

Human Wharton's Jelly-Derived Stem Cells Display a Distinct
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Compared to Bone Marrow-Derived Stem Cells

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Human Wharton's jelly-derived stem cells display a distinct immunomodulatory and pro-regenerative transcriptional signature compared to bone marrow-derived stem cells

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| Abstract: | <p>Mesenchymal stromal cells (MSCs) are multipotent stem cells with immunosuppressive and trophic support functions. While MSCs from different sources frequently display a similar appearance in culture, they often show differences in their surface marker and gene expression profiles. Although bone marrow is considered the 'gold standard' tissue from which to isolate classical MSCs (BM-MSC), MSC-like cells are currently also derived from more easily accessible extra-embryonic tissues such as the umbilical cord. In this study we defined the best way to isolate MSCs from the Wharton's jelly of the human umbilical cord (WJ-MSC) and assessed the mesenchymal and immunological phenotype of BM-MSC and WJ-MSC. Moreover, the gene expression profile of established WJ-MSC cultures was compared to two different bone marrow-derived stem cell populations (BM-MSC and multipotent adult progenitor cells or MAPC®). We observed that explant culturing of Wharton's jelly matrix is superior to collagenase tissue digestion for obtaining mesenchymal-like cells, with explant isolated cells displaying increased expansion potential. While being phenotypically similar to adult MSCs, WJ-MSC show a different gene expression profile. Gene ontology analysis revealed that genes associated with cell-adhesion, proliferation, and immune system functioning are enriched in WJ-MSC. In vivo transplantation confirms their immune modulatory effect on T cells, similar to BM-MSC and MAPC. Furthermore, WJ-MSC intrinsically overexpress genes involved in neurotrophic support and their secretome induces neuronal maturation of SH-SY5Y neuroblastoma cells to a greater extent than BM-MSC. This signature makes WJ-MSC an attractive candidate for cell-based therapy in neurodegenerative and immune-mediated central nervous system disorders such as multiple sclerosis, Parkinson's disease or amyotrophic lateral sclerosis.</p> |
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Human Wharton's jelly-derived stem cells display a distinct immunomodulatory and pro-regenerative transcriptional signature compared to bone marrow-derived stem cells

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Running title: transcriptome of WJ- & BM-derived stem cells

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List of non-standard abbreviations:

KO-DMEM/F12: Knock Out™ Dulbecco's modified Eagle's medium with F-12

DMEM-LG: DMEM with low glucose

BM-MSC: human bone marrow-derived MSCs

WJ-MSC: human Wharton's jelly-derived MSCs

MAPC: multipotent adult progenitor cells

NT2/D1: NTERA-2 clone D1 cell line

PD: population doublings

CPD: cumulative population doublings

FC: fold change

GO: gene ontology

Abstract

Mesenchymal stromal cells (MSCs) are multipotent stem cells with immunosuppressive and trophic support functions. While MSCs from different sources frequently display a similar appearance in culture, they often show differences in their surface marker and gene expression profiles. Although bone marrow is considered the ‘gold standard’ tissue from which to isolate classical MSCs (BM-MSC), MSC-like cells are currently also derived from more easily accessible extra-embryonic tissues such as the umbilical cord. In this study we defined the best way to isolate MSCs from the Wharton’s jelly of the human umbilical cord (WJ-MSC) and assessed the mesenchymal and immunological phenotype of BM-MSC and WJ-MSC. Moreover, the gene expression profile of established WJ-MSC cultures was compared to two different ~~adult MSC bone marrow-derived stem cell~~ populations ~~derived from bone marrow~~ (BM-MSC and multipotent adult progenitor cells or MAPC[®]). We observed that explant culturing of Wharton’s jelly matrix is superior to collagenase tissue digestion for obtaining mesenchymal-like cells, with explant isolated cells displaying increased expansion potential. While being phenotypically similar to adult MSCs, WJ-MSC show a different gene expression profile. Gene ontology analysis revealed that genes associated with cell-adhesion, proliferation, and immune system functioning are enriched in WJ-MSC. In vivo transplantation confirms their immune modulatory effect on T cells, similar to BM-MSC and MAPC. Furthermore, WJ-MSC intrinsically overexpress genes involved in neurotrophic support and their secretome induces neuronal maturation of SH-SY5Y neuroblastoma cells to a greater extent than BM-MSC. This signature makes WJ-MSC an attractive candidate for cell-based therapy in neurodegenerative and immune-mediated central nervous system disorders such as multiple sclerosis, Parkinson’s disease or amyotrophic lateral sclerosis.

Introduction

Mesenchymal stromal cells (MSCs) represent a population of multipotent progenitor cells defined by their unique capacity to self-renew and differentiate into typical mesenchymal cell types such as osteoblasts, adipocytes, and chondrocytes [1,2]. Classically, MSCs were isolated from bone marrow (BM-MSC) and characterized as a plastic adherent, fibroblast-like cell population with clonogenic colony forming ability, distinguishing them from hematopoietic cells [3,4]. Aside from their supportive function during haematopoiesis, MSCs secrete a variety of growth factors, immunomodulatory cytokines, and other trophic mediators, which makes them an attractive therapeutic strategy for applications in inflammatory and cytodegenerative conditions [5-7]. Because the isolation yield as well as *in vitro* expansion efficiency and differentiation potential of BM-MSC is often limited [8-12], isolation of MSC-like cells from other tissues of adult [13-15] and fetal [16-18] origin is being explored.

