N	

Note: Data presented in this table are adapted from Ref. 44.

in our data set. Correspondingly, analysis and sorting experiments demonstrated that LepR expression was conserved in human primary MSCs with about 90% of CFU-Fs in human BM being LepR$^+$. However, as LepR belonged to the group I markers, CFU-F enrichment rates could not be improved by including LepR in the sorting panel.

Thus, sorting of cells expressing the low-affinity nerve growth factor receptor CD271 and exhibiting low expression/absence of CD140a expression (CD271$^+$/CD140a$^{low/-}$) enabled the enrichment of human adult primary BM-MSCs with the highest purity reported for hematopoietically active adult BM thus far. Furthermore, this marker combination could also be used to isolate MSCs from older adults, since they showed a similar distribution pattern compared to younger adults, even though CFU-F frequencies were lower.

In contrast to our findings in human adults, CD140a has been reported as a positive MSC selection marker for human fetal BM and also for murine CFU-Fs. Pinho *et al.*[43] reported that the combination of CD51 (integrin α_V) and CD140a highly enriched for clonogenic cells in human fetal BM. MSC isolation based on CD140a and Sca1 expression from murine BM was reported by Morikawa *et al.*[46] Our own preliminary data (Ghazanfari *et al.*, unpublished results) furthermore showed, in agreement with Pinho *et al.*,[43] that human fetal BM CD140a$^+$ cells had a higher CFU-F frequency compared to CD140a$^-$ cells. Furthermore, and also in accordance with previously published data,[47] we

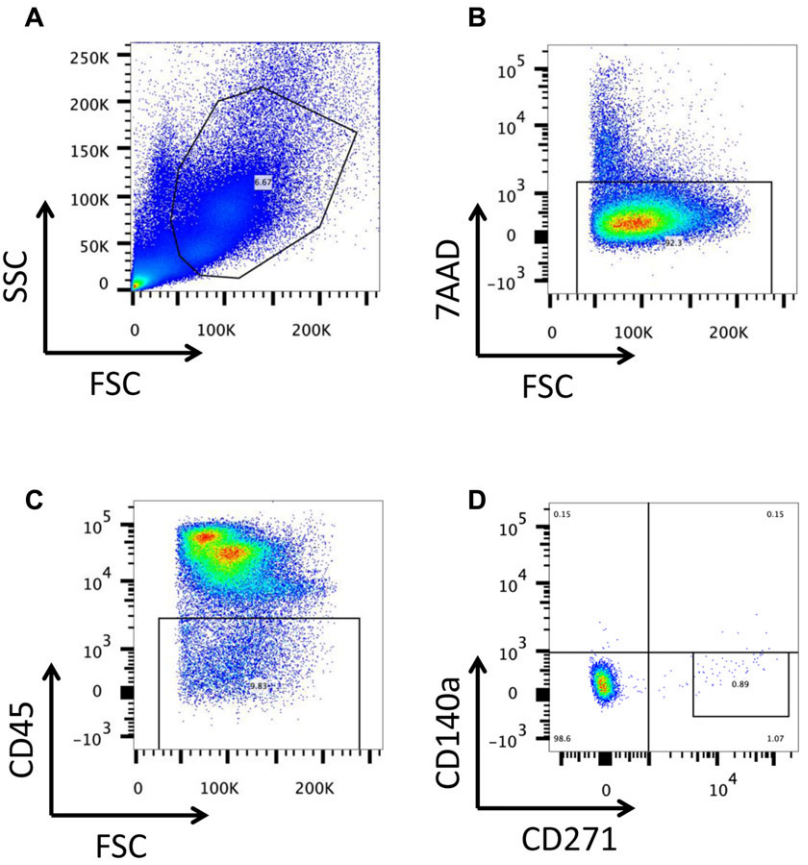

Figure 1. Gating strategy of isolating Lin$^-$ CD45$^-$ CD271$^+$ CD140a$^{low/-}$ cells. Freshly isolated lineage-depleted human bone marrow mononuclear cells were stained with antibodies against CD271, CD45, and CD140a, and Lin$^-$ CD45$^-$ CD271$^+$ CD140a$^{low/-}$ cells were sorted by FACS following (A) forward/side scatter gating, (B) dead cell exclusion (7-AAD gating), (C) hematopoietic cell exclusion, and (D) gating on CD140a$^-$ cells. Gates for the CD45/CD271/CD140a sorts from lineage-depleted bone marrow were set according to the corresponding fluorescence-minus-one controls for CD45 (C), CD140a, and CD271 (D). The figure shows a representative set of FACS plots of experiments reported previously by Li *et al.*[44]

