Human *versus* porcine mesenchymal stromal cells: phenotype, differentiation potential, immunomodulation and cardiac improvement after transplantation

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Abstract

Although mesenchymal stromal cells (MSCs) have been applied clinically to treat cardiac diseases, it is unclear how and to which extent transplanted MSCs exert their beneficial effects. To address these questions, pre-clinical MSC administrations are needed for which pigs appear to be the species of choice. This requires the use of porcine cells to prevent immune rejection. However, it is currently unknown to what extent porcine MSCs (pMSCs) resemble human MSCs (hMSCs). Aim of this study was to compare MSC from porcine bone marrow (BM) with human cells for phenotype, multi-lineage differentiation potential, immune-modulatory capacity and the effect on cardiac function after transplantation in a mouse model of myocardial infarction. Flow cytometric analysis revealed that pMSC expressed surface antigens also found on hMSC, including CD90, MSCA-1 (TNAP/W8B2 antigen), CD44, CD29 and SLA class I. Clonogenic outgrowth was significantly enriched following selection of CD271+ cells from BM of human and pig (129 ± 29 and 1961 ± 485 fold, respectively). hMSC and pMSC differentiated comparably into the adipogenic, osteogenic or chondrogenic lineages, although pMSC formed fat much faster than hMSC. Immuno-modulation, an important feature of hMSC, was clearly demonstrated for pMSC when co-cultured with porcine peripheral blood cells stimulated with PMA and pIL-2. Finally, pMSC transplantation after myocardial infarction attenuated adverse remodelling to a similar extent as hMSC when compared to control saline injection. These findings demonstrate that pMSCs have comparable characteristics and functionality with hMSCs, making reliable extrapolation of pre-clinical pMSC studies into a clinical setting very well possible.

Keywords: pMSC ● hMSC ● cellular therapy ● CD271+ cells ● immunomodulation ● myocardial infarction

Introduction

Studies on bone marrow (BM) cell transplantation after acute myocardial infarction (MI) or for chronic myocardial ischaemia are accumulating [1–5]. Most studies showed significant improvement in cardiac function, however, these were only modest when compared with control patients [6, 7]. Two main mechanisms have been put forward to be responsible for the observed improvement in myocardial perfusion. First, BM cells might differentiate into endothelial cells, smooth muscle cells or cardiomyocytes. However, an active participation of these transplanted cells in regeneration of tissue has not been convincingly demonstrated. Second, transplanted BM cells secrete paracrine factors, which promote angiogenesis, exert cytoprotective effects, recruit resident cardiac stem cells, reduce inflammatory

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responses and decrease fibrosis and stiffness of the scar [8–13]. BM comprises a heterogeneous population of cells, however, many of the beneficial activities have been ascribed to the mesenchymal stromal cell (MSC).

For better understanding and improvement of cellular therapy, extended pre-clinical studies are most important. The porcine heart shows close similarities with the human heart in terms of size, structure and function. In recent years, pre-clinical studies in pigs focussed on issues such as differences in the number of MSCs injected, autologous *versus* allogeneic cells injected, time of delivery of cells after MI and route of delivery [14–16]. Although culture-expanded porcine MSCs (pMSCs) were used in these studies, the characterization and knowledge on their functional potential has lagged behind, and no direct comparison between hMSC and pMSC was made. Reliable extrapolation of pre-clinical data to the clinical situation highly depends on similarities between hMSC and pMSC.

In 2006, the International Society of Cell Therapy formulated an international definition of MSC to standardize isolation and characterization of MSC [17]. The minimal criteria include: (*i*) adherence to plastic under standard culture conditions; (*ii*) expression of CD105, CD73, CD90 and a lack in expression of CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR; (*iii*) differentiation *in vitro* towards osteoblasts, adipocytes and chondrocytes. Additional features are the immunosuppressive potential *in vitro* and *in vivo* [18–21], and the possibility to enrichment MSC by selecting CD271+ cells. The immune-modulatory potential of MSC is applied in clinical trials to prevent and treat graft-*versus*-host disease and prevent graft rejection [19–21]. This capacity further intensifies the interest for MSC in cellular therapy. Upon selecting CD271+ cells from fresh BM, an enrichment in MSC frequency can be obtained, which therefore can be used as a marker for MSC [22–24].

Aim of our study was to compare pMSC *versus* hMSC in terms of phenotyping, multi-lineage differentiation potential and CD271+ and CD271– enrichment of MSC. Furthermore, we compared the immune-modulatory capacity of pMSC and hMSC *in vitro* after co-culturing with peripheral blood (PB) mononuclear cells (PBMNC). Finally, we compared the effect of pMSC *versus* hMSC transplantation on cardiac function after MI.

Materials and methods

Isolation, expansion and determination of frequency of MSC from BM

pMSC: Mononuclear cells (MNC) were obtained after ficoll separation (1.077 g/ml; GE Healthcare, Uppsala, Sweden) from BM of pigs ($\pm$6-month-old landrace pigs, 55–85 kg). Animal experiments were performed according to the '*Guide for Care and Use of Laboratory Animals*', and were approved by the Animal Ethical Experimentation Committee, Utrecht University. MNC were plated in 0.1% gelatin-coated 6-well plates (5×10^5 MNC/cm^2) as previously described [25] and cultured in medium 199 (Lonza, Verviers, Belgium) supplemented with 10% heat inactivated foetal bovine serum (HI FBS) (HyClone, Logan, UT, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (P/S) (PAA Laboratories GmbH, Clbe, Germany), 20 μg/ml endothelial cell growth factor (Roche Diagnostics, Indianapolis, IN, USA) and 8 IU/ml heparin (Leo Pharma, Breda, The Netherlands) at 37°C under 5% CO$_2$/95% air atmosphere. Additional medium for expansion: α-MEM (Gibco, Paisley, UK) with HI FBS, P/S, L-ascorbic acid-2-phosphate (0.2 mM) and bFGF (1ng/ml). After 48–72 hrs, non-adherent cells were removed and adherent cells refreshed with medium.

hMSC: hMSC were obtained from healthy BM donors after informed consent and expanded at the GMP facility of the Stem Cell Laboratory of the UMC Utrecht.

CFU-F assays: 0.5, 1.0, 2.5, 5 or 10 $\times$ 10^6 cells were plated in T25, either after ficoll separation or after lysing red blood cells from the BM (lysing buffer 8.818 g NH$_4$Cl, 1.062 g KHCO$_3$, 0.03952 g Na$_2$EDTA, for 10 min. on ice), and cultured for an additional 10 days. Subsequently, adherent cells were washed twice with PBS and fixed with ice-cold methanol for 15 min. at 4°C. To visualize colonies, cells were stained with Giemsa (Sigma-Aldrich, Zwijndrecht, The Netherlands) diluted 1:8 with H$_2$O for 15 min. at room temperature (RT) and washed twice with H$_2$O. CFU-F colonies containing at least 50 cells were scored using an inverted microscope (Zeiss, Munich, Germany).

