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Chapter 1:

Direct comparison of Wharton Jelly and bone marrow derived mesenchymal stromal cells to enhance engraftment of cord blood CD34+ transplants.

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Abstract

Co-transplantation of CD34+ hematopoietic stem and progenitor cells (HSPC) with mesenchymal stromal cells (MSC) enhances HSPC engraftment. For these applications, MSC are mostly obtained from bone marrow. However, MSC can also be isolated from the Wharton's jelly (WJ) of the human umbilical cord. This source, regarded to be a ‘waste product’, enables relatively low cost MSC acquisition without any burden to the donor. Here, we evaluated the ability of WJ MSC to enhance HSPC engraftment. First, we compared cultured human WJ MSC with human bone marrow-derived MSC (BM MSC) for in vitro marker expression, immunomodulatory capacity and differentiation into three mesenchymal lineages. Although we confirmed that WJ MSC have a more restricted differentiation capacity, both WJ MSC and BM MSC expressed similar levels of surface markers and exhibited similar immune inhibitory capacities. Most importantly, co-transplantation of either WJ MSC or BM MSC with CB CD34+ cells into NOD-SCID mice showed similar enhanced recovery of human platelets and CD45+ cells in the peripheral blood and a 3-fold higher engraftment in the BM, blood and spleen six weeks after transplantation when compared to transplantation of CD34+ cells alone. Upon co-incubation, both MSC sources increased the expression of adhesion molecules on CD34+ cells, although SDF-1-induced migration of CD34+ cells remained unaltered. Interestingly, there was an increase in CFU-GEMM when CB CD34+ cells were cultured on monolayers of WJ MSC in the presence of exogenous thrombopoietin, and an increase in BFU-E when BM MSC replaced WJ MSC in such cultures. Our results suggest that WJ MSC is likely to be a practical alternative for BM MSC to enhance CB CD34+ cell engraftment.
Introduction

Cord blood (CB) is used as an alternative source for hematopoietic stem-and-progenitor cell (HSPC) transplantation. However, the successful outcome of CB transplantation is limited by the relatively low number of transplantable HSPC in these grafts, which results in delayed hematopoietic recovery post-transplant. Double CB transplantation in this respect increases the number of transplantable HSPC, but the time to recovery of donor neutrophils and platelets in the peripheral blood post-transplant is still inferior to transplantation of bone marrow (BM) or mobilized peripheral blood (mPB) grafts. One method to overcome this CB associated disadvantage is to enhance the engraftment of HSPC by co-transplantation of accessory cells such as mesenchymalstromal cells (MSC). MSC were first identified in BM as multipotentcells and characterized largely by in vitro attributes. These included their ability to differentiate into mesodermal cells, such as adipocytes, chondrocytes and osteoblasts, their adherence to plastic and their expression of specific cell surface markers. In addition, MSC have the capacity to modulate immune responses. Interestingly, in animal models, co-transplantation of human CB-derived CD34+ cells with human MSC was shown to improve hematopoietic engraftment. Both local and systemic mechanisms may play a role in this latter process, for example, by the MSC promoting homing to the bone marrow or its vasculature or releasing proangiogenic, immunomodulatory or growth factors that promote engraftment. Although originally identified in cultures obtained from bone marrow aspirates, MSC can also be isolated from other sources such as adipose tissue, compact bone, amniotic fluid, cord blood, the umbilical cord or the placenta. MSC cultured from Wharton’s Jelly (WJ MSC) of the umbilical cord display unique characteristics such as a greater expansion capacity and faster in vitro growth compared to BM MSC. Moreover, WJ MSC have some logistical advantages over BM MSC. Notably, the umbilical cord is considered a waste product and WJ MSC can therefore be obtained from this source at relatively low cost and without burden to the donor. The WJ could therefore be a promising source for the clinical application of MSC. With this in mind, we set out to compare the effect of co-transplantation of human CB-derived CD34+ cells with either BM or WJ MSC on hematopoietic engraftment in immune deficient NOD-SCID mice. Furthermore, we assessed whether co-transplantation of WJ MSC that were autologous to the CB CD34+ cells affected this engraftment when compared to co-transplantation with allogeneic WJ MSC.