have shown that there was a progressive decrease of CD140a-expressing cells in murine nonhematopoietic cells from 45% at E17.5 (embryo day 17.5) to 21% at P8 (postnatal day 8) and 1.2% at P17.[44] Taken together, these data suggest that CD140a expression on MSCs is developmentally regulated, possibly pointing to a differential function of PDGFα and/or its receptor during different stages of development.

Adult BM cells are by far the most frequently used source for MSC isolation, and our studies were able to define the phenotype of adult primary MSCs as Lin$^-$ CD45$^-$ CD271$^+$ CD140a$^{low/-}$. Sorting on low/negative CD140a expression in combination with CD271 allowed isolation of a (nearly) pure population of the putative stromal stem/progenitor cell population.[44] Lin$^-$ CD45$^-$ CD271$^+$ CD140a$^{low/-}$ cells showed a typical MSC morphology with cytoplasmic vacuoles and large, immature nuclei, and they expressed signature primary MSC markers, such as CD90, CD105, CD140b, and STRO-1, while lacking expression of endothelial and hematopoietic markers, such as CD31, CD34, and CD45 (Fig. 2). CD140$^-$ cells also showed a relatively higher expression of pluripotent proteins (Nanog, Oct4, and Sox2), cell cycle regulators (p21 and p16), hematopoiesis-supporting proteins (VCAM1, SPP1, CXCL12, and angiopoietin 1), and differentiation proteins (ALPL, PPARγ, and ACAN) compared to CD140a$^+$ cells. Cell cycle analysis furthermore revealed that the majority of