Extra-embryonic tissues are of great interest for stem cell isolation, as they are an abundant source for cells without ethical concerns. Several studies indicate that stromal cells derived from the umbilical cord matrix or Wharton's jelly (WJ-MSC) show MSC-like features, including adherence to plastic substrates, and a similar surface phenotype and differentiation capacity [19-21]. Furthermore, WJ-MSC display immune regulatory properties [22-24] and improve tissue regeneration in several disease models [25,26]. Interestingly, previous studies defined that cells from extra-embryonic/fetal origin are superior to adult MSC, showing broader differentiation potential, enhanced expansion potential, and higher cell isolation yields [10,27,28]. Accordingly, they are presented as an intermediate between embryonic stem cells and adult MSC-like stem cells [29], being derived from a more primitive tissue source than adult MSC. In contrast, other studies suggest that MSC-like cells derived from different tissues are similar, because they originate from a common progenitor cell [30-32].

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3 While MSC-like cells from different tissue sources share a substantial degree of similarity in
4 their phenotype, variation in marker expression profile and lineage-specific commitment have
5 been reported [33,34]. As such, it is questionable whether cells from different tissues are truly
6 similar stem cells. More likely, they show a gene expression profile related to their functions
7 in the tissue of origin.
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12 In this study, our aim was to define the relationship between Wharton's jelly and adult bone
13 marrow-derived stem cell populations and to describe the unique gene expression signatures
14 of the perinatal WJ-MSC. We first compared two isolation methods for obtaining Wharton's
15 jelly stromal cells, being the enzymatic digestion of cord matrix and micro-dissected tissue
16 fragment cultures (explants). Next, our established WJ-MSC cultures were morphologically
17 and phenotypically compared to BM-MSC. Both cell types were assessed for their growth
18 characteristics, mesenchymal differentiation capacity, and surface marker expression. Next,
19 we performed gene expression profiling of WJ-MSC compared to bone marrow-derived
20 MSCs and multipotent adult progenitor cells (MAPC). Ingenuity pathway analysis was used
21 to determine the molecular signature of the differentially expressed genes in WJ-MSC.
22
23 Collectively our data show that WJ cells resemble BM-derived MSCs in culture, but differ at
24 the transcriptional level, with WJ cells overexpressing immune regulatory molecules and
25 neurotrophic factors. In vivo, WJ- but also BM-MSC and MAPC exert local immune
26 modulatory effects on T cells after subcutaneous transplantation in acute rat experimental
27 autoimmune encephalomyelitis. Furthermore, conditioned medium of WJ-MSC more potently
28 induced neurite outgrowth of differentiated SH-SY5Y compared to BM-MSC. These findings
29 indicate that stem cells derived from the Wharton's jelly are a different type of MSC
30 displaying intrinsic immune modulatory and trophic support functions. These characteristics
31 make WJ-MSC a promising candidate for therapeutic application in inflammation driven
32 neurodegenerative diseases.
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Materials and methods

Ethical approval

The collection and experimental use of human cells and tissues was approved by the Medical Ethical Committees of Hasselt University and Ziekenhuis Oost-Limburg (Genk). Umbilical cord tissues were obtained from cesarean sections after informed consent. Animal experiments were performed in accordance with institutional guidelines and are approved by the Hasselt University Ethical Committee for Animal Experiments.

Isolation and culture of WJ-MSC

Human umbilical cords (n=10) were obtained aseptically from full-term uncomplicated pregnancies with planned cesarean section and processed for stem cell isolation within 24 hours. Cells were isolated using the explant isolation method, as previously described [33], with minor modifications. Cord blood was removed and the remaining tissue was aseptically stored in sterile phosphate-buffered saline (PBS; Lonza, Verviers, Belgium) supplemented with 1% penicillin-streptomycin (P/S; 10.000:10.000; Gibco™; ThermoFisher Scientific, Waltham, MA, USA) and 0.2% Fungizone® (250 µg/ml; Gibco™). Tissues were kept in PBS during processing. First, the umbilical arteries and vein were removed and the remaining matrix was cut into ± 2 mm³ fragments (explants). Next, the explants were cultured in Knock Out™ Dulbecco's modified Eagle's medium with F-12 (KO-DMEM/F12; Gibco™) supplemented with 1% P/S, 1% GlutaMAX™ (L-glut; 200 mM; Gibco™) and 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany). Cultures were kept at 37°C in a humidified atmosphere containing 5% CO₂ and were left undisturbed for 10 days, to allow migration of cells from attached explant tissue. Non-attached explants were discarded and medium was renewed every three days. At 80-90% confluence, cells were harvested using Stempro® accutase (Gibco™). WJ-MSC intended for functional assays were harvested

