Primary Mesenchymal Stem and Progenitor Cells from Bone Marrow Lack Expression of CD44

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Running title: Native mesenchymal stem cells do not express CD44

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Key words: Mesenchymal stem cells, CD44, microarray, Bone Marrow, Flow cytometry.

Background: Natural phenotype of mesenchymal stem cells (MSCs) has not been well-characterized.

Results: MSCs from bone marrow naturally are CD44−, however, in vitro cultivation results in acquisition of CD44 expression on the cells.

Conclusion: Native MSCs in bone marrow lack CD44 expression.

Significance: Our findings highlight natural phenotype of MSCs and open new possibilities for prospective isolation of MSCs from bone marrow.
SUMMARY

Despite significant progress in our understanding of mesenchymal stem cell (MSC) biology during the last years, much of the information is based on experiments using in vitro culture-selected stromal progenitor cells. Therefore, the natural cellular identity of MSCs remains poorly defined. Numerous studies have reported that CD44 expression is one of the characteristics of MSCs in both human and mice, however, we here have prospectively isolated bone marrow stromal cell subsets from both human and mouse bone marrow by flow cytometry and characterized them by gene expression analysis and function assays. Our data provide functional and molecular evidence suggesting that primary mesenchymal stem and progenitor cells of bone marrow reside in the CD44- cell fraction in both mice and humans. The finding that these CD44- cells acquire CD44 expression after in vitro culture provides an explanation to the previous misconceptions concerning CD44 expression on MSCs. In addition, the other previous reported MSC markers including CD73, CD146, CD271, CD106/VCAM1 are also differentially expressed on those two cell types. Our microarray data revealed distinct gene expression profile of the freshly CD44- cells and the cultured MSCs generated from these cells. Thus, we conclude that bone marrow MSCs physiologically lack expression of CD44, highlighting the natural phenotype of MSCs and open new possibilities to prospectively isolate MSCs from the bone marrow.

INTRODUCTION

Mesenchymal stem cells (MSCs) was originally isolated from bone marrow (BM) by their capacity to generate colony-forming unit-fibroblast (CFU-F) in vitro (1). Although there has been significant progress in understanding of the biological features of MSCs, much of the information has been obtained from in vitro studies on culture-expanded cells, which may not represent the phenotype of MSCs in vivo (2-5). Multi-color fluorescence activated cell sorting (FACS) have been fundamental for definition and prospective isolation of different cell populations of the hematopoietic system over the last 20 years. Recent development of FACS-based protocols for the isolation and characterization of MSCs directly from BM opens the possibility to better identify and characterize non-hematopoietic cell compartments in the BM. In mice, platelet derived growth factor receptor α (PDGFRα), stem cell antigen-1 (SCA1), CD51 and Nestin are expressed on freshly isolated BM stromal cell populations enriched with MSCs (6-8). In human, several surface proteins including Stro-1, CD271 and CD146 may be used as markers for mesenchymal stem and progenitor cells (9-14). In addition, expression of markers such as CD105, CD90 and CD49A have been diversely reported to be characteristic of MSCs (15). Among those, CD44 has been reported to be highly expressed on in vitro expanded MSCs from both humans and mice (16-22). CD44 is an adhesion molecule existing in different isoforms that interact with multiple ligands such as hyaluronan, selectins, collagen and fibronectin (23). It is widely expressed in multiple cell types including hematopoietic cells, cancer stem cells (24).

In the present study, by using multi-color FACS, microarray analysis and CFU-F assay, we have found that while freshly isolated MSCs from human and mouse BM express the surface markers previously reported to mark early mesenchymal progenitors, they lack expression of CD44. Further characterization of the cells revealed that the CD44- cells displayed little or no CFU-F activity whereas the CD44+ cells contain almost all the clonogenic cells with multilineage differentiation potentials.
However, *in vitro* culture of the CD44^- MSCs and progenitor cells resulted in their conversion to a CD44 positive phenotype, providing an explanation to the previous observations suggesting CD44 as a marker for MSCs. Furthermore, the cultured MSCs derived from the fresh CD44^- stromal cells display distinct gene expression profiles of cell adhesion molecules and growth factors as well as cytokines. These findings highlight the importance of *in vivo/ex vivo* analysis of mesenchymal cells for identifying their physiological properties and suggest that CD44 expression can be used as a negative rather than a positive marker for prospective isolation of MSCs from BM.

**EXPERIMENTAL PROCEDURES**

**Subject:** BM aspirates were obtained from iliac crest of normal young adult volunteers following informed consent according to the procedures approved by local ethics committee at Karolinska Institute (Stockholm, Sweden). Mouse bones were obtained from adult (3-4 month old) normal FVB/N mice. Animal procedures were performed with approval from the ethics committee at Linköping University (Linköping, Sweden).

**FACS isolation and analysis of human BM MSCs.** Mononuclear cells from BM aspirates of healthy adult volunteers were isolated by Ficoll-Hypaque (Lymphoprep, Axis-Shield PoC AS) density centrifugation. The CD45^- CD235^- cells were enriched by negative selection using CD45 and CD235 microbeads and magnetic-activated cell sorting (MACS, Miltenyi Biotec). The cells were then stained with anti-human CD271 CD146, CD105, CD106, CD73, STRO-1, CD29, CD45 and Glycophorin A/CD235. Anti-human CD19 was included in the staining in order to exclude possible contamination of B cells in the sorted stromal cells. For information about the antibodies used in the study, see supplemental information. Dead cells were excluded by propidium iodide (PI) staining. The cells were analyzed and sorted on FACS Aria II Sorp (BD).

