CD146 expression on mesenchymal stem cells is associated with their vascular smooth muscle commitment

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Abstract

Bone marrow mesenchymal stem cells (MSCs) are plastic adherent cells that can differentiate into various tissue lineages, including osteoblasts, adipocytes and chondrocytes. However, this progenitor property is not shared by all cells within the MSC population. In addition, MSCs vary in their proliferation capacity and expression of markers. Because of heterogeneity of CD146 expression in the MSC population, we compared CD146Low and CD146High cells under clonal conditions and after sorting of the non-clonal cell population to determine whether this expression is associated with specific functions. CD146Low and CD146High bone marrow MSCs did not differ in colony-forming unit-fibroblast number, osteogenic, adipogenic and chondrogenic differentiation or in vitro haematopoietic-supportive activity. However, CD146Low clones proliferated slightly but significantly faster than did CD146High clones. In addition, a strong expression of CD146 molecule was associated with a commitment to a vascular smooth muscle cell (VSMC) lineage characterized by a strong up-regulation of calponin-1 and SM22 expression and an ability to contract collagen matrix. Thus, within a bone marrow MSC population, certain subpopulations characterized by high expression of CD146, are committed towards a VSMC lineage.

Keywords: CD146 • vascular smooth muscle cell • mesenchymal stem cells • proliferation • differentiation

Introduction

Mesenchymal stem/stromal cells (MSC) are multipotent progenitors that give rise to skeletal cells (osteoblasts, chondrocytes, haematopoietic-supportive stromal cells) and adipocytes [1–4]. They are relatively easy to expand, and their progenitor nature is evaluated by colony-forming unit-fibroblast (CFU-F) assay [5]. Although MSCs are often characterized after a period of culture, recently, many studies have aimed to define their native form in bone marrow aspirates. These works emphasize different membrane molecules as native MSC markers. Among them, CD200, a marker for MSC appeared to be a good marker to reproducibly purify native MSCs. The CD146 expression by BM-MSCs depended on the environment: up-regulated under normoxia and down-regulated under hypoxia. This O2-related expression was associated with the in situ localization: CD146 expressing reticular cells were located in perivascular regions, whereas cells close to the bone surface were CD146−[10]. In addition, adipose-tissue perivascular cells with MSC properties, such as pericytes of microvessels and capillaries, were identified to have high CD146 expression, whereas other cells from tunica adventitia from large vessels lacked CD146[11, 12]. Therefore, the MSC expression of CD146 is heterogeneous and may depend on the tissue and the molecular environment. This observation suggests functional differences.
Therefore, in this study, we investigated BM-MSC functions in terms of CD146 expression. We compared sorted and clonogenic CD146−/Low and CD146High cells after in vitro expansion and examined the different properties of MSC such as osteogenic, chondrogenic, adipogenic and vascular smooth muscle cell (VSMC) differentiation; haematopoiesis support; proliferation; CFU-F formation; transcriptome and phenotype to distinguish between these two BM-MSC subpopulations.

Material and methods

Preparation of single cell-derived clonal-cultured MSCs and sorted MSCs

Human BM-MSCs were isolated by culture from the BM of healthy donors obtained during the preparation of allogeneic haematopoietic stem cell grafts. This tissue is considered waste material in France and does not require informed consent for use, in accordance with French ethical and legal regulations. Briefly, BM-MSCs were obtained from unprocessed BM without red blood lysis nor density-gradient method and seeded at 5 × 10^4 cells/cm² into a 150-cm² flask with minimum essential medium α (αMEM, Life Technologies, Saint Aubin, France) supplemented with 10% foetal calf serum (FCS, Lonza, Levallois-Perret, France) and 10 μg/ml ciprofloxacin (Bayer, Puteaux, France). For all MSC cultures, the medium was renewed twice a week until cells reached confluence (P1). Cells were then detached with trypsin (Invitrogen), counted (CFU-F) of MSCs were selected and cells were expanded. After expansion, MSC clones were detached by use of trypsin (Invitrogen), counted and submitted to phenotypic characterization. Cytometry was used to select clones on the basis of CD146 expression: CD146 mean fluorescence, MSC clones were detached by trypsin (Gibco, Life Technologies) [13, 14].