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3 between passage 2 and passage 8. For microarray analysis, WJ-MSC RNA (n=5) was
4 collected at the third passage in TRIzol™ Reagent (ThermoFisher Scientific) and frozen on
5 dry ice. To compare growth of explant-derived cells in different media, WJ-MSC were
6 harvested from the same cord and cultured in parallel in KO-DMEM/F12 or DMEM with low
7 glucose (1 g/L; DMEM-LG; Gibco™) containing the supplements described above.
8 Enzymatic isolation was performed as described previously [35], with the difference that
9 blood vessels were removed prior to digestion. To compare isolation methods, both explant
10 and enzymatically isolated cells from the same donor were cultured in DMEM-LG with
11 supplements.
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25 *Culture of bone marrow-derived stem cells*

26 Human bone marrow-derived MSC (n=7) were purchased from Lonza and cultured according
27 to manufacturer's instructions. For microarray analysis, BM-MSC RNA (n=5) was collected
28 at the third passage using TRIzol. Human MAPC pellets (n=5; research grade
29 MultistemMAPC®) were obtained from ReGenesys (ReGenesys bvba, Leuven, Belgium). For
30 this, MAPC were grown in culture stacks, then harvested and centrifuged after which the
31 pellet was collected in TRIzol and frozen on dry ice. MAPC for transplantation purposes were
32 provided as frozen stocks (n=2). Cells were immersed in water bath until partially thawed and
33 then inverted in DMEM low glucose (1 g/L) (Gibco™) supplemented with 10% FBS
34 (Gibco™) and centrifuged at 600 g for 8 minutes. Supernatant was removed and cells were
35 dissolved in new medium for counting. Cells were washed twice, and then suspended in
36 sterile saline solution (0.9% sodium chloride; Baxter i.v. fluids, Val d'Hony-Verdifarm,
37 Beringen, Belgium) for animal injection (*vide infra*).
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56 *Culture of NTERA-2 cl.D1 and SH-SY5Y cell lines*

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3 NTERA-2 cl.D1 (NT2/D1) was purchased from LGC standards (Molsheim, France; ATCC®
4 CRL-1973™) and cultured according to the recommended conditions. The SH-SY5Y
5 neuroblastoma cell line (Sigma-Aldrich, St. Louis, Mo, USA) was grown in DMEM/F12
6 (Gibco™) supplemented with 2 mM L-glutamine (Gibco™), 1% P/S and 10% FBS which
7 will be referred to as standard SH-SY5Y medium. The cells were seeded at a density of $5 \times$
8 10^3 cells/cm² and incubated at 37°C in a humidified atmosphere containing 5% CO₂. The
9 culture medium was changed every 3-4 days. After reaching 70-80% confluence, the cells
10 were harvested using 0.05% Trypsin with EDTA (Gibco™) and expanded.
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23 *Preparation of conditioned medium of MSC subtypes*

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25 In order to prepare the conditioned (secretome-containing) medium of WJ-MSC (CM WJ-
26 MSC) and BM-MSC (CM BM-MSC) for the neurite outgrowth assay, these cell types were
27 seeded at a density of 2×10^4 cells/cm² in their standard culture medium (*vide supra*). After
28 24 hours, the medium was changed to 1 ml/5 cm² SH-SY5Y medium containing 0.1% FBS.
29 48 hours later, the medium was collected, centrifuged at 300g, aliquoted and stored at -80°C
30 for later use.
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41 *Culture kinetics*

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43 The proliferative capacity of WJ-MSC and BM-MSC was assessed by calculating cell
44 doubling kinetics for each passage. Cell numbers were determined by means of Fuchs-
45 Rosenthal hemocytometer counts after trypan-blue exclusion (Biochrom AG). Population
46 doublings (PD) were calculated using the formula $PD = 3.32 (\log (nH) - \log (nS))$, where nS
47 is the number of seeded cells and nH the number of harvested cells. To yield cumulative
48 population doubling levels (CPD), the PD value was added to the sum of population
49 doublings of the previous passages: $PD_x = 3.32 (\log (nH_x) - \log (nS_x)) + PD_{x-1}$, with x being
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3 the passage number. Cumulative population doublings were calculated over a period of 70 ± 5
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5 days. For WJ-MSC, cumulative population doublings were calculated after the first passage,
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7 since the initial seeding number was unknown and the number of plastic adherent cells could
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9 only be determined after this time point.
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11 12 13 14 *Analysis of colony formation*

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16 Colony formation was assessed by seeding WJ-MSC in 6-well plates (Greiner Bio-One,
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18 Frickenhausen, Germany) at 50 cells / cm². Cells were cultured for 10 days, with medium
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20 change every 3 days. Then, the cultures were fixed with 4% paraformaldehyde (PFA; Sigma-
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22 Aldrich) and stained with 0.5% crystal violet (Sigma-Aldrich) in methanol (VWR, Heverlee,
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24 Belgium). After washing twice with PBS and air-drying, colony formation was examined with
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26 a Primo Vert phase contrast microscope (Carl Zeiss, Jena, Germany).
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31 32 *Multi-lineage differentiation*

