

# Novel Markers for the Prospective Isolation of Human MSC

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**ABSTRACT:** The isolation of mesenchymal stem cells (MSC) from primary tissue is hampered by the limited selectivity of available markers. So far, CD271 is one of the most specific markers for bone marrow (BM)-derived MSC. In search of additional markers, monoclonal antibodies (mAbs) with specificity for immature cells were screened by flow cytometry for their specific reactivity with the rare CD271<sup>+</sup> population. The recognized CD271<sup>+</sup> populations were fractionated by fluorescence-activated cell sorting and the clonogenic capacity of the sorted cells was analyzed for their ability to give rise to CFU-F. The results showed that only the CD271<sup>bright</sup> but not the CD271<sup>dim</sup> population contained CFU-F. Two-color flow cytometry analysis revealed that only the CD271<sup>bright</sup> population was positive for the established MSC markers CD10, CD13, CD73, and CD105. In addition, a variety of mAbs specific for novel and partially unknown antigens selectively recognized the CD271<sup>bright</sup> population but no other BM cells. The new MSC-specific molecules included the platelet-derived growth factor receptor- $\beta$  (CD140b), HER-2/erbB2 (CD340), frizzled-9 (CD349), the recently described W8B2 antigen, as well as cell-surface antigens defined by the antibodies W1C3, W3D5, W4A5, W5C4, W5C5, W7C6, 9A3, 58B1, F9-3C2F1, and HEK-3D6. In conclusion, the described markers are suitable for the prospective isolation of highly purified BM-MSC. These MSC may be used as an improved starting population for transplantation in diseases like osteogenesis imperfecta, cartilage repair, and myocardial infarction.

**KEYWORDS:** mesenchymal stem cells; bone marrow; MSC; prospective isolation

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Ann. N.Y. Acad. Sci. 1106: 262–271 (2007). © 2007 New York Academy of Sciences.  
doi: 10.1196/annals.1392.000

## INTRODUCTION

Mesenchymal stem/stromal cells (MSC) are self-renewing cells that can give rise to mesodermally derived tissues including bone, cartilage, muscle, stromal cells, tendon, and connective tissue.<sup>1-6</sup> Cultured MSC are well-characterized plastic-adherent, spindle-shaped cells that express a panel of key markers including CD105 (endoglin, SH2), CD73 (ecto-5' nucleotidase, SH3, SH4), CD166 (ALCAM), CD29 ( $\beta$ 1-integrin), CD44 (H-CAM), CD90 (Thy-1), and STRO-1 but are negative for CD45, CD34, and HLA-DR.<sup>7,8</sup> Depending on the source and on the culture conditions, they additionally express CD349 (frizzled-9), SSEA-4, Oct-4, nanog-3, and nestin.<sup>9</sup>

In contrast to cultured MSC, only little information exists about the features of the tissue precursor cell that can give rise to plastic-adherent cells. In addition, a strict terminology to distinguish between these cell types is lacking. In an attempt to render the terminology more precisely, the International Society for Cellular Therapy (ISCT) proposed in a position paper, that cultured MSC should be designated "multipotential mesenchymal stromal cells," whereas the term "mesenchymal stem cells" should be reserved for cells from primary tissues that can give rise to "colony-forming units–fibroblasts (CFU-F)" *in vitro* and tissue repopulation with multilineage differentiation capacity *in vivo*.<sup>10</sup>

To date, only a few markers have been developed and proved to be suitable for the isolation of MSCs from primary tissues. Markers that meet established criteria for their positive selection include STRO-1, CD73 (ecto-5' nucleotidase, SH3, SH4), CD105 (endoglin, SH2), and CD271 (low-affinity nerve growth receptor), whereas CD45 and glycophorin A (CD235) are used for the negative selection of MSC.<sup>11-18</sup> To identify new MSC-specific markers, more than 200 monoclonal antibodies (mAbs) of our collection were screened for their reactivity with CD271-positive BM cells. Antibodies fractionating the CD271-positive BM population in at least two populations were used to isolate candidate populations by fluorescence-activated cell sorting (FACS). The clonogenic potential of the sorted cells was analyzed by scoring their ability to give rise to CFU-F. Using this approach, 15 novel MSC-specific markers were identified.

