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Expression of blood group genes by mesenchymal stem cells

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Summary

Incompatible blood group antigens are highly immunogenic and can cause graft rejections. Focusing on distinct carbohydrate- and protein-based membrane structures, defined by blood group antigens, we investigated human bone marrow-derived mesenchymal stem cells (MSCs) cultured in human serum. The presence of H (CD173), ABO, RhD, RhCE, RhAG, Kell, urea transporter type B (SLC14A1, previously known as JK), and Duffy antigen receptor of chemokines (DARC) was evaluated at the levels of genome, transcriptome and antigen. *Fucosyltransferase-1* (*FUT1*), *RHCE*, *KEL*, *SLC14A1 (JK)* and *DARC* mRNA were transcribed in MSCs. *FUT1* mRNA transcription was lost during differentiation. The mRNA transcription of *SLC14A1 (JK)* decreased during chondrogenic differentiation, while that of *DARC* increased during adipogenic differentiation. All MSCs synthesized SLC14A1 (JK) but no DARC protein. However, none of the protein antigens tested occurred on the surface, indicating a lack of associated protein function in the membrane. As A and B antigens are neither expressed nor adsorbed, concerns of ABO compatibility with human serum supplements during culture are alleviated. The H antigen expression by GD2dim+ MSCs identified two distinct MSC subpopulations and enabled their isolation. We hypothesize that $GD2^{dim+}$ H^{+} MSCs retain a better 'stemness'. Because immunogenic blood group antigens are lacking, they cannot affect MSC engraftment *in vivo*, which is promising for clinical applications.

Keywords: stem cell transplantation, mesenchymal cells, blood groups, H antigen, CD173.

The bone marrow (BM) harbours a variety of adult stem cells, among which mesenchymal stem cells (MSCs), also called mesenchymal stromal cells, constitute <0·01% of all BM-derived mononuclear cells (Phinney & Prockop, 2007). Clinically useful for immunomodulation, multipotent differentiation and paracrine effector secretion (Dominici *et al*, 2001; Herzog *et al*, 2003; Satija *et al*, 2009; Schaefer *et al*, 2009; Siegel *et al*, 2009), MSCs are utilized in immunomodulatory therapies (Le Blanc *et al*, 2004, 2008; Uccelli *et al*, 2006; Ball *et al*, 2007) and regenerative medicine (Horwitz *et al*, 1999, 2001). Characterization of MSC subpopulations and their involvement in graft compatibility and immunogenicity may be instrumental in better defining their therapeutic potential in clinical trials.

Several distinct subpopulations sharing various degrees of MSC-like properties are collectively labelled MSCs. Molecules encoding surface structures like CD271, CD49a, W7C5, W8B2, C15, CDCP1, CD340, CD349 and SSEA1/4 may be used to define such subpopulations, while the neural ganglioside GD2, expressed exclusively on MSCs in the BM, is particularly promising as a unifying marker (Quirici *et al*, 2002; Giesert *et al*, 2003; Buhring *et al*, 2004; Martinez *et al*, 2007; Phinney & Prockop, 2007).

Membrane structures of blood group antigens, like the Duffy (FY) antigen receptor of chemokines (DARC) (Hadley & Peiper, 1997; Swardson-Olver *et al*, 2002), the ammonia transporter RhAG (Marini *et al*, 2000) or the urea trans-

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porter type B (SLC14A1, previously termed JK) (Shayakul & Hediger, 2004; Wester *et al*, 2008), and the RhBG and RhCG glycoproteins (Zidi-Yahiaoui *et al*, 2005; Han *et al*, 2006), function as receptors or transporters. Investigating these structures may allow classification of MSCs by morphological or genetic criteria, possibly implying membrane functionality. Because alloantibodies play a pivotal role in organ-allograft rejection (Colvin & Smith, 2005; Terasaki & Cai, 2008; Tobian *et al*, 2008), immunization induced by MSC antigens may result in the alteration or even rejection of a cellular graft is of particular concern for promising MSC applications in regenerative medicine without immunosuppressive therapy.

Focusing on carbohydrate- and protein-based membrane structures that are defined as blood group antigens, we investigated the characteristics of undifferentiated and differentiated MSCs derived from BM and cultured in medium free of animal serum.