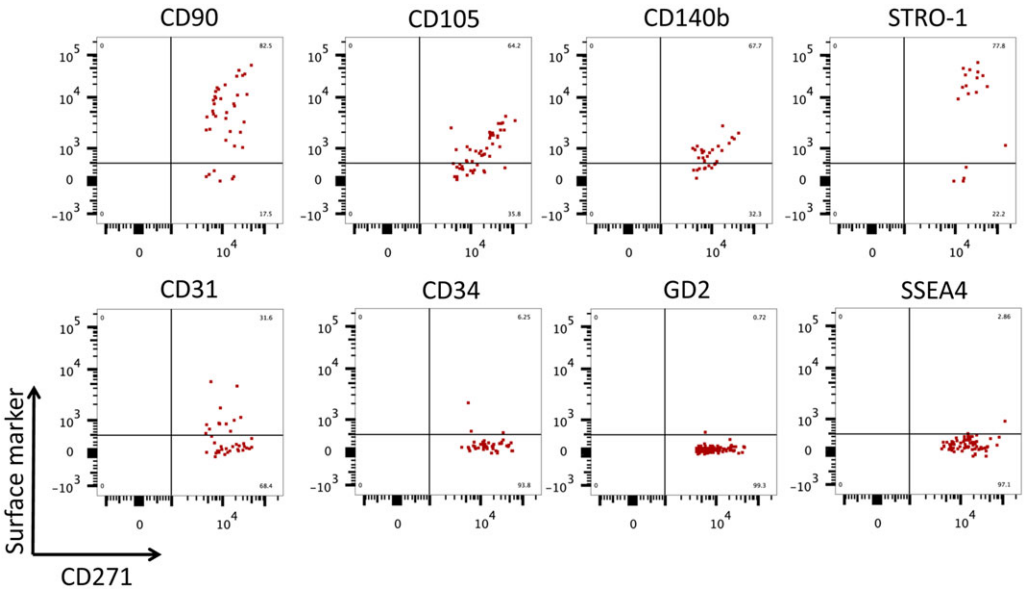

Figure 2. Surface molecules expression in primary Lin$^-$ CD45$^-$ CD271$^+$ CD140a$^{low/-}$ cells. Lineage-depleted, freshly isolated human bone marrow mononuclear cells were stained with antibodies as indicated and analyzed by flow cytometry. Representative plots of CD271 expression (*x*-axis) versus expression of the indicated marker (*y*-axis) are shown after forward/side scatter gating, exclusion of dead cells (7AAD), and gating on CD45$^-$ and CD140a$^-$ cells. Red dots indicate coexpression of the markers listed on top (i.e., CD105, CD90, CD140b, STRO-1, CD31, CD34, SSEA4, and CD34, respectively) on CD45$^-$ CD271$^+$ CD140a$^-$ cells. The figure shows one representative set of FACS plots of a total of three independent experiments previously reported by Li *et al.*[44]

the CD140a$^{low/-}$ cells were in the G_0 phase of the cell cycle. Cultured stromal cells derived from sorted CD140a$^{low/-}$ cells showed a typical MSC surface marker profile and demonstrated robust multilineage differentiation potential both *in vitro* and, importantly, *in vivo* in xenotransplantation experiments using immunodeficient NSG mice.[44] Furthermore, the hematopoiesis-supporting capacity of CD140a$^{low/-}$ cells was evaluated in coculture expansion experiments with human cord blood CD34$^+$ cells. Here, we could demonstrate that coculture with CD140a$^{low/-}$ cells showed a better preservation of the CD34$^+$ phenotype, a higher yield of CD34$^+$ cells, and an increased total cell production compared to cultures without stromal support. Moreover, CD34$^+$ cells expanded on CD140a$^{low/-}$ feeder cells were capable of efficient long-term hematopoietic multilineage reconstitution in immunodeficient mice.

Taken together, we have—on the basis of comparative gene expression analysis—identified a number of novel markers for human MSCs, one of which in combination with CD271 allows for prospective isolation of a distinct population of immature, quiescent stromal stem/progenitor cells with multilineage differentiation potential and potent hematopoietic support capacity. Ongoing studies now aim to investigate the phenotype and function of these cells in normal and diseased BM and, furthermore, ongoing experiments seek to address whether human adult primary MSCs fulfill stringent stem cell criteria (i.e., *in vivo* self-renewal and differentiation as tested in serial transplantation experiments).

Last, there is increasing evidence indicating that even highly enriched primary MSCs might be heterogeneous in relation to ontogeny, phenotype, and functional characteristics. Tormin *et al.*,[15] for example, demonstrated that CD146 expression on primary MSCs correlated with *in situ* localization. While subendothelial sinusoidal cells and marrow reticular cells primarily displayed the CD271$^+$ CD146$^+$ MSC phenotype, bone lining CD271$^+$ MSCs were predominantly CD146$^{low/-}$. Thus, CD146 expression allowed discrimination between perivascularly and endosteally localized MSCs.[15] This finding was later confirmed by Sivasubramaniyan and coworkers, who furthermore identified CD56 as a positive marker for

endosteally localized MSCs.[48] Regarding differences in CFU-F potential, Mabuchi *et al.* demonstrated that single CD271$^+$ THY-1$^+$–derived CFU-Fs could be divided into three functionally distinct groups termed RECs (rapidly expanding MSC clones), MECs (moderately expanding MSC clones), and SECs (slowly expanding MSC clones). RECs exhibited robust multilineage differentiation and self-renewal potency, and were therefore considered to be the most primitive and undifferentiated population.[49] Last, in addition to CD146 and THY-1, other markers, such as MSCA-1 and SUSD2, have also been used to isolate subpopulations of MSCs,[50] and furthermore, differences in differentiation potential of single-sorted BMSC-derived CFU-Fs point to a possible hierarchical structure of the primary MSC compartment.[15] Here, our current ongoing single-cell gene expression experiments will hopefully contribute to further clarify this important aspect of primary MSC biology.

Current approaches to the study of murine MSCs: prospective isolation and genetic models

Prospective isolation of murine BM-MSCs based on surface markers

Although not to the same extent as human cells, murine culture–derived mesenchymal stromal cells have been studied intensively, as illustrated by a large numbers of published papers. Nevertheless, relatively little is known about the surface marker phenotype of the primary MSCs in murine BM, which would be one approach to better characterize this important cell population. However, in contrast to the human system, genetic approaches to identify and manipulate stromal elements *in vivo* can be utilized in animal models, providing powerful tools for the study of the BM stromal and the hematopoietic microenvironment (see below).