Materials and methods

Umbilical cord blood (CB) and umbilical cord collection. CB was drawn from the umbilical vein at birth at >36 weeks gestation after written informed consent from the mother at hospitals in the Netherlands according to Netcord–FACT standards and with ethical permission from the medical ethical board of the Leiden University Medical Center (LUMC), Leiden, The Netherlands. Blood was collected by gravity drainage into Macopharma collection bags containing 21 ml Citrate Phosphate Dextrose Adenine-1 (Macopharma, Utrecht, The Netherlands). The blood was stored at 4°C and processed within 48 hours of collection. Umbilical cords were collected concomitantly with the CB in a sterile container containing PBS with 1% (v/v) antibiotic/antimycotic mix (Life Technologies, Woerden, The Netherlands).
CD34+ cell purification. Mononuclear cells were isolated from CB using a sterile Ficoll density gradient (1.077 g/cm³, Pharmacy LUMC, Leiden, The Netherlands). The CD34+ cell fraction was isolated from the mononuclear cell fraction by double CD34+ cell selection using immunomagnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the isolated CD34+ cell fraction was verified by flow cytometry (Beckman Coulter, Woerden, The Netherlands) using CD45-PITC and CD34-PE antibodies (all Beckman Coulter, ISHAGE protocol 27), and was higher than 90% for all CD34+ cells used throughout the experiments. Cells were cryopreserved in IMDM with 10% (v/v) DMSO and 4% (w/v) human serum albumin (Pharmacy LUMC) and stored at -150°C until use.

MSC isolation and culture. Umbilical cord-derived MSC: MSC were isolated with an explant method as described in DeBruyn et al. 28 Briefly, the cords were cut into 5 cm segments and then longitudinally and the vein and arteries tissue removed. The segments were placed on 10 cm culture dishes (Greiner, Alphen a/d Rijn, The Netherlands) with the inside of the cord, i.e. the Wharton Jelly, facing the bottom of the plate. MSC medium (DMEM supplemented with 15% (v/v) FBS and 1% (v/v) Antibiotic-Antimycotic solution, all Life Technologies) was added to the plate until the segments were submerged in medium. The culture plates were placed in a humidified incubator at 37°C and 5% CO2. Medium was refreshed every 3 days. After 10 days, the segments were removed and the MSC adhering to the plate were grown to confluence and passaged into culture flasks. Since we did not separate the (sub)amnion an the Wharton Jelly, we cannot exclude that a small part of the MSC derived from the (sub)amnion. However in line with the original description of this method by the De Bruyn et al 28 we decided to use the cells WJ MSC throughout the manuscript. BM-derived MSC: BM was collected from patients undergoing knee or hip replacement surgery at the LUMC with informed consent of the donor and with ethical permission from the medical ethical board of the Leiden University Medical Center (LUMC). Mononuclear cells were isolated from the BM suspensions by gradient centrifugation with Ficoll (1.077 g/ml, pharmacy LUMC) and loaded into culture flasks containing DMEM with 10% (v/v) FBS and 1% (v/v) Penicillin and Streptomycin (all Life Technologies). After overnight culture in a humidified incubator at 37°C and 5% CO2, non-adhering cells were washed from the flask with PBS. Adherent cells were grown to confluence and passaged. After 3 passages, cells were cryopreserved in FBS with 10% (v/v) DMSO (pharmacy LUMC). The MSC that were used throughout this study were between passage 3 and 6.

Flow cytometry. Flow cytometry analysis for cell surface marker expression was performed with a Beckman Coulter FC500 or a BD FACSCalibur running CXP or CellQuest Pro software respectively. Isolated CD34+ cells were analyzed for the expression of CD34 and CD45 (both from Beckman Coulter) and MSC were analyzed for the expression of CD105, CD90, CD80, CD73, CD45, CD34, CD31, HLA-ABC and HLA-DR (all from BD Biosciences).

Differentiation of UC and BM MSC into mesodermal lineages. The WJ MSC and BM MSC were analyzed for their ability to differentiate into adipocytes, chondrocytes and osteoblasts as described previously. 29,30 In brief, MSC were cultured in specific adipogenic, chondrogenic and osteogenic differentiation media. After 21 days, the osteogenic cultures were analyzed for the presence of osteoblasts by staining of calcium deposits with Alizarin red and alkaline phosphatase with fast blue. In the adipogenic cultures, lipid droplets were visualized with Oil red O staining and, in the chondrogenic cultures, cells were stained with Toluidine blue. We used an arbitrary scoring system that
assesses the degree of differentiation in the cultures. Cultures showing no differentiated cells were scored as 0, a few differentiated cells as 1, moderate differentiation as 2 and full differentiation as 3 (Figure 1).

**Gene expression of adipogenic and osteogenic differentiation cultures.** BM MSC and WJ MSC were cultured for 2 weeks in specific adipogenic and osteogenic differentiation media or normal MSC medium (control). The cells of the cultures were lysed and RNA was isolated using the DirectZol RNA miniprep kit (Zymo Research, Irvine, USA). cDNA was subsequently prepared using the High Capacity RNA to cDNA kit (Life Technologies, Grand Island, USA). Expression of osteogenic and adipogenic genes was analyzed using TaqMan Gene Expression assays (see below) and the ViiA 7 Real-Time PCR System (Life Technologies). Relative expression of the genes was calculated with the ΔΔCt method normalized to RPL13a. Osteogenic genes: Runt-related transcription factor 2 (RUNX2), Osterix, (OSX), Osteocalcin (OC), Bone Morphogenetic Protein 2 (BMP2). Adipogenic genes: Peroxisome Proliferator-Activated Receptor-gamma (PPARg), Fatty Acid Binding Protein 4 (FABP4), Perilipin (PLIN).

