Isolation of human mesenchymal stem cells (MSCs)

Bone marrow (BM) was collected into a sterile heparinized syringe from each subject. MSCs were separated from whole BM using a density gradient technique (Colter *et al.*, 2001). 5 ml BM was suspended in 10 ml phosphate buffered saline (PBS; Lonza, Walkersville, USA) and laid over 15 ml of a density gradient (Lymphoflot composed of 9.1 % sodium diatrizoate and 5.7 % polysaccharide; Biotest, Dreieich, Germany). After 20 minutes at 1,000 x g centrifugation without braking the mononuclear cells were harvested, washed twice with PBS, transferred to 75 cm2 or 185 cm2 culture flasks (Nunc, Wiesbaden, Germany) at 10^5 cells per cm^2 and incubated at 37 °C in 5 % humidified CO2 using standard culture medium composed of α-MEM (Lonza), 100 U/ml penicillin-streptomycin (Lonza) and 10 % human serum (pooled from 7 blood group AB donors). For the antigen adsorption assay, human sera of blood group AB and O were used. After 24 hours the non-adherent cells were removed and the adherent cells were cultured in standard culture medium. Reaching subconfluency, the cells were detached from flasks with trypsin/EDTA (Lonza), washed with PBS and transferred to new flasks.

Differentiation of MCSs by in vitro culture

MSCs are functionally characterized by in vitro differentiation assays (Pittenger *et al.*, 1999). We documented the differentiation potential into three mesenchymal lineages: adipogenic, osteogenic and chondrogenic differentiation (Fig. S1). The MSCs were treated with adipogenic medium, osteogenic medium or chondrogenic medium as described (Ji *et al.*, 2004; Kern *et al.*, 2006) or standard culture medium as a control. After differentiation, the cells were detached from the flask bottoms by trypsinization and either frozen in RLT buffer (RNeasy Mini;

Qiagen, Hilden, Germany) at -80 °C for isolation of RNA within 4 weeks or stained to visualize the in vitro adipogenesis, osteogenesis or chondrogenesis.

Adipogenic differentiation was performed using the hMSC Adipogenic BulletKit (PT-3004, Lonza). 10^3 cells/cm^2 were seeded and cultured in standard culture medium. Reaching subconfluency, the medium was changed to the induction medium. The medium was alternated twice per week between the induction and maintenance media of the kit for 21 days. For staining of the lipid vacuoles, the cells were washed twice with PBS and incubated for 30 minutes with 2 ml 10 % formalin. After washing with distilled H2O, the cells were incubated for 2 minutes with 2 ml of 60 % isopropanol (Bio Whittaker, Verviers, Belgium). The isopropanol was removed and 2 ml filtered oil red O, which was composed of 3 parts of 300 mg oil red O powder (Sigma) dissolved in 100 ml 99 % isopropanol (Sigma) and 2 parts deionized water, were put onto the cells for 5 minutes (Fig. S1, panel A). The cells were counterstained with 2 ml hematoxylin (Sigma) for 1 minute.

Osteogenic differentiation was performed using "osteogenic medium" composed of standard culture medium with 10^{-8} M dexamethasone, 0.2 mM ascorbic acid and 10 mM β-glycerolphosphate (Sigma). 10^3 cells/cm^2 were seeded and cultured in standard culture medium. Reaching subconfluency, the medium was changed to the osteogenic medium, which was replaced by fresh medium twice per week for 21 days. For staining of the osteogenic differentiated cells, we used a staining kit (Alkaline Phosphatase, Sigma). After washing with PBS, the cells were incubated for 1 minute with 2 ml citrate fixative composed of 4.6 mmol/L citric acid, 2.3 mmol/L sodium citrate, 3 mmol/L sodium chloride with surfactant, 66.6% acetone

and 3% formaldehyde at pH 3.6. The fixative was removed and 2 ml alkaline-dye composed of 0.5 ml sodium nitrite, 0.5 ml FRV-alkaline solution, 0.5 ml naphtole AS-BI alkaline solution and 22.5 ml deionized water were put onto the cells for 30 minutes (Fig. S1, panel B). The cells were counterstained with 2 ml hematoxylin for 1 minute.