With regard to the phenotype of primary MSCs in murine BM, Koide *et al.*[51] reported that the marker combination PDGFRα$^+$ Sca-1$^+$ CD45$^-$ TER119$^-$ identified a population of cells in adult murine BM that was highly enriched for CFU-Fs. This population furthermore fulfilled *in vitro* trilineage differentiation potential criteria, both at the bulk and single-cell levels.[46] Other markers for murine MSCs include CD105 and CD51, of which CD105 has been demonstrated to be a marker for a primitive lineage-restricted progenitor population with limited self-renewal capacity.[52,53] Staining of BM cells with CD51 and PDGFRα allowed for identification of a double-positive population with sphere-forming clonogenic activity that was enriched for the highest levels of HSC regulatory genes.[43] Furthermore, CD140a- and CD51-expressing nonhematopoietic mouse BM stromal cells overlap with the Nestin$^+$ cell population; they contain the majority of fibroblastic CFUs and have *in vivo* sphere-forming self-renewal capacity.

LepR has recently been reported as a marker that highly enriches murine BM-MSCs.[45] The LepR$^+$ populations overlap with PDGFRα- and CD51-expressing cells and account for 94% of BM CFU-Fs.[45] Furthermore, LepR$^+$ perivascular stromal cells have been shown to be the major source of SCF and CXCL12 in murine BM.[54,55] Finally, negative markers, such as CD44, have been identified that—in combination with other markers—can be utilized to enrich for murine clonogenic stromal cells.[27]

Identification and characterization of murine BM-MSCs using a genetically engineered mouse model

Recent developments in genetic engineering have enabled the generation of mouse models that allow researchers to specifically label and thus prospectively isolate and manipulate well-defined mesenchymal cell populations in murine BM. The Nestin–GFP transgenic mouse was the first model that was reported to label BM multipotent mensenchymal stromal cells.[14] Using mice expressing GFP under the Nestin promotor, a rare population of Nestin$^+$ perivascular cells innervated by sympathetic nervous system fibers and relatively highly expressing β_3 adrenergic receptor and CXCL12 was demonstrated to meet the criteria defining MSCs, including generation of ectopic bone and BM upon transplantation of clonal mesenspheres. Later, two distinct populations within Nestin–GFP cells were identified: Nestin–GFP^{bright} cells that were rare cells containing most of the colony-forming activity and that were localized close to arterioles, and Nestin–GFP^{dim} cells, which were more abundant and associated with sinusoids.[56]

Other transgenic mouse models utilize site-specific Cre–Lox recombination technology for Mx1–cre, Prx1–cre, and Osx–cre labeling. Mx1, in addition to hematopoietic cells, labels also

nonhematopoietic, nonendothelial osteogenic cells in the bone that contribute to bone repair.[57] Prx1–cre labels cells derived from the somatic lateral plate mesoderm, which includes a wide variety of mesenchymal lineage cells (osteoblasts, osteocytes, chondrocytes, perivascular stromal cells, including $LepR^+$ cells, CAR cells, and periosteal cells).[55] Osx, also known as Sp7, is a key transcription factor required for osteoblastic differentiation, and Osx-expressing cells are thought to be committed progenitors that contribute to bone remodeling in adult mice.[57] Furthermore, perinatal Osx-expressing cells have been shown to possess CFU-F activity, as well as trilineage differentiation capacity *in vitro*.[58]

A recent study showed that bone morphogenetic protein antagonist gremlin 1 defines a population of osteochondroreticular stem cells in the murine BM.[59] Using a genetic tracing model, a population of highly pure $alphaV^+$ Thy^- $6C3^-$ $CD105^-$ $CD200^+$ skeletal stem cells has been mapped to their downstream progenitors of bone, cartilage, and stromal tissue. It was furthermore demonstrated that mesenchymal lineage commitment could be modulated by external cues, such as injury or disease.[53]

However, despite this considerable progress in identifying important stromal cell populations in murine BM using genetically modified mouse models, it is not quite clear yet whether and to what extent these different cell populations overlap, and important open questions regarding the biology of MSCs and their subpopulations remain to be addressed in the future.