Materials and methods

Isolation and differentiation of human MSCs

Bone marrow was obtained under sterile conditions from patients without metabolic or neoplastic diseases during orthopaedic operations. All patients gave written informed consent. The study was approved by the Institutional Review Board (IRB) of the University Hospital Tübingen (IRB no. 95/2005V).

Detailed isolation and characterization protocols and further Materials and Methods specifications are available online (see Supporting information online) (Pittenger *et al*, 1999; Colter *et al*, 2001; Flegel *et al*, 2002; Ji *et al*, 2004; Kern *et al*, 2006; Muruganandan *et al*, 2009).

Characterization of MSCs by surface antigens

CD antigens other than blood group antigens: MSCs were stained using monoclonal antibodies specific for 30 distinct MSC surface antigens and controls. Blood group antigens: MSCs were analysed using antibodies to H; A and B; D, C, E, c and e; K and k; Jk^a and Jk^b; and Fy^a and Fy^b. We evaluated patients who were known to be positive or negative for these antigens. Immunocytochemistry: to explore the expression of the H and GD2 antigens, the MSCs were cultivated and stained in chamber slides using anti-H and anti-GD2 antibodies. Fluorescence labelling was performed with Alexa 594-labelled (anti-H) and Alexa 488-labelled (anti-GD2) antibodies. Nuclei were stained with 4′,6-Diamidino-2-phenylindole (DAPI).

Western blotting for blood group proteins

The protein expression of SLC14A1 (JK) and DARC by undifferentiated MSCs and adipogenic, osteogenic, and chondrogenic differentiated MSCs was analysed by Western blot.

Molecular biology for blood group genes

Blood group genotyping: commercial blood group genotyping kits for ABO, CDE, Kell, Jk and Fy were used (Inno-Train Diagnostik, Kronberg, Germany). MSCs, peripheral blood mononuclear cells (PBMNCs), and KG-1a (myeloblast, chronic myeloid leukaemia) and SK-MEL-28 (melanoma) cell lines were genotyped for blood groups. Patient's genotypes corresponded to their serological blood groups (data not shown). Qualitative reverse transcription polymerase chain reaction (RT-PCR): we determined the transcription of blood group genes by testing for the mRNA in MSCs using RT-PCR. Quantitative RT-PCR (qRT-PCR): to quantify the influence of differentiation on the blood group expression at the transcriptome level, the mRNA expression of *SLC14A1 (JK)* and *DARC* genes was evaluated by qRT-PCR in adipogenic, osteogenic and chondrogenic differentiated as well as in undifferentiated MSCs. The expression of peroxisome proliferator-activated receptor-γ (*PPARG*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was determined as controls.

Immunohaematology

Red blood cell (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. Adsorption of H, A or B antigens to MSCs: after four passages of cultivation in AB serum or O serum the MSCs from an O donor were analysed by flow cytometry using antibodies to the H, A and B antigens.

Statistics

We used the two-tailed Mann–Whitney *U*-test and considered $P < 0{\cdot}05$ statistically significant.

Results

MSCs were isolated from whole BM by a density gradient technique (Colter *et al*, 2001) and characterized functionally by *in vitro* differentiation assays for their potential to differentiate into the adipogenic, osteogenic or chondrogenic mesenchymal lineage (Fig S1) (Pittenger *et al*, 1999). We confirmed the known surface antigen pattern by flow cytometry of undifferentiated MSCs. They were positive for CD10, CD29, CD44, CD59, CD71, CD73, CD90, CD105, CD106, CD130, CD140a, CD140b, CD146, CD166, CD 271, GD2, W8B2 and HLA class I; and negative for negative for CD14, CD15, CD31, CD34, CD43, CD45, CD56, CD93, CD117, CD133, CD243 and HLA class II (Fig S2).