For clonal studies, total BM cells were seeded in 24-well plates at 1–2 × 10^4 cells/well in αMEM supplemented with 10% FCS and ciprofloxacin. Wells were screened every day starting at day 7 to identify wells with one clone. After 10–14 days, the wells containing only one colony (CFU-F) of MSCs were selected and cells were expanded. After expansion, MSC clones were detached by use of trypsin (Invitrogen), counted and submitted to phenotypic characterization. Cytometry was used to select clones on the basis of CD146 expression: CD146 mean fluorescence intensity was used to determine CD146Low and CD146High clones. The doubling population number was calculated by considering the number of CFU-Fs as the number of initiating cells at day 0.

For non-clonal studies, BM cells were seeded at 1000 cells/cm² and differentiated for 21 days in inductive medium (αMEM, 10% FCS, dexamethasone 100 nM, ascorbic acid 2-phosphate 50 μM, sodium phosphate monobasic 3 mM/l glycerophosphate 10 mM). Cell mineralization was then evaluated by alizarin red staining (40 mM alizarin red solution) [19]. For both differentiation assays, a control test involved cells grown in αMEM with 10% FCS for 21 days. For RT-PCR analysis of in vitro differentiation assays, MSCs were seeded in 6-well plates (2 × 10^4 cell/cm²) and allowed to

Flow cytometry

Clonal BM-MSCs were phenotyped by flow cytometry with the FITC- or phycoerythrin-conjugated antibodies anti-Cd90, anti-Cd45, anti-Cd13 (Beckman-Coulter, Villepinte, France), anti-Cd73, anti-Cd105 and anti-Cd146 (clone P1H12) (Becton-Dickinson, Le Pont de Claix, France) and Allophycocyanin-conjugated anti-Cd146 (clone 541-10B2; Miltenyi, Bergisch Gladbach, Germany) or isotype control monoclonal antibodies. Samples were analysed by use of an ADPCyan flow cytometer and Kaluza software (Beckman-Coulter).

CFU-F assay

To evaluate the frequency of CFU-Fs generated, clonal BM-MSCs were seeded at 8 cells/cm² for 10 days. Cultures were then washed with PBS, fixed with methanol and stained with Giemsa (Oxoid, Dardilly, France); colonies with more than 50 cells were counted.

Incorporation of 5-ethyl-2′-deoxyuridine (EdU)

During last 3 days of culture, non-clonal and clonal BM-MSCs were incubated with 5 μM Click-IT EdU (Molecular Probes, Life Technologies). After treatment, clonal BM-MSCs were stained with APC-conjugated anti-CD146 monoclonal antibody, fixed, permeabilized and treated with a Click-IT reaction cocktail for detection following the manufacturer’s recommendations. Samples were analysed by use of an ADPCyan flow cytometer and Kaluza software (Beckman-Coulter).

VSM differentiation

Non-clonal BM-MSCs from different donors were cultured in a 25 cm² flask with αMEM + 10% FCS + 10 μg/ml ciprofloxacin with or without 2 ng/ml fibroblast growth factor 2 (FGF-2) [15] or transforming growth factor β1 (TGF-β1; R&D Systems, Minneapolis, MN, USA) for 10 days [16, 17].

Immunofluorescence

Clonal MSCs or VSM-differentiated MSCs were fixed, permeabilized and incubated with purified primary antibodies mouse anti-human calponin-1 (anti-CNN1; Abcam), rabbit anti-human smooth muscle 22α (anti-SM22α; Abcam) or isotype control (Abcam) overnight at 4°C. After a wash with PBS, cells were incubated with secondary goat antimouse or anti-rabbit-alexa 488 (Invitrogen). Samples were analysed under a fluorescence microscope Olympus (Tokyo, Japan) IX71, ×20 objective.