Conclusions and outlook

Nonhematopoietic mesenchymal stromal cells have generated a considerable interest owing to their potential for clinical cell therapy as well as their important role in bone and BM physiology. In order to precisely study this important cell type, it is essential to exactly define its phenotype *in situ*, as cultured stromal cells have been demonstrated to differ considerably from their progenitors. We recently reported the phenotype of the primary MSCs in human adult BM, which now allows the study of important properties of this putative stem cell population in normal and diseased BM. This information, together with the results obtained from advanced genetic murine models, will help us to understand the complex interactions of hematopoietic and nonhematopoietic cells in the BM microenvironment. Furthermore, recently developed stromal xenotransplantation techniques will allow the application of genetic manipulation approaches with human cells, ideally performed in xenotransplantation settings using as-yet nonexistent humanized mice with human-specific stromal cytokines. With regard to the clinical use of cultured MSCs, information about the primary MSC phenotype can be used to better standardize the starting material, thus improving MSC product quality. Furthermore, novel nonadherent sphere culture techniques[60] might pave the way for the development of MSC production methods that better conserve the immature phenotype and potency than current standard adherent cultures. Finally, novel label-free sorting techniques based on biophysical properties have been recently reported for the sorting of MSC subpopulations in culture.[61] Other alternatives are ultrasound-based techniques, which are currently being investigated in our laboratory[62,63] and which can possibly be applied to purify pre- and postculture clinical MSC products.

Acknowledgments

This work was supported by funds from the HematoLinné and StemTherapy Program, the Swedish Cancer Foundation, the Swedish Childhood Cancer Foundation, Gunnar Nilsson's Cancer Foundation, Gunnel Björk's Testament, ALF (government public health grant), and the Skåne County Council´s Research Foundation. We apologize to all authors whose work could not be cited in the review because of space restrictions.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Tavassoli, M. & W.H. Crosby. 1968. Transplantation of marrow to extramedullary sites. *Science* **161:** 54–56.
2. Friedenstein, A.J., R.K. Chailakh & K.S. Lalykina. 1970. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet.* **3:** 393–403.
3. Friedenstein, A.J. *et al.* 1974. Stromal cells responsible for transferring microenvironment of hematopoietic tissues. Cloning *in vitro* and retransplantation *in vivo*. *Transplantation* **17:** 331–340.
4. Friedenstein, A.J. 1976. Precursor cells of mechanocytes. *Int. Rev. Cytol.* **47:** 327–359.