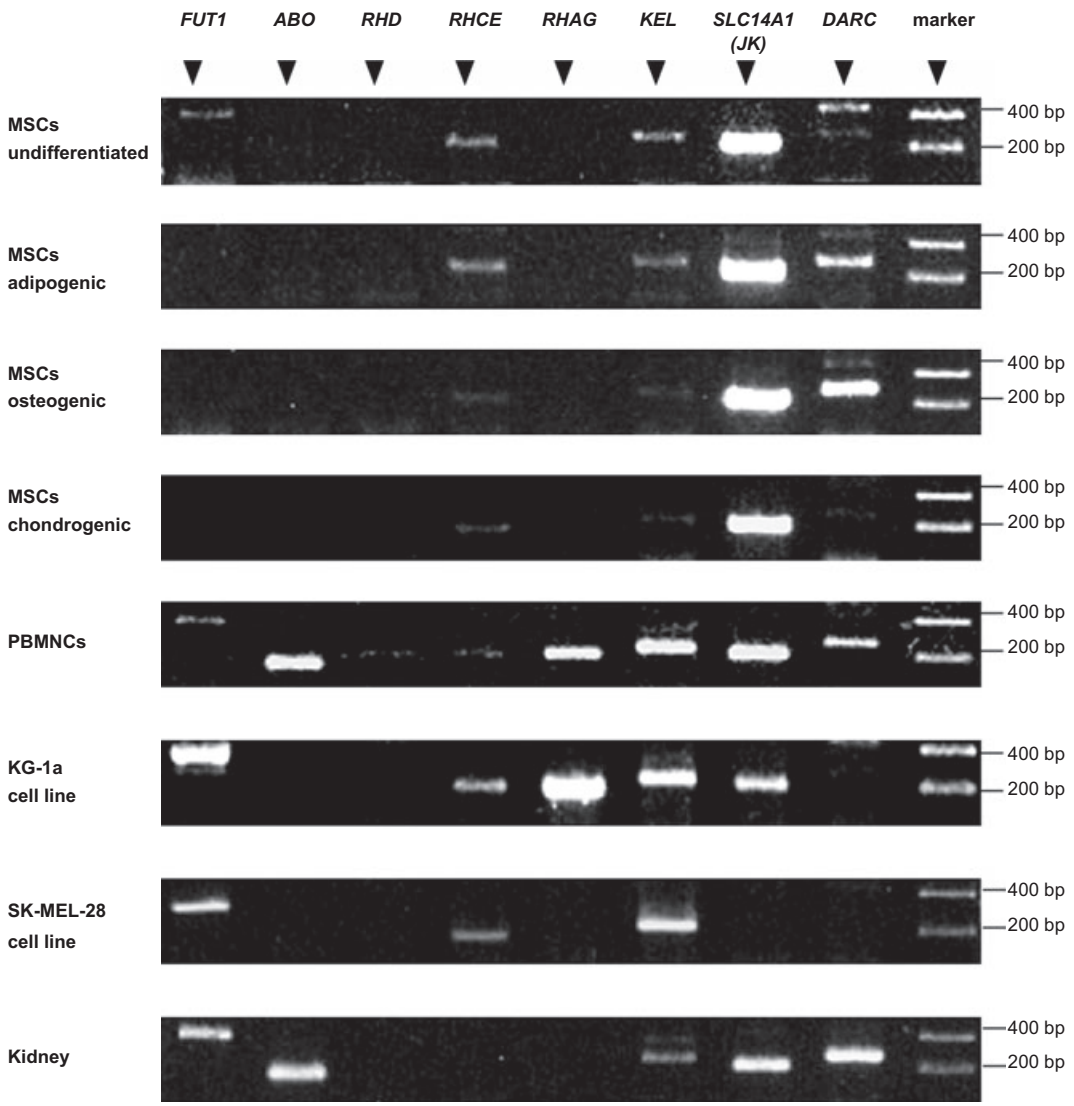

Fig 1. Blood group mRNA expression by undifferentiated MSCs and three differentiated human MSC lineages at passages 1 and 2. RNA from peripheral blood mononuclear cells (PBMNCs), two human cell lines and kidney are tested as controls. Representative results are shown. Comparable results were obtained with five donors for the MSCs panels; two experiments for PBMNCs and the two cell lines; and more than 10 experiments with kidney RNA.

Transcription of blood group mRNA in MSCs

MSCs expressed *FUT1*, *RHCE*, *KEL*, *SLC14A1 (JK)* and *DARC* mRNA. No mRNA for *ABO*, *RHD* and *RHAG* was found (Fig 1). The native, undifferentiated MSCs expressed *FUT1* mRNA, whereas under adipogenic, osteogenic and chondrogenic differentiation no expression of *FUT1* mRNA was detected.