Antibodies for flow cytometric analysis and sorting

For the screening of cultured MSC from human and pig, a panel of more than 50 antibodies (Abs) was used that contained the following commercially available Abs against human CD3, CD13, CD14, CD19, CD29, CD31, CD34, CD44, CD45, CD49e, CD55, CD71, CD73, CD90, CD117, CD133, CD146, HLA-ABC, HLA-DR [Becton Dickinson (BD), Franklin Lakes, NJ, USA], CD49b (Biolegend, San Diego, CA, USA), CD105 (Ancell Corp, Bayport, MN, USA), CD166 (clone 3A6; RDI, Concord, MA, USA), CD235a (Dako, Glostrup, Denmark), CD271 (Clone 20.4 against Low-affinity NGFR; Miltenyi Biotec, Bergisch Gladbach, Germany), Sca-1 (BD), ALP (clone B4-78-c; Hybridoma Bank, Iowa city, IA, USA), KDR (R&D Systems, Minneapolis, MN, USA), SSEA-4 (clone MC-813-70; Hybridoma Bank), W8B2 [human mesenchymal stem cell antigen-1 (MSCA-1); Biolegend], W4A5B5 (Biolegend). Additionally, a series of Abs showing reactivity with the CD271 positive subpopulation in human BM with putative MSC specificity was used; W3C4E11 (CD349; frizzled-9), W5C4, and 39D5 (CD56 epitope expressed on MSC but not on NK cells) or MY31 (CD56 on MSC and NK cells), W1C3, W6B3H10 (CD133), 24D2E2 (CD340), W5C5A8, 58B1A3, CH3A4A7AR (CD340), 67D2 (CD164), W7C5 (CD109), 67A4 (CD324), 28D4D0 (CD140b), HEK-3D6C9, W3D5A9 and CUB1 (CD318) [24]. Binding of the non-conjugated Ab was detected using isotype (IgG, IgG1, IgM or IgG3) PE-conjugated goat anti-mouse antisera (Southern Biotechnology Associates, Inc, Birmingham, AL, USA). Additional Abs which specifically recognize porcine cells (Ab Serotec, Düsseldorf, Germany) were directed against CD45 (clone K252-1E4), CD31 (clone LCI-4), SLA class I (clone JM1E3) or SLA class II DR (clone 2E9/13). The Abs against CD49d (Thermo Scientific, Rockford, IL, USA) and CD49f (GoH3, Becton Dickinson) had been reported to show cross reactivity with porcine protein. Flow cytometric analysis was performed on a FACS Calibur (Becton Dickinson, Biosciences, San Jose, CA, USA), or a Cytomics FC 500 (Beckman Coulter, Fullerton, CA, USA), while for cell sorting the FACS Aria (Becton Dickinson) was used. At least three different MSC donors were

analysed, and sorting of CD271+ cells was performed at least three times from different BM samples. 1×10^6 per BM sample and 1×10^4 cells per MSC culture were measured within a 'live cell' gate based on light scatter properties. To calculate the enrichment factors for the CFU-F after single or double Ab labelling, we divided the frequency of CFU-F in the sorted fraction by the frequency of CFU-F in the starting material, *i.e.* the MNC fraction after ficoll separation of the BM aspirates.

Osteogenic and adipogenic differentiation potential

Three different protocols were used for induction towards osteogenesis and adipogenesis; protocol A (21 days, previously used for murine MSC as well as hMSC [25, 26]; protocol B (18 days, previously used for hMSC [27]; and protocol C (10 days, for hMSC, developed by Miltenyi Biotec, Bergisch Gladbach, Germany). All differentiations were performed in triplicate in 24-well plates (2.5×10^4 cells/cm²), using MSC of passage ≥4.

Protocol A: *Osteogenic differentiation:* MSC were differentiated in basic medium (α-MEM (Gibco, Paisley, UK) supplemented with L-ascorbic acid-2-phosphate (280 μM), dexamethasone (10^{-7} M) and 5 mM β-glycerophosphate (from day 7 onwards). *Adipogenic differentiation:* Similar as for osteogenic differentiation, but medium was additionally supplemented with indomethacin (50 μM), insulin (10ug/ml) and 1-methyl-3-isobutylxanthine (IBMX, 5 μM).

Protocol B: *Osteogenic differentiation:* MSC were cultured in DMEM (Gibco, Paisley, UK) supplemented with L-ascorbic acid-2-phosphate (50 μM), dexamethasone (0.1 μM) and β-glycerol phosphate (10 mM). *Adipogenic differentiation:* MSC were cultured for 48–72 hrs in induction medium [DMEM supplemented with dexamethasone (1 μM), indomethacin (100 μM), insulin (10 μg/ml) and IBMX (500 μM)], followed by a 24-hr culturing in maintenance medium. This alternate culturing in induction and maintenance medium was repeated two times, accomplished by a culturing period of 1 week in maintenance medium.

Protocol C: MSC were cultured in media manufactured by Miltenyi [nonhaematopoietic (NH) stem cell media] for osteogenic and adipogenic differentiation.

Microscopic observations during osteogenic and adipogenic induction: To determine the extent of differentiation, cells were checked twice a week and classified as indicated in Table 2 for adipogenic or osteogenic differentiation.

Histochemical staining and quantification: After differentiation, cells were washed with PBS, fixed with 4% formalin for 10 min., and alkaline phosphatase expression was determined with a substrate solution of naph-tolphosphate AS-MX (0.2 mg/ml) and Fast Blue RR Salt (0.6 mg/ml) in TRIS buffer (0.1 M pH 8.9) with MgSO4 (0.3 mg/ml). After washing with PBS, cells were decolourized with ethanol/sodium hydroxide, and its supernatant was measured spectrophotometrically (550 nm, Bio-Rad microplate reader model 550). To detect calcium deposition, the fixated cells were stained with Alizarin Red S (2% in distilled water, pH of 5.5 with ammonium hydroxide) for 2–5 min., rinsed with PBS, and quantified by extracting Alizerin Red S from the cells with a 10% Cetylpiridimium ClPO4 buffer and measurement at 550 nm. Adipocytes were fixed, stained with Oil-Red-O (30 mg Oil-Red-O/ml 60% isopropanol) for 10 min., decolourized with ethanol, and extracts were measured spectrophotometrically at 550 nm.