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34 Adipogenic, osteogenic, and chondrogenic differentiation of both BM-MSC and WJ-MSC
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36 was performed using the corresponding hMSC differentiation bullet kits[®] from Lonza,
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38 according to the manufacturer's instructions. Subsequent histochemical analysis was
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40 performed as previously described [33].
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45 46 *Flow cytometry*

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48 WJ-MSC and BM-MSC were harvested, washed with FACS buffer (PBS supplemented with
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50 2% FBS) and incubated in the dark for 30 minutes at 4°C with fluorescently labeled
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52 antibodies (see Table S1) or corresponding isotype controls. Stained samples were washed
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54 and analyzed on a FACSCalibur instrument using CellQuest software (BD Biosciences,
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56 Erembodegem, Belgium).
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Microarray analysis

For transcriptome analysis, total RNA from five different donors of WJ-MSC, BM-MSC and MAPC was isolated using the MagMAX™-96 for Microarrays Total RNA Isolation Kit (Ambion®; ThermoFisher ScientificLife Technologies). Sample quality and concentration were measured using the Agilent 2100 bioanalyzer (Agilent Technologies) and NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) respectively. Next, RNA was converted to cDNA, fragmented and labeled, and subsequently hybridized to Affymetrix GeneChip Human Gene 1.0 ST arrays using the Ambion WT Expression Kit and GeneChip WT Terminal labeling and hybridization kits, carried out in a GeneChip hybridization oven 645 (all Affymetrix UK Ltd.). Hybridized chips were stained and washed in a GeneChip fluidics station 450 and scanned using a GeneChip Scanner 3000 7G (both Affymetrix). All steps were carried out according to standard Affymetrix protocols. Quality control was performed using GeneChip Operating software (GCOS 1.4; Affymetrix). Scanned images were visually inspected and raw intensity CEL files were generated. Subsequently, Bioconductor packages running under the R platform were used to analyze the gene expression data [36,37]. Using the oligo package [38], raw intensity files were pre-processed to obtain robust multichip average (RMA) expression values. Next, differences in gene content between two cell types were identified using an unpaired two-sided t-test. Genes were considered differentially expressed with a fold change (FC) cutoff value ≥ 2 and a Benjamini-Hochberg (BH) corrected p value < 0.05 . Data were visualized in R as volcano plots and venn diagrams using the limma package [39]. A heatmap was generated using the heatplus package [40] and principal component analysis (PCA) was plotted using the prcomp function. For data visualization, variance-based non-specific filtering was performed using the genefilter package to remove probe sets exhibiting the smallest variations in expression across the

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3 samples [41]. For pathway analysis, the full unfiltered data sets were used. Gene ontology
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5 analysis was performed using QIAGEN's Ingenuity® Pathway Analysis (IPA® , QIAGEN
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7 Redwood City, www.qiagen.com/ingenuity) and the Database for Annotation, Visualization
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9 and Integrated Discovery (DAVID) [42,43]. For both programs, the significance of
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11 association between data set and canonical pathway was determined using built-in Fisher's
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13 exact test to measure the gene enrichment in annotation terms [44]. Overrepresented
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15 biological functions and canonical pathways with a Fisher exact P value < 0.05 were
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17 considered significant. The raw data files have been deposited in NCBI's Gene Expression
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19 Omnibus [45], accessible through GEO Series accession number GSE77685.
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Quantitative PCR (qPCR)

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~~Total RNA from additional stem cell donors and the NT2/D1 cell line was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics, Vilvoorde, Belgium) according to manufacturer's instructions. RNA quality was assessed with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) according to standard procedures. 1 µg of RNA of both microarray samples and additional donors was reverse transcribed to cDNA using the Reverse transcription system (Promega, Leiden, The Netherlands) according to manufacturer's protocol. To validate the microarray results, qPCR was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems®; Life Technologies) with universal cycling conditions (20 seconds 95°C, 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C). The qPCR reaction was performed in micro AMP Fast Optical 96 well reactions plates (Applied Biosystems®; Life Technologies) and consisted of fast SYBR green master mix (Applied Biosystems®; Life Technologies), 10 mM of forward and reverse primers (Eurogentec, Seraing, Belgium), nuclease free water (Promega) and 12.5 ng cDNA template in a total reaction volume of 10 µl. Non template controls contained~~

~~nuclease free water instead of cDNA. Primers are listed in Table S2, and were designed using Primer 3 [46]. Primers for POU5F1 (OCT4), SOX2, NANOG, SMAD3 were as previously described by Gao et al. [47]. Primers for KLF4 and MYC were obtained from the RTprimer database (RTprimerDB ID3109 & ID8577; <http://medgen.ugent.be/rtprimerdb/>; [48]). Relative quantification of gene expression was calculated using qBasePLUS® software (Biogazelle, Zwijnaarde, Belgium). Data were normalized to the most stable reference genes and results were presented as fold changes to the WJ-MSC sample group.~~

BDNF ELISA

Supernatant from WJ- and BM-MSC cultures was collected after 7 days and snap frozen. BDNF was detected using the ChemiKine™ Brain Derived Neurotrophic Factor Sandwich ELISA kit (Chemicon®, Millipore, Overijse, Belgium) following the manufacturer's protocols. Data are presented as mean concentration (pg/ml) from 3 independent experiments.