Chondrogenic differentiation was assessed using the hMSC Chondrogenic Differentiation BulletKit (PT-3003, Lonza). Its Chondrogenic Basal Medium requires freshly added TGF-beta 3 (130-094-006; Miltenyi Biotec, Bergisch Gladbach, Germany) as a growth factor. 2.5 x 10^5 cells were transferred into a 15 ml tube. After 200 x g centrifugation 0.5 ml Chondrogenic Basal Medium with TGF-beta 3 was added and replaced by fresh medium twice per week for 28 days. The cultured cell suspension formed a solid pellet in the tube. For staining of the chondrogenic differentiated cells, the chondrocyte pellet was removed after 28 days, stained with 1 % Alcian Blue 8GX (Serva, Heidelberg, Germany) in 3 % acetic acid at pH 2.5 and squeezed on a glass slide (Fig. S1, panel C). For RNA extraction, the chondrogenic differentiation was performed in 185 cm^2 culture flasks (Nunc). 10^3 cells/cm^2 were seeded and cultured in standard culture medium. Reaching subconfluency, Chondrogenic Basal Medium was added and replaced by fresh medium twice per week. After 21 days, the cells were detached by trypsinization and frozen in RLT buffer at -80 °C for isolation of RNA within 4 weeks.

Characterization of MSCs by surface antigens

MSCs were detached from flasks at subconfluency with trypsin/EDTA and washed with PBS. Cells were incubated for 30 minutes in the dark at 4 °C with primary and secondary antibodies. For flow cytometry, cells were suspended in 50 µl PBS with 1 % bovine serum

albumin (Sigma), 0.1 % fetal calf serum (FCS, Lonza) and 0.1 % sodium azide (Sigma). The antibodies were used at 1 µg/10^6 cells or according to the manufacturer's instructions. The cell suspensions in 300 µl were analyzed for 10,000 events (FACScan with BD CellQuest Pro software; BD Biosciences, San Jose, CA). 7-aminoactinomycin D (7-AAD; BD Bioscience, San Jose, CA) was used to exclude dead cells.

CD antigens other than blood group antigens. Monoclonal antibodies were mouse antihuman phycoerythrein (PE)-conjugated or non-labeled anti-CD10, -CD14, -CD15, -CD29, -CD31, -CD34, -CD43, -CD44, -CD45, -CD56, -CD59, -CD71, -CD73, -CD90, -CD105, -CD106,-CD117, -CD130, -CD140a, -CD140b, -CD146, -CD166, -GD2, -HLA class I and -HLA class II (BD Biosciences); -CD93 (R&D Systems, Wiesbaden, Germany); -CD133 and -CD271 (Miltenyi Biotec); -CD243 (Millipore, Schwalbach, Germany); and -W8B2 (BioLegend, Uithoorn, Netherlands). PE-conjugated or non-labeled IgG1, -IgG2a and -IgM antibodies (BD Bioscience) were used as isotype matched controls. Secondary antibody was a polyclonal PE-conjugated goat antimouse Ig (BD Bioscience). Staining and flow cytometry analysis was performed at passage 1 (P1) as it is also done in our clinical routine.

Blood group antigens. For each blood group system, MSCs from antigen positive and negative donors were analyzed. No donors were available who typed negative for H (Bombay phenotype 0h), k or Fy^b. Monoclonal antibodies were fluoresceinisothiocyanat (FITC)-conjugated anti-A (mouse anti-human clone NaM87-1F6, BD Bioscience), anti-B (mouse anti-human clone NaM9-2E11, Jaques Boy, Reims, France) and anti-D (human anti-human clone NaTH109-1G2, Jaques Boy); PE-conjugated or non conjugated anti-DARC (mouse anti-human

clone 358307, R&D Systems); and non-conjugated anti-H (mouse anti-human clone BRIC231, AbD Serotec, Düsseldorf, Germany), anti-K (human anti-human clone K1.1.21HM.EF, BAG, Lich, Germany), anti-C (human anti-human clone MS24, Jaques Boy), anti-c (human anti-human clone MS33, Jaques Boy), anti-E (human anti-human clone H98-3E7, Jaques Boy), anti-e (human anti-human clones MS21 and MS63, Jaques Boy), anti-Jk^a (human anti-human clone MS15, Jaques Boy) and anti-Jk^b (human anti-human clone MS8, Jaques Boy). A polyclonal antibody was the non-conjugated anti-cellano (k antigen; human anti-human, lot no. 280408 and 300707, Serac, Bad Homburg, Germany).

FITC-conjugated IgG3κ (BD Bioscience) and IgG (Rockland, Gilbertsville, PA); PE-conjugated IgG2a (R&D Systems); and non-labeled IgG1κ, IgG2a (BD Bioscience), IgG and IgM (AbD Serotec) were used as isotype matched controls.