Osteogenic, adipogenic and chondrogenic differentiation

For osteogenic differentiation, cells were seeded at 5 × 10^3 cells/cm² and differentiated for 21 days in inductive medium (αMEM + 10% FCS, dexamethasone 100 nM, ascorbic acid 2-phosphate 50 μM, sodium phosphate monobasic 3 mM/l glycerophosphate 10 mM). Cell mineralization was then evaluated by alizarin red staining (40 mM alizarin red solution) [18]. For adipogenic differentiation, cells were seeded at 2 × 10^3 cells/cm² and differentiated for 21 days in medium (αMEM, 10% FCS, dexamethasone 1 μM, 3-isobutyl-1-methylxanthine 0.45 mM, indomethacin 60 μM), then stained with 5 μg/ml Nile Red solution (lipid vacuole staining) and 2 μg/ml bisBenzimide solution (nuclear staining) [19]. For both differentiations, a control test involved cells grown in αMEM with 10% FCS for 21 days. For RT-PCR analysis of in vitro differentiation assays, MSCs were seeded in 6-well plates (2 × 10^3 cell/cm²) and allowed to
reach confluence before treatment. Medium was changed twice a week. For osteoinduction, MSCs were stimulated with 50 ng/ml recombinant human bone morphogenetic protein 4 (BMP4; Stemgent, Cambridge, UK) in αMEM+2% FCS for 21 days. For adipogenesis and chondrogenesis, MSCs were cultivated in adipogenic differentiation medium (Miltenyi Biotec, Bergisch Gladbach, France) for 10 days and chondrogenic differentiation medium (Miltenyi Biotec) + 10 ng/ml recombinant human TGF-β3 (R&D Systems) for 21 days.

Co-culture of clonal BM-MSCs and CD34+ cells

CD146−/Low and CD146High clonal BM-MSCs were seeded in 12-well plates in duplicate at 2 × 10^5 cells/well in 1 ml culture medium. After 24 hrs, BM-MSCs were co-cultured with 4 × 10^5 CD34+ cells in 2 ml Myelocult medium containing 10 μM hydrocortisone (Stem Cell Technologies, Grenoble, France). At days 7, 14, 21 and 28, non-adherent cells were collected, counted and assayed for haematopoietic progenitor content by use of the methyl-cellulose semisolid culture type (Miltenyi Biotec). The total number of clonogenic progenitors was calculated (number of colonies/number of seeded cells in methyl-cellulose) × total number of cells in the co-culture). Colony-forming unit-granulocytes (Gs), CFU-macrophages (Ms), CFU-granulocyte macrophages (GMs) and burst-forming unit-erythroids (BFU-Es) were counted.

Affymetrix microarrays

After clonal BM-MSC culture and CD146 expression analysis, the RNA in non-clonal and clonal CD146−/Low and CD146High MSCs from four donors was extracted by use of the RNeasy Kit (Qiagen, Hilden, Germany). RNA purity and integrity were checked by use of Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The RNA integrity number was >9 for all samples used for microarray assays. Biotinylated cRNA was amplified by the small sample labelling protocol (TwoCycle amplification kit, Affymetrix, Santa Clara, CA, USA) and hybridized on GeneChip Hu gene 1.0 ST oligonucleotide microarrays (Affymetrix). Expression signal values and P-values were obtained for each probe set by use of Partek Genomics Suite (Partek, St. Louis, MO, USA) by the Robust Multichip Averaging algorithm in normalization. Principal component and gene expression analysis involved use of MeV. The accession number in the Geo data set in Medline is available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48540.

RNA extraction/cDNA synthesis and quantitative PCR (qPCR)

RNA was extracted by use of the AllPrep ARN/ADN/protein kit (Qiagen, Valencia, CA, USA). cDNA synthesis was performed with 1 μg total RNA with the High Capacity cDNA reverse-transcription kit (Applied Biosystems, Life Technologies) and random hexamers. qPCR was performed on diluted cDNA (equivalent to 25 ng of starting purified RNA) with SsoFast EvaGreen Supermix (BioRad) and 500 nM forward and reverse primers in a total volume of 20 μl on a CFX Real Time System (Bio-Rad, Marnes-la-Coquette, France) at 95°C, 3 min. and 40 cycles of denaturation (95°C, 10 sec.) and primer hybridization and amplification (60°C, 30 sec.). Primer sequences are in Table 1. Each primer couple displayed interpretable PCR efficiencies (95–105%). Melting curves and an appropriate No-RT control were used to validate amplification specificity. Data were analysed by use of the Bio-Rad CFX manager (threshold=0.2) and exported to DataAssist Software v3.0 (Applied Biosystems). Gene expression was calculated by the 2−ΔΔCt (or 2−ΔACt) method with PPIA used as an appropriate reference gene because it had the lesser M score.

Cell contraction assay

CD146High/Low clones were incubated in collagen matrices in 24-well plates for 24 hrs during which stress developed. To initiate contraction, we released collagen gels from the sides of the culture dishes. After 5 hrs, the surface area of the matrix contracted was expressed as a percentage of its initial surface area.