Neurite outgrowth assay and immunocytochemistry

To evaluate CM-induced neurite outgrowth, a two-step protocol based on retinoic acid (RA) and BDNF exposure, previously described by Encinas *et al.* [46] and implemented for CM-induced neurite outgrowth by Gervois *et al.* [47] was used. SH-SY5Y cells were seeded at a density of 2.5×10^4 cells/cm² in standard SH-SY5Y medium on 50 µg/ml collagen type I-coated glass coverslips. After 24 hours, the medium was changed to standard SH-SY5Y medium supplemented with 10 µM RA (Sigma-Aldrich) and 1% FBS instead of 10% to induce neuronal differentiation. These cells were then kept in culture for 5 days with one medium change after 3 days. Next, the cells were washed with PBS and either CM WJ-MSC, CM BM-MSC or DMEM/F12 supplemented with 50 ng/ml BDNF or 10 µM RA (Immunotools, Friesoythe, Germany) and 0.1% FBS, was added to the RA-induced SH-SY5Y

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3 cells to stimulate neuronal maturation. The cells were kept in culture for 7 days and the
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5 culture medium or CM was changed every 2-3 days. Samples of each differentiation step were
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7 fixed with 4% PFA and immunostainings were performed according to a standardized
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9 protocol for immunofluorescence as described previously [48]. The fully BDNF-differentiated
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11 SH-SY5Y cells will be used as a reference for neurite outgrowth of matured neuroblastoma
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13 cells. The influence of RA, BDNF, CM BM-MSC and CM WJ-MSC on neurite outgrowth of
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15 SH-SY5Y cells was evaluated by measuring the mean length of the longest neurite on neurite-
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17 bearing cells that were stained for 1h with mouse anti-human β -III tubulin (1/2000; clone
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19 2G10; Sigma-Aldrich) using Fiji software [49] with the NeuronJ plugin [50]. At least 100
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21 neurites were counted in each sample. The primary antibody was detected with an Alexa fluor
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23 555-conjugated donkey anti-mouse secondary antibody (30 min incubation, 1/500; clone
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25 A31570; Invitrogen™; ThermoFisher Scientific). The appropriate isotype control (IgG2a;
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27 clone MG2a-53; Biolegend, London, UK) was used instead of the primary antibody in an
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29 equal concentration (500 ng/ml) to verify the staining specificity.
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36 *Acute EAE induction and subcutaneous stem cell transplantations*

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38 Lewis rats (Janvier Labs, Le Genest-Saint-Isle, France) were housed four per cage, in a
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40 controlled environment at 22–24 °C with 55% humidity, on a 12 hour light/dark cycle, with
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42 ample cage enrichment and access to rodent's chow and water *ad libitum*. Acute EAE
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44 induction was performed as previously described [51]. Briefly, 8 weeks old female Lewis rats
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46 were injected subcutaneously in both foot pads with 100 μ l immunization fluid, consisting of
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48 250 μ g/ml guinea pig myelin basic protein (MBP; generated in-house), 2.5 mg/ml H37RA
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50 heat-killed mycobacterium tuberculosis (Difco, Detroit, MI, USA) and 60 μ l Complete
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52 Freund's adjuvant (Sigma-Aldrich). Stem cell transplantations were performed on -1 dpi, 3
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54 dpi and 6 dpi, via subcutaneous injection in both hind limbs (100 μ l each side, lateral to
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3 femur) of 1×10^6 human WJ-MSC, ~~or~~ BM-MSC, or MAPC (n=8 animals each) in 200 μ l of
4 sterile saline solution, or with saline only (control, n=48). Nine days post immunization the
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6 rats (n=3 per group) were sacrificed by perfusion with Ringer's solution (generated in-house)
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8 for lymph node collection.
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11 12 13 14 *Restimulation of lymphocytes*

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16 MBP-specific lymphocytes were obtained 9 days post immunization by isolation of the
17 popliteal lymph nodes (LN). LN (n=3 rats per group) were segregated into single cell
18 suspensions using a 70 μ m strainer and 5 ml syringe plunger (both Greiner Bio-One). Cell
19 suspensions were separated using ficoll density gradient centrifugation (Histopaque®-1077;
20 Sigma-Aldrich) and the mononuclear layer was aspirated for subsequent enrichment of
21 antigen-specific lymphocytes. For this, 2×10^5 cells were cultured for 48 hours in round-
22 bottom 96-well plates (Greiner Bio-One) in restimulation medium, consisting of RPMI 1640
23 (Gibco™) supplemented with 0.5% P/S, 1% sodium pyruvate (Gibco™), 1% non-essential
24 amino acids (Gibco™), 20 μ M 2-mercaptoethanol (Sigma-Aldrich), 2% inactivated
25 autologous serum (Lewis rat serum, generated in-house), and 10 μ g/ml MBP. For control
26 conditions MBP was omitted.
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43 *[³H]-thymidine incorporation*