Secondary antibodies were a FITC or PE-conjugated rat anti-mouse anti-IgG1κ (BD Bioscience) for the anti-H primary antibody, FITC-conjugated goat anti-mouse Ig (BD Bioscience) for the non conjugated anti-DARC antibody and an FITC-conjugated rabbit anti-human anti-IgG (Jaques Boy) for all other primary antibodies. Staining and flow cytometry analysis was performed at passages 2 or 3 (P2 or P3).

Red blood cells (RBC) of known blood group phenotype were used as positive and negative controls for the antibody specificities used in flow cytometry (Flegel *et al.*, 2002). A total of 125 µl packed RBCs were incubated in 2 ml 5 % formaldehyde solution overnight while shaking to prevent agglutination. A 10 % RBC suspension was used for flow cytometric analysis.

Immunocytochemistry for H and GD2. The MSCs were cultivated on chamber slides (Nunc) to examine the expression of the H and GD2 antigens on MSCs. Cells were fixed in 4 % paraformaldehyde for 20 minutes, washed with PBS, suspended in PBS containing 5 % FCS and incubated overnight at 4 °C with the unlabeled mouse anti-human anti-H antibody at 1:10 dilution (BRIC231) or a mouse anti-human IgG isotype control at 1:5 dilution (BD Biosciences). After washing with PBS, the Alexa 594-labeled goat anti-mouse antibody at 1:200 dilution (Invitrogen, Carlsbad, CA) was added and incubated 4 hours at room temperature. This procedure was repeated with the unlabeled mouse anti-human anti-GD2 antibody at 1:25 dilution (BD Biosciences) or a mouse anti-human IgG2a isotype control at 1:25 dilution (BD Biosciences) and the Alexa 488-labeled goat anti-mouse antibody at 1:200 dilution (Invitrogen). After washing with PBS, mounting medium containing 4′,6-Diamidino-2-phenylindole (VectaShield with DAPI; Vectorlabs, Burlingame, CA) was added and the cells were mounted under cover slips. The cells were documented with a fluorescence microscope (Axiovert 200 immunofluorescence microscope with Axiovision software; Zeiss, Göttingen, Germany).

Western blotting for blood group proteins

Differentiation of MSCs may possibly affect the expression of blood group proteins, even if antigens may be lacking on the surface. We analyzed the expression of Kidd and DARC proteins by western blot in undifferentiated MSCs and in adipogenic, osteogenic, and chondrogenic differentiated MSCs. Western blotting was performed with protein lysates from 1 x 10^6 cells. Protein concentrations were measured using the Bradford assay. For each lane, 100 µg of proteins were subjected to SDS-PAGE in a 12.5 % SDS gel and transferred to polyvinylidene fluoride membranes (PVDF; Millipore, Schwalbach, Germany) by tank blotting.

Membranes were blocked in blocking buffer composed of PBS with 5 % non-fat milk in PBST (0.05 % Tween 20 PBS at pH 7.4) for 1.5 h and were then incubated at 4 °C overnight with polyclonal mouse anti-human antibodies against the full length FY protein (DARC) at 1:1,000 dilution (Abnova, Heidelberg, Germany) and against the Kidd protein at 1:1,000 dilution (Abnova) as well as the monoclonal mouse anti-human GAPDH at 1:1,000 dilution as loading control (Chemicon, Nürnberg, Germany). The DARC and Kidd antibodies were diluted in 5 % non-fat milk in PBST, and GAPDH antibody was diluted in 0.66 % I-Block (Tropix; Applied Biosystems, Weiterstadt, Germany). Subsequently, the membranes were incubated for 2 hours at room temperature with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Tropix) at 1:10,000 dilution in blocking buffer and thereafter exposed to the chemoluminescence substrate CDP-Star (Tropix) for 30 minutes. Signal intensities were visualized with a CCD camera system (Biometra, Goettingen, Germany).

Molecular biology for blood group genes

Blood group genotyping. Genomic DNA was isolated with the peqGOLD Blood DNA Mini Kit (PEQLAB, Erlangen, Germany) for genotyping of MSCs. PCR with sequence specific primers (PCR-SSP) was done using blood group typing kits for AB0, Rhesus-CDE, Kell, Kidd and Duffy (Inno-Train Diagnostik, Kronberg, Germany). For each analyzed blood group allele, MSCs from antigen positive and negative donors were used as controls, the only exception being 0_h.