**Figure 1:** Scoring system of the level of differentiation of WJ MSC or BM MSC into 3 mesodermal lineages. The amount of staining was assessed for all different donors after differentiation cultures and compared to reference stains to determine the level of staining. 0=no differentiated cells, 1= <20% differentiated cells, 2= <60% differentiated cells, 3= >60% differentiated cells.
Immune inhibition of adult PBMC and CB MNC by WJ MSC and BM MSC. To analyze the effect of MSC on the proliferation of mononuclear cells obtained from adult peripheral blood (PBMC), $1 \times 10^5$ PBMC were cultured for 5 days in 24 well plates with αCD3αCD28 beads (Life Technologies) alone or in combination with different concentrations of BM- or WJ-derived MSC in a fully humidified incubator at 37°C and 5% CO₂. Cell proliferation was measured by $^3$H-thymidine incorporation.

Co-culture of MSC and CB CD34⁺ cells with TPO. MSC obtained from BM and WJ were thawed and plated into a 24 well plate at 1.25x10⁵ cells/well and grown overnight in MSC medium (DMEM with 10% (v/v) FCS). After 24h, the MSC were irradiated (10 Gy) and the cells were washed twice with PBS. CB CD34⁺ cells were added to the wells at 10⁵ cells/well and cultured in expansion medium with Nplate (50ng/ml, TPO analog, Amgen, Breda, the Netherlands) as described previously. After 10 days of culture, the hematopoietic cells were harvested by collecting all non adherent cells by aspirating the supernatant, washing the plates with PBS and spinning down the collected cell suspension. The cells were counted and analyzed for the expression of CD34-PE, CD61-PE-Cy7 and CD45-FITC (all Beckman Coulter) by flow cytometry. The hematopoietic stem and progenitor cells (HSPC) that were cultured were subsequently analyzed for their capacity to generate myeloid colonies in Methocult (Stemcell Technologies, Vancouver, Canada) as described previously.

Transwell migration experiments. Four different cell suspensions were prepared. These were identical to the in vivo experiment described below. After 30 minutes of incubation of CB CD34⁺ cells with MSC, part of the cell suspension was analyzed for the expression of CD34, CD11a, CD11b, CD184, CD49e and CD49d (antibodies all from Beckman Coulter) using flow cytometry. The remaining cells were used for migration experiments in transwell plates (Costar, (VWR) Amsterdam, The Netherlands). 6.5 mm diameter with 5 μm pore filters. The lower compartment of the well and the filter were coated with 2 ng/ml fibronectin (Sigma, St Louis, USA) for 15 minutes at 37°C. The lower compartments of the plates were loaded with IMDM and 100ng/ml SDF-1α (R&D Systems, Abingdon, UK). All cells were placed in the upper compartment of the plate and incubated for 5 hours at 37°C and 5% CO₂ in a humidified incubator. After incubation, the numbers of CD34⁺ cells that were harvested from the lower compartment were counted to determine the proportion of cells that migrated.

Transplantation in NOD-SCID mice. Female 5-6-week old NOD-SCID mice (Charles River, l’Arbresle, France) were kept in micro-isolator cages in laminar flow racks in the LUMC animal facilities. The animal ethical committee of the LUMC approved all animal experiments. NOD SCID mice received 3.5 Gy total body irradiation 24 hours before transplantation. Mice were transplanted with 1x10⁵ CB-derived CD34⁺ cells alone or in combination with 1x10⁶ MSC through tail vein injections. Peripheral blood was collected from the tail vein at weekly intervals starting 3 weeks after transplantation. Blood collection and human platelet measurements were performed as described previously [32]. Briefly, human platelets were stained with a non-cross reactive mouse-anti-human CD41-PE and mouse anti human CD45-PC7 (both Beckman Coulter). Erythrocytes were lysed with IOTest3 lysing solution (Beckman Coulter) for 10 min. at room temperature. Flow-Count™ fluorospheres (Beckman Coulter) were added to the cells to enable analysis of the absolute number of circulating human platelets. Analysis was performed by flow cytometry (FC500, Beckman Coulter) using CXP software. Six weeks after transplantation, mice were sacrificed and bone marrow cells were obtained by flushing femurs with IMDM.
Next, human cell engraftment and multilineage chimerism were analyzed by flow cytometry using goat-anti-mouse-CD45-PE (LCA, Ly-5, 30-F11, Pharmingen, Erebodegem, Belgium), mouse-anti-human CD45-FITC, CD33-FITC, CD34-PE, CD19-PE (all from Beckman Coulter) and the appropriate isotype controls. Erythrocytes were lysed with IO Test3 Lysing solution (Beckman Coulter). Analysis was performed by flow cytometry (FC500, Beckman Coulter) using CXP software.