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45 The proliferation of LN cells was assayed ex vivo by ³H-thymidine incorporation. Following
46 48 hours of re-stimulation, 1 μ Ci [³H]thymidine (PerkinElmer, Waltham, MA, USA) was
47 added to the lymphocyte cultures for an additional 18 hours. Next, cells were collected with
48 an automated harvester (PerkinElmer) and incorporation of radioactivity was quantified using
49 a β -plate liquid scintillation counter (PerkinElmer). Relative proliferation is given as
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3 stimulation index, calculated as the radioactivity of the stimulated sample divided by the
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5 average radioactivity of unstimulated samples.
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10 *Statistical analysis*

11 Statistical analyses were performed using Graphpad Prism 5 (GraphPad Software Inc., La
12 Jolla, CA, USA). Data sets were tested for normal distribution using D'Agostino and Pearson
13 omnibus normality test. A one-way analysis of variances (ANOVA, Tukey post hoc) and two-
14 tailed unpaired t-test were used to test for significant differences. Results are reported as mean
15 with standard deviation (SD). The Kruskal-Wallis (Dunns post hoc) and Mann-Whitney U
16 analysis were used for data sets which did not pass normality. Results are reported as median
17 with interquartile range (IQR). Differences were considered statistically significant at P
18 values < 0.05.
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Results

Explant-derived WJ-MSC show a mesenchymal phenotype

MSC-like cells were isolated from the Wharton's jelly of the umbilical cord using explant culturing. Approximately seven days after isolation, fragments of cord tissue attached to the culture surface and WJ-MSC started migrating out of the tissue (**Figure 1A**). After an additional seven days of culture, cells were harvested and seeded for further expansion and characterization. By then, cultures were explant free and cells were grown to 80% confluence before harvesting. WJ-derived cells were plastic adherent and consisted of a mixed population of smaller triangular shaped cells and larger cells with fibroblast-like morphology (**Figure 1B**). When cultures were grown to full confluence, WJ-MSC became spindle-shaped and formed colonies (**Figure 1C**). In addition, cells were able to form colonies when cultured at low density (**Figure 1C, inset**). WJ-MSC cultures kept growing for 23.4 ± 1.8 CPD and 81.0 ± 7.3 days until replicative senescence occurred. Of note, culturing WJ-MSC in KO-DMEM/F12 resulted in higher growth rates compared to parallel cultures in DMEM-LG (**Figure S1A**).

An alternative way to isolate cells from the Wharton's jelly comprises the enzymatic digestion of the cord matrix to release the cells [35,52]. When comparing both isolation methods, we observed that WJ-MSC obtained using the explant approach had a significantly higher growth rate compared to enzymatically isolated cells (parallel cultures; **Figure 1D**). The latter displayed an aberrant morphology compared to standard cultures, having a large myofibroblast-like appearance (**Figure S1B**). This was reflected in their MSC marker profile (**Figure 1E**), showing a decreased CD73 and CD90 expression upon prolonged culture (increasing passage). In addition, replicative senescence occurred more early in enzymatically isolated WJ cells, reflected by a lower growth rate and earlier growth arrest.

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3 Next, we compared the growth characteristics, phenotype, and differentiation capacity of our
4 established Wharton's jelly explant cultures with those of classical bone marrow-derived
5 MSCs. The cells used for phenotypical characterization were harvested between passage 2
6 and passage 8. For culture kinetics analysis, cultures were grown until replicative senescence.
7
8 To compare cell yield between the two cell types, cumulative population doublings were
9 calculated over a period of 70 ± 5 days. In this time frame, all cells were still growing without
10 any signs of replicative decline. Compared to BM-MSC (n=7), our established WJ-MSC
11 cultures (explant isolated + KO-DMEM/F12; n=10) showed significant higher CPD after 70
12 days of culturing (**Figure 1F**).

13
14 Flow cytometry analysis demonstrated that WJ-MSC display a specific set of surface antigens
15 (**Table 1**). WJ cells expressed the three main mesenchymal markers CD73, CD90 and CD105.
16 All cultures were negative for HLA-DR and the hematopoietic markers CD14, CD19, CD34
17 and CD45. No expression of the pluripotency factors OCT4 , SOX2 and NANOG was
18 detected by flow cytometry or qPCR (**Figure S1C&D** respectively). In addition, WJ-MSC
19 expressed several surface adhesion molecules and immunological markers, being either co-
20 stimulatory or inhibitory in nature. Compared to BM-MSC, they expressed significant higher
21 levels of CD10, CD146, **Integrin- α 4** (CD49d), **ICAM-1** (CD54), CD200 and PD-L2.
22 Furthermore, both cell types expressed HLA class I molecules and co-stimulatory and
23 inhibitory molecules (e.g. HLA-ABC, HLA-E, PD-L1 and TLR-4) involved in immune cell
24 communication. In addition, WJ-MSC expressed low levels of IFN- γ R1 (CD119) and CXCR3
25 (CD183) receptors. Stem cell subpopulation-related markers CD146, CD117, CD271 and
26 CD133 could be found in several samples, yet donor variability was observed for both MSC
27 types, as shown by the high standard errors. Furthermore, WJ-MSC differentiate into the three
28 classical mesenchymal cell types [33]. End stage differentiation analysis by
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2 immunohistochemistry is shown in **Figure S2** for adipocyte, osteoblast and chondrocyte
3 formation.
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10 ***Transcriptional analysis reveals WJ-MSC as a distinct type of MSCstem cell***