Qualitative RT-PCR. We evaluated the possible influence of the differentiation on the blood group expression on transcriptome level. The mRNA expression of blood group antigens by adipogenic, osteogenic, and chondrogenic differentiated as well as undifferentiated MSCs was determined by RT-PCR.

Total RNA from 1 – 5 x 10^5 MSCs, that have been kept frozen at -80 °C for less than 4 weeks in RLT buffer, was extracted (RNeasy Mini Kit) using the spin protocol for the isolation of total RNA from animal cells. Approximately 100 ng of the total RNA were reverse transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). The resultant cDNA was used as template for PCR-SSP. We devised intron-spanning primer pairs forthe genes of the fucosyltransferase 1 (FUT1), AB0-glycosyltransferase and of the RhAG, RhD, RhCE, Kell, Kidd and Duffy (DARC) proteins (Table S1). Amplification was performed according to the PCR-SSP protocol for red cell antigens (Inno-Train Diagnostik): A total volume of 10 µl PCR mix contained 3 µl redPCR, 0.08 µl Taq DNA polymerase (5 U/µl), 0.2 µl primer mix (final concentration 300 nM each, forward and reverse primers), 2 µl cDNA and H_2O to 10 µl total volume. Thermocycling conditions comprised an initial denaturation step of 120 seconds at 94 °C; 5 cycles for 20 seconds at 94 °C and 60 seconds at 70 °C; 10 cycles for 20 seconds at 94 °C, 60 seconds at 65 °C and 45 seconds at 72 °C; 20 cycles for 20 seconds at 94°C, 50 seconds at 61 °C, 45 seconds at 72 °C; final step 5 minutes at 72 °C. PCR products were analyzed by gel electrophoresis in 2 % agarose stained with ethidium bromide.

The specificity of the RT-PCR-system was proven with reverse transcribed human kidney RNA (Stratagene, La Jolla, CA) as positive control for *FUT1*, *AB0*, *Kell*, *Kidd* and *DARC* and as negative control for *RHAG, RHCE* and *RHD*. RNA from peripheral blood mononuclear cells (PBMNCs), and a human acute myelogenous leukemia cell line KG-1a (CCL-246.1;

American Type Culture Collection (ATCC), Manassas, VA) served as positive controls for *FUT1*, *AB0*, *RHAG*, *RHD*, *RHCE*, *Kell*, *Kidd* and *DARC*. RNA from the human malignant melanoma cell line SK-MEL-28 (HTB-72; ATCC) was used as negative control for *Kidd* and *DARC*. RNA was isolated and reverse transcribed as described above.

Quantitative RT-PCR (qRT-PCR). To quantify the influence of differentiation on the blood group expression at the transcriptome level, the mRNA expression of *Kidd* and *DARC* genes was evaluated by qRT-PCR in adipogenic, osteogenic and chondrogenic differentiated as well as in undifferentiated MSCs. Moreover, the expression of peroxisome proliferator-activated receptor (PPAR-γ), a master regulator of differentiation in BM-MSCs (Muruganandan *et al.*, 2009), and the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was evaluated in all samples. For detection of PPAR-γ and GAPDH, the ready-to-use amplification primer mixes for RT-PCR (search-LC, Heidelberg, Germany) were applied (LightCycler; Roche).

For *Kidd* and *DARC* the same primers as in the qualitative RT-PCR were used in with a LightCycle FastStart Master Sybr Green I kit (Roche). The final concentrations were 300 nM primers and 3 mM $MgCl_2$. For optimization of PCR efficiency and specificity the protocols for each target were adapted: A touchdown PCR protocol was used for *Kidd* with an annealing time of 10 seconds and the annealing temperature decreasing from 68 °C to 58 °C by 0.5 °C every second cycle followed by 16 seconds extension time and 10 seconds melting time. For *DARC* the annealing temperature of 68 °C for 10 seconds was kept constant, and 7 seconds extension time and 10 seconds melting time were used.

For relative quantification of gene expression, the expression of each target gene was normalized to the expression of GAPDH in the same sample. The expression of the respective genes and the specificity of the RT-PCR were also verified by gel electrophoresis of the RT-PCR products.

Immunohematology

RBC blood group phenotyping of the MSC donors. RBC samples of the MSC donors were typed for antigens of the blood group systems ABO (A and B), H (H antigen), Rhesus (D, C, E, c, e), Kell (K and k), Kidd (Jk^a and Jk^b), and Duffy (Fy^a and Fy^b) by standard serological methods.