## METHODS

### *Isolation of Bone Marrow (BM) Mononuclear Cells*

BM was harvested at the Hospital for Workers Compensation from the femur shaft of patients undergoing a total hip replacement after approval of the ethics committee of the University of Tübingen. Approximately 25 mL of BM cells were collected and mixed with 5000 U of heparin (Sigma-Aldrich, Taufkirchen, Germany). Mononuclear cells were recovered by Ficoll Histopac (Biochrom

KG, Berlin, Germany) density gradient fractionation ( $750 \times g$  for 20 min at RT) and remaining erythrocytes lysed in ammonium chloride solution (Cell Systems, Remagen, Germany) for 10 min at  $4^\circ\text{C}$ .

### *CFU-F Assay*

The CFU-F assay was performed by plating either  $1 \times 10^5$  unselected BM mononuclear cells or  $5 \times 10^3$  FACS-selected BM cells into gelatine-coated T-25 flasks (0.1% gelatin/ $\text{H}_2\text{O}$  solution; CellSystems) in the presence of a serum replacement medium, (Knockout<sup>TM</sup> D-MEM; Invitrogen, Karlsruhe, Germany) containing 5 ng/mL human basic fibroblast growth factor (bFGF; CellSystems<sup>9</sup>). After 14 days of culture, adherent cells were washed twice with PBS, fixed with methanol (5 min, RT), and air-dried. To visualize and enumerate MSC CFU-F, the cells were stained with May-Grünwald-Giemsa solution (1 to 20 diluted with deionized water, 5 min, RT), washed twice with deionized water, and air-dried. CFU-F colonies were typically between 1 and 8 mm in diameter and were scored macroscopically.

### *Generation of mAbs Reactive with Human MSC*

The mAbs W1C3 (IgG1), W3C4 (CD349 IgM<sup>9,19,20</sup>), W3D5 (IgG2a), W4A5 (IgG1), W5C4 (IgG2b), W5C5 (IgG1), W7C6 (IgG1), and W8B2 (IgG1<sup>21</sup>) with unknown specificities were raised by immunization of 6- to 8-week-old female Balb/c mice (Charles River WIGA, Sulzfeld, Germany) with the retinoblastoma cell line WERI-RB-1. The mAbs HEK-3D6 (IgG2a) and F9-3C2F1 (IgG1) were raised by immunization with the human embryonic kidney cell line HEK-293, mAb 58B1 (IgG1) was raised by immunization with the megakaryocytic cell line UT-7, mAb 9A3G9 (IgG1) by immunization with the breast carcinoma cell line DU.4475, mAb 39D5 (IgG1, CD56) by immunization with the CD34-positive hematopoietic cell line KG-1a,<sup>22</sup> and mAbs 24D2 (IgG1, CD340<sup>23</sup>) and 28D4 (IgG2b, CD140b<sup>24,25</sup>) by immunization with NIH-3T3 cells transfected with the full-length coding sequence of human HER-2 or PDGF-RB, respectively. The isotypes of mAbs were determined by enzyme-linked immunosorbent assay (ELISA) (Boehringer Mannheim, Mannheim, Germany). The properties of the mAbs are summarized in TABLE 1.

For immunization, the mice were injected five times intraperitoneally with  $10^7$  cells at 2-week intervals. The spleen was removed 4 days after the final boost prior to fusion with the SP2/0 myeloma cell line (German Collection of Microorganisms and Cell Cultures; DSMZ). Suspended spleen cells ( $\sim 10 \times 10^7$  cells) and  $3 \times 10^7$  myeloma cells were mixed, centrifuged, and washed in serum-free RPMI-1640 medium. One milliliter of polyethylene glycol (PEG; Sigma-Aldrich) was added drop-by-drop to the pellet of the cell

**TABLE 1. Antigen specificity and isotypes of mAbs used in the study**

mAb	Antigen	Isotype
W1C3	unknown	IgG1
W3C4	frizzled-9 (CD349) <sup>9, 19, 20</sup>	IgM
W3D5	unknown	IgG2a
W4A5	unknown	IgG1
W5C4	unknown	IgG2b
W5C5	unknown	IgG1
W7C6	unknown	IgG1
W8B2	unknown <sup>21</sup>	IgG1
9A3	unknown	IgG1
24D2	HER-2/erbB2 (CD340) <sup>19, 20, 23</sup>	IgG1
28D4	PDGF-RB (CD140b) <sup>24, 25</sup>	IgG2b
39D5	N-CAM (CD56; epitope NOT on NK cells) <sup>22</sup>	IgG1
58B1	unknown	IgG1
F9-3C2F1	unknown	IgG1
HEK-3D6	unknown	IgG2a