Statistics. All statistics were done using IBM SPSS Statistics (version 20, www.ibm.com/SPSS_Statistics). Results are presented as mean ± S.E.M. To test for statistical significance, a Mann-Whitney test or one way ANOVA was used. Results were considered to be significant if the p-value was equal to or less than 0.05.

Results

**WJ-derived MSC have similar marker expression but limited differentiation potential compared to BM-derived MSC**

Following isolation and subsequent expansion, the phenotype of the WJ MSC was determined and compared to BM MSC (Figure 2A). Similar to BM MSC, WJ MSC expressed HLA-ABC, CD73, CD90 and CD105 and lacked expression of the pan hematopoietic marker CD45 and the endothelial/hematopoietic marker CD31. A small population of BM MSC expressed HLA-DR (BM MSC 6.2±4.2% vs WJ MSC 0.2±0.1%); while a small subset of WJ MSC expressed CD34 (WJ MSC 3.8±0.3% vs. BM MSC 0.6±0.4%). We next analyzed the ability of BM MSC and WJ MSC to differentiate into adipocytes, chondrocytes and osteoblasts. The majority of BM-derived MSC isolates had the capacity to differentiate into chondrocytes and adipocytes and half of the BM MSC isolates differentiated into osteoblasts. In contrast, more than 25% of the WJ MSC isolates (4 out of 18) showed adipocytes generation and less than 15% of the WJ MSC isolates (2 out of 18) showed osteoblast differentiation (Figure 2B and C). Moreover, in these respective 4 and 2 cultures adipocyte and osteoblast differentiation was sporadic (grade 1). Chondrogenic differentiation occurred in half of the WJ isolates (9 out of 18). Thus, although BM MSC and WJ MSC are phenotypically similar, WJ MSC are limited with respect to their capacity to differentiate into mature mesodermal cell types as shown by their lower degree of differentiation compared to BM MSC.

For one MSC donor of each type of MSC, these functional differences were also investigated by the comparison of differentiation specific gene expression. As shown before, no osteoblast or adipocyte specific staining was observed in the WJ MSC culture, whereas positive staining for both cell types could be found in the BM MSC cultures (Figure 3A). The expression of the adipocyte related genes peroxisome proliferator-activated receptor-γ (PPARG), fatty acid binding protein 4 (FABP4) and perilipin (PLIN) was clearly upregulated in the BM MSC cultures, while WJ MSC only showed upregulation of PPARG (Figure 3B). For osteoblast related genes this method showed to be unreliable since osteogenic differentiated BM MSC did not show any increase in osteogenic gene expression, despite the presence of osteogenic specific staining (Figure 3A and 3C).
Figure 2: Characterization of the Wharton’s Jelly MSC. A: Expression of cell surface markers by WJ MSC and BM MSC. The percentage of cells (±SEM; n=10) that express the respective markers is shown. B: Ability of WJ MSC (n=18 different isolates) and BM MSC (n=23 different isolates) to differentiate into adipocytes (Oil Red O), osteoblasts (alkaline phosphatase and calcium deposition) and chondrocytes (toluidine blue staining). 0=no differentiated cells, 1=<20% differentiated cells, 2=<60% differentiated cells, 3=>60% differentiated cells (see also Figure S1). The bar represents the mean of all experiments. C: Representative images of differentiation cultures after incubation with cell differentiation specific stains.
Figure 3: differentiation cultures of BM and WJ MSC, A: Staining of the differentiation cultures for cell specific markers/deposits of osteoblasts (alizarin red and alkaline phosphatase) and adipocytes (oil red O), B: Relative expression of adipogenic genes by BM MSC and WJ MSC after adipogenic differentiation cultures. Shown is the difference in up or down regulation of the genes by MSC in differentiation cultures compared to cultures in normal MSC medium (2^ΔΔCt, normalized to RPL13a), C: Relative expression of osteogenic genes by BM MSC and WJ MSC after osteogenic differentiation cultures. Shown is the difference in up or down regulation of the genes by MSC in differentiation cultures compared to cultures in normal MSC medium (2^ΔΔCt, normalized to RPL13a).
WJ MSC and BM MSC inhibit T cell proliferation of PBMC stimulated with αCD3αCD28 beads