**FACS isolation and analysis of mouse MSCs.** The BM mononuclear cells from femurs, tibias and iliac crest of FVB/N mice were isolated using a standard protocol which was tested in our laboratory without affecting cell surface marker expression. The bones were first crushed in PBS+10%FBS (PAA) in order to obtain maximal cells in BM endosteal region prior to enzyme treatment. The marrow cells were collected and the bone fragments were then treated with 0.1% Collagenase II (CLS II Worthin gton Biochemical) and 0.05% trypsin-EDTA for 45 min at 37°C. The tubes were shaken every 10 min during incubation. The treatment was stopped by adding ice cold FBS to reach a final concentration of 20% FBS, subsequently wash the bones by PBS+10% FBS. The cells were collected and filtered via 70 µm cell strainer (BD). The bone and marrow cells were pooled and spun down at 300g for 10 min and then resuspended in PBS+10% FBS. The stromal cells were first enriched by depleting hematopoietic cells using purified rat anti-mouse antibodies against CD45 and LIN (TER119, B220, CD4, CD8, GR1 and MAC1) and subsequently using sheep anti-rat Dynal beads (Invitrogen). The endothelial cells and the residual hematopoietic cells were visualized by CD31 and goat-anti-rat tricolor antibody and/or CD45 and TER119. The dead cells were excluded by PI staining. The CD44^- and CD44^+ stromal cells were gated or analyzed based on fluorescent minus one (FMO) controls for CD44 expression on FACS aria II Sorp (BD). For information about the antibodies used in the study, see Supplemental information.

**CFU-F assay.** The stromal cells (CD45-LIN-CD31^-CD44^+ and CD45-LIN-CD31^-CD44^-)
from normal mouse and human BM were sorted and plated into 96-well plates or 12-well plates containing complete Mesencult medium in Mesencult® Proliferation Kit for mouse (#05511) and human (#05411), respectively (Stem cell Technologies, Vancouver, Canada) in hypoxic (1% O2) for 10-12 days (mouse) or 12-14 days (human). The mouse cells were seeded at a density of 10, 40, 100 and 200 cells/well for the CD44- cells and 100, 200, 400, 1000 and 2000 cells/well and the human cells were plated at 2, 5, 10 cells/well for the CD44- cells and 10, 50, 100 and 1000 cells/well for the CD44+ cells. The complete Mesencult medium was prepared by mixing one part of MSC stimulatory Supplements and 4 parts of Mesencult Basal Medium for mouse cells and one part of MSC stimulatory Supplements and 9 parts of Mesencult Basal Medium for human cells (Stem cell Technologies, Vancouver, Canada). The colonies were stained with Giemsa (Sigma) and scored under inverted microscope (Leica DMIL, Leica Microsystems, Germany). Cluster of more than 50 cells was counted as one colony and the images were taken using Leica Application Suite software.

Quantitative RT-PCR. Cells were sorted directly into buffer-RLT (Qiagen) and frozen at −80°C. RNA extraction and DNase treatment was performed with the RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions for samples containing less than 10⁵ cells. Eluted RNA samples were reverse transcribed using SuperScript III and random primers (Invitrogen) according to protocol supplied by the manufacturer. Real-time quantitative PCR (Q-PCR) reactions were performed by mixing 2 x TaqMan universal PCR master mix, 20 x TaqMan primer/probe mix, RNase-free H2O and 2.5 µl of cDNA for a final reaction volume of 10 µl. For information about Assays-on-Demand probes, see below list of probes used for Q-PCR.

Cell Cycle Analysis. The analysis was performed as described(25). BM mononuclear cells from wild-type FVB/N mice were initially stained with antibodies against CD45, lineage cells and CD44. After incubation with the cell surface antibodies, the cells underwent fixation with a Cytofix/Cytoperm kit (BD Biosciences) and staining of PE-anti-KI67 and DAPI. Analysis was performed on a FACS ARIA II SORP (BD Biosciences).

In vitro differentiation assays. The assay was performed on culture-expanded cells at passage 2-4. The cells were under stimulation with differentiation media for 3-4 weeks. The media was changed every 2-3 days. For osteoblast differentiation, the cells were cultured in complete alpha MEM medium or DMEM containing 10 % FBS, 10nM HEPES (1M), 100 U/mL of penicillin, 100 µg/mL streptomycin, 50µg/ml ascorbic acid (Sigma), 1-5 x10⁻⁷ M dexamethasone (Sigma) and 10mM glycerol phosphate were used. The cells were fixed with 10 % formalin or ice-cold methanol and the calcium deposit were verified by using 1% Alizarin Red S (Sigma) (pH 4.1) or von Kossa staining (26). For the von Kossa silver nitrate staining method, cultures were fixed in cold methanol for 15–20 min. After rinsing, the fixed plates were incubated with 5% silver nitrate solution under UV light using a UVllinker (UVitec, Cambridge, UK). Mineralized nodules were seen as dark brown to black spots. On the other hand, for alizarin red S (sodium alizarin sulphonate) staining, 1 % alizarin red S (Sigma) was prepared in distilled water and the pH was adjusted to 4.1–4.3 using 0.5 % ammonium hydroxide. Cultures were stained with alizarin red S for 10–15 min after the fixation. After removal of unincorporated excess dye with distilled water, the
mineralized nodules were labeled as red spots. For adipogenesis, the cultures were incubated in DMEM Glumax (Gibco) supplemented with 10% FBS, 10nM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 5-10µg/mL insulin (Sigma), 0.5 mM isobutylmethylxanthine (Sigma), 1-5 x 10^{-6} M dexamethasone (Sigma). The cells were fixed with 10% formalin and stained with 0.3% Oil Red O (Sigma) in methanol (Sigma). The chondrocyte differentiation was induced in monolayer culture, the cells were cultured in 12-well plates in complete DMEM with high glucose 4.5 g/L containing 10^{-7} M dexamethsane, 1% ITS (Sigma), 2mM sodium pyruvate (Sigma), 0.35mM proline (Sigma) and 10ng/ml TGF-β3 (R&D System) or complete chondrocyte differentiation medium (mixed by StemXVivo Human/Mouse Chondrogenic Supplement (Catalog # CCM006) and StemXVivo Human/Mouse Chondrogenic Base Media (Catalog # CCM005) in 1/100 ration (R&D system). The chondrocyte differentiation was verified by staining of proteoglycan with both 0.1% Toluidine blue (Sigma) (pH 2.0 to 2.5) or 1% Alcian blue in 3% acetic acid solution (pH 2.5). Then removed the excess dye and washed three times with distilled water. Then plates were mounted with Clear Mount™ Mounting solution (Invitrogen, CA, USA). The images were taken using bright field using Leica Appication Suite software.