Quantitative SLC14A1 (JK) and DARC mRNA expression in MSCs

Because the largest mRNA amounts were detected for *SLC14A1 (JK)* and *DARC* (Fig 1), we quantified these mRNAs by qRT-PCR. *SLC14A1 (JK)* mRNA was reduced in chondrogenic differentiated MSCs relative to undifferentiated MSCs (Table I). Increased *DARC* mRNA was detected in adipogenic differentiated MSCs; some osteogenic differentiated MSCs had also hugely increased *DARC* mRNA, although there was much variation among the donors. For comparison, *PPARG* mRNA was increased, particularly in adipogenic differentiated MSCs. The overall *SLC14A1 (JK)* mRNA amounts exceeded that of *PPARG* mRNA, while *DARC* mRNA amounts were low.

Intracellular SLC14A1 (JK) and DARC proteins in MSCs

Following the detection of small quantities of *SLC14A1 (JK)* and *DARC* mRNA by qRT-PCR, we explored the presence of these proteins by Western blot. Some SLC14A1 (JK) protein

Table I. Quantitative expression of *SLC14A1 (JK), DARC and PPARG* mRNA.

	Relative mRNA (mean ± SD)*					
MSCs	*SLC14A1 (JK)* ($n = 7$)†	*P*	*DARC* ($n = 6$)	*P*	*PPARG* ($n = 7$)	*P*
Undifferentiated	1·0 ± 0·9	0·001	1·0 ± 0·9	0·002	1·0 ± 0·9	0·004
Adipogenic	0·4 ± 0·2		5·6 ± 3·3		5·7 ± 4·8	
Osteogenic	0·3 ± 0·4	0·038	123·9 ± 216·8		2·2 ± 1·9	0·018
Chondrogenic	0·1 ± 0·1		0·3 ± 0·6		1·2 ± 1·9	

*The mRNA expression was normalized to a mean = 1·0 for undifferentiated MSCs in each of the three genes. mRNA in undifferentiated MSCs relative to *GAPDH* expression was $1{\cdot}4 \pm 1{\cdot}3 \times 10^{-2}$ for *SLC14A1 (JK)*, $4{\cdot}8 \pm 4{\cdot}5 \times 10^{-6}$ for *DARC* and $3{\cdot}6 \pm 3{\cdot}4 \times 10^{-3}$ for *PPARG*. The two-tailed Mann–Whitney *U*-test was used and $P < 0{\cdot}05$ considered statistically significant.
†*SLC14A1 (JK)* mRNA in osteogenic differentiation was $n = 6$.

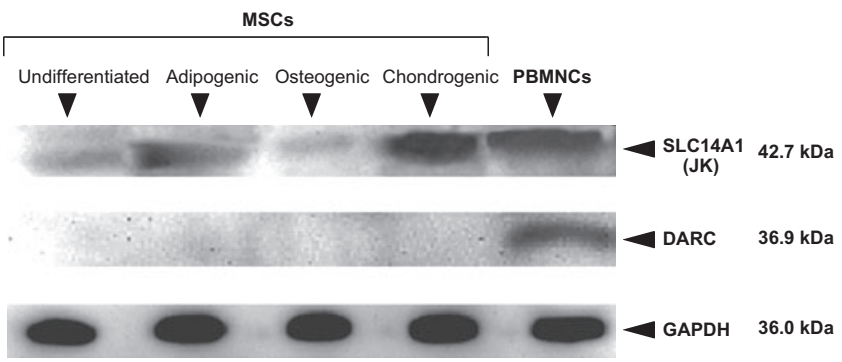

Fig 2. Expression of SLC14A1 (JK) and DARC protein in MSCs. Western blots for SLC14A1 (JK) and DARC proteins are depicted along with controls for the housekeeping protein GADPH. Representative results are shown. Comparable results were obtained in experiments with MSCs from three different donors.

was found in all MSC populations, while no DARC protein was detectable (Fig 2).

Blood group antigens on the MSC surface

We examined the expression of blood group antigens during several passages. The antigens A, B, D, C, E, c, e, K, k, Jk^a, and Jk^b, as well as the DARC protein could not be detected by flow cytometry (Fig 3A), an established sensitive technique for RBCs (Flegel *et al*, 2002).