Antigen adsorption assay. We evaluated the possible adsorption of the antigens A and B from the standard culture medium, which contains human serum. Soluble blood group substances, like A and B substance, occurs in human serum of blood group AB and may be adsorbed to the surface of the cells during culture. Starting with the initial isolation from BM until the final analyses, MSCs from one donor each of blood group O and A were cultured with 10 % human serum of blood group AB as we generally did for MSCs or with 10 % human serum of blood group O as negative control. After 4 passages of cultivation in AB serum or O serum the cells were analyzed by flow cytometry using anti-A, anti-B and anti-H antibodies.

Legends to the supplementary figures

Figure S1. Differentiation capacity of MSCs. The MSC cultures were induced to differentiate in vitro and are depicted for adipogenesis (panel A), osteogenesis (panel B) and chondrogenesis (panel C). The lipid vacuoles are stained in red, alkaline phosphatase in pink-violet and mucopolysaccharides in blue-bluish green. The adipogenesis and osteogenesis panels are counterstained with hematoxylin. Comparable results were obtained with MSCs from 20 donors. The controls for undifferentiated MSCs are not shown.

Figure S2. Surface antigens of undifferentiated MSCs. The expression was tested for 26 CD antigens, as well as GD2, W8B2 and 2 types of HLA antigens. The histograms overlays show the fluorescence with the specific antibody (solid) and with the isotype control (line). Comparable results were obtained with MSCs from 20 donors.

Figure S3. Blood group antigens on RBCs: Kell and ABO. The expression was tested for the antigens K and k (cellano) for the Kell blood group system, and antigens A, B and O (H antigen) for the ABO and H blood group systems. The histograms overlays show the fluorescence with the specific antibody (solid) and with the isotype control (line). Representative results are shown from 2 to 4 independent experiments.

Figure S4. Blood group antigens on RBCs: Duffy, Kidd and Rhesus. The expression was tested for the major antigens of the FY (DARC), JK and RH blood groups. The histograms overlays show the fluorescence with the specific antibody (solid) and with the isotype control (line). Representative results are shown from 2 independent experiments.

Reference List

Colter DC, Sekiya I & Prockop DJ (2001) Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. *Proc Natl Acad Sci U S A* **98**, 7841-7845.

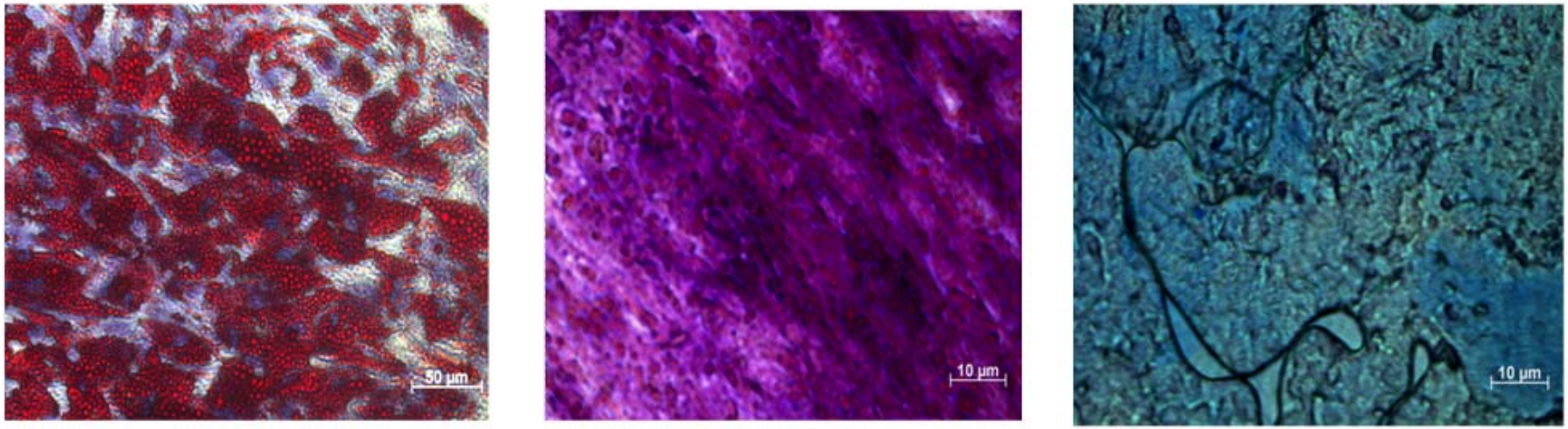
Flegel WA, Curin-Serbec V, Delamaire M, Donvito B, Ikeda H, Jorgensen J, Kumpel B, Le Pennec PY, Pisacka M, Tani Y, Uchikawa M, Wendel S & Wagner FF (2002) Section 1B: Rh flow cytometry. Coordinator's report. Rhesus index and antigen density: an analysis of the reproducibility of flow cytometric determination. *Transfus Clin Biol* **9**, 33-42.