**Microarray analysis.** RNA is extracted from the sorted CD45 'LIN'CD44' and CD44' subsets or culture-expanded MSCs at passage 1-3, labeled and amplified according to Affymetrix™ GeneChip Expression Analysis Technical Manual. Chips are scanned using GeneChipTM Scanner 3000. Human Genome U133 plus 2.0 Chips are normalized using invariant set normalization and probe level expression values are calculated using the PM-MM model provided by the dCHIP software (www.dchip.org) for dCHIP analysis. For Gene set enrichment analysis (GSEA), the data are normalized by RMAExpress software (http://rmaexpress.bmbolstad.com). GSEA of the microarray data were performed according to the instructions (http://www.broadinstitute.org/gsea/index.jsp). Gene sets tested included gene ontology (c5.all.v2.5.symbols.gmt), BioCarta (c2.biocarta.v2.5.symbols.gmt) and KEGG (c2.kegg.v2.5.symbols.gmt). After collapsing, there are 20606 genes left from microarray datasets and there are 1164 remaining gene sets after gene set size-filtering (min=15, max=500). Gene sets with a nominal p-value < 0.05 and false discovery rate (FDR) < 0.25 were considered to be significantly enriched. Those genes occurring in the ranked list before the point at which a maximal GSEA enrichment score is achieved are referred to as the leading edge subset and thus are responsible for the core enrichment observed for a given gene set. Within each gene set, the farther the position of a gene to the left (red) implies a higher correlation with CD44 negative phenotype, and the farther to the right (blue) implies a higher correlation with genes down-regulated upon CD44 expression.

**Biochemical pathways analysis of the microarray data.** The lists of 2-fold changed genes from the microarray data were applied to the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics resources v6.7 (http://david.abcc.ncifcrf.gov/tools.jsp) for for mapping KEGG pathways(27).

**Statistical analysis.** The unpaired $t$ test or Mann-Whitney test was used to compare the differences between the cell types based on the data distribution. All reported $p$-values were obtained using the Graph Pad Prism 4.0 software and less than 0.05 was considered statistically significant. The frequencies of
CFU-Fs were calculated by either Pearson in Excel or L-Calc software (Stem cell Technologies INC).

RESULTS

Freshly isolated mesenchymal stem and progenitor cells of mouse BM lack expression of CD44.

Since accumulated evidence has suggested that CD44 is a common positive marker on expanded MSCs (28), we wanted to investigate if this protein could be used to purify and characterize primary MSCs ex vivo. We first analyzed CD44 expression in BM stromal cells (CD45-LIN-CD31+) of mouse BM by FACS using an antibody recognizing all forms of CD44 (clone IM7) (Figure 1A). This revealed that CD44 was expressed on a significant subfraction (45 ± 7 %) of BM stromal cells. However, upon investigation of co-expression of CD44 with MSC-associated cell surface markers including SCA1, PDGFRα/CD140a, CD51/integrin αv (6,7,29), we found that the cells expressing these MSC markers were exclusively detected in the CD44- cell fraction (Figure 1B). VCAM1/CD106 and CD105 have been reported to be expressed on cultured MSCs (30). However, analysis of the expression of these markers on freshly isolated BM cells suggested that even though the CD44- stromal cells display high expression of VCAM1/CD106 and CD105, the expression of these surface molecules is also detected on the CD44+ stromal cells (Figure 1B). These data suggest that the phenotypically defined MSCs are enriched in the CD44- cells of mouse BM.

To investigate the functional properties of the CD44+ and CD44- stromal cells, we sorted the cells (Figure 1A) and evaluated their clonogenic potential by limiting dilution CFU-F assay (Figure 1C-1D). The frequency of CFU-Fs in the CD44+ cells was 1/167 whereas no CFU-F could be detected in the CD44- cells when plated at any of the indicated cell densities. The CFU-Fs generated from the CD44- cells were fibroblast-like consistent with immature phenotype of mesenchymal progenitor cells (Figure 1E). In some of the experiments, CD45-LIN+ cells were sorted for CFU-F assay as controls. However, no colonies were observed from 200,000 cells plated. These data indicate that the CD44+ cells contain almost all CFU-Fs in mouse BM whereas CD44 expression marks the cells lacking colony-forming capacity.

Nestin and fibromodulin (Fmod) have been reported to mark primary MSCs in mouse (31) and human (32). Consistent with the finding of enrichment of CFU-Fs in the CD44- cell fraction, Q-PCR analysis showed that Nestin and FmoD mRNA were enriched in the CD44+ cells and almost undetectable in the CD44- cells (Figure 1F). In addition, the CD44+ cells expressed higher levels of mRNA encoding matrix protein and growth factors including collagen type I (Col1a1), nephroblastoma overexpressed gene (Nov), Angiopoietin like-1 (Angptl1) and insulin growth factor 1 (Igfl), all reported to be expressed in BM MSCs (7,33,34) (Figure 1F). In all, these data provide molecular support for the idea that the CD44- cell population is enriched with MSCs and progenitors.

Another feature commonly associated with stem cell populations is quiescence under steady state conditions. In order to investigate the cell cycle status of the CD44+/7 cells in mouse BM, we performed cell cycle analysis by simultaneous staining of KI67 and DAPI revealing that while 0.6 % of the CD44+ cells were in G0, around 15% of the CD44- cells resided in a dormant state (G0) (Figure 2A-2B). Correspondingly, the absolute majority of the CD44+ cells were accumulated in cycling (G1 and S/G2/M) stages (Figure 2), indicating that a proportion of the CD44- cells remained quiescent whereas most of the CD44+ cells were in active cell cycle. This was supported by Q-PCR analysis showing upregulation of
the cell cycle inhibitor genes $p21^{Cip}$ and $p27$ in the CD44$^+$ cells, compared to the CD44$^+$ cells (Figure 2C). These data suggest that the physiologically quiescent cells reside in the CD44$^-$, not the CD44$^+$ cells.