Because *FUT1* mRNA, encoding the fucosyltransferase enzyme that synthesizes the H antigen, was found in native MSCs (Fig 1), we explored H antigen expression by flow cytometry. Whereas the histogram plots were not indicative, the flow cytometric dot plots revealed a small subpopulation of native MSCs that was positive for the H antigen (anti-CD173; Fig 3B, red dots in the lower right quadrant). The myeloblast KG-1a cells showed a similar wide variability in H antigen expression, although the fraction of strongly H positive cells was much larger than in MSCs. Thus, the flowcytometric dot plots showed a cell population stained by anti-H representing a distinct subpopulation of viable H antigen positive MSCs and KG-1a cells.

MSCs might adsorb A and B antigens to their cell surfaces from the standard culture media containing human serum with soluble A and B substances, as with AB serum. However, A and B antigens were not detected on MSCs after cultivation in AB serum after four passages, indicating no adsorption of the respective blood group substances to the surface of the MSCs (Fig 3C).

Antigen H is expressed on the surface of $GD2^{dim+}$ MSCs

Immunocytochemical staining of undifferentiated MSCs identified MSC subpopulations differing by GD2 expression, which were dubbed $GD2^{bright+}$ and $GD2^{dim+}$ (Fig 4). To corroborate our results with flow cytometry (Fig 3B), we detected some MSCs that expressed the H antigen (CD173). Double staining of GD2 antigen by green fluorescence and the H antigen by red fluorescence revealed that the $GD2^{bright+}$ cells expressed no H antigen, although a $GD2^{dim+}$ H^+ MSC subpopulation was identified.