Ji JF, He BP, Dheen ST & Tay SS (2004) Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. *Stem Cells* **22**, 415-427.

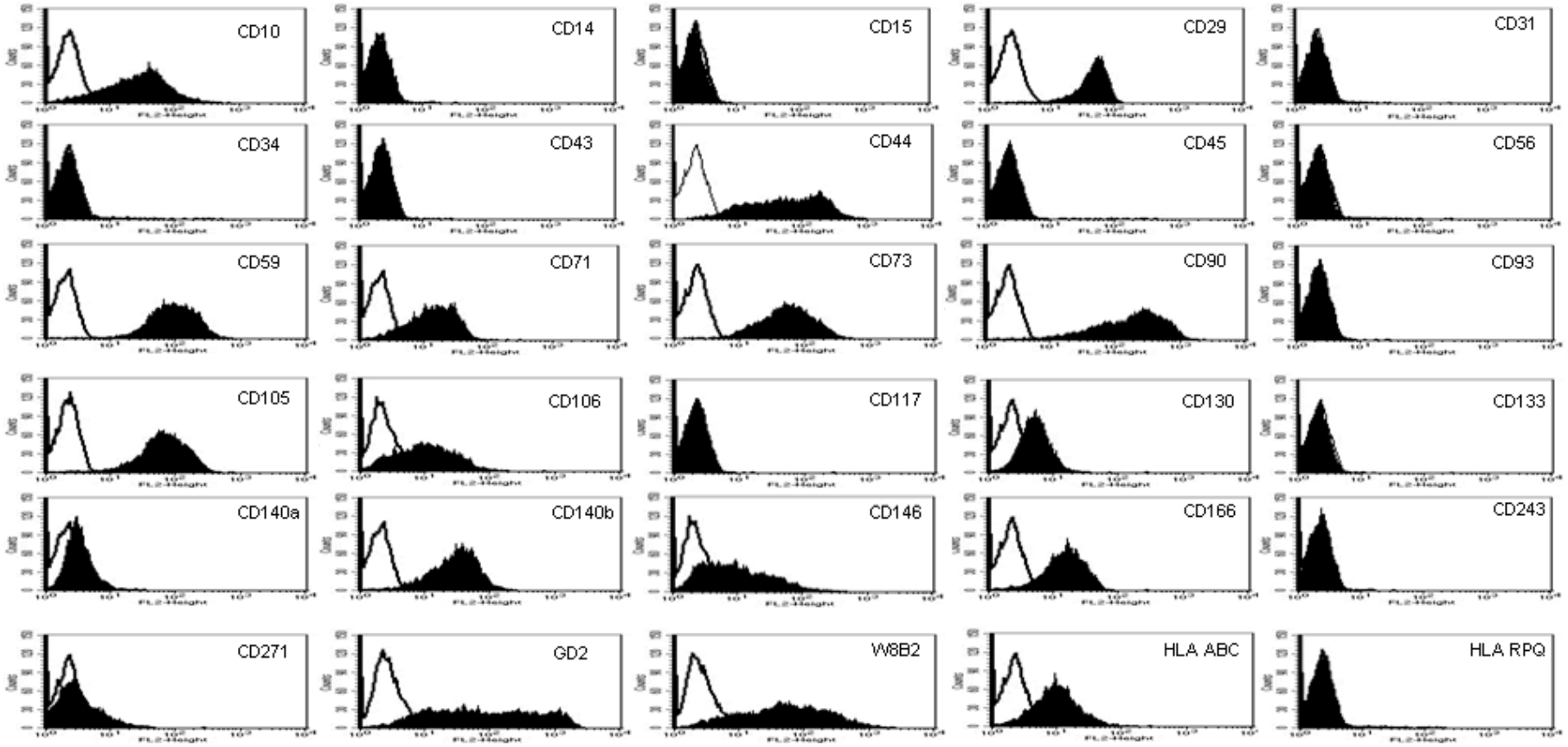
Kern S, Eichler H, Stoeve J, Kluter H & Bieback K (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* **24**, 1294-1301.