5. Sacchetti, B. *et al.* 2007. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* **131:** 324–336.
6. Friedenstein, A.J., R.K. Chailakhyan & U.V. Gerasimov. 1987. Bone marrow osteogenic stem cells: *in vitro* cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet.* **20:** 263–272.
7. Owen, M. & A.J. Friedenstein. 1988. Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found. Symp.* **136:** 42–60.
8. Caplan, A.I. 1991. Mesenchymal stem cells. *J. Orthop. Res.* **9:** 641–650.
9. Doorn, J. *et al.* 2012. Therapeutic applications of mesenchymal stromal cells: paracrine effects and potential improvements. *Tissue Eng. Part B Rev.* **18:** 101–115.
10. Le Blanc, K. *et al.* 2008. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* **371:** 1579–1586.
11. Bianco, P. *et al.* 2013. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat. Med.* **19:** 35–42.
12. Srivastava, A. *et al.* 2016. Part 1: Defining unproven cellular therapies. *Cytotherapy* **18:** 117–119.
13. Deans, R.J. *et al.* 2016. Part 5: Unproven cell therapies and the commercialization of cell-based products. *Cytotherapy* **18:** 138–142.
14. Mendez-Ferrer, S. *et al.* 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* **466:** 829–834.
15. Tormin, A. *et al.* 2011. CD146 expression on primary non-hematopoietic bone marrow stem cells is correlated with *in situ* localization. *Blood* **117:** 5067–5077.
16. Keating, A. 2012. Mesenchymal stromal cells: new directions. *Cell Stem Cell* **10:** 709–716.
17. Friedenstein, A.J. *et al.* 1974. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the *in vitro* colony assay method. *Exp. Hematol.* **2:** 83–92.
18. da Silva Meirelles, L., P.C. Chagastelles & N.B. Nardi. 2006. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J. Cell Sci.* **119:** 2204–2213.
19. Beltrami, A.P. *et al.* 2007. Multipotent cells can be generated *in vitro* from several adult human organs (heart, liver, and bone marrow). *Blood* **110:** 3438–3446.
20. Arufe, M.C. *et al.* 2009. Differentiation of synovial CD-105^{+} human mesenchymal stem cells into chondrocyte-like cells through spheroid formation. *J. Cell. Biochem.* **108:** 145–155.
21. Ahrens, N. *et al.* 2004. Mesenchymal stem cell content of human vertebral bone marrow. *Transplantation* **78:** 925–929.
22. Horwitz, E.M. *et al.* 2005. Clarification of the nomenclature for MSC: the international society for cellular therapy position statement. *Cytotherapy* **7:** 393–395.
23. Dominici, M. *et al.* 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8:** 315–317.
24. Tormin, A. *et al.* 2009. Characterization of bone marrow-derived mesenchymal stromal cells (MSC) based on gene expression profiling of functionally defined MSC subsets. *Cytotherapy* **11:** 114–128.
25. Pevsner-Fischer, M., S. Levin & D. Zipori. 2011. The origins of mesenchymal stromal cell heterogeneity. *Stem Cell Rev.* **7:** 560–568.
26. Churchman, S.M. *et al.* 2012. Transcriptional profile of native CD271^{+} multipotential stromal cells: evidence for multiple fates, with prominent osteogenic and Wnt pathway signaling activity. *Arthritis Rheum.* **64:** 2632–2643.
27. Qian, H., K. Le Blanc & M. Sigvardsson. 2012. Primary mesenchymal stem and progenitor cells from bone marrow lack expression of CD44 protein. *J. Biol. Chem.* **287:** 25795–25807.
28. Rombouts, W.J. & R.E. Ploemacher. 2003. Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. *Leukemia* **17:** 160–170.
29. Battula, V.L. *et al.* 2009. Isolation of functionally distinct mesenchymal stem cell subsets using antibodies against CD56, CD271, and mesenchymal stem cell antigen-1. *Haematologica* **94:** 173–184.
30. Harichandan, A., K. Sivasubramaniyan & H.J. Buhring. 2013. Prospective isolation and characterization of human bone marrow-derived MSCs. *Adv. Biochem. Eng. Biotechnol.* **129:** 1–17.
31. Simmons, P.J. & B. Torok-Storb. 1991. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood* **78:** 55–62.
32. Quirici, N. *et al.* 2002. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp. Hematol.* **30:** 783–791.
33. Jones, E.A. *et al.* 2002. Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheum.* **46:** 3349–3360.
34. Delorme, B. *et al.* 2008. Specific plasma membrane protein phenotype of culture-amplified and native human bone marrow mesenchymal stem cells. *Blood* **111:** 2631–2635.
35. Gronthos, S. *et al.* 2003. Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J. Cell Sci.* **116:** 1827–1835.
36. Veyrat-Masson, R. *et al.* 2007. Mesenchymal content of fresh bone marrow: a proposed quality control method for cell therapy. *Br. J. Haematol.* **139:** 312–320.
37. Aslan, H. *et al.* 2006. Osteogenic differentiation of noncultured immunoisolated bone marrow-derived CD105^{+} cells. *Stem Cells* **24:** 1728–1737.
38. Battula, V.L. *et al.* 2007. Human placenta and bone marrow derived MSC cultured in serum-free, b-FGF-containing medium express cell surface frizzled-9 and SSEA-4 and give rise to multilineage differentiation. *Differentiation* **75:** 279–291.
39. Sivasubramaniyan, K. *et al.* 2013. Prospective isolation of mesenchymal stem cells from human bone marrow using novel antibodies directed against Sushi domain containing 2. *Stem Cells Dev.* **22:** 1944–1954.
40. Liu, L. *et al.* 2006. *Ex vivo* expansion and *in vivo* infusion of bone marrow-derived Flk-1^{+}CD31^{-}CD34^{-}mesenchymal stem cells: feasibility and safety from monkey to human. *Stem Cells Dev.* **15:** 349–357.
41. Jones, E.A. *et al.* 2006. Optimization of a flow cytometry-based protocol for detection and phenotypic characterization of multipotent mesenchymal stromal cells