Next, we compared *in vitro* immunomodulatory properties of WJ MSC and BM MSC in co-cultures with un-stimulated and αCD3αCD28-stimulated PBMC. In this setting, MSC were not immunogenic themselves, since co-incubation with MSC did not lead to proliferation of unstimulated PBMC. Moreover, WJ MSC and BM MSC inhibited proliferation of stimulated PBMC and this reduction was MSC dose dependent (Figure 4, p<0.0001 for all ratios of MSC and PBMC compared to stimulated PBMC alone). Interestingly, co-culture of BM MSC and PBMC at a 1:1 ratio resulted in decreased inhibition of proliferation (43±6%) as compared to a 1:2 ratio of BM MSC (70±4%, p<0.001). Additionally, at this 1:1 ratio, WJ MSC were more inhibitory than BM-MSC (Figure 4; 68±4% vs. 43±6% inhibition respectively, p<0.00005).

![Figure 4: WJ and BM MSC inhibit T cell proliferation. BM or WJ MSC were mixed with 1x10^5 PBMC at different ratios and stimulated with αCD3αCD28 beads for 5 days. Proliferation of the PBMC was measured by ([3H] thymidine incorporation on day 5 and compared to a control without MSC. MSC obtained from both sources significantly inhibited the proliferation of the PBMC in a dose dependent manner (n=8, *p<0.0001 for all ratios; at a 1:1 ratio of MSC:PBMC comparing WJ MSC with BM MSC **p<0.00005).](image-url)
Co-transplantation of MSC enhances the engraftment of CB-derived CD34+ cells in NOD SCID mice irrespective of the donor source

To evaluate and compare the effects of the different MSC on HSC engraftment in vivo, we co-transplanted human CB-derived CD34+ cells and human MSC into sublethally irradiated NOD SCID mice (n=3 experiments with 3 different donors). Additionally, autologous WJ MSC (i.e. MSC generated from the umbilical cord of the CD34+ cell donor), and allogeneic WJ MSC (MSC generated from the umbilical cord of another donor) were compared with co-transplantation of allogeneic BM derived MSC and transplantation of CD34+ cells alone. Starting from 3 weeks after transplantation until the mice were sacrificed at week 6, we analyzed the peripheral blood (PB) of the mice for the presence of human platelets (Figure 5A) and human CD45+ cells at weekly intervals (Figure 5B). All recipient mice had detectable levels of human platelets in their peripheral blood as early as week 3. Co-transplantation of MSC from all sources resulted in higher levels of circulating platelets compared to transplantation of CD34+ cells alone (CD34+ cells alone: 57±31 plt/µl PB vs. with BM MSC: 304±135 plt/µl PB, p=0.073, with autologous WJ MSC: 610±244 plt/µl PB, p<0.05 or with allogeneic MSC: 556±390 plt/µl PB, p<0.05). At 6 weeks after transplantation, platelet levels were on average 5-fold higher in the CD34+ and MSC co-transplanted groups when compared to the platelet levels in recipients of CD34+ cells alone (CD34+ cells alone: 790±216 plt/µl PB vs. BM MSC and CD34+ cells: 4146±1586 plt/µl PB, p<0.005, autologous WJ MSC and CD34+ cells: 4649±1203 plt/µl PB, p<0.005 and allogeneic WJ MSC and CD34+ cells: 5546±1654 plt/µl PB, p<0.05). Similarly, co-transplantation of WJ MSC significantly increased human CD45+ cells in the PB from week 4 onwards as compared to transplantation of CD34+ cells alone (40.6±22.7x10³ and 45.1±17x10³ cells/ml for CD34+ cells with autologous and allogeneic WJ MSCs vs CD34+ cells alone respectively, p<0.05). Slower recovery of circulating human leukocytes was observed with co-transplanted BM MSC as compared to co-transplanted WJ MSC. Co-transplantation of BM MSC increased circulating CD45+ cells 5 weeks after transplantation reaching levels similar to co-transplanting WJ MSC at this time point (69.7±21.9x10³ versus 12.6±3.9x10³ CD45+ cells/ml for CD34+ cells with BM MSC or for CD34+ transplantation only, p<0.05). Six weeks after transplantation, the mice were sacrificed and the bone marrow was analyzed for the presence of human hematopoietic cells. Co-transplantation of MSC obtained from both WJ and BM increased the frequency of human CD45+ cells in the bone marrow by at least 2-fold as compared to transplantation of CD34+ cells alone (26.7±5.5% human CD45+ cells vs. BM MSC 61.8±7.1%, p<0.0005, with autologous WJ MSC: 67.7±4.8%, p<0.0001 or with allogeneic WJ MSC 68.9±3.6%, p<0.0001). A similar pattern was observed for human CD45+ cell chimerism in spleen and peripheral blood. Co-transplantation of MSC and CD34+ cells increased chimerism in the spleen by at least 3-fold compared to transplantation of CD34+ cells alone (Figure 5D). CD34+ cells alone: 7.5±1.5% vs. co-transplantation with BM MSC: 23.9±4.6%, p<0.05, with autologous WJ MSC: 34.1±4.5%, p<0.005 or with allogeneic WJ MSC: 32.2±4.3%, p<0.001) and in the blood at least 5-fold (Figure 5E, CD34+ cells alone: 4.2±1.0% human CD45+ cells vs. co-transplantation with BM MSC: 21.7±5.6%, p<0.05, with autologous WJ MSC: 24.7±4.5% or with allogeneic WJ MSC: 26.5±6.6%, p<0.005). BM cells harvested from the femurs of the mice expressed similar percentages of the common myeloid marker CD33, the lymphoid markers CD19 and CD3 and the stem/progenitor cell marker CD34 irrespective of the co-transplantation of MSC (Figure 5F). In our model, autologous and allogeneic WJ MSCs enhanced total human CD45+ cell reconstitution to a similar extent.
Co-culture of WJ MSC and CB CD34+ cells with TPO enhances CFU-GEMM formation, while BM MSC enhances BFU-E formation