Quantitative polymerase chain reaction for detecting osteogenic and adipogenic genes: Total RNA was isolated using TriPure (Roche,

Indianapolis, IN, USA), according to the instructions of the manufacturer. From 500 ng RNA, free of genomic DNA, cDNA was generated (iScript™ cDNA Synthesis Kit; Bio Rad, Hercules, CA, USA). For quantitative RT-PCR (qPCR) (Bio-Rad, MyiQ™System), cDNA was mixed with iQ™SYBR® Green Supermix (Bio-Rad) and specific forward and reverse primers (Invitrogen Ltd, Paisley, UK). Primers used for pig; for β-actin as a housekeeping gene, forward ATC CAC GAG ACC ACC TTC AA, reversed TGA TCT CCT TCT GCA TCC TG, for the adipogenic genes, aP2, forward AAC CCA ACC TGA TCA TCA CTG, reversed TCT TTC CAT CCC ACT TCT GC, and PPARγ2, forward AGG AGC AGA GCA AAG AGG, reversed AGA GTT ACT TGG TCA TTC AGG. For the osteogenic genes; osteocalcin, forward CAG GAG GGA GGT GTG TGA G, reversed TGC GAG GTC TAG GCT ATG C, alkaline phosphatase, forward CCA AAG GCT TCT TCT TGC TG, reversed TGT ACC CGC CAA AGG TAA AG, and osteopontin, forward AAG GAC AGT CAG GAG ACG AG, reversed TCA ATC ACA TTG GAA TGC TC. After normalization for β-actin (housekeeping), relative gene expression was calculated by ΔΔCt as previously described.

Chondrogenic differentiation potential

2.5×10^5 MSC were pelleted into 15 ml tubes *via* centrifugation ($800 \times g$ for 5 min.), and cultured in chondrogenic medium (high glucose (4.5 g/l) DMEM with 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenous acid, 5.33 μg/ml linoleic acid, 1.25 mg/ml bovine serum albumin (ITS+, Collaborative Research, Cambridge, MA, USA), 0.1 μM dexamethasone, 10 ng/ml TGF-β1, 50 μg/ml ascorbate 2-phosphate, 2 mM pyruvate and P/S) [24]. At harvest, the pellets were fixed in 4% formaldehyde, paraffin embedded, sectioned and analysed after staining with haematoxylin, fast green and safranin-O to visualize collagen accumulation and proteoglycans.

Immunomodulatory effect of MSC on PB

MNC were obtained after ficoll separation from porcine and human PB (pPBMNC and hPBMNC, respectively), and stained with carboxyfluorescein diacetate succinimidyl ester (CFSE, 0.6 μM/ml, 10 min. 37°C; Molecular Probes, Invitrogen Ltd). In 96-well plates, 2×10^4 pPBMNC or hPBMNC were maximally stimulated with phorbol-12-myristate-13-acetate (PMA; 50 ng/ml) in combination with pIL-2 or hIL-2 (150 IU/ml), respectively, or with OKT3 (anti-CD3 Ab; 2 ug/ml) in case of hPBMNC. Stimulation was performed in the presence or absence of MSC (2×10^4). After 6 days of culture CFSE expression of PBMNC was measured by flow cytometry [18, 28].

Myocardial infarction and MSC injection

All animal procedures were approved in accordance with the Guide for the Care and Use of Laboratory Animals, with prior approval of the Animal Ethical Experimentation Committee, Utrecht University. In NOD/SCID mice (male, aged 9–12 weeks), MI was induced by left coronary artery (LCA) occlusion as described previously [29]. Human or porcine MSCs were injected (500,000 cells per animal) in the border zone by two injections of 5 μl [30]. Cardiac parameters were determined at baseline and at 28 days post-MI. End-diastolic volume (EDV), end-systolic volume (ESV) and ejection fraction were determined 4 weeks post-MI by high resolution MRI (9.4T; Bruker Biospin, Rheinstetten, Germany) as described [31]. Analysis

was performed using Q-mass for mice digital imaging software (Medis, Leiden, The Netherlands) by a blinded investigator. Heart sections of mice at 28 days post-MI were used to quantify the fibrotic scar using picrosirius red staining with circularly polarized light microscopy [32].

Characterization of transplanted cells

Characterization of transplanted cells by immunofluorescence was performed on 7 μm cryosections. Mouse anti-integrin-b1 (1:50; SC-53711; SantaCruz, Santa Cruz, California, USA) and rabbit anti-troponin- I (1:100; ab47003; Abcam, Cambridge, USA) were used as primary Abs, followed by Alexa-labelled (Invitrogen) secondary Abs against mouse and rabbit. Sections were fixed in acetone, blocked with 2% bovine serum albumin (BSA) and incubated overnight with primary Ab. After incubation with secondary Ab for 1 hr, sections were washed in PBS and mounted in Fluoromount (Southern Biotech) after counterstaining with DAPI (Invitrogen).

Statistical analysis

Data are presented as mean $\pm$ SEM. Differences between groups were analysed by Mann–Whitney U, using a significance level of $P < 0.05$ (SPSS Statistics v17, Chicago, IL, USA).

Results

Isolation, expansion and frequency of MSC derived from porcine BM

Colonies were found 7 to 12 days after plating MNC suspension from porcine BM (Fig. 1A). After expansion of both human and
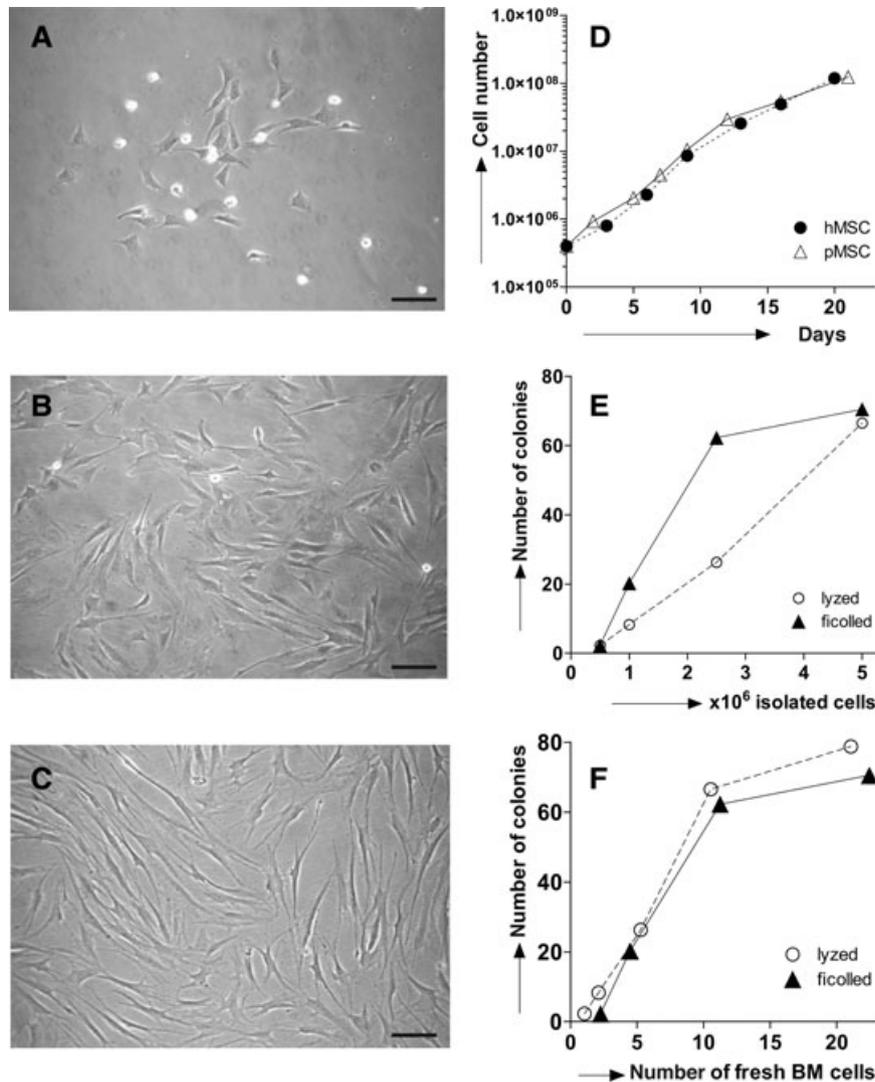
Fig. 1 Isolation and expansion of pMSC and hMSC. Colonies of different size appeared after plating porcine BM (**A**). At passage >3 pMSC (**B**) showed to be less elongated and spindle-shaped than hMSC (**C**). Growth potential of hMSC and pMSC (**D**). CFU-F assays of pMSC showing more colony outgrowth in ficolled BM than lysed BM after plating 1.0 and 2.5 $\times$ 10^6 cells (**E**). Normalized number of colonies to the original amount of fresh BM cells showing equal numbers of CFU-F between both isolation methods (**F**). Scale bar: 50 μm.