Statistical analysis

Data are expressed as mean ± SEM of (n) separate experiments. Statistical comparisons involved Student’s T-test and GraphPad Prism software (La Jolla, CA, USA). Differences were considered statistically significant at P < 0.05.

Results

CFU-F and proliferation potential of MSCs and CD146 expression

From BM, we expanded non-clonal MSCs or clonal MSCs and selected them by CD146 expression (CD146−/Low and CD146High, Fig. 1A). For non-clonal cells, CD146 expression was heterogeneous (Fig. 1Aa), but clonogenic cells showed homogenous negative-to-low or high expression of CD146 (CD146−/Low and CD146High) (Fig. 1Ab and c). Clonal CD146−/Low and CD146High fractions produced a similar number of CFU-Fs (20 ± 5 versus 27 ± 19, respectively; Fig. 1B). In addition, sorted non-clonal CD146−/Low and CD146High cells did not differ in generating CFU-Fs (Fig. S1A and B).

Proliferation potential was slightly but significantly higher for clonal CD146−/Low than CD146High cells (population doubling number: 20.9 ± 0.7 versus 20.3 ± 0.6, P = 0.016) at week 4 (Fig. 1C) and was similar with sorted non-clonal CD146−/− cells (Fig. S1C). As confirmed by flow cytometry, the proportion of cycling cells (percentage of EdU-incorporated cells) was significantly higher in the clonal CD146−/Low than CD146High fraction (43% versus 24%, Fig. 1D). Of note, CD146 expression was enhanced in clonal CD146−/Low cells after proliferation period but did not reach the level detected in the clonal CD146High population.

Functional assessment of CD146−/Low and CD146High MSCs

To test whether CD146−/Low and CD146High clones exhibited differences in multilineage differentiation capacity, we tested their ability to...
differentiate into osteoblasts, adipocytes and chondrocytes in vitro. CD146^{−/Low} and CD146^{High} cells did not differ in differentiating into osteoblasts and adipocytes with alizarin red and Nile red staining, respectively (Fig. 2Aa–f). To confirm these data, we quantified osteoblasts by expression of Runx-related transcription factor 2 (Runx2), osterix (Osx), alkaline phosphatase (ALPL) and distal-less homeobox 5 (DLX5); adipocytes by expression of peroxisome proliferator-activated nuclear receptor γ 2 (PPARγ2) and fatty acid binding protein 4 (FABP4); and chondroblasts by expression of collagen 10 A1 (COLL10A1) differentiation genes by transcriptomic analysis (Fig. 2Ba–c). This difference could be explained by the fact that clones underwent more culture during the selection of populations (Fig. S2Aa–e). As a result, the expression of CNN1 was significantly up-regulated in CD146^{High} clonal MSCs (Fig. 3B b). To confirm these data, we performed transcriptome analysis by qRT-PCR of mRNA from CD146^{High} and CD146^{Low} populations derived from clones (Fig. 3A). Interestingly, the expression of CD49a (α1-integrin subunit) was up-regulated in CD146^{High} clones. After their expansion, cells were subjected to gene array analysis. We obtained a list of genes up-regulated in CD146^{High} clones and identified a high expression of CD146, thus confirming our discrimination between studied clones. In this list, we found keratins (KRT7, KRT34, KRT81), CNN1 and LIM-calponin homology domain (LIMCH1) linked to a VSM lineage; insulin-like growth factor binding protein 2 (IGFBP2) expressed in VSMCs; inhibin A (INHBA), a basement membrane protein; and SPARC-related modular calcium binding 1 (SMOC1) and other genes not yet characterized (Fig. 3B, see accession no. GSE48540 in Material and methods).

Because the CD49a molecule is up-regulated in the VSMC commitment program [20, 21], we focalized on VSMC-specific molecules in CD146^{−/Low} and CD146^{High} cells such as CNN1, which was significantly up-regulated in CD146^{High} clones (Fig. 3B b). To confirm these data, we performed transcriptome analysis by qRT-PCR of mRNA from CD146^{High} cells and identified a high expression of CD146, thus confirming our discrimination between studied clones. In this list, we found keratins (KRT7, KRT34, KRT81), CNN1 and LIM-calponin homology domain (LIMCH1) linked to a VSM lineage; insulin-like growth factor binding protein 2 (IGFBP2) expressed in VSMCs; inhibin A (INHBA), a basement membrane protein; and SPARC-related modular calcium binding 1 (SMOC1) and other genes not yet characterized (Fig. 3B, see accession no. GSE48540 in Material and methods).