Several functional characteristics of MSC might play a role in their observed ability to facilitate engraftment. MSC have been shown previously to support the growth of human CB CD34+ cells. \(^{33-37}\) Furthermore, we have shown that transplantation of TPO expanded CB CD34+ enhanced early platelet repopulation while retaining long term hematopoietic engraftment capacity in NOD-SCID mice. \(^{31,32}\) Combining these 2 potential engraftment enhancing strategies, we compared the capacity of human WJ MSC and BM MSC to support differentiation and expansion of human CB-derived CD34+ cells in the presence of exogenous TPO. To this end, CD34+ cells were cultured for 10 days on monolayers of MSC obtained from different sources in the presence of exogenous TPO and analyzed for expansion of total MNC and CFU formation of the cultured CD34+ cells.

Cultures of CD34+ cells on either BM MSC or WJ MSC monolayers did not enhance the TPO induced expansion of total nucleated cells (Figure 6A, WJ MSC 4.8±0.9 fold expansion and BM MSC 4.1±1.5 fold expansion vs. no stromal support 4.0±0.8 fold expansion) over this time period. Additionally, the ratio between the 3 main cell subpopulations that are typically formed when CB CD34+ are cultured with TPO, namely residual CD34+ cells (rCD34+), CD34-CD61- cells (Lin-) and CD34-CD61+ cells (CD61+), \(^{38}\) were similar between cultures without stromal support and those on MSC mono-layers from different sources (Figure 6B). To investigate the differentiation potential of the remaining hematopoietic stem and progenitor cells (HSPC) in the TPO induced cultures, we next analyzed their colony-forming capacity in CFU assays. To this end, HSPC were first separated from the MSC after co-culture and subsequently cultured in semi-solid cultures in the presence of cytokines. Interestingly, HSPC derived from cultures on BM MSC monolayers exhibited an increased capacity to form BFU-E colonies (Figure 6C, BM MSC 42.8±10.2 BFU-E/1000 rCD34+ cells compared to no MSC 14.5±6.1 BFU-E/1000 rCD34+ cells, p<0.05). HSPC cultured in the presence of WJ MSC gave rise to higher numbers of CFU-GEMM (35.8±9.9 colonies/1000 CD34+ cells vs. 20.6±6.3/1000 CD34+ without stromal support; Figure 6C, right panel, n=5 experiments, p<0.05).

Figure 5: Co-transplantation of MSC and CD34+ cells enhances peripheral blood recovery and bone marrow engraftment in NOD SCID mice. A: Human platelet recovery in the PB of the mice after transplantation. B: Human CD45+ cell recovery in the PB of the mice after transplantation. C: Percentage of human CD45+ cells in the BM of the mice 6 weeks after transplantation D: Percentage of human CD45+ cells in the spleen of the mice 6 weeks after transplantation. E: Percentage of human CD45+ cells in the blood of the mice 6 weeks after transplantation. Bars represent the mean of all mice F: Lineage differentiation of the human CD45+ cells in the BM 6 weeks after transplantation. The total number of human CD45+ cells in the femurs off all mice was analyzed for the expression of lymphoid markers CD19 and CD3, myeloid marker CD33 and stem/progenitor cell marker CD34. (n=3 experiments with 3 different CB donors/MSC isolates)
Figure 6: Co-culture of BM or WJ MSC with CB CD34+ cells supports the expansion of CB CD34+ cells in the presence of TPO. CB CD34+ cells obtained from different donors (n=5) were cultured with TPO for 10 days in the presence or absence of MSC obtained from different sources. Next, the composition of the expanded cells was analyzed using flow cytometry and the capacity to form myeloid colonies was analyzed with CFU assays. A: Fold expansion (depicted as the total number of hematopoietic nucleated cells after culture divided by the number of input cells) in the absence or presence of stromal cells. B: Percentage of the 3 major populations of the total hematopoietic cells observed after expansion in the absence or presence of stromal support from MSC from different sources; rCD34+ = residual CD34 cells, Lin- = CD34-CD61-Lineage cells, and CD61+ = CD34-CD61+ cells. C: Colony-forming capacity of TPO expanded CD34+ cells after culture in the absence or presence of stromal support. (*p<0.05).
Incubation of CB-derived CD34+ cells with MSC does not significantly alter their migration towards CXCL12, but increases the expression of adhesion markers