Taken together, the phenotypically, functionally and molecularly defined BM mesenchymal stem and progenitor cells naturally do not express CD44.

**Human BM MSCs are enriched in CD44 mesenchymal cells.**

To investigate whether our findings from mouse studies hold true in human, we analyzed CD44 expression in normal human BM stromal cells of healthy donors and characterized the CD44$^{+/−}$ cell subsets by multi-color FACS, colony assay and global gene expression analysis. Within CD45$^+$CD235$^+$CD31$^-$ cells, the majority of the cells express CD44 on the cell surface while less than 10% of them are CD44$^-$ (Figure 3A-3B). Since CD146 and CD271 have been reported to be expressed on freshly isolated human BM MSCs (10,11,14,35), we analyzed their expression in relation to that of CD44 in CD44$^+$CD235$^+$CD31$^-$ cells. Interestingly, while the majority of the CD44$^+$ cells are positive for both CD146 and CD271 only around 4% of the CD44$^-$ cells express these markers (Figure 3A-3B). Unique co-expression of CD146 and CD271 in the CD44$^-$ cells was also reflected in similar frequencies of the CD44$^-$ cells to that of the CD146$^+$CD271$^+$ cells in BM (Figure 3B-3D). These data suggest that the absolute majority of the phenotypically defined MSCs reside in the CD44$^-$ cells in human BM.

To test their clonogenic capacity, we sorted the CD44$^{+/−}$ subsets directly from human BM by FACS (Figure 3A) and performed limiting dilution assay of CFU-Fs. We found that the CFU-F frequency in the CD44$^+$ cells reached 1/14, 94-fold higher than that (1/1314) in the CD44$^-$ cells (Figure 3E). Although the proportion of CD44$^−$ cells is much smaller than that of the CD44$^+$ cells in the BM, the higher recovery of CFU-Fs from the CD44$^-$ cells suggest that they still contain the majority of the CFU-Fs (Figure 3F). However, it is important to note that the most of the colonies formed from the CD44$^+$ cells were observed when the cells were plated at higher, nonclonal density (2000 cells/cm²) (Figure 3E). This could lead to an overestimation of the CFU-F frequency and the possibility of that the CFU-F frequency may not reflect the frequency of the clonogenic cells in this cell population according to previous observation (2). Moreover, while the freshly sorted CD44$^+$ cells were highly proliferative and generated fibroblast-like cells when plated in culture (Figure 3G), the colonies generated from the freshly sorted CD44$^+$ cells displayed a dramatically reduced expansion capacity and could not be replated after the secondary culture. This together with growth characteristics of the cells suggests that most of the clonogenic cells in human BM are naturally CD44$^-$.

Multipotency is a key stem cell feature for MSCs. We next performed *in vitro* differentiation assay on expanded cells generated from freshly sorted CD44$^+$ cells to test their multilineage differentiation potentials. This revealed that the CD44$^-$ cells could generate adipocytes, osteoblasts and chondrocytes *in vitro* (Figure 3H). The differentiation assay could not be performed with the freshly sorted CD44$^+$ cells since they could not be sufficiently expanded in culture. These data suggest that the CD44$^-$ cells contain a major part of the MSCs in human BM.

Furthermore, FACS analysis of the reported MSC-associated markers CD73, CD29, VCAM1 and STRO1 on BM cells indicated that majority of the CD44$^-$ stromal cells from human BM expressed these surface antigens (Figure S1). Surprisingly, the CD44$^-$
cells displayed low expression CD105, presenting a discrepancy from what has been reported for cultured MSCs(15).

**Human CD44 mesenchymal cells display MSC-associated molecular phenotype.**

To further investigate molecular properties of the CD44- and CD44+ cells from human BM, we performed microarray analysis on freshly sorted human BM CD45CD235CD31- CD44+-/- subsets. dCHIP analysis revealed 929 genes that are more than 4-fold differentially expressed in the CD44- as compared to the CD44+ cells. Among genes reported to be related to MSC properties, we noted downregulation of cell cycle progression genes and upregulation of the cell cycle inhibitor genes including CDKN1A(p21), GAS1 and GAS6 in the CD44- cells as compared to CD44+ cells (Figure 4A and S2-S3), suggesting a relatively quiescent status of the CD44- cells. Importantly, the CD44- cells expressed higher levels of a set of cytokines and growth factors including KIT ligand (KITLG), vascular endothelial growth factor (VEGFC), Jagged-1 (JAG1), Angiopoietin-like 4 (ANGPTL4), ANGPT1 and CXCL12 as well as extracellular matrix proteins (ECM) such as laminin α4 (LAMA4), fibronectin (FN1), fibromodulin (FMOD), necdin (NDN) and collagen type I (COL1A1), all reported to be enriched in human BM MSCs (32-34,36) and shown to be important for hematopoiesis (36,37). On the contrary, expression of these genes is undetectable or low in the CD44+ cells (Figure 4A-4C). These data provide comprehensive views into potential functions of the CD44- and CD44+ subsets and important hints for further investigation of the role of human BM stromal cells in homeostasis and diseases.