High expression of CD146 in MSCs is associated with commitment to VSM lineage

The levels of the MSC markers CD90, CD73 and CD105 were similar in CD146^{−/Low} and CD146^{High} populations derived from clones (Fig. 3A). Interestingly, the expression of CD49a (α1-integrin subunit) was up-regulated in CD146^{High} clones. After their expansion, cells were subjected to gene array analysis. We obtained a list of genes up-regulated in CD146^{High} clones and identified a high expression of CD146, thus confirming our discrimination between studied clones. In this list, we found keratins (KRT7, KRT34, KRT81), CNN1 and LIM-calponin homology domain (LIMCH1) linked to a VSM lineage; insulin-like growth factor binding protein 2 (IGFBP2) expressed in VSMCs; inhibin A (INHBA), a basement membrane protein; and SPARC-related modular calcium binding 1 (SMOC1) and other genes not yet characterized (Fig. 3B, see accession no. GSE48540 in Material and methods).

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Table 1 List of primers

|     | Forward primer                              | Reverse primer                          |
|-----|--------------------------------------------|----------------------------------------|
| ALPL| 5′-CCTGGAGCTCAGAGCTCAAA-3′                 | 5′-ACTGGT GAGACCCCATCC-3′              |
| DLX5| 5′-GCCACAAACCAGCCAGAA-3′                   | 5′-GGGAGGTACGTAGCTTCTCAGACC-3′         |
| RUNX2| 5′-GCCCAAACATCTAGATGTGC-3′                 | 5′-CACGCGGCTGCAACAAGAC-3′              |
| OSX | 5′-CTCCCTGCGACTGCTCCTCA-3′                 | 5′-GGCCAGCCTGATCCCTGTT-3′              |
| PPARγ2| 5′-GATACAGTCTGCTGAAACATACCA-3′            | 5′-CCACGGAGCTGATCCCAA-3′               |
| FABP4| 5′-CCTGGAGCTCAGAGCTCAAA-3′                 | 5′-ACTGGT GAGACCCCATCC-3′              |
| COL10A1| 5′-GGTATAGCAGTAAAGGAGGAGCA-3′              | 5′-GGGCGAGCTTGTGCCTTACCT-3′            |
| CNN1| 5′-CCTGTCCTGCTGCTACTCA-3′                  | 5′-GGGAGGTACGTAGCTTCTCAGACC-3′         |
| SM22A| 5′-TTGGATCCGACATGCGCCAAACAG-3′             | 5′-AGATCATCAGTTAGAAAGGCTAGGSC-3′       |
| NANO| 5′-TGGACACTGCTGTAATCCCT-3′                 | 5′-CGTGTAGTATTAGCTCCAAACAT-3′          |
| SOX2| 5′-CCATCCACATCTCAGCAAA-3′                  | 5′-CCACGGAGCTGATCCCAA-3′               |
| OCT4A| 5′-AGTGAGGGCAACCTGCGAGA-3′                 | 5′-GGCCAGCCTGATCCCTGTT-3′              |
| ELN | 5′-AACAGCGCTTCCGCCC-3′                     | 5′-GGGAGGTACGTAGCTTCTCAGACC-3′         |

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CD146High clones expressed CNN1 as compared with CD146Low clones (77.3 ± 7% versus 16.6 ± 14.7% of CNN1-positive cells, P < 0.0001; Fig. 4A). Finally, after VSMC phenotype analysis in clones, we sought to test CD146High and CD146Low subpopulations at a functional level. Thus, cells were assessed for collagen matrix contraction capacity [22, 23]. Interestingly, CD146High clones contracted more matrix than CD146Low clones at 5 hrs after contraction initiation (51.7 ± 9.4% versus 63.3 ± 2.1% of initial surface of matrix; Fig. 4B). Therefore, CD146High MSCs had more VSMC characteristics than CD146Low clones.