Hematopoietic stem cell homing to the marrow is the primary step for their engraftment and relies on their adhesive and migratory capacities. We investigated whether MSC change the migratory characteristics of CB CD34+ cells towards CXCL12 in transwell migration studies. To this end, CB CD34+ cells and MSC were incubated together for 30 minutes to mimic the time that CD34+ cells and MSC are in the same tube prior to infusion. Aliquots of the cell suspensions were placed in transwell plates while others were analyzed in parallel for adhesion marker expression. Additionally, the effect of the presence of either autologous or allogeneic WJ MSC on the migration of CD34+ cells was tested (Figure 7A). Autologous WJ MSC tended to increase CD34+ migration when compared to the migration of CD34+ cells alone; this difference however, was not significant (11.3±5.2% vs 6.8±2.2% for CD34+ cell migration with or without autologous WJ MSC). Next, we investigated the expression of several adhesion markers known to be involved in homing to the BM. In this respect, we analyzed the expression of CD11a, CD11b, CD49d, CD49e and the CXCL12 receptor CD184 (CXCR4) on CD34+ cells after 30 minutes incubation with MSC (Figure 7B). Regardless of the MSC source, incubation of CB CD34+ cells with MSC seemed to induce a general increase in marker expression. However, even between MSC from the same source, expression levels varied considerably. Therefore, only CD49d and CD49e expression was significantly increased after incubation with BM MSC and allogeneic WJ MSC respectively (p<0.05).

Figure 7: In vitro homing characteristics of and adhesion molecule expression of CD34+ in the presence of MSC obtained from different sources. A: Migration of CD34+ cells towards SDF-1α in the presence of MSC obtained from WJ or BM. B: Expression of adhesion markers and CXCR4 on CB-derived CD34+ cells after incubation with WJ MSC or BM MSC.
Discussion

In the present study, we compared the effect of human MSC obtained from WJ and BM on the engraftment of CB-derived CD34+ cells in an immune-deficient murine transplant model. The recovery of human platelets and CD45+ cells in the peripheral blood of these mice was significantly enhanced by co-transplantation of either WJ or BM MSC from 3 weeks onwards. At 6 weeks post-transplantation, the percentage of human CD45+ cells in the BM, spleen and peripheral blood was at least 3-fold higher when MSC were co-transplanted with CD34+ cells compared to transplantation of CD34+ cells alone. MSC obtained from BM and WJ were comparable in their capacity to enhance the engraftment of CB-derived CD34+ cells. Although the mechanism is so far not determined, MSC induced CD34+ cell engraftment has been suggested to be associated with their immune-modulatory capacity. MSC from different tissues including WJ are known to modulate immunological responses. At high levels of IFN-γ and TNF-α, MSC have an anti-inflammatory effect for which secreted indoleamine 2,3-dioxygenase (IDO) is one of the proposed mediators. Conversely, in steady state conditions at low levels of IFN-γ and TNF-α, allogeneic MSC are able to stimulate an immunological response. The absence of inflammatory signals directly after transplantation could therefore not only diminish the immunosuppressive effect of the co-transplanted MSC but may even have an immune activating effect. The immunosuppressive properties of MSC, however, are most likely to mediate a beneficial effect on the outcome of HCT e.g. by exhibiting a prophylactic effect on the occurrence or severity of Graft versus Host Disease or host mediated graft rejection. In agreement, a study in which mononuclear cells (MNC) obtained from two CB units were transplanted demonstrated that either removal of the immune competent cells from the graft or MSC co-transplantation alleviated single CB donor dominance and improved overall engraftment. In our in vitro experiments MSC from the BM and WJ were equally potent in inhibiting the proliferation of αCD3αCD28 stimulated PBMC. However, as we use immune deficient NOD SCID mice for our in vivo transplantation experiments and transplants without any immune competent cells, these immune modulating qualities do not seem to be instrumental for the similar engraftment enhancing effect of both MSC sources since. Alloimmunization between the recipient and the transplanted cells or between the CD34+ cell purified HSC graft and the co-transplanted allogeneic MSC are therefore not likely to occur. In agreement, no difference in the engraftment enhancing capacity of autologous and allogeneic WJ MSC was found. Since immune related components cannot be assessed in our model, the marked increase of engraftment must therefore be caused by other MSC-derived factors.