**Acquisition of CD44 expression on mesenchymal stem and progenitor cells during in vitro culture.**

The finding that the CD44- stromal cells contain essentially all MSCs is completely on the contrary to what has been reported for culture-expanded MSCs. In order to test whether expression of CD44 was due to in vitro manipulation of the cells, we cultured the freshly isolated CD44- MSCs from mouse and human BM and analyzed CD44 expression on the cells after culture at different passages. As previously reported, these cells acquired CD44 expression on their surface after in vitro expansion early at the first passage and remained at a high level during the later passages (>98.7±1.5%) (Figure 5). We have developed multiple cell clones from single CFU-F generated from mouse CD44- cells in the limiting dilution assays and all of the clones were positive for CD44 in culture at early and later passages (passage 5 to 14) (Figure 5B). However, acquisition of CD44 expression on the MSCs upon culture did not affect proliferation capacity of the cells (Figure 5D). These data suggested that CD44 was dramatically up-regulated on mesenchymal stem and progenitor cells during in vitro culture and CD44+ phenotype of expanded MSCs does not reflect the true cellular identity of the primary MSCs and progenitor cells.
In vitro cultivation induce extensive changes in gene expression in human MSCs. The induction of dramatic changes in CD44 expression and proliferation characteristics upon cultivation of the CD44+ BM stromal cells prompted us to search for molecular mechanisms underlying culture-related changes in the MSCs. To this end, we performed microarray experiments to compare gene expression patterns in cultured MSCs at passage 1-3 to that of the freshly sorted CD44- mesenchymal cells (CD45-CD235-CD31-CD44+) from the same donors. dCHIP analysis of the microarray data revealed that 2708 genes were more than 2-fold differentially expressed in the CD44+ cultured MSCs as compared to the freshly sorted CD44- cells. Several of the changes appeared to be consistent between two different donors and over three passages. Among the differentially expressed genes we identified a large number of surface antigens in addition to CD44 (Figure 6). These included cell adhesion receptors such as integrins (ITGA3, ITGAE, ITGB5 and ITGA6) and CD109, ADAM12, CD151, CD59, CD248, some of which have been reported to be expressed on cultured MSCs (30,38). While MSC-associated markers such as CD73 and CD146 are upregulated, NGFR/CD271, VCAM1, CD36 and EPOR are downregulated in the cultured MSCs compared to the freshly sorted CD44- cells. Importantly, in keeping with our FACS data, CD44 is dramatically upregulated in the cultured MSCs. In addition, we observed dramatic upregulation of hyaluronan synthase (HAS1, HAS2), growth factor and matrix protein genes including VEGF, WNT5A,WNT5B, FN1, LAMAB1, BDGF and Collagens in the cultured MSCs (Figure 7). Microarray data-based signal pathway-mapping illustrated upregulated (≥2-fold) genes for the WNT signal, Focal adhesion and MAPK signal pathways in the cultured MSCs expressing CD44 (Figure S4, S5). On the contrary, CXCL12, MPO and EGR1 are significantly downregulated in the cultured MSCs. Hence, we conclude that in vitro cultivation of human MSCs results in dramatic and consistent changes in gene expression patterns.

DISCUSSION

In the present study, we have prospectively isolated the CD44+ and CD44- stromal cells from both human and mouse BM by multi-color FACS and characterized them phenotypically, functionally and molecularly. In striking contrast to the previous finding of high expression of CD44 on culture-expanded MSCs, we have uncovered that native mesenchymal stem and progenitor cells lack CD44 expression. This finding is of large importance for the development of methods for isolation of BM MSCs both for experimental and clinical purposes.

SCA1, PDGFRa and Nestin have recently been shown to be expressed on freshly isolated mouse BM MSCs (6,31,45). We here report that the BM stromal cells positive for those markers are enriched in the CD44- cells in mice. Similarly, the majority of the previously defined CD271+CD146+ MSCs (14) from human BM do not express CD44. Furthermore, we also show that the commonly used positive MSC markers CD73, CD106, CD29 and STR01 are highly expressed on the freshly sorted CD44- cells. These data together with the finding of enrichment of CFU-F activities in the CD44- cells strongly support CD44 negative phenotype of the MSCs. This result can be further corroborated by the cell cycle analysis and molecular data showing enrichment of quiescent cells and the cells with multilineage differentiation potential in the CD44- cells. Another interesting finding in the present study is that the CD44+ stromal cells express higher levels of hematopoiesis-regulating growth factors and matrix proteins including CXCL12, KIT Ligand, Angiopoietin
1, VEGF, JAG1, LAMA4 and FN1, compared to the CD44+ cell fraction. Thus, this cell population might play an important role for maintenance of normal hematopoiesis in vivo. Taken together, our data strongly suggest that CD44+ stromal cells are enriched with multipotent mesenchymal stem and progenitor cells. Although MSCs are only a small fraction of the CD44+ cells, given the fact that CD44 is widely expressed in hematopoietic cells and endothelial cells, using this marker alone or in combination with other negative markers including CD45 and CD235 in human and in combination with a positive marker such as SCA1 in mice would significantly facilitate prospective isolation of MSCs from BM.

Most importantly, our data provide clear evidence for alteration of MSC phenotype during in vitro manipulations of the cells, emphasizing the importance of prospective isolation of MSCs in order to uncover the nature and therapeutic potentials of the cells. The functional consequence for acquisition of CD44 expression on the expanded MSCs during therapeutic use is unclear. However, since the acquisition of CD44 expression already occurred at the first passage and remained on the cells at the later passages during in vitro expansion, it is likely that stem cell growth characteristics such as sustainable expansion capacity of the MSCs remained after acquiring CD44 expression. In addition, the cells expanded in culture from the CD44+ cells could give rise to adipocytes, chondrocytes and osteoblasts, suggesting maintenance of multilineage differentiation potential of the cells after acquisition of CD44 expression on the surface. Hence, expression of CD44 per se does not appear to reduce the MSC potential of the cultured cells in vitro. However, it was reported that the culture-expanded MSCs displayed reduced or loss of homing capacity to BM although highly expressed CD44 (6). On the contrary, primary MSCs showed more efficient homing to BM compared to the cultured MSCs (46). Loss of BM homing capacity of the cultured MSCs might be due to culture-induced changes in expression of adhesion receptors including CD44 and CXCR4 on the cells, which might lead to unwanted entrapment in other organs (6,38). CD44 exists in multiple isoforms on different type of the cells and CD44 expression as well as its binding capacity towards their ligands are regulated by different cytokine stimulation and other environmental changes (47). It has been shown that Early growth response 1(EGR1) regulate CD44 transcription via binding to CD44 promoter in B cells (48). However, our gene expression data suggest that the level of EGR1 transcript is dramatically reduced (by 14 fold) in the cultured MSCs while these cells acquire CD44 expression. Hence, apparently the functional linkage between those two molecules in the MSCs require further investigations.