Effect of FGF-2 and TGF-β1 on MSC proliferation, CD146 expression and VSMC commitment

Because TGF-β1 is a key cytokine for inducing VSMC differentiation [16, 17, 24–27], we assessed the effect of TGF-β1 on CD146 expression in MSCs and VSMC differentiation. In addition, we used FGF2, a cytokine with mitogenic effect that promotes proliferation of undifferentiated MSCs in vitro [15, 28]. From crude bone marrow-derived MSCs treated with FGF2 or TGF-β1, we

Fig. 1 Mesenchymal stem cells (MSC) proliferation is higher with CD146Low than CD146High expression. (A) Flow cytometry of CD146 expression in MSCs derived from non-clonal (a) or clonal (b–c) conditions. Several clones were analysed for CD146 expression, and one CD146Low clone and one CD146High clone among 20 clones are shown. (B) Colony-forming unit-fibroblast (CFU-F) number in clones seeded at 8 cells/cm² in F25 flasks at 10 days. Data are mean ± SEM CFU-Fs for 100 seeded cells (n = 6). (C) Population doubling number calculated as Log2 (cell expansion) in CD146Low and CD146High clonal MSCs expanded for 4 weeks (n = 17 for CD146Low and n = 23 for CD146High). Data are mean ± SEM. (D) Flow cytometry of CD146 population with 5-ethynyl-2′-deoxyuridine (EdU) incorporation in CD146Low and CD146High clonal MSCs for 3 days. Data are representative of three independent experiments.

Fig. 2 CD146Low and CD146High cells show similar mesenchymal stem cells (MSC) characteristics. (A) Osteogenic or adipogenic differentiation of non-clonal and CD146Low/High clonal MSCs stained with alizarine red (a–c) or Nile red (d–f). Images are representative of three independent experiments. (B) Quantitative RT-PCR and transcriptome analysis of osteoblast (RUNX2, OSX, ALPL and DLX5), adipocyte (PPARγ2, FABP4) and chondroblast (COLL10A1) marker genes in non-clonal (NC) or clonal CD146Low/High MSCs (Low or High) at day 0 (D0) and after 21 days of differentiation (D21). (C) CD34+ haematopoietic stem cells (4 × 10⁵) were cultured on clonal CD146Low or CD146High MSCs. At days 7, 14, 21 and 28, non-adherent haematopoietic cells were counted and tested for haematopoietic progenitor content (a). Data are mean ± SEM number of CFU-Granulocytes (b), CFU-Macrophages (c), CFU-Granulocyte Macrophages (d) and BFU-Erythroid (e). Data are mean ± SEM (n = 4).
analysed MSC proliferation and CD146 expression over 21 days. First, as compared with untreated cells, FGF2-treated MSCs showed enhanced proliferation (16.6 ± 1.6 versus 12.2 ± 2.1 population doubling). In contrast, proliferation was slowed with TGF-β1 (9.5 ± 2.8 population doubling, Fig. 5Aa). Moreover, as determined by flow cytometry with EdU incorporation, the proportion of cycling cells (EdU-incorporated cells) was significantly lower with TGF-β1 than FGF2 treatment (27.8% versus 49.2%, Fig. 5Ab and c). Second, CD146 expression was lower in MSCs with than without FGF2 treatment (9.9 ± 2.8 population doubling, Fig. 5Aa), but higher with TGF-β1 treatment (33.1 ± 12.5, Fig. 5B). In parallel and in agreement with Figure 5B, the median fluorescence intensity (MFI) of CD146 staining on EdU-incorporated cells was up-regulated with TGF-β1 as compared with FGF2 (12.1 versus 4.7), so CD146 expression was enhanced by TGF-β1 treatment (Fig. 5A b and c). Furthermore, the presence of a CD146+ population after TGF-β1 treatment can be explained by our use of unfractioned BM whole MSC populations in terms of CD146 expression.

To confirm the VSMC commitment by these treated cells, we tested CNN1 expression in all conditions. Untreated MSCs expressed a basal level of CNN1, which was up-regulated by TGF-β1 as compared with FGF-2 (Fig. 5Ca–c). The expression of SM22α, an early VSMC marker, was strong and stable in all conditions (Fig. 5Cd–f). Therefore, similar to the behaviour of single cell-derived CD146High clones, a high level of CD146 expression in the MSC population induced by TGF-β1 was associated with low proliferation and commitment to a VSMC lineage.
Discussion

Soluble agents as well as cell density, spatial distribution of cells, and solid components of culture (such as extracellular matrix molecules) are increasingly considered critical to cell fate determination and heterogeneity of cultured MSCs. Some experiments suggested that even when derived from a single cell, the progeny of MSCs can be conditioned to behave differently [29, 30]. This heterogeneity could be a result of extensive ex vivo culturing, in vivo heterogeneity and plasticity of phenotype reflecting the natural repertoire of MSCs.