Table 1 Surface antigens expression on hMSC and pMSC

	Surface antigen	Human	Pig
Minimal definition of hMSC	CD73	+++	nc
	CD90	+++	+++
	CD105	+++	nc
	CD34	−	−
	hCD45/pCD45	−	−
	CD14	−	−
	CD19	−	nc
Additional positive markers on hMSC	CD44	+++	+++
	CD166	+++	−
	CD29	+++	+++
	CD49b	+	nc
	CD49d	+	++
	CD49f	+	+
	CD49-e	+++	− CND
	HLA/SLA class I	+++	+++
	HLA/SLA class II	−	−
	NGFR or CD271	±	±
Candidate markers to enrich for primary MSC and 'Buhring antibodies'	Stro-1	++	±
	W4A5B5	+	±
	W8B2B10	+	+
	58B1	++	++
	W5C4	++	++
	W3C4	++	+
	hCD31/pCD31	−	−
Endothelial markers	CD146	+++	+
	SSEA-4	++	nc
Other stem cell markers	SSEA−1	−	−
	c-kit	−	−
	Sca-1	−	−

Human (h) or pig (p) specific antibody; nc: no cross reactivity; CND: cross reactivity not determined; +++ almost all cells are positive; ++ 50–90% of cells are positive; + 10–50% positive cells; ± 1–9% of cells are positive; − less than 1% positive cells.

pig MSC, pMSC [$P > 3$, pMSC (Fig. 1B)] seemed to be less elongated and spindle-shaped than hMSC (Fig. 1C). No differences in growth potential were observed between hMSC and pMSC during a period of 3 weeks (Fig. 1D, population doubling times of 2.5, and 2.4 days, respectively). To determine pMSC frequency, CFU-F assays were performed (Fig. 1E). Together with a higher starting amount of porcine BM cells, an increasing number of colonies was formed. Moreover, outgrowth of ficolled BM cells resulted in a 2-fold higher number of colonies compared to that of lysed BM cells at a dose of 1 and 2.5×10^6 cells. However, after correction for the original number of fresh BM cells used, both isolation methods resulted in equal numbers of CFU-F (Fig. 1F).

Selection of CD271+ cells from human as well as porcine BM enrich for MSC frequency

Selection of CD271+ cells from human BM resulted in a significant increase in hMSC frequency following culture in a CFU-F assay (data not shown), as described before for hMSC [22, 24]. Also from porcine BM, the outgrowth of CD271+ cells resulted in a significant higher number of colonies, as compared to unsorted BM cells (Fig. S1). Although not statistically different, the number of colonies cultured from the CD271− fraction of porcine BM was less than the non-manipulated porcine BM.

Immunophenotypic characterization by flow cytometry

Culture-expanded pBM cells expressed several markers that are characteristic for hMSC (Table 1). The pMSC were positive for CD90, CD44, CD29, CD49d, CD49f, SLA class I, and negative for haematopoietic markers such as CD45, CD34, CD14. In addition, pMSC were negative for the endothelial marker CD31, positive for CD146, and had no expression of SLA class II and CD166. Unfortunately, no cross-reactivity was found on PB of pig for CD105, CD73, CD19, CD49b, ALP and SSEA4 Abs. CD271 and W8B2, antigens used to enrich primary MSC from fresh BM, were equally expressed on culture-expanded pMSC and hMSC. Other candidates, which have been used in the past to select primary MSC, such as W4A5 and Stro-1 [33, 34], were also expressed on culture-expanded MSC, however in lower percentages on pMSC than on hMSC.

Adipogenic differentiation potential

Several differentiation protocols are used for hMSC differentiation, but optimal conditions for differentiation of pMSC are currently not known. Therefore we included three different protocols and determined its effect on MSC differentiation. As for hMSC, diversity in differentiation potential was also observed for pMSC. Differentiation efficiency depended on the protocol used, but was also donor dependent (Table 2). Protocol A showed the largest potential for inducing adipogenic differentiation for both

Table 2 Diversity in adipogenic or osteogenic differentiation for human and pig MSC

hMSC	Adipogenic differentiation			Osteogenic differentiation		
Protocol	A	B	C	A	B	C
donor 1: P4	+++	ND	ND	−	ND	ND
donor 2: P3	++++	+++	(+)	++	+	(+)
donor 3: P3	++++	+++	++	++	−	+++
donor 4: P7	+++	++	ND	+++	−	ND
donor 5: P4	+	++	(+)	(+)	+	+++
donor 6: P6	+++	++++	++	++	(+)	(+)
donor 7: P5	++++	++	(+)	+++	++	++
pMSC	**Adipogenic differentiation**			**Osteogenic differentiation**		
Protocol	A	B	C	A	B	C
donor 1: P3	+++	ND	ND	−	ND	ND
donor 2: P6	++++	++	++++	−	−	ND
donor 3: P17	++	+++	+	+	−	+++
donor 5: P9	(+)	+	+	(+)	+	+++
donor 6: P4	++++	+++	+++	++	+++	(+)
donor 7: P6	+++	+	(+)	++++	+++	+++
donor 8: P3	+	++	(+)	++	++	(+)

Large variations were found within one protocol (donor variation), as well as within one particular donor when differentiated with protocol A, B or C (protocol variation). −: no differentiation observed; (+): very less differentiation; +: some differentiation; ++: obvious differentiation; +++: strong differentiation; ND: not determined.