11 Differential gene expression between extra-embryonic WJ-MSC, adult BM-MSC, and MAPC
12 was determined using Affymetrix Genechip microarray analysis. Global differences in gene
13 expression profiles were visualized by unsupervised hierarchical clustering and principal
14 component analysis. As shown in **Figure 2A&B**, the biological samples of each cell type
15 cluster together and form separate groups, indicating that functionally distinct stem cell
16 populations were obtained. Based on hierarchical clustering and Pierson's Correlation, WJ-
17 MSC are a unique group of MSCsstem cells, more closely related to BM-MSC than MAPC.
18 Furthermore, clustering shows that MAPC are also distinct from BM-MSC. Volcano plots
19 providing an indication for these differences are given in **supplementary Figure S3**. The
20 number of differentially expressed genes in WJ-MSC (cutoff: false discovery rate 0.05 and
21 Log FC 1) are depicted in **Figure 2A inset**. Comparing WJ-MSC to BM-MSC, the expression
22 of 795 genes was altered, of which 382 genes were upregulated and 413 genes were
23 downregulated. Compared with MAPC, a total of 1625 genes were altered, of which 797 were
24 upregulated and 828 were downregulated in WJ-MSC. The top 30 most differentially
25 expressed genes within the WJ cells are listed in **Table 2 & 3**. Of note, WJ-MSC highly
26 express multiple cell adhesion molecules and cytokines, such as *ITGA2*, *DSG2*, *DSC3*, *IL1A*,
27 *IL1B*, *IL8* and *CD200*, compared to BM-MSC and MAPC. A selection of these differentially
28 expressed gene categories is given in **Table 4**.
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51 Interestingly, WJ-MSC displayed 411 transcripts that were differentially regulated in
52 comparison to both BM-MSC and MAPC (**Figure 2A inset**), of which are 272 genes up-
53 regulated and 106 genes down-regulated. To further elucidate the distinctive gene expression
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3 profiles, differential gene pools were screened for their related molecular signature using gene
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5 ontology and pathway analysis software.
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8 9 ***Gene ontology analysis identifies specialized gene expression within WJ-MSC***

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11 To determine the differences between the three cell types, we analyzed their gene expression
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13 patterns in more detail. Gene ontology (GO) analysis was performed to link the differentially
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15 regulated transcripts with their biological and molecular functions. For the analysis we
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17 focused on the WJ group (n= 411 transcripts, differentially expressed versus both BM-MSC
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19 and MAPC). Enriched canonical pathways and functional groups are summarized in **Table 5**
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21 and **supplementary Table S32**.
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25 The most predominant of the overrepresented processes and functions of WJ-MSC include
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27 those pertaining to cell movement, cell-cell interactions and adhesion, cell death and survival
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29 as well as cardiovascular system development and hepatic cell functioning. Additionally,
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31 molecules related to embryonic development were enriched. ~~Interestingly, amongst the three~~
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33 ~~stem cell types, no differential expression of pluripotency markers *OCT4*, *SOX2* and *NANOG*~~
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35 ~~was observed (Table 4). This does not necessarily mean that these transcription factors are not~~
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37 ~~expressed. Indeed, our flow cytometry and PCR data indicated a low negative expression of~~
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39 ~~these transcription factors in WJ-MSC cultures (Figure S1C&D).~~ In contrast, *KLF4* was
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41 significantly higher expressed in BM-MSC compared to the other cell types. MAPC on the
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43 other hand showed enrichment of genes related to the cell cycle regulatory machinery,
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45 including cyclin-dependent kinases, such as *CDK1* (see **Table 3**).
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53 ***The gene expression signature of WJ-MSC indicates an active cell modulatory potential***
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55 ***through cell-cell contact and secretion of molecules***
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When focusing on the differentially expressed gene pools within WJ-MSC, a variety of cytokines and trophic factors such as *IL8*, *IL6*, *IL1*, *CXCL8*, *BDNF*, *NTF3*, *TGFB2*, *VEGFA*, *FGF2*, and *LIF* as well as adhesion and immune regulatory molecules, including *ICAM1*, *VCAM1*, *CD200*, *COX-2*, *PD-L1*, *CD49B*, *HLA-ABC*, *HLA-G*, *HLA-E*, and *HLA-F* were intrinsically overrepresented (Table 4). Moreover, BDNF protein levels were significantly increased in the supernatant of naive WJ cultures compared to bone marrow MSC (Figure 3). In contrast, BM-MSC and MAPC overexpressed TLR4 mRNA. Additionally, PTGES and HGF and KLF4 were ~~was~~ found to be specifically increased in BM-MSC and COX-1 was upregulated in MAPC. Expression of IDO-1, a T cell proliferation inhibitor [53], was not detected.