To study the different heterogenous subpopulations of MSCs and define specific markers, we focused on CD146, an endothelial and subendothelial marker well described in the MSC literature. CD146, also known as melanoma cell adhesion molecule (Mel-CAM, MCAM) or MUC18 belongs to the immunoglobulin superfamily. In normal adult tissues, CD146 is primarily expressed by vascular endothelium and smooth muscle cells [31–33]. Because of the heterogeneity of CD146 expression in MSC populations, we compared CD146<sub>Low</sub> and CD146<sub>High</sub> cells from clonal and sorted MSCs after culture expansion to determine whether this expression is associated with specific functions. We sorted CD146<sub>High</sub> and CD146<sub>Low</sub> cells with excellent purity from eight healthy donors after primary culture. CD146<sub>High</sub> and CD146<sub>Low</sub> cells did not differ in CFU-F frequency, cell expansion or expression of MSC markers (CD90, CD73, CD44, CD105, CD13, CD166) or adherence molecules (CD106, CD49d, CD49f; data not shown). However, this approach has two potential issues: (i) the surface re-expression of CD146 in sorted CD146<sup>+</sup> cells after a short period of culture and (ii) the low number of cells obtained after sorting not being amenable to functional studies. To bypass these issues and to have a sufficient quantity of stable CD146<sup>+</sup> and CD146<sup>+</sup> cells to compare, we generated single cell-derived CD146<sup>+</sup> clonogenic MSCs [7]. After expansion of these clonogenic MSCs, most clones expressed CD146 at a moderate level (52% of CD146<sup>int</sup>); 21% were CD146<sub>Low</sub> and 27% were CD146<sup>High</sup>. In this study, we compared clones with extreme levels of CD146 expression, CD146<sub>Low</sub> and CD146<sup>High</sup>.

As previously found, CD146<sub>Low</sub> and CD146<sup>High</sup> MSCs did not differ in CFU-F number or osteogenic, chondrogenic and adipogenic differentiation capacity [10]. Recently, CD146 knockdown in human MSCs was found to disturb proliferation and osteogenic differentiation [34]. This disagreement has a few explanations. First, use of RNA interference or ectopic overexpression can have adverse effects on MSC signalling, whereas in our study, we used ‘untouched’ physiological clones expressing different levels of CD146 depending on their fate in culture. Second, we used clones with high population doubling number, whereas the previous authors used MSCs passaged only once.

CD146<sup>High</sup> and CD146<sub>Low</sub> clones showed similar in vitro haematopoietic-supportive activity, which agrees with Tormin et al.,
who demonstrated no difference in stroma-supporting capacity between CD271+/CD45−/CD146−/Low and CD271+/CD45−/CD146+/High fractions in long-term culture initiating cells [10]. However, in a xenotransplantation model, contrary to the CD146− fraction, culture-expanded CD146+ cells could re-establish the haematopoietic microenvironment [7]. Such discrepancies could be explained by the experimental conditions. We and Tormin et al. both used in vitro approach whereas Sacchetti et al. performed their experiments in vivo.

In addition, we found proliferation slightly but significantly higher for CD146+/-Low than CD146+/-High MSCs. Accordingly, FGF2 treatment decreased CD146 expression but increased proliferation, as was previously described [7]. Conversely, TGF-β1 treatment was associated with increased CD146 expression and VSMC differentiation. The association of CD146 and the VSMC differentiation lineage is reinforced by the fact that the CD146 promoter contains the gene regulation element CarGbox, which is present in all promoters of key genes for the VSMC lineage (αSM-actin, SM22α, CD49a) [33]. Alpha1 integrin subunit (CD49a) is one of the SMC-specific marker genes [35]. In our study, CD49a was up-regulated in CD146+/-High clonal MSCs as was the specific VSMC marker CNN1 at the gene and protein level. In addition, early VSMC markers SM22α and ELN were up-regulated in CD146+/-High MSCs. Also, CD146+/-High MSCs exhibited better potential for contraction of collagen matrix than did CD146+/-Low counterparts. Vascular smooth muscle cells are multipotential cells with the ability to differentiate into osteoblasts (resulting in vascular calcification), chondroblasts and adipocytes depending on the inducers (BMP, oxidative stress, etc.) [36]. Because CD146+/-High MSCs share some properties with VSMCs (e.g. phenotype and matrix contraction potential), they would also exhibit multipotentiality. Therefore, CD146 expression by MSCs seems to be a characteristic and predictive marker for commitment towards the VSMC lineage.