human and porcine MSC. Adipocytes were clearly formed in pMSC cultures during induction with protocol A, B or C as indicated by the accumulation of fat droplets. Interestingly, differentiating pMSC also started to excrete fat (Fig. 2A arrow and right panel), which did not occur in hMSC (Fig. 2B; intracellular accumulation). The optimal differentiation for pMSC was already reached at 7–10 days (Fig. 2C), while an additional period of 10–14 days was needed for hMSC (Fig. 2E). Protocol A, B or C did not show significant differentiation differences for pMSC or hMSC, confirming microscopic observations (Table 2). Due to fat excretion after the optimal differentiation period, intracellular lipid accumulation within pMSC had decreased at week 3 as compared to hMSC differentiation. Therefore, quantitative measurements following decolourization of Oil-Red-O-stained adipocytes are an underestimation of the actual fat that was formed by pMSC (Fig. 2D and F). Undifferentiated hMSC (data not shown) and adipogenic-induced human fibroblasts (Fig. 2G) did not show any positive staining. PPARγ2 and aP2 mRNA expression, indicative of adipogenic differentiation, were increased in all three protocols (Fig. 2H). Based on aP2 mRNA expression, protocol A and B displayed the highest induction. PPARγ2 expression levels were not statistically significant between the three protocols.

Osteogenic differentiation potential

By combining the results of all differentiated donors, protocol A and C showed a trend for optimal osteogenic differentiation in hMSC (Fig. 3C and D), and protocol C for pMSC (Fig. 3A and B), confirming the observed microscopic data (Table 2). This was further confirmed by alkaline phosphatase (ALP) staining (data not shown). Osteogenic differentiation of human fibroblasts did not result in the formation of osteoblasts (Fig. 3E). Osteopontin and ALP mRNA expression after osteogenic induction of pMSC were highly increased (Fig. 3F), in favour of protocol B. Osteocalcin expression was enhanced after induction with protocol C, but not after induction with protocol A or B.

Chondrogenic differentiation potential

After 3 weeks of induction, chondrogenic differentiation of pMSC resulted in pellets that were larger than those from hMSC (Fig. 4). Since cell proliferation does not further occur during these culture conditions, the increase in pellet size is due to the formation of additional extracellular matrix or hypertrophic cell growth. Fast green staining, indicating collagen accumulation, was found for
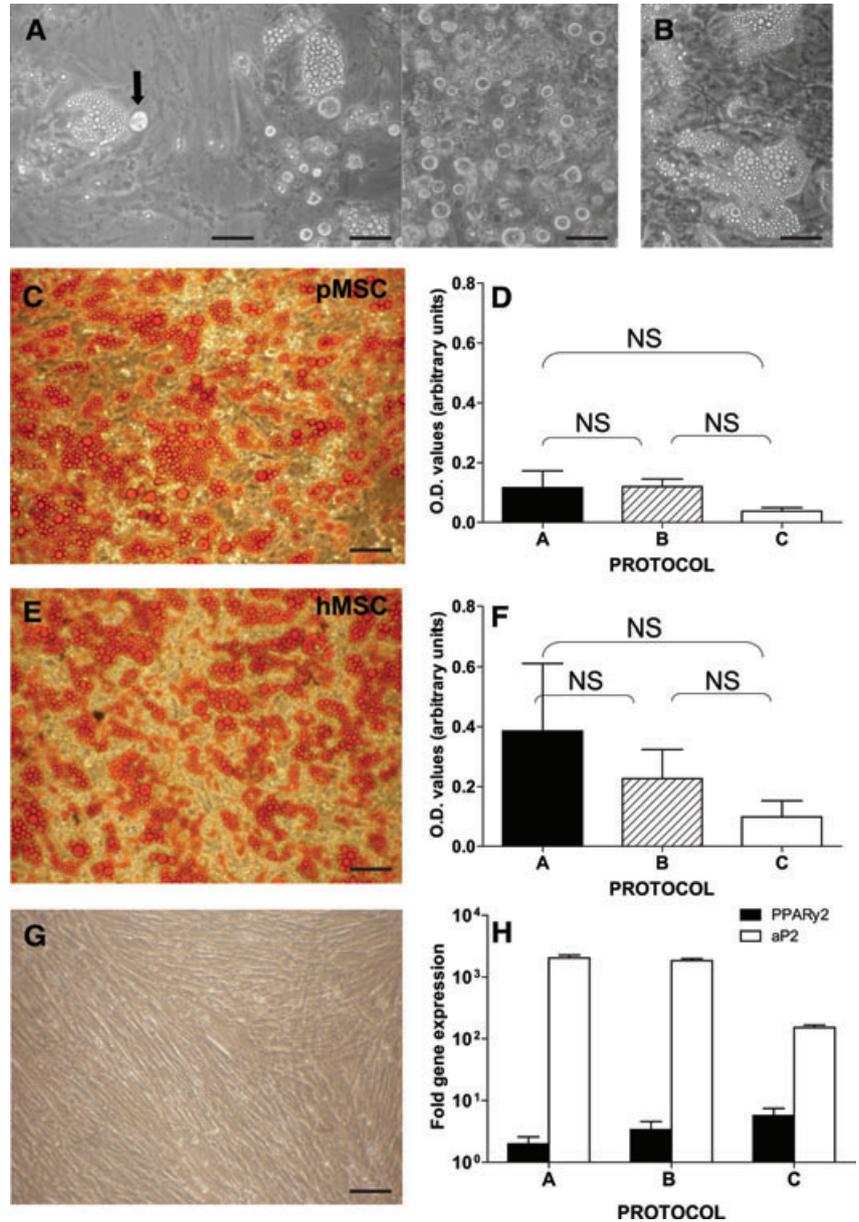
Fig. 2 Differentiation of pMSC and hMSC towards adipocytes. Representative porcine donor showing fat accumulation (**A**) and large amounts of fat excretion during differentiation (arrow and right part of A). During hMSC differentiation, fat was accumulated but *not* excreted (**B**). Consequently, fat accumulation during pMSC differentiation had decreased at week 3 as compared to hMSC differentiation (**C** and **E**). Quantitative measurements of Oil-Red-O-stained adipocytes ($n = 3$–5) by pMSC and hMSC (**D** *versus* **F**). Human fibroblasts were not able to differentiate into adipocytes (**G**). mRNA levels of PPARγ2 and aP2 ($n = 4$) are increased upon adipogenic differentiation in pMSC (**H**). Scale bars: 25 μm (**A** and **B**) and 50 μm (**C**, **E**, **G**).

both human (left in Fig. 4A and B) and pig MSC (Fig. 4C–F). One particular pMSC donor showed massive chondrogenic differentiation, with clear lacunes formed (Fig. 4C and D).