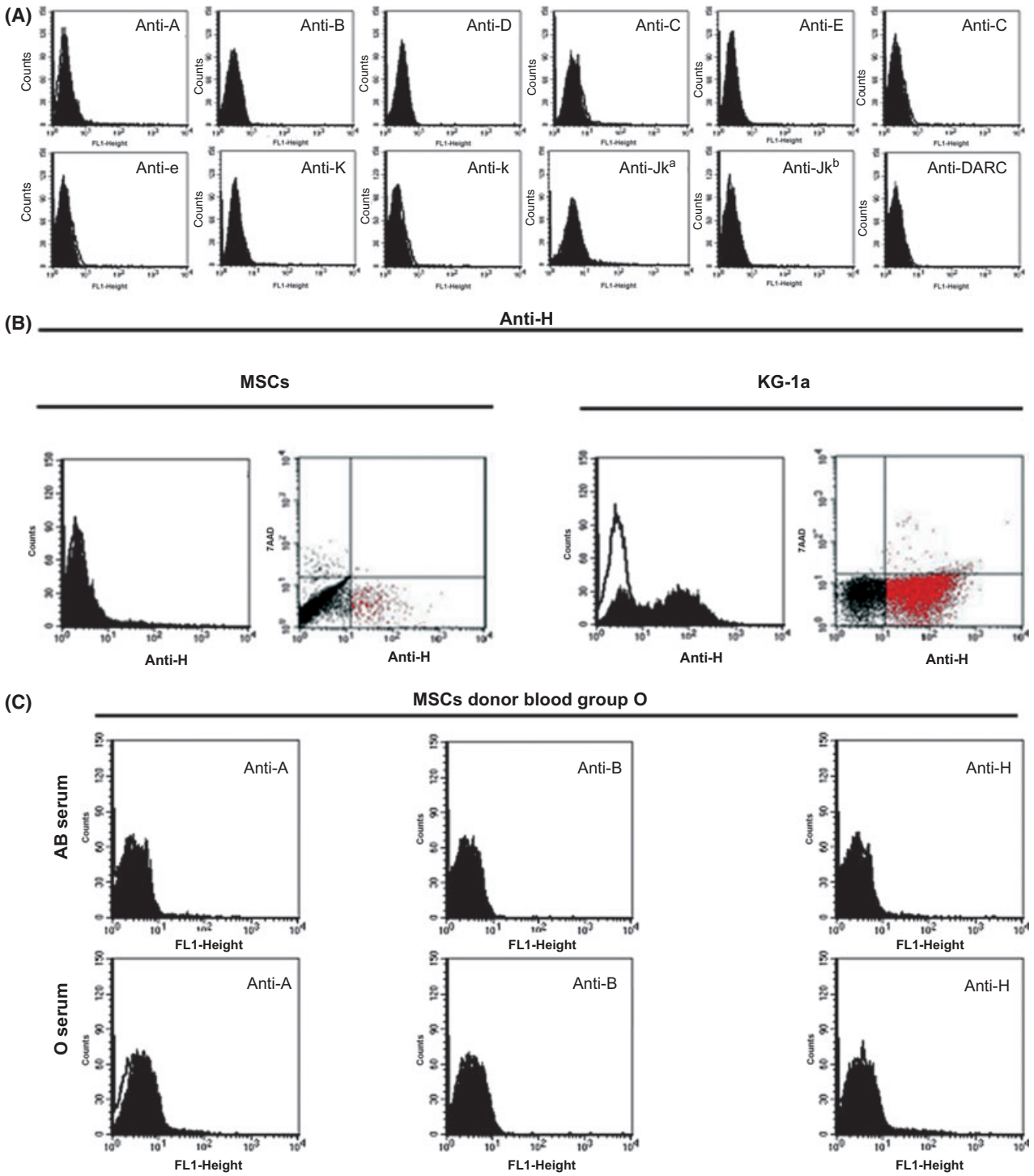

Fig 3. Flow cytometry analyses of blood group antigen expression on MSCs. Expression of all 12 blood group antigens tested was lacking from the surface of MSCs (panel A). Representative results are shown for undifferentiated MSCs. Comparable results were obtained with three donors for up to six passages each (P1–P6). H antigen expression is detected in a small subpopulation of MSCs (panel B, red dots in the lower right quadrant of the flow cytometry dot plot), which is much more prominent in KG-1a cells tested for comparison. A representative result from more than 10 experiments with undifferentiated MSCs from different donors is shown. MSCs from a blood group O donor were cultivated in AB serum for four passages and tested for absorption of A, B and H substances (panel C); MSCs with O serum are shown as a control. Comparable results were obtained with MSCs from a blood group A donor. All histogram plots depict the specific antibody profile (solid) and the isotype control staining (line).

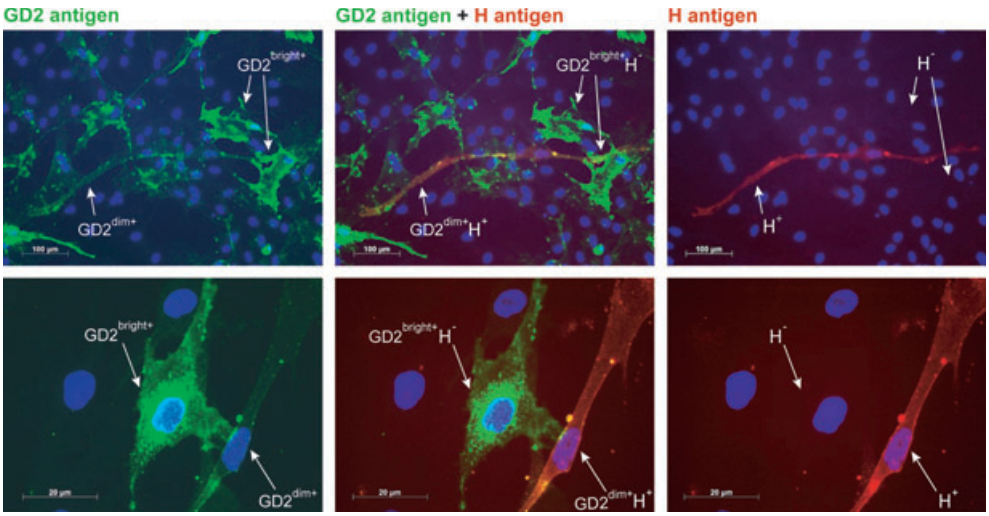

Fig 4. Identification of MSC subpopulations by GD2 and H antigen expression. Two immunocytochemistry slides from different donors (upper and lower panels) are stained for GD2 (green fluorescence) and H antigens (red fluorescence). The fluorescence for GD2 (left panels); GD2 and H antigens (middle panels); and H (right panels) are shown. Nuclei are stained blue (DAPI). Comparable results were obtained with MSCs from two additional donors.