Alternatively, MSC may play a direct role in regenerating the bone marrow niche, first by homing to the marrow and differentiating into stromal tissues and second by inducing the proliferation and differentiation of HSC. Concerning the first option, our studies show that the differentiation capacity of WJ MSC is variable and limited and thus unlikely to be the cause for their engraftment stimulating effect. Corroborated by a study from Bosch et al., this also makes WJ MSC less interesting candidates for therapeutic bone or cartilage regeneration. Concerning the second option, WJ MSC have been shown to support the growth of CB-derived CD34+ cells ex vivo. As we previously showed that TPO expanded CB CD34+ cells contributed to both improved platelet recovery and BM engraftment, we combined these 2 different mechanisms in an in vitro experiment and investigated whether MSC could further enhance TPO-induced effects on CD34+ cultures. In this regard additional presence of MSC did not change TPO induced expansion and neither was the composition of the formed subpopulations changed. CFU cultures of the
expanded cells did however show that the MSC have a different effect on the types of cells that are formed after expansion. Cultures of HSPC on BM MSC monolayers exhibited an increased capacity to form BFU-E colonies while HSPC cultured in the presence of WJ MSC gave rise to higher numbers of CFU-GEMM. These observations might be of conceptual importance since CFU-GEMM are correlated with the presence of more primitive stem cells. 46 Hence, WJ MSC in this respect might have a better potential to preserve the more immature CD34+ cells in culture with TPO than BM MSC. Other studies have shown that the co-culture of MSC, including WJ MSC, can enhance the fold expansion of both total nucleated cells and CD34+ cells. 33,35,37 These studies used a cocktail of cytokines including SCF, Flt3L and TPO in their culture protocol. Adding SCF and Flt3L to the expansion medium can significantly enhance the fold expansion of the cells, 47 and the absence of these cytokines could therefore explain the lack of TNC and CD34+ cell expansion in our experiments.

However, for both the MSC induced marrow niche regenerating mechanisms to become relevant, homing of MSC with the HSPCs to the bone marrow is necessary. This has so far not been convincingly shown. 11,48,49 Although co-localization of MSC and CD34+ cells in the pelvis has been reported [49], 48 MSC are more often detected in various organs, but not in the BM 11 and entrapment of MSC in the lungs has been described as a possible explanation for the lack of homing of the MSC. 49 MSC-induced homing of CD34+ cells to the HSC niche might be another explanation for increased engraftment by local (e.g. by cell to cell contact in the marrow) or systemic (e.g. by paracrine factors production) support. Our in vitro studies, with brief exposure of CD34+ cells to MSC could not consistently show enhanced migration of CD34+ cells towards CXCL12. However, in the presence of MSC, CD34+ cells upregulate surface markers that are associated with their homing to or retention in the BM marrow (e.g. CD11a, CD11b, CD184, CD49e and CD49d). 39,50,51

In conclusion, our data support the use of human WJ MSC as an alternative source to enhance the engraftment of human CB-derived CD34+ cells. Although MSC induced homing of HPSC to the BM, seems an interesting explanation from our studies, for a real life estimate of MSC role in engraftment support other mechanisms also need to be considered. Engraftment in this regard is likely dependent on (interacting) factors like the immune status of the recipient and hematopoietic immune (activating) cells in the transplants. However, the immune-deficient recipient mice and the CD34+ isolated stem cell transplant that were used in our studies, makes MSC induced immunomodulation an unlikely explanation for the observed enhanced engraftment in vivo. Our in vitro experiments, however, do suggest that WJ MSC may serve as an alternative source to BM MSC for immunomodulatory applications. A general advantage of WJ MSC is that the umbilical cord can be regarded as a waste product, and that WJ MSC can therefore be obtained at relatively low cost without harm or risk for the donor. Since co-transplantation of allogeneic MSC did not show particular disadvantages, 52-54 and while co-transplantation of autologous MSC with donor hematopoietic stem cells might even inhibit engraftment, 55 our study does not support the paired use and banking of CB CD34+ cells and WJ MSC of the same umbilical cord. However, concurrent collection of the umbilical cord with CB collection enables sharing the logistics, tissue typing and virological testing for two products from only one donor with additional saving of costs and effort to obtain MSC from BM. This new CB cord strategy would therefore create a relative cheap, off the shelf MSC product that can be provided by tissue banks to hospitals. The value of such an approach however will eventually depend on the therapeutic efficacy of MSC and more specifically WJ MSC.
References

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