![Fig. 5](image_url)

**Fig. 5** Effect of fibroblast growth factor 2 (FGF2) and transforming growth factor β1 (TGF-β1) on mesenchymal stem cells (MSC) proliferation, CD146 expression and vascular smooth muscle cell commitment. (A) MSCs from crude bone marrow were treated or not with FGF-2 or TGF-β1 for 21 days. Doubling population number was calculated as Log2 (cell expansion). Data are mean ± SEM from four independent experiments (a). Flow cytometry of MSC population with EdU incorporation in MSCs treated with FGF2 (b) or TGFβ1 (c) from days 3 to 10 of culture. (B) Flow cytometry quantification of CD146 expression (FI) calculated as median fluorescence intensity (MFI) CD146/isotype control between MSCs treated or not with FGF2 or TGF-β1. (C) Immunofluorescence of protein levels of CNN1 (a–c) and SM22α (d–f) in MSCs treated or not for 10 days with FGF2 or TGFβ1. Images are representative of four independent experiments.
Interestingly, the VSMCs were previously related to BM stromal cells with haematopoietic sustaining activities [37]. Indeed, the in vitro model of long-term haematopoiesis requires a layer of stromal cells generated by Dexter-like culture systems [38, 39]. This type of culture constrains BM stromal adherent cells to shift towards the MSC lineage [38, 40]. Despite some reports of osteoblastic localization for the HSC niche, others demonstrated a vascular situation [41]. In this latter context, HSCs are associated with parasinusoidal myoid cells [40, 42, 43]. All of these cells express αSM-actin. In foetal and adult human BM, αSM-actin-expressing cells form haematopoietic niches [44]. Sacchetti et al. described CD146+ cells expressing VSMC linage markers (αSM-actin, CUNN1, Desmin) with the ability to form a haematopoietic microenvironment [7]. Recently, Peault’s group showed in humans that only CD146+ cells could maintain in vitro HSCs with long-term repopulating potential [45]. Moreover, in agreement with our results, in vitro investigations of haematopoiesis-sustaining properties revealed no differences between CD146+/Low and CD146+/High stromal cells; only in vivo experiments showed differences. Overall, the CD146+/High MSCs represent a fraction of whole BM-MSCs with few detectable differences as compared with CD146+/Low MSCs. Nevertheless, the differences concerning the commitment towards a VSMC lineage and a high CD146 expression in the MSC population reflect the start of this commitment.

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Conflicts of interest

The authors declare no conflicts of interest.

Author contribution

N.E. performed the research, N.E. and P.B. designed the research study, F.G. and M.G. contributed reagents and tools. N.E., P.B. and F.D. wrote paper and L.S. revised it.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Sorted CD146+/Low and CD146+/High MSCs. (A) Crude bone marrow cells were seeded at 5 × 10^6 cells/cm² in a flask for 21 days. MSCs were sorted into CD146+/Low and CD146+/High cells. (B) CFU-F number from sorted MSCs seeded at 8 cells/cm² in a 25 flask for 10 days. Data are mean ± SEM number of CFU-Fs for 100 cells seeded (n = 8). (C) Population doubling number of non-clonal sorted CD146+/Low and CD146+/High cells after 21 days of culture. Data are mean ± SEM (n = 6).

Figure S2. Expression of stemness genes and VSMC-specific markers by clonal CD146+/Low/High MSCs. (A) qRT-PCR analysis of mRNA expression of NANOG, OCT4A and SOX2 in non-clonal MSCs (NC), CD146+Low and CD146+High clones. (B) qRT-PCR analysis of mRNA expression of SM22α and ELN in non-clonal MSCs (NC), CD146+Low and CD146+High clones. Data are mean ± SEM from three independent experiments.

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