Phenotyping of RBCs by flow cytometry

As a control for antigen detection by flow cytometry, blood group antigens on RBCs were confirmed using standard serological techniques (Fig S3 and S4).

Discussion

The application of BM-derived MSCs, utilized with immunomodulating therapies and for repair of the musculoskeletal system, may provide a means for avoiding the use of immunosuppressive drugs that is often necessary with other cellular therapies. However, allogeneic MSCs can induce alloantibody production, as shown in an animal model (Beggs *et al*, 2006). Blood group antigens are potent activators of the host immune system and can lead to antibody-mediated destruction of not only RBCs, but also nucleated cells and organs (Colvin & Smith, 2005).

We investigated the expression of clinically important blood group antigens by MSCs at the levels of transcription, intracellular proteins and presence of antigens on the surface (Table II). MSCs were isolated and cultivated using pooled allogeneic human serum, employed in several studies evaluating the clinical utility of MSCs (Bieback *et al*, 2009, 2010; Turnovcova *et al*, 2009). Under Good Manufacturing Practice conditions, the *ex vivo* cultivation and expansion of MSCs should be free of animal serum, avoiding the immunization to fetal calf serum (FCS) known to occur in patients receiving MSCs cultured in FCS-containing media (Sundin *et al*, 2007).

Blood group status can be determined at the genomic level, a technology increasingly performed in the clinical setting using blood group genotyping platforms. Our serological and genotyping results were equivalent indicating blood group genotyping is reliable. Because most antigens are not expressed on MSCs, blood group genotyping is currently not needed to characterize undifferentiated BM–MSCs for clinical use. While we detected mRNA transcription of the *FUT1*, *RHCE*, *KEL*, *SLC14A1 (JK)* and *DARC* genes, no *ABO*, *RHD* or *RHAG* mRNA occurred in MSCs. mRNA transcription varied among subpopulations of MSCs that differentiated into adipogenic, osteogenic and chondrogenic lines.

Table II. Summary of mRNA and protein expression by clinically important blood group genes in human bone marrow-derived mesenchymal stem cells (MSCs).

Blood group system		Parameter of MSCs	
Gene	Major antigens	Transcriptome mRNA in cells	Proteome/antigen on cell surface
FUT1	H	+	+
ABO	A and B	−	−
RHAG	RhAG	−	−
RHD	D	−	−
RHCE	C, E, c, and e	+	−
KEL	K and k	+	−
SLC14A1 (JK)	Jk^a and Jk^b	+	−
DARC	Fy^a and Fy^b	+	−

Trypsin affects the detection of few clinically relevant blood group proteins. However, Rhesus, Kell, Kidd and Duffy, and the carbohydrate-based antigens are known to be resistant to trypsin treatment. Therefore, we used trypsin to detach the MSCs from the culture flasks in our study.

MSCs did not express *ABO* mRNA and consequently neither A nor B antigen was detectable on their surface. Both results indicate that ABO compatibility is not required for the human

serum used in MSC culture. If supplies of AB serum become short with anticipated increases in clinical stem cell culture and its applications increase as anticipated, it can be replaced by serum of other blood groups. In addition, because the soluble A and B antigens occurring in AB serum were also not adsorbed onto MSCs, use of AB serum for culture is unlikely to interfere with the ABO compatibility of MSCs *in vivo*.

The key enzyme for synthesizing the H antigen is the β-D-galactoside 2-α-L-fucosyltransferase (FUT1), which is in turn a prerequisite for the generation of the A and B antigens. *FUT1* and H antigen are expressed by KG-1a cells and immature $CD34^+$ haematopoietic progenitor cells but absent in mature lymphocytes (Cao *et al*, 2001). We detected the H antigen on a $GD2^{dim+}$ subpopulation of undifferentiated MSCs, whereas no *FUT1* mRNA was found in differentiated MSCs. H is a strong sugar-based antigen and avid poly- and monoclonal antibodies are available, to facilitate the identification as well as the purification and clinical testing of the newly defined $GD2^{dim+}$ H^+ and $GD2^{bright+}$ H^- cells occurring among undifferentiated MSCs (Fig 4). Based on this technical feature, the H antigen may become instrumental for the identification of immature MSCs, which may differ in 'stemness' from the remaining MSCs in the BM. FUT1 is also involved in the biosynthesis of Globo H, a potential tumour-associated antigen in human breast cancer stem cells (Chang *et al*, 2008).