from human bone marrow. *Cytometry B Clin. Cytom.* **70:** 391–399.
42. Isern, J. *et al.* 2013. Self-renewing human bone marrow mesenspheres promote hematopoietic stem cell expansion. *Cell Rep.* **3:** 1714–1724.
43. Pinho, S. *et al.* 2013. PDGFRα and CD51 mark human Nestin^{+} sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J. Exp. Med.* **210:** 1351–1367.
44. Li, H. *et al.* 2014. Low/negative expression of PDGFR-α identifies the candidate mesenchymal stromal cells in adult human bone marrow. *Stem Cell Rep.* **3:** 965–974.
45. Zhou, B.O. *et al.* 2014. Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell* **15:** 154–168.
46. Morikawa, S. *et al.* 2009. Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. *J. Exp. Med.* **206:** 2483–2496.
47. Qian, H. *et al.* 2013. Molecular characterization of prospectively isolated multipotent mesenchymal progenitors provides new insight into the cellular identity of mesenchymal stem cells in mouse bone marrow. *Mol. Cell. Biol.* **33:** 661–677.
48. Sivasubramaniyan, K. *et al.* 2012. Phenotypic and functional heterogeneity of human bone marrow– and amnion-derived MSC subsets. *Ann. N.Y. Acad. Sci.* **1266:** 94–106.
49. Mabuchi, Y. *et al.* 2013. LNGFR^{+}THY-1^{+}VCAM-1^{hi+} cells reveal functionally distinct subpopulations in mesenchymal stem cells. *Stem Cell Rep.* **1:** 152–165.
50. Busser, H. *et al.* 2015. Isolation and characterization of human mesenchymal stromal cell subpopulations: comparison of bone marrow and adipose tissue. *Stem Cells Dev.* **24:** 2142–2157.
51. Koide, Y. *et al.* 2007. Two distinct stem cell lineages in murine bone marrow. *Stem Cells* **25:** 1213–1221.
52. Chan, C.K. *et al.* 2009. Endochondral ossification is required for haematopoietic stem-cell niche formation. *Nature* **457:** 490–494.
53. Chan, C.K. *et al.* 2015. Identification and specification of the mouse skeletal stem cell. *Cell* **160:** 285–298.
54. Ding, L. *et al.* 2012. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* **481:** 457–462.
55. Ding, L. & S.J. Morrison. 2013. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* **495:** 231–235.
56. Kunisaki, Y. *et al.* 2013. Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* **502:** 637–643.
57. Park, D. *et al.* 2012. Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. *Cell Stem Cell* **10:** 259–272.
58. Mizoguchi, T. *et al.* 2014. Osterix marks distinct waves of primitive and definitive stromal progenitors during bone marrow development. *Dev. Cell* **29:** 340–349.
59. Worthley, D.L. *et al.* 2015. Gremlin 1 identifies a skeletal stem cell with bone, cartilage, and reticular stromal potential. *Cell* **160:** 269–284.
60. Isern, J. *et al.* 2013. Self-renewing human bone marrow mesenspheres promote hematopoietic stem cell expansion. *Cell Rep.* **3:** 1714–1724.
61. Lee, W.C. *et al.* 2014. Multivariate biophysical markers predictive of mesenchymal stromal cell multipotency. *Proc. Natl. Acad. Sci. U.S.A.* **111:** E4409–E4418.
62. Dykes, J. *et al.* 2011. Efficient removal of platelets from peripheral blood progenitor cell products using a novel micro-chip based acoustophoretic platform. *PLoS One* **6:** e23074.
63. Lenshof, A. *et al.* 2014. Efficient purification of CD4^{+} lymphocytes from peripheral blood progenitor cell products using affinity bead acoustophoresis. *Cytometry A* **85:** 933–941.