Comparing gene expression patterns and surface marker expression in the freshly isolated and cultured MSCs revealed that in vitro manipulation of MSCs resulted in dramatic overall changes in gene expression patterns. We here provided new evidence for changes in expression of adhesion receptors and signalling molecules. While CD105, integrin α1/CD49A, α3/CD49C, α4/CD49D, α5/CD49E, α6/CD49F, CD151 and CD109 are upregulated on the cultured MSCs, expression of other cell surface antigens such as VCAM1, ICAM4, CD36, EPOR and PTHGR are reduced in the culture-expanded MSCs, as compared to the freshly sorted CD44+ stromal cells. These changes could possibly result in changes in differentiation potential and other cellular processes of the cultured MSCs since those factors have been reported to be important for regulating lineage differentiation of human MSCs (reviewed in (39))(40-43). In addition, the changes in expression cell surface receptors including CD44 could result in the
upregulation of multiple signal molecules related to cell adhesion, growth factor pathways in the cultured MSCs, however, the latter could in turn lead to further changes of the cell surface receptor expression through inside-out signal transduction.

CD44 function is controlled by its posttranslational modifications such as sialofucosylations. It has been shown that the CD44 glycoform bearing alpha-2,3-sialyl modifications on the cultured MSCs is not reactive with BM vascular E-selectins, which resulted in poor osteotropism after systemic transplantation of the cells. However, this can be rescued by converting the glycoform to selectin-binding glycoform of CD44 (49). On the other hand, it was reported that CD44 on the cultured MSCs contributed to migration of the cells into injured kidney via interaction with hyaluronic acid at sites of injury (17,50). It is important to note that hyaluronic acid is also upregulated in many solid cancers (24). Positive contribution of cultured MSCs to breast cancer development has been reported (51). Therefore, although MSCs hold great promise for cancer therapy(52), safety issue should be seriously concerned before clinical use of culture-expanded MSCs for gene or drug delivery.

In summary, we have here provided phenotypic, functional and molecular evidence that BM mesenchymal stem and progenitor cells physiologically do not express CD44 in both humans and mice. However, in vitro culture could result in acquisition of CD44 expression on their surface and changes in expression of cytokine, growth factor, matrix protein and other signalling molecules. These findings highlight importance of ex vivo analysis of the cells and provide clear evidence for true cellular identity of MSCs and progenitor cells.

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FOOTNOTES:
Author Contributions: HQ designed and performed research, collected, analyzed and interpreted data, wrote the manuscript and did final approval of the manuscript. KLB performed research, analyzed data and did final approval of the manuscript. MS designed research, analyzed and interpreted data, wrote the manuscript and did final approval of the manuscript.

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Abbreviations: MSCs, Mesenchymal stem cells; BM, Bone marrow; FACS, fluorescent activated cell sorting; CFU-F, colony-forming unit-fibroblast.

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Figure Legends

Figure 1. Clonogenic mesenchymal stem and progenitor cells lack expression of CD44 in mouse BM. (A) One representative FACS profile shows analysis of CD44 expression in CD45-LIN’CD31’ cells. The numbers in the panels are mean % of the CD44+/− cells, from 10 experiments. (B) FACS analysis of expressions of SCA1, CD51, CD90.1, CD105, VCAM1/CD106 and PDGFRα/CD140a in CD45’LIN’CD44+/− cells. The numbers in the panels indicated mean percentages of the gated cells within CD45’LIN’CD31’ cells. The data were from 3-10 experiments. (C) Limiting dilution of CFU-Fs in the CD44+/− cells. The cells were plated at densities of 10, 50, 100, 200 cells for the CD44− cells and 200, 500, 1000, 2000 cells per well for the CD44+ cells in 96-well plates. The cell dose yielding 37.5% negative wells for CD44− cells was 167, indicated by dashed line. The 95% confidence interval (CI) bands are shown in dotted lines. There was no CFU-Fs observed from the CD44+ cells at any of the doses. (D) The frequencies of CFU-Fs in the CD44+ and CD44− cells calculated by L-Calc (Stem Cell Technologies). Data were mean ± 95% CI, from 3 experiments. nd, Not detectable. (E) Morphology of Giemsa stained CFU-Fs derived from the CD44− cells. (F) Q-PCR analysis of expressions of MSC-associated genes. Data were 3 independent sorting experiments. Each dot represents mean of triplicate measurements in each experiment. MSC associated genes include Fmod, Igf1, Nov, Nes, Coll1 and Angptl1. The differences between the two cell types were compared by unpaired one-tailed t test.

Figure 2. Quiescent mesenchymal cells reside in the CD44− stromal cell fraction. (A) One representative FACS profile of cell cycle analysis of the CD45’LIN’CD44+ and CD44− cells by KI67 and DNA staining. Cell-cycle status within the defined the CD45’LIN’CD44+ and CD44− cells was determined by simultaneous two-parameter analysis with DNA content versus KI67 expression. Numbers in quadrants show the percentages of the gated CD44+ and CD44− cells in each of the cell-cycle phases (G0, G1, and S/G2/M). (B) Mean cell-cycle distribution of total CD44+ and CD44− cells. Data were from 2 experiments. (C) Q-PCR analysis of cell cycle regulator genes p21, p27 and Cdk6 in the CD44+ and CD44− cells. The data were normalized to endogenous Hprt expression, from 2 independent sorting experiments. Every dot represents mean of triplicate measurements for each gene. The differences between the two cell populations are indicated in the panels.