Among the three Rh-associated ammonia transporters, RhBG and RhCG are expressed on many nucleated cells, but not on RBCs (Handlogten *et al*, 2005). The third, RhAG, recently recognized as blood group system no. 30, is found on RBC and required for membrane integration of the two proper Rhesus proteins, RhD and RhCE (Huang, 1998; Mouro-Chanteloup *et al*, 2002; Daniels *et al*, 2009). Because *RHAG* is not transcribed in MSCs, the lack of the RhCE protein on the surface, despite some *RHCE* transcription, can be explained by the known posttranslational dependence of RhCE on RhAG. Considering the erythroid-restricted expression of RhAG (Zidi-Yahiaoui *et al*, 2005), our data confirm the non-erythroid character of MSCs.

Prichett *et al* (2000) detected the human urea transporter HUT11 (SLC14A1; JK), the protein carrying the Jk antigen, in 'explant cultures of human bone' containing osteoblasts and an unknown fraction of MSCs. We confirmed a reduced *SLC14A1 (JK)* transcription in differentiating MSCs. However, there was no good correlation with *PPARG* transcription, which is a marker for adipogenic differentiation (Wu *et al*, 2010): *SLC14A1 (JK)* was least transcribed in chondrogenic MSCs, while *PPARG* transcription in chondrogenic MSCs equaled undifferentiated MSCs. In conclusion, *PPARG* and *SLC14A1 (JK)* seem to be regulated independently in MSCs. *DARC* transcription is increased under adipogenic and apparently osteogenic differentiation. It occurs however in such low copy numbers that, not surprisingly, no DARC protein was found on the MSC surface. The Kell blood group protein, a zinc endopeptidase, is expressed on erythroid and many non-erythroid cells (Russo *et al*, 2000). We found some *KEL* transcription in MSCs but no KEL protein on the MSC surfaces, which is consistent with the immature stem cell character of MSCs.

Allogeneic MSCs can be immunogenic and contribute to rejection of haematopoietic stem cells, inducing a memory T-cell response in the host, as shown in a non-myeloablative murine transplantation setting (Nauta *et al*, 2006). Interferon-γ induced human leucocyte antigen (HLA) class II antigen expression may contribute to alloimmunization by MSCs (Chan *et al*, 2006), which may warrant further investigation. However, we could not detect such HLA class II antigen expression on undifferentiated MSCs during our culture with human serum. Among the clinically most relevant and potentially immunizing blood group proteins, only the SLC14A1 (JK) protein could be detected in the cytoplasm, but notably not on the surface of MSCs. Our data suggest that, even in the non-immunosuppressed graft recipient, a rejection or malfunction of the cellular graft due to an immunization against these blood group proteins and their antigens may not be expected. This lack of immunogenic blood group antigens on MSCs is a plus for promising clinical applications.

In further characterizing human MSCs, the data presented here suggests not only practical aspects of current GMP production, but also compatibility of these cells in the clinical setting of stem cell transplantation and regenerative medicine.

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Disclosure of conflict of interest

The authors declare no competing interests relevant to this article.

Authorship contributions

R.S. initiated the study and supervised the performance of the experiments; R.S., Ma.S. and W.A.F. provided the study concept and design; R.S., R.A.K., G.S. and Mi.S. collected samples; T.K. provided study material; C.G. designed primers; R.S., G.S., M.A.R., U.H.-K., M.A. and M.B. collected data; R.S., T.K., Mi.S. assembled data; L.D. performed statistical analyses; R.S., Ma.S., R.A.K., G.S., C.G. and W.A.F. interpreted data; R.S., H.N. and W.A.F. discussed data; C.G., L.D. and

H.N. contributed to manuscript writing; R.S. wrote drafts and W.A.F. the final version of the manuscript.

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Supporting Information

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

Fig 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).

Fig S2. Surface antigens of undifferentiated MSCs. The expression was tested for 26 CD antigens, as well as GD2, W8B2 and two types of HLA antigens.

Fig 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.

Fig 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.

Appendix S1. Detailed description of all methods.

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