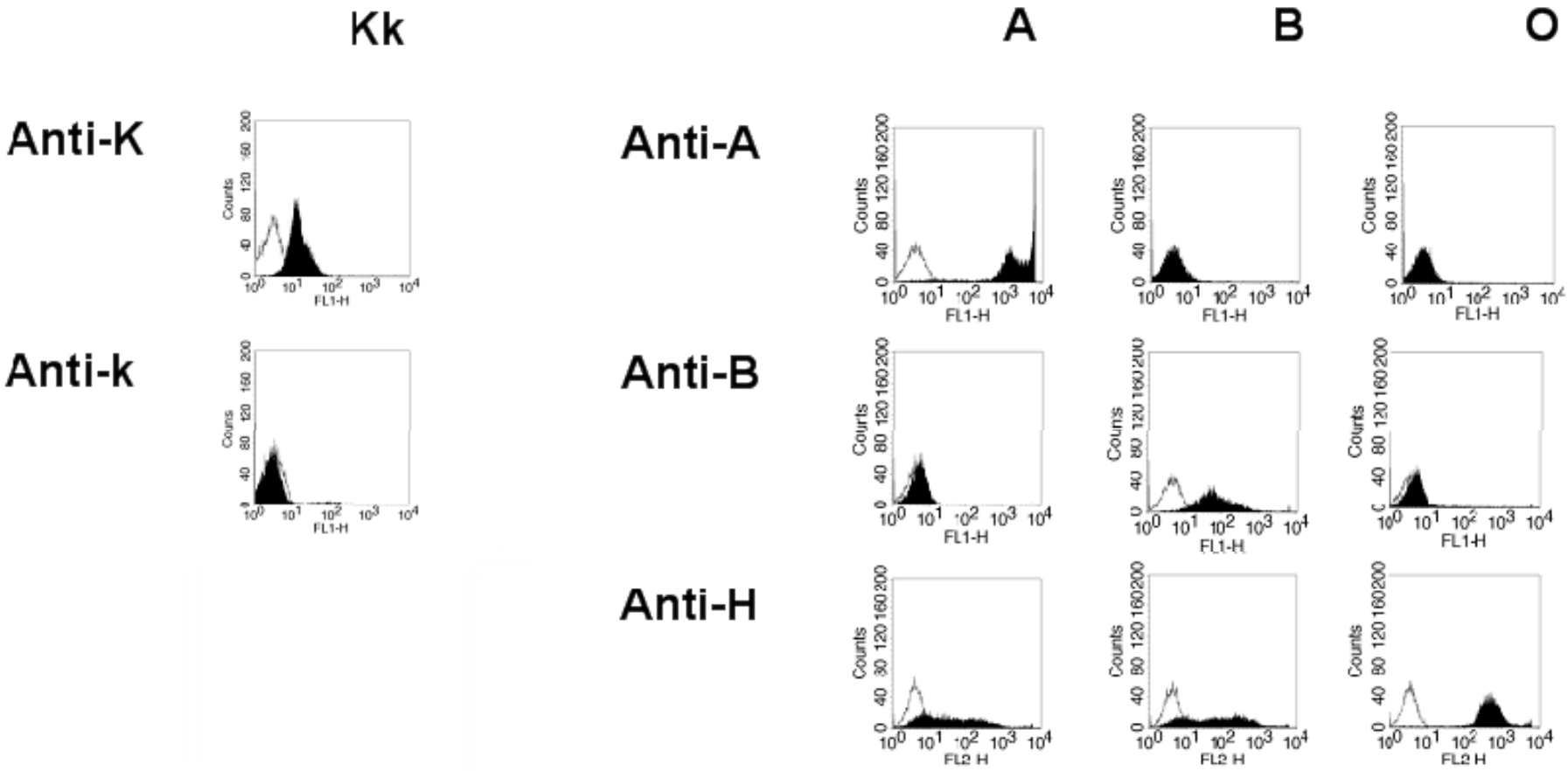
Muruganandan S, Roman AA & Sinal CJ (2009) Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program. *Cell Mol Life Sci* **66**, 236-253.