Figure 3. Human BM mesenchymal stem and progenitor cells primarily reside in CD44− stromal cell fraction. The data were from 5 analysis experiments with 4 healthy donors. (A) FACS profiles show CD44, CD271, CD146 expression in human BM CD45−CD235−CD31’ cells and sorting of the CD44+/− cells. The stromal cells were first enriched by MACS prior to staining of the antibodies. (B) Percentages of the CD44+ and CD44− cells within stromal cells in BM. (C) Expressions of CD146 and CD271 in the CD44− and CD44+ cells. (D) The frequencies of CD146−CD271’ cells, CD44− and CD44+ cells in BM. (E) Limiting dilution of CFU-Fs in the CD44+/− cells. The cell dose yielding 37.5% negative wells were indicated by dashed lines on X-axis. (F) CFU-F recovery from the CD44+/− cells of BM. (G) Morphology of Giemsa stained CFU-Fs. (H) In vitro differentiation of the CD44− cells. The adipocytes were identified by oil red stainings. The osteoblasts were confirmed by both Alizarin red and von konnssa stainings. The chondrocytes were identified by both Toluidine blue and Alcian blue.
Figure 4. Microarray data provide molecular evidence for immature phenotype of the CD44+ cells from human BM. (A) dCHIP analysis of gene expressions of cytokines, growth factors and ECM proteins in the CD44+ and CD44- cells from normal human BM. Clustering shows the genes being up-regulated more than 4-fold in the CD44+ cells compared to the CD44- cells. Red represents high and blue low expression. The numbers in the heatmap are expression values of each indicated genes. (B-C) GSEA analysis of significantly up-regulated gene sets in the CD44- cells. The green curves plot the ES (enrichment score). Black vertical dashed lines specify the maximum ES score. Significantly enriched data sets are defined according to GSEA default settings (p < 0.001 and FDR < 0.25). The heatmap shows expressions of the genes in the leading edge subsets (only top 40 genes were shown for clarity if more than 40 genes in the leading edge subsets). (B) Enrichment of genes in the gene sets of extracellular matrix region in the CD44- cells. (C) Enrichment of the gene set of extracellular matrix structural constituents in the CD44- cells. The data were from 8 microarray platforms and 2 sorting experiments, normalized by RMAExpress software. See also in Figure S1-S2 and Table S1-S3.

Figure 5. Acquisition of CD44 expression in CD45-LIN-CD44- MSCs and progenitor cells after culture. The freshly sorted CD44- cells from human and mouse BM were cultured and the expanded cells were analysed by FACS for CD44 expression. (A) CD44 expression in the expanded mouse stromal cells after 8-10 days culture. (B) CD44 expressions in the cultured cells at later passages (passage 5-15) derived a single CD44- mouse cell clone. The red lines indicated expression of CD44 and the blue line indicated the isotype control stainings. (C) CD44 expression in the cultured human BM stromal cells. The freshly sorted CD45-CD235-CD31-CD44- cells from BM of healthy humans were cultured for 14 days. A15, 16 and A17 indicated different donors. The red lines indicated expression of CD44 and the blue line indicated the isotype control stainings. (D) Fold expansion of the cells generated from the freshly sorted CD44- cells during culture. Limited number (10-50 cells) of the sorted CD44- cells were plated in culture and the cells generated from the culture were counted and calculated for fold expansion. The data were mean ± SEM, from 3 independent experiments on BM from 3 healthy volunteers. X-axis indicates the number of the passages (p).

Figure 6. Culture-induced alteration of cell adhesion molecule expression in MSCs from human BM. The microarray data on the freshly sorted CD44- mesenchymal cells and on the culture-expanded MSCs (acquired CD44 expression) derived from the fresh CD44- cells of the same donors were analysed by dCHIP software. The microarray experiments on the culture-expanded MSCs were performed at passage (p) 1-3. The ‘a’ and ‘b’ indicated replicate samples at each passage (p1-p3) from the indicated donors (a15 or a17). Clustering shows the genes being up-regulated more than 3-fold in the cultured cells compared to the freshly sorted CD44- cells. Red represents high and blue low expression. The numbers in the heatmap are expression values of each indicated genes and the fold changes in expression of the receptors are calculated based on the mean expression values from each cell type. The data were from 10 microarray platforms (6 arrays of the cultured cells and 4 arrays of the fresh cells) and 2 sorting experiments on 2 healthy volunteers, normalized by RMAExpress software.
Figure 7. Differential expression of growth factor, matrix protein and signaling molecules in freshly sorted MSCs and the cultured MSCs of human BM. Gene expressions of cytokines, growth factors and ECM proteins in the freshly sorted CD44+ mesenchymal cells and the culture-expanded MSCs (acquired CD44 expression) derived from the freshly sorted CD44- cells of the same donors were analysed by dCHIP software. The microarray experiments on the culture-expanded MSCs were performed at passage (p) 1-3. The ‘a’ and ‘b’ indicated replicate samples at each passage (p1-p3) from the indicated donors (a15 or a17). Clustering shows selected genes being up-regulated more than 8-fold in the cultured cells compared to the freshly sorted CD44- cells. For clarity, only part of the growth factors and signalling molecules are shown in the panel. Red represents high and blue low expression. The numbers in the heatmap are expression values of each indicated genes and the fold changes are calculated based on the mean expression values from each cell type. The data were from 10 microarray experiments (6 arrays of the cultured cells and 4 arrays of the fresh cells) and 2 sorting experiments on 2 healthy volunteers, normalized by RMAExpress software. See also in Figure S4 and Figure S5.
Fig. 1 (Qian, et al.)

A

CD45/LIN

CD31

PI

PI-

CD31-

100

101

102

103

104

APC-A: CD106

APC-A

CD31-

100

101

102

103

104

APC-Cy7-A: CD44

APC-Cy7-A

1.39

33.4

37.3

29.8

B

CD106

CD105

PI:CD45-LIN-

PI-

SCA1

FSA

CD44

CD44-

CD45-LIN-

CD31-

100

101

102

103

104

APC-A: CD106

APC-A

CD31-

100

101

102

103

104

APC-Cy7-A: CD44

APC-Cy7-A

1.39

33.4

37.3

29.8

C

% negative response

Cell doses

D

Frequency of CFU-Fs

nd

E

10 μm

F

Col1a1

P = 0.01

Igf1

P = 0.035

Nes

P = 0.005

Pparg

P = 0.0027

Fmod

P = 0.047

Angpt1

P = 0.01

Relative expression to Hprt
Fig. 2 (Qian, et al.)

A

G1  S/G2/M

G0

DAPI

B

Cell cycle distribution (% of CD44+ cells)

CD44+

CD44-

P < 0.0001

P = 0.008

ns, P = 0.065

C

Relative expression to Hprt

p21

P = 0.016

p27

P = 0.008

Cdk6

ns

CD44+  CD44-

CD44+  CD44-

CD44+  CD44-

CD44+  CD44-
Fig. 5 (Qian, et al.)