Additionally, when searching GO databases for increased biological processes and molecular functions within this differentially regulated gene pool of 411 transcripts, pathways involved in cell movement, cell-cell signaling, as well as cellular adhesion were enriched (Table 5 & S3S2).

Validation of microarray results

~~To confirm our microarray results, a selection of genes (see Tables 2&3_4 and Figure 4) was subjected to quantitative expression analysis. Consistent with the microarray profiles, qPCR analysis confirms the distinct expression pattern of 27 genes amongst the three stem cell types (Figure S45). Genes including ICAM1, CD200, BDNF and LIF amongst others were overexpressed in WJ-MSC. Moreover, BDNF levels were significantly increased in the supernatant of naive WJ cultures compared to bone marrow MSC (Figure 53). As with the microarray results, BM-MSC and MAPC overexpressed TLR4 mRNA. Additionally, PTGES and KLF4 were found to be specifically increased in BM-MSC and COX-1 was upregulated in MAPC. The mRNA levels of four pluripotency associated transcription factors, namely~~

~~OCT4, SOX2, NANOG and MYC were unaltered between all cell types. Our qPCR data confirm the reliability of our microarray findings.~~

MSC secretome promotes neurite outgrowth in SH-SY5Y cells

To verify the neurotrophic activity of WJ- and BM-MSC, conditioned medium was collected and used to mature differentiating neuroblastoma cells (SH-SY5Y). Neurite outgrowth gradually increased during the differentiation process (**Figure 4A-F**) as was also demonstrated quantitatively (**Figure 4G**). RA treatment ($45.2 \pm 0.4 \mu\text{m}$) significantly increased the mean neurite length compared to controls ($36.38 \pm 4.36 \mu\text{m}$; $P < 0.05$) (**Figure 4A, B**) but prolonged RA exposure (**Figure 4C**) did not additionally improved neurite outgrowth ($45.14 \pm 3.6 \mu\text{m}$). Quantification of neurite outgrowth after β -III tubulin staining showed a significant longer mean neurite length ($P < 0.001$) for cells treated with CM BM-MSC ($66.79 \pm 3.24 \mu\text{m}$), CM WJ-MSC ($90.71 \pm 5.58 \mu\text{m}$) and BDNF ($82.32 \pm 5.11 \mu\text{m}$) (**Figure 4D-F** respectively) compared to control, RA-induced, and RA-maturated SH-SY5Y cells (**Figure 4A, B and C** respectively). BDNF- and CM WJ-MSC-maturated SH-SY5Y cells have significantly longer neurites than CM BM-MSC-maturated cells ($P < 0.01$ and $P < 0.001$ respectively). No significant difference in mean neurite length could be observed between CM WJ-MSC and BDNF maturated SH-SY5Y cells.

Subcutaneous stem cell transplantation in acute EAE exerts local immune modulatory effects on T cells

To verify the immune suppressive phenotype of the stem cells, in vivo transplantation in acute EAE was performed. In order to assess potential effects on peripheral immune cell activation, stem cells were repeatedly transplanted subcutaneously near the immunization site one day before disease induction, and on day 3 and day 6 during EAE immune system priming. On

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3 day 9, lymphocytes were harvested from local lymph nodes and assessed for sensitivity to the
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5 immunizing antigen. MBP-specific proliferation of LN cells (mainly T cells) was measured
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7 and compared between treated and control animals (**Figure 5**). LN derived T cells from all
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9 treatment groups showed a significantly reduced reactivity towards MBP. These data show
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11 that WJ-, BM-MSc, and MAPC treated EAE animals exhibited a reduced lymphocyte
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13 proliferation upon restimulation with MBP, indicating that local application of stem cells
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15 interferes to some extent with their activation.
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Discussion

MSCs are widely investigated as candidates for tissue engineering and cell transplantation in inflammatory degenerative diseases due to their differentiation capacity, their extensive immune modulatory properties as well as trophic support functions [54]. The human umbilical cord has gained much attention over the years as an alternative source of multipotent MSCs. Furthermore, many reports attribute a broader expansion and differentiation potential to these cells than adult tissue-derived stem cells [10,29]. To investigate these extensive biological features of umbilical cord-derived WJ-MSCs in comparison to adult bone marrow-derived stem cells, we first identified the most optimal method to isolate and expand the WJ-MSCs. Afterwards, we assessed their phenotype and defined their whole genome transcriptional profile.

We found that the explant isolation technique was superior as compared to enzymatic culturing. The explant cell morphology more closely resembled that of fibroblast-like MSCs from bone marrow and the cells exhibited better growth rates than their enzymatic counterparts. Moreover, enzymatic cultures presented with earlier growth arrest and the spontaneous appearance of large myofibroblast-like cells. We