Immunomodulatory effect on stimulated peripheral blood

Stimulation of human PB with OKT3 resulted in blast-like cells with a significant increase in size and granularity as compared to the non-stimulated cells (Fig. 5A–D) and showed several cell divisions at day 6 of stimulation (Fig. 5D, gates a-e). Non-stimulated cells were small, with no proliferation induced and a high CFSE staining (Fig. 5A and B). Combined stimulation of human and porcine PB with PMA and IL-2 (human and pig) resulted in a significant number of dividing cells (Fig. 5F and H, respectively); 52% hPBMNC divided 4 times, and 72% pPBMNC divided 5 times. In the presence of hMSC (10%) or pMSC (13%), division rates were significantly lower than in absence of MSC (Fig. 5G and I).
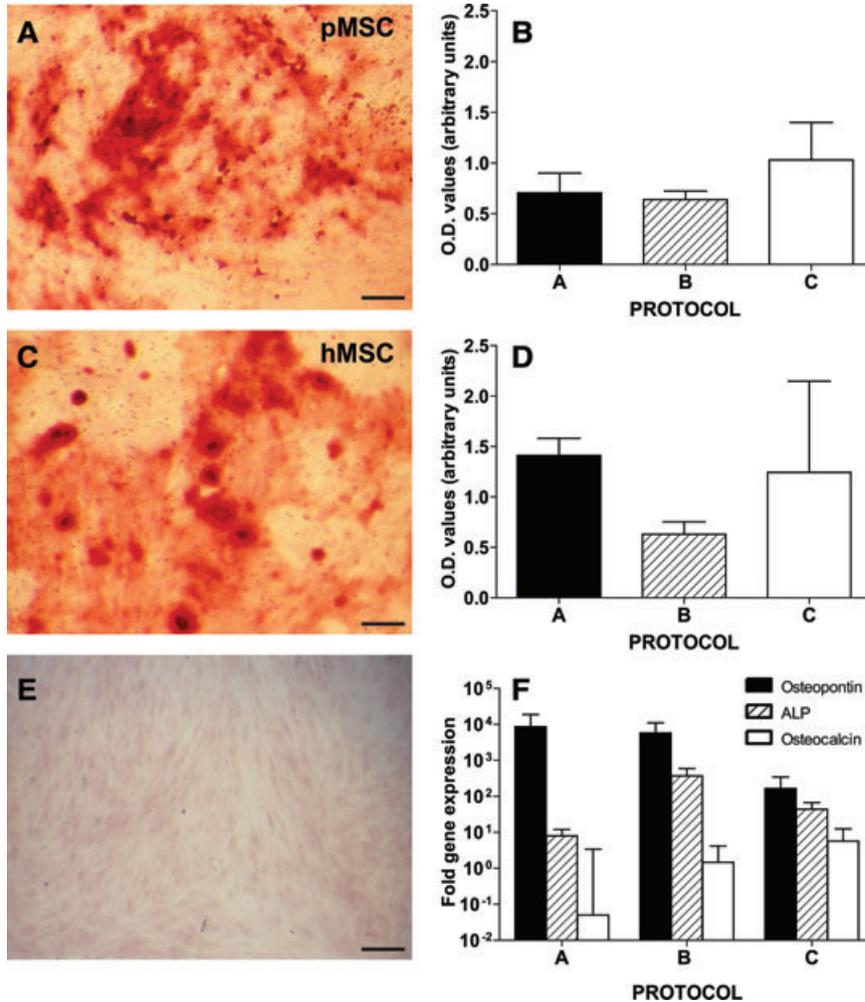
Fig. 3 Differentiation of pMSC and hMSC towards osteoblasts. Representative pictures of Alizarin Red S staining of pMSC and hMSC differentiation towards osteoblasts (**A** and **C**). After induction of differentiation ($n = 3$–5 per protocol) large differences were observed within one protocol (donor variation), but also among the protocols as illustrated by the variation in quantitative measurements of the different protocols (**B**, pMSC; **D**, hMSC). Human fibroblasts were not able to differentiate into osteoblasts (**E**). mRNA levels of osteopontin, alkaline phosphatase (ALP) and osteocalcin ($n = 4$) upon osteogenic differentiation in pMSC (**F**). Scale bar: 50 μm.

Cardiac function and remodelling after MSC transplantation

MI was induced and mice received injections of either PBS ($n = 5$), pMSC ($n = 4$) or hMSC ($n = 4$) in the border zone of the infarct. In all groups, both EDV and ESV increased significantly post-MI when compared to baseline (Fig. 6A and B). However, pMSC- and hMSC-injected animals exhibited less cardiac remodelling, demonstrated by a significantly smaller EDV (132.4 ± 18.0 μl pMSC; 137.6 ± 18.3 μl hMSC; $P < 0.05$) and ESV (105.8 ± 19.7 μl pMSC; 115.9 ± 18.1 μl hMSC; $P < 0.05$). Cardiac function significantly declined in all groups (Fig. 4C), which was most pronounced in PBS-injected animals (EF 11.7 ± 1.0%). Compared to PBS, pMSC transplantation significantly conserved EF (21.4 ± 4.8%; $P < 0.05$). Although borderline significant, this was similar in hMSC-injected animals (16.4 ± 2.6%; $P = 0.05$). Surviving MSCs were very low at 28 days post-MI and mainly

resided in the infarcted area (Fig. S2). In accordance with less adverse remodelling, area of myocardial fibrosis (scar area) was less in MSC-transplanted animals (Fig. S3).

Discussion

Although pre-clinical studies in pigs have been performed to improve our understanding of the effects of MSC therapy [15, 16, 35, 36], the characterization of pMSC has lagged behind. The comparison of pMSC with hMSC is necessary to reliably extrapolate pre-clinical data on pMSC therapy, which highly depends on similarities between porcine and human MSC. This is illustrated by the fact that clear differences in murine MSC were found depending on mice strains, required growth media, growth rates and presence of surface epitopes [37]. The expression of markers on
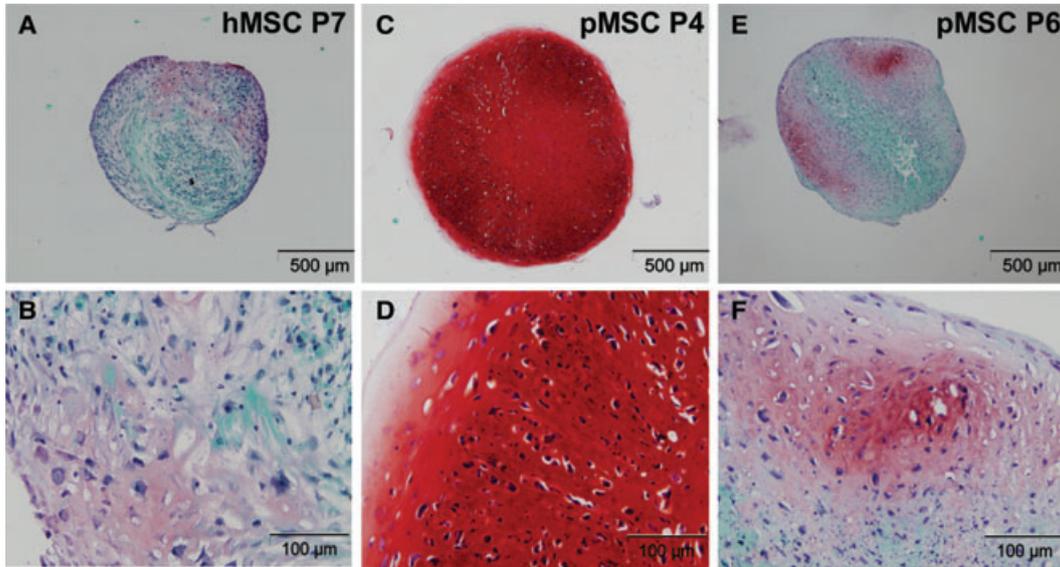
Fig. 4 Differentiation of pMSC and hMSC towards chondrocytes. pMSC show impressive differentiation towards chondrocytes (**C** and **F**), forming larger pellets than differentiated hMSC (**A** and **B**) and more extracellular matrix formation (**B**, **D**, **F**) illustrated by intense collagen staining (fast green staining) and proteoglycans (safranin-O).