A  **Mouse**

![Diagram of Mouse Culture](image)

Freshly sorted CD44^+ cells

B  

| #1 | #2 | #3 | #4 |
|----|----|----|----|

CD44%

C  **Human**

From CD45^−/CD235^−/CD31^−/CD44^− cells

| A15 | A16 | A17 |
|-----|-----|-----|

CD44%

D  

Fold expansion of the CD44 cells

| P1 | P2 | P3 | P4 | P5 |
|----|----|----|----|----|

Passages
| Gene name      | Fold change |
|---------------|-------------|
| ITGA6         | 28          |
| CD9           | 41          |
| ADAM12        | 30          |
| MET           | 85          |
| PTPN13        | 6           |
| CD109         | 61          |
| NTSE/CD73     | 90          |
| ITGA3         | 6           |
| CEECAM1       | 7           |
| CD44          | 14          |
| CD99          | 11          |
| CD248         | 12          |
| ANPEP/CD13    | 23          |
| CD151         | 9           |
| ITGAE         | 4           |
| MCAM/CD146    | 4           |
| ITGAE         | 18          |
| BMPR2         | 6           |
| FZD2          | 13          |
| ITGB5         | 6           |
| GHR           | -10         |
| CD53          | -5          |
| CEACAM8       | -214        |
| CD300A        | -3          |
| NGFR/CD271    | -4          |
| PTHR1         | -3          |
| FRZB          | -19         |
| VCAM1         | -12         |
| CD82          | -3          |
| EPOR          | -5          |
| ICAM4         | -14         |
| CD36          | -53         |
| SPN           | -11         |
| TFRC          | -3          |
**Figure 7. (Qian et al.)**

| Gene name      | Fold change |
|----------------|-------------|
| CD44fresh      | 500         |
| CD44a15_P1a    | 282         |
| CD44a15_P1b    | 417         |
| CD44a15_P2a    | 497         |
| CD44a15_P2b    | 10          |
| CD44a17_P3a    | 10          |
| CD44a17_P3b    | 10          |
| CD44a17_P3c    | 9           |
| CD44a17_P3d    | 9           |
| CD44a17_P3e    | 10          |
| CD44a17_P3f    | 10          |
| CD44a17_P3g    | 10          |
| CD44a17_P3h    | 9           |
| CD44a17_P3i    | 9           |
| CD44a17_P3j    | 10          |
| CD44a17_P3k    | 10          |
| CD44a17_P3l    | 10          |
| CD44a17_P3m    | 9           |
| CD44a17_P3n    | 9           |
| CD44a17_P3o    | 10          |
| CD44a17_P3p    | 10          |
| CD44a17_P3q    | 10          |
| CD44a17_P3r    | 9           |
| CD44a17_P3s    | 9           |
| CD44a17_P3t    | 10          |
| CD44a17_P3u    | 10          |
| CD44a17_P3v    | 10          |
| CD44a17_P3w    | 9           |
| CD44a17_P3x    | 9           |
| CD44a17_P3y    | 10          |
| CD44a17_P3z    | 10          |
| CD44a17_P3a    | 9           |
| CD44a17_P3b    | 9           |
| CD44a17_P3c    | 10          |
| CD44a17_P3d    | 10          |
| CD44a17_P3e    | 10          |
| CD44a17_P3f    | 9           |
| CD44a17_P3g    | 9           |
| CD44a17_P3h    | 10          |
| CD44a17_P3i    | 10          |
| CD44a17_P3j    | 10          |
| CD44a17_P3k    | 9           |
| CD44a17_P3l    | 9           |
| CD44a17_P3m    | 10          |
| CD44a17_P3n    | 10          |
| CD44a17_P3o    | 9           |
| CD44a17_P3p    | 9           |
| CD44a17_P3q    | 10          |
| CD44a17_P3r    | 10          |
| CD44a17_P3s    | 10          |
| CD44a17_P3t    | 9           |
| CD44a17_P3u    | 9           |
| CD44a17_P3v    | 10          |
| CD44a17_P3w    | 10          |
| CD44a17_P3x    | 9           |
| CD44a17_P3y    | 9           |
| CD44a17_P3z    | 10          |
| CD44a17_P3a    | 10          |
| CD44a17_P3b    | 10          |
| CD44a17_P3c    | 9           |
| CD44a17_P3d    | 9           |
| CD44a17_P3e    | 10          |
| CD44a17_P3f    | 10          |
| CD44a17_P3g    | 9           |
| CD44a17_P3h    | 9           |
| CD44a17_P3i    | 10          |
| CD44a17_P3j    | 10          |
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| CD44a17_P3r    | 10          |
| CD44a17_P3s    | 10          |
| CD44a17_P3t    | 9           |
| CD44a17_P3u    | 9           |
| CD44a17_P3v    | 10          |
| CD44a17_P3w    | 10          |
| CD44a17_P3x    | 9           |
| CD44a17_P3y    | 9           |
| CD44a17_P3z    | 10          |
| CD44a17_P3a    | 10          |
| CD44a17_P3b    | 10          |
| CD44a17_P3c    | 9           |
| CD44a17_P3d    | 9           |
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| CD44a17_P3n    | 10          |
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| CD44a17_P3w    | 10          |
| CD44a17_P3x    | 9           |
| CD44a17_P3y    | 9           |
| CD44a17_P3z    | 10          |
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| CD44a17_P3l    | 9           |
| CD44a17_P3m    | 10          |
| CD44a17_P3n    | 10          |
| CD44a17_P3o    | 9           |
| CD44a17_P3p    | 9           |
| CD44a17_P3q    | 10          |
| CD44a17_P3r    | 10          |
| CD44a17_P3s    | 10          |
Primary Mesenchymal Stem and Progenitor Cells from Bone Marrow Lack Expression of CD44
Hong Qian, Katarina Le Blanc and Mikael Sigvardsson

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