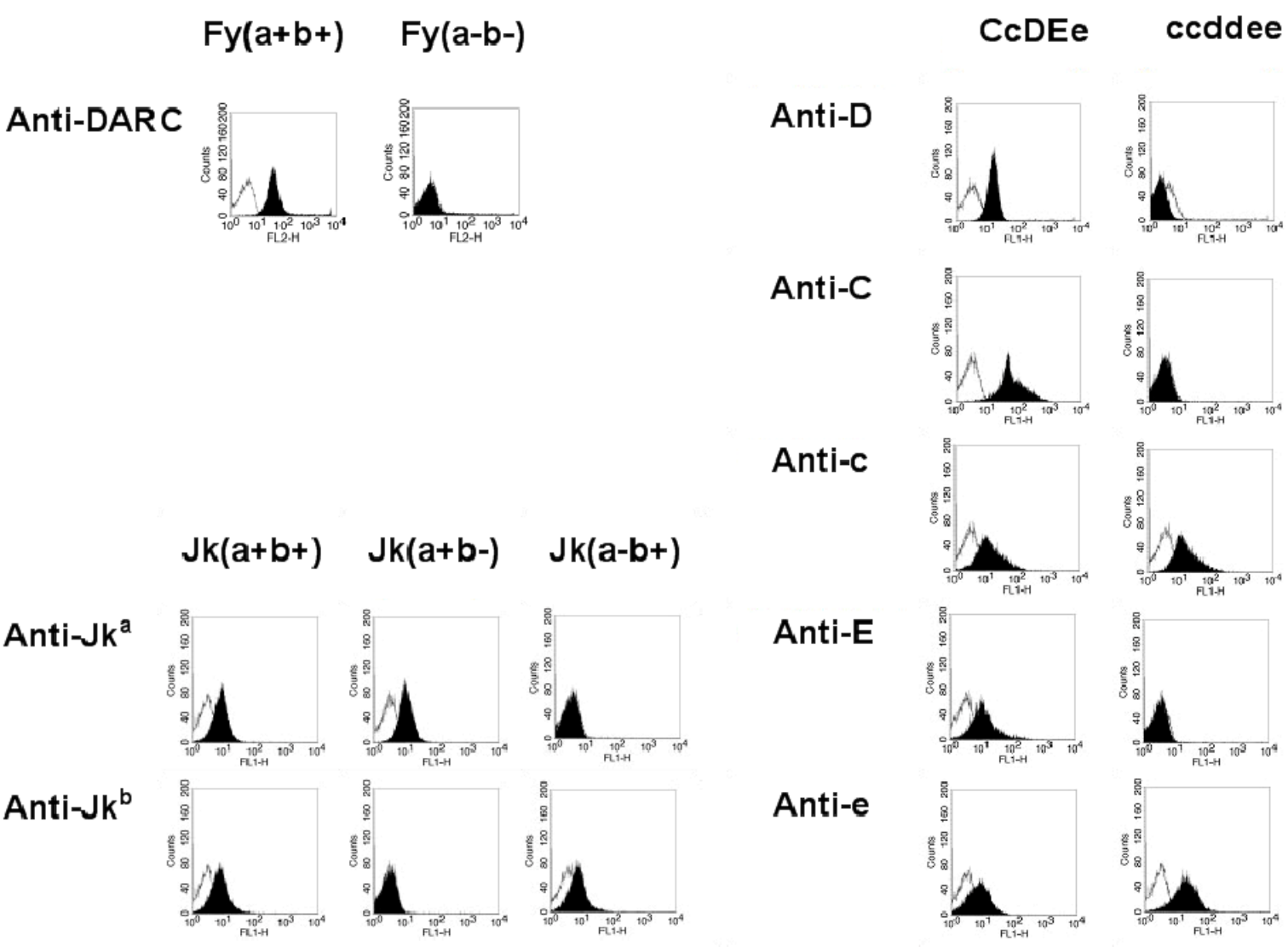
Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S & Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147.



A B C


CD10
CD14
CD15
CD29
CD31
CD34
CD43
CD44
CD45
CD56
CD59
CD71
CD73
CD90
CD93
CD105
CD106
CD117
CD130
CD133
CD140a
CD140b
CD146
CD166
CD243
CD271
GD2
W8B2
HLA ABC
HLA RPQ


Kk
A
B
O
Anti-K
Anti-k
Anti-A
Anti-B
Anti-H
Counts
FL1-H
FL2-H


Fy(a+b+)
Fy(a-b-)
Anti-DARC
CcDEe
ccddee
Anti-D
Anti-C
Anti-c
Jk(a+b+)
Jk(a+b-)
Jk(a-b+)
Anti-Jk^a
Anti-Jk^b
Anti-E
Anti-e
Counts
FL1-H
FL2-H