hMNC stimulated with PMA + h-IL2			pMNC stimulated with PMA + p-IL2		
%dividing	-hMSC	+hMSC	%dividing	-pMSC	+pMSC
1st div	28.9	52.6	1st div	7.0	23.3
2nd div	8.3	22.2	2nd div	4.9	22
3rd div	7.6	11.6	3rd div	5.7	18.8
4th div	51.8	10.3	4th div	9.2	21.8
			5th div	72.4	12.6
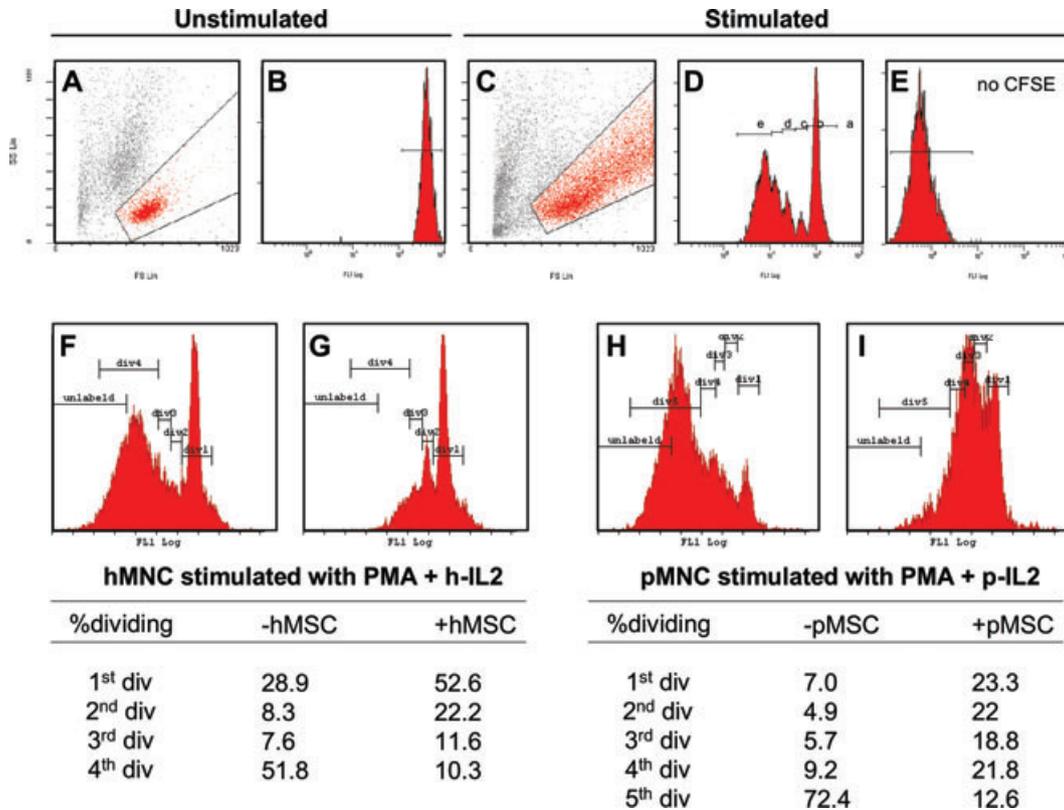
Fig. 5 pMSC suppress proliferation of T cells from pig. Unstimulated human PBMNC were small and CFSE staining was high (**A** and **B**). Stimulation resulted in an increase in size and proliferation (**C** and **D**). Unstained stimulated cells showed no CFSE staining (**E**). Combinational stimulation (PMA and IL-2) of hPBMNC (**F**) and pPBMNC (**H**) resulted in significant numbers of cells in 4th or 5th division after six days, which was markedly decreased in the presence of hPBMNC or pPBMNC (**G** and **I**).
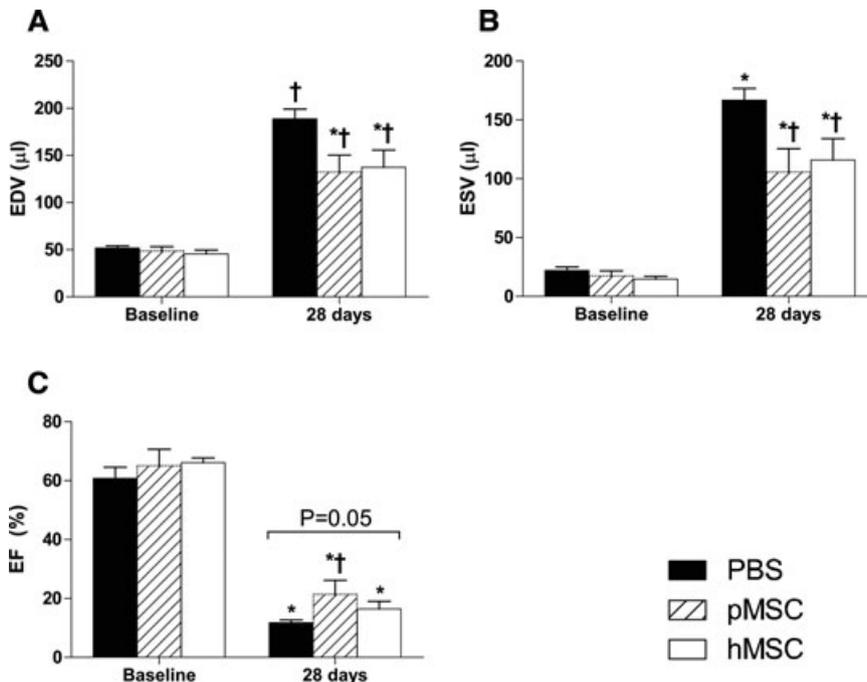
Fig. 6 Cardiac geometry and function after MSC transplantation. Animals injected with pMSC and hMSC exhibited less cardiac remodelling as indicated by significantly smaller EDV (**A**) and ESV (**B**) when compared to PBS-injected animals 28 days post-MI. pMSC transplantation improved EF when compared to PBS (**C**), with hMSC showing borderline significant improvements ($n = 4$–5/group, *$P < 0.05$ *versus* baseline, †$P < 0.05$ *versus* PBS).

pig MSC, and differentiation to osteoblasts, adipocytes and chondrocytes have been described before [38–40]. However, a direct comparison of pMSC with hMSC for all the described MSC features is still lacking.