NOTE. The indicated murine mAbs against human cell-surface antigens were generated as described in the "Methods" section.

mixture that was agitated for 3 min at 37°C. The resulting hybridoma cells were suspended in 50 mL RPMI-1640 containing 10% fetal bovine serum (FBS) and hypoxanthine-aminopterin-thymidine (HAT; Sigma-Aldrich) and plated into four 24-well plates (Greiner, Nürtingen, Germany). Culture supernatants from growing hybridoma cells reacting with the cell lines used for immunization but not with peripheral blood (PB) cells were considered to select the corresponding hybridoma cells. Hybridoma cells secreting antibodies reacting with CD271-positive BM cells were cloned twice by limiting dilution and cultured in the presence of hypoxanthine-thymidine (HT; Sigma-Aldrich). Growing clones were expanded in 75 cm<sup>2</sup> culture flasks and gradual removal of HT was achieved by adding HT-free RPMI-1640 medium.

### *Staining of Cells, Immunofluorescence Analysis, and Cell Sorting*

For immunofluorescence analysis, BM cells were washed twice with phosphate-buffered saline (PBS) containing 1% of FBS and 0.01% of NaN<sub>3</sub> (FACS buffer) and incubated with polyglobin to block the nonspecific binding. In the next step, the cells were incubated with culture supernatants of the indicated antibodies for 15 min on ice. After washing, the cells were incubated with a F(ab)<sub>2</sub> fragment of goat anti-mouse secondary antibody conjugated with R-phycoerythrin (PE; Dako Cytomations, Glostrup, Denmark) for additional 15 min. After washing, the cells were then incubated with excess amounts of mouse IgG (1:20 diluted with PBS or FACS buffer; Southern Biotech,

Birmingham, AL) to block free binding sites of the secondary step antiserum. In the final step, cells were stained with CD271-APC (Miltenyi Biotech, Bergisch, Germany), and analyzed on a FACSCanto flow cytometer (Becton Dickinson, Heidelberg, Germany). Antibodies selectively reacting with CD271-positive populations were also used to stain cells for preparative purposes. In these cases, BM cells were stained with the indicated antibody and CD271-APC using the above described four-step labeling protocol and fractionated on a FACSaria high-speed cell sorter (Becton Dickinson). The collected cells were used to determine the CFU-F capacity.

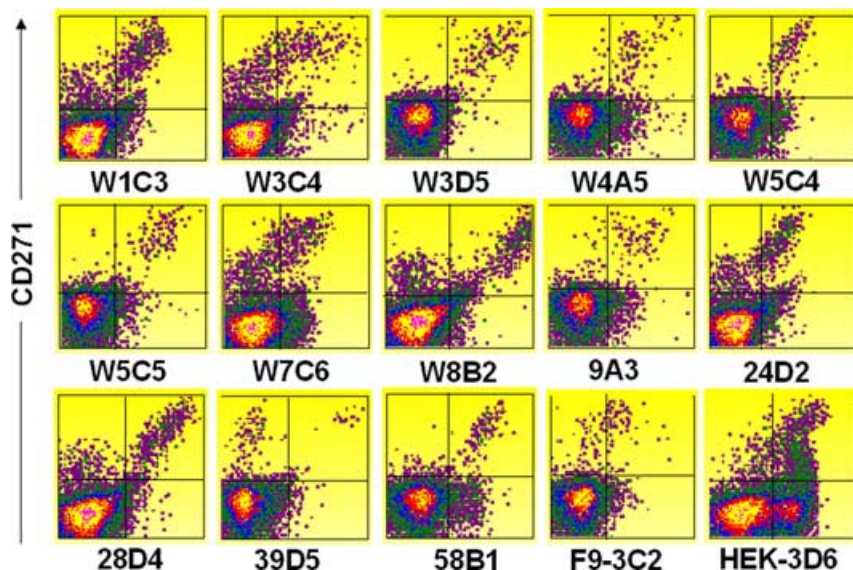
## RESULTS

### *Identification of mAbs Reactive with Human BM CD271<sup>+</sup> Cells*

Screening of >200 inhouse generated mAbs for reactivity with CD271<sup>+</sup> BM cells revealed that 15 mAbs were able to fractionate the heterogeneous CD271<sup>+</sup> population into CD271<sup>bright</sup> and CD271<sup>dim</sup> cells. mAbs (14/15) exclusively recognized CD271<sup>bright</sup> but not CD271<sup>dim</sup> cells, and only mAb HEK-3D6 detected both cell types (FIG. 1). The highest selectivity and staining intensity for CD271<sup>bright</sup> cells was recorded for mAbs W8B2, W5C5, W3D5, and 28D4 (CD140b). mAbs W1C3, W5C4, and 24D2 (CD340) were almost equally selective but stained the cells at lower intensity. mAbs F9-3C2F1, and 39D5 (CD56 epitope not expressed on NK cells) detected only fractions of CD271<sup>bright</sup> cells. Finally, mAbs W3C4 (CD349), W7C6, W4A5, HEK-3D6, 9A3G9, and 58B1 additionally recognized rare CD271<sup>-</sup> cell populations.

### *Clonogenic MSC Reside in the CD271<sup>bright</sup> but Not in the CD271<sup>dim</sup> Population*

To determine the clonogenic potential of mAb-defined CD271<sup>+</sup> BM populations, CFU-F assays of fractionated cells were performed. Staining of BM cells with antibodies against CD271 and CD140b revealed that CD140b is exclusively expressed on CD271<sup>bright</sup> cells (FIG. 2 A), and culture of fractionated cells showed that only CD271<sup>bright</sup>CD140b<sup>+</sup> cells but not CD271<sup>dim</sup>CD140b<sup>-</sup> cells gave rise to CFU-F (FIG. 2 B). Similar results were found in the case of CD271<sup>bright</sup>W8B2<sup>+</sup>, CD271<sup>bright</sup>HEK-3D6<sup>+</sup>, CD271<sup>bright</sup>CD349<sup>+</sup>, and CD271<sup>bright</sup>CD56<sup>+</sup>, cells (not shown). As mAbs W5C5, W3D5, W1C3, W5C4, and 24D2, F9-3C2F1, W7C6, W4A5, 9A3G9, and 58B1 exclusively detect CD271<sup>bright</sup> but not CD271<sup>dim</sup> cells and as clonogenic cells are only found in the CD271<sup>bright</sup> fraction, it is very likely that these antibodies specifically stain clonogenic cells as well.



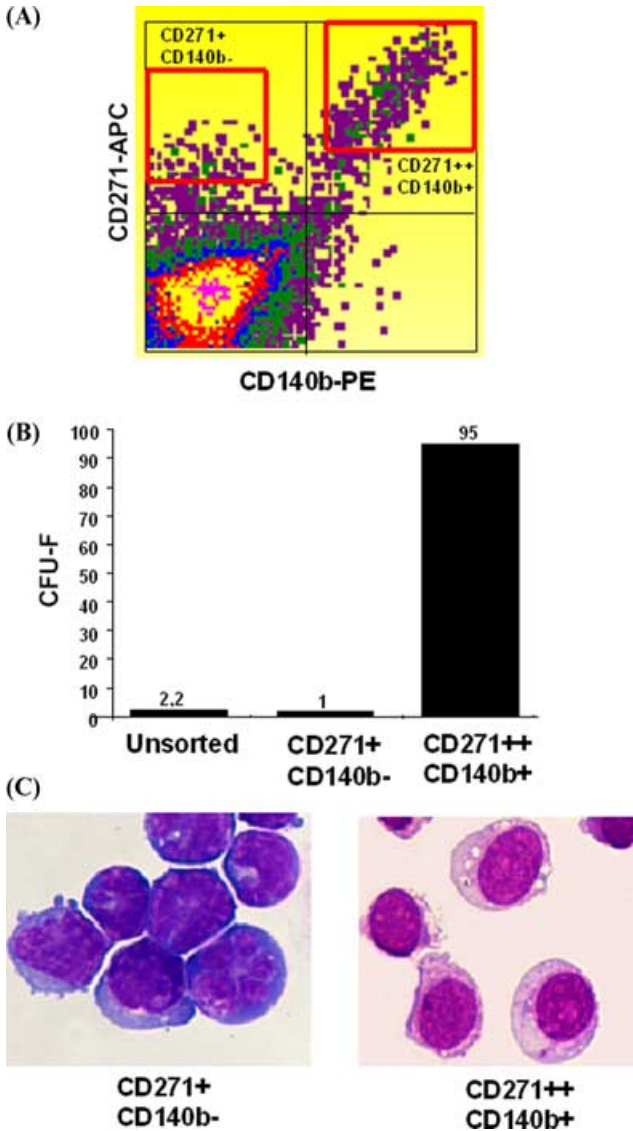
**FIGURE 1.** Reactivity profiles of mAbs with CD271<sup>+</sup> BM cells. BM cells were labeled with the mAbs using indirect immunofluorescence (PE) staining followed by direct staining with CD271-APC, as described in the “Methods” section. Cells were analyzed on a FACSCanto or sorted on a FACSAria flow cytometer. Data were processed using the FCS Express software (DeNovo, Thornhill, Ontario, Canada).

### *Morphology of Sorted CD271<sup>bright</sup>CD140b<sup>+</sup> MSC*

To compare the morphological features of CD271<sup>bright</sup>CD140b<sup>+</sup> and CD271<sup>dim</sup>CD140b<sup>-</sup> populations, the cells were sorted and stained with May-Grünwald-Giemsa. As shown in FIGURE 2 C, CD271<sup>bright</sup>CD140b<sup>+</sup> cells consist of cells (MSC) with a relatively bright nuclear staining and a high cytoplasmic content. In contrast, CD271<sup>dim</sup> cells show an immature lymphoblastoid appearance and a darker nuclear staining. In conclusion, CD271<sup>bright</sup> cells are not only functionally but also phenotypically and morphologically distinct from CD271<sup>dim</sup> cells.

## DISCUSSION

Until present, CD271 was described to be the most selective marker for the characterization and purification of human BM-MSC.<sup>14</sup> By screening of a large number of our antibodies for reactivity with CD271<sup>+</sup> cells, 15 mAbs could be identified that detect a subpopulation of CD271<sup>+</sup> cells at high selectivity. Further analysis showed that 14/15 mAbs detected only CD271<sup>bright</sup> cells and that only these cells gave rise to CFU-F, whereas CD271<sup>dim</sup> cells were



**FIGURE 2.** Morphology and clonogenic capacity of sorted BM cells. (A) Display of BM cells stained with CD271-APC and CD140b-PE. Cells were stained as described in the “Methods.” (B) Clonogenic capacity of sorted CD271<sup>bright</sup>CD140b<sup>+</sup> and CD271<sup>dim</sup>CD140b<sup>-</sup> BM cells. Cells in windows shown in (A) were sorted on a FACSAria cell sorter (Becton Dickinson) and defined cell numbers were plated on gelatine-coated flasks and cultured serum-free, b-FGF containing medium.<sup>9</sup> Fourteen days after culture, the colonies were enumerated. CFU-F numbers were equalized to 5,000 plated cells. (C) Morphology of sorted CD271<sup>bright</sup>CD140b<sup>+</sup> and CD271<sup>dim</sup>CD140b<sup>-</sup> BM cells stained with May-Grünwald-Giemsa.

functionally, morphologically, and phenotypically distinct from CD271<sup>bright</sup> cells. Because the mAbs (except mAb HEK-3D6) presented here recognize only the clonogenic CD271<sup>bright</sup> population, they represent novel and improved tools for the selection of MSC. In this context, particular emphasis should be placed on mAbs W3D5, W5C5, and W8B2 because they combine the favorable features of the selective recognition and a bright staining of CD271<sup>bright</sup> cells. Another promising candidate is mAb 39D5. This reagent recognizes a CD56 epitope that is not expressed on NK cells but on a fraction of highly clonogenic CD271<sup>bright</sup> cells.<sup>22</sup> In conclusion, the antigens recognized by the mAbs are novel candidate targets for the prospective isolation of MSC. The selected cells may be used either directly after isolation or after *in vitro* expansion for transplantation in diseases like osteogenesis imperfecta, cartilage repair, and myocardial infarction.

#### ACKNOWLEDGMENTS

This work was supported by (i) the Federal Ministry for Education and Research (BMBF), BioProfile Stuttgart/Neckar-Alb; project 0313048A: Generierung von Antikörpern gegen Frizzled Rezeptoren (GPCRs) zur Isolierung und Charakterisierung von Stammzellen, by (ii) the Federal Ministry for Education and Research (BMBF), BioProfile Stuttgart/Neckar-Alb; project 0313668B: Entwicklung eines bioartifiziellen Leberreaktors mit allogenen humanen Hepatozyten, by (iii) the Deutsche Forschungsgemeinschaft (DFG) supported Sonderforschungsbereich SFB-685 (Immunotherapy: Molecular Basis and Clinical Applications) project Z2: Core Facility for Cell sorting, and by (iv) the intramurally supported Fortüne project F1282751: Herstellung und Charakterisierung monoklonaler Antikörper gegen Frizzled-Rezeptoren für die Stammzellisolierung und Tumoreliminierung.

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