In our study, we therefore compared the immune-phenotype of pMSC and hMSC, as well as their multi-lineage differentiation potential using various differentiation protocols. Moreover, we demonstrated for the first time that isolation of CD271 (NGFR)+ cells from fresh porcine BM enrich for MSC outgrowth, and that both pMSC and hMSC have immunosuppressive properties. Finally, we showed that pMSC transplantation improved cardiac function and attenuated remodelling to similar extent as hMSC after MI in mice.

Small morphological differences were observed in culture; pMSC seemed to be less elongated and spindle-shaped than hMSC. Both cell types exhibited similar growth expansion potential following culture. Concerning the surface markers studied, hMSC and pMSC showed similar expression for most of them. However, for several markers we did not find cross-reactivity (CD105, CD73, CD19, CD49b, ALP and SSEA4), since most of the Abs are directed to human. Unfortunately, we could not find porcine-reactive Abs for most of these epitopes.

It is known that a significant enrichment in MSC can be obtained by selecting CD271+ cells from fresh human BM. We show for the first time that selecting CD271+ cells from porcine BM resulted in enrichment of MSC. This surface antigen seems to represent a true MSC marker present on primary MSC, which is well conserved among species and decreases in expression following culture-expansion [41]. In the same report, cross-reactivity

on MSC for other Abs was shown, including CD271, W8B2, W4A5, CD56, W3C4 (CD349), W5C4 and 58B1. In line with our findings, this might suggest that the minimal criteria for MSC as defined in 2006 need further adjustments [17].

We used three different protocols for adipocytes and osteoblast differentiation, because a general consensus on the optimal differentiation conditions for these cells was not known. Therefore, three culture media were selected, which are commonly used for both porcine and human MSC differentiations to make a fair comparison possible. We showed that pMSCs were able to differentiate to the osteogenic, adipogenic and chondrogenic lineages. Adipogenic differentiation kinetics of pMSC were faster than that of hMSC, and fat accumulated initially in the adipocytes itself, but was subsequently secreted. This secretion of fat was not observed in hMSC differentiation. From our results we can conclude that pMSCs do not need other induction-stimuli for osteoblast/adipocyte differentiation than those used for hMSCs. Nevertheless, variations in differentiation potential for pMSC as well as for hMSC donors were observed among and within protocols.

Diversities in differentiation potential are already known for human MSC as well as for mice MSC [42], depending on the heterogeneity of the starting population and their subsequent expansion. Attempts to define the starting population more precisely with limited differentiation potential for specific applications, thereby enriching for naïve MSC or a population of choice, have been described previously [23, 33, 43–46]. Recently, human BM cells were selected, expressing CD271 and W8B2, with no expression for CD56, which differentiated preferentially to adipocytes in

contrast to CD271+W8B2+CD56+ cells, showing primarily chondrocytic differentiation [23].

In the present study, we demonstrate that pMSCs, like hMSCs, also have potent immunosuppressive capacities. The immunosuppressive effect of MSC has been ascribed to a non-specific anti-proliferative effect [47]. However, if host MSC and allogeneic donor BM cells were combined for transplantation, engraftment of transplanted cells was enhanced [48]. When transplanted MSC and BM cells were both of an allogeneic donor, engraftment was not significantly improved, suggesting that MSCs are not intrinsically immuno-privileged and trigger immune responses *in vivo.* This underlines the need for autologous pMSC in pre-clinical models in order to prevent an immune response, thereby enhancing engraftment of donor cells.

We also compared the effect of pMSC with hMSC transplantation on functional improvement after MI *in vivo*, another important feature of MSC. Transplantation of pMSC significantly improved cardiac function and attenuated adverse remodelling to a similar extent as hMSC transplantation, accompanied by less myocardial fibrosis. Since we could hardly see transplanted cells connecting with viable myocardial cells, we do not think that physical and electronic integration is the explanation of the observed effects, rather a paracrine benefit for the endogenous cells. Although the exact mechanism (paracrine effects or differentiation) remains a matter of debate [49, 50], pMSC performed equally well as hMSC. Therefore, we indirectly assumed that pMSC and hMSC might produce the same (cocktail of) growth factors.

In conclusion, we found that pMSCs were similar in phenotype and multi-lineage differentiation, which did not require different osteoblast/adipocyte differentiation protocols when compared to hMSC, although some phenotypical differences do exist between the MSC populations. We demonstrated for the first time that isolation of CD271+ (NGFR+) cells from fresh porcine BM enriches for MSC and that pMSCs have comparable immunosuppressive properties *in vitro*. Finally, we showed that pMSC transplantation after MI exhibited a similar cardiac improvement as hMSC *in vivo*.

These findings demonstrate that pMSCs are comparable with hMSCs, making reliable functional and safety extrapolation of future porcine pre-clinical studies on cellular therapy to the clinical situation possible.

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Author contributions

W.N., M.O., H.R., D.F., S.J., D.S. and B.N. performed the research; W.N., P.D. and J.S. designed the research study; W.N., M.O., D.F., S.J., D.S. and J.S. analysed the data; W.N., M.O., H.R., H.B. and J.S. wrote the manuscript; A.M., H.B., P.D. and J.S. approved the final manuscript.

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Conflict of interests

The authors confirm that there are no conflicts of interest.

Supporting Information

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

Fig. S1 Selection of CD271+ cells enrich for MSC. CD271+ cells were selected from porcine bone marrow using flow cytometry (**A**). CFU-F assay showing more colonies (**B**) after selection for CD271 compared to both unselected and CD271-negative bone marrow ($n = 6$–10, *$P < 0.05$ *versus* baseline, †$P < 0.05$ *versus* CD271– cells).

Fig. S2 Surviving MSCs at 28 days after transplantation. Representative immunofluorescent picture at 28 days post-MI, showing very few surviving human MSCs after transplantation (**A**) and at higher magnifications (**B–D**). MSCs (β1-integrin, green) showed no troponin I (red) staining and a connection with surviving myocytes was not observed. Scalebar 20 μm.

Fig. S3 Cardiac fibrosis. Picrosirius red staining ($n = 4$–5/group) showed a significant smaller scar size in the infarct of MSC-treated animals compared to PBS-treated animals at 28 days post-MI ($P < 0.05$). Scalebar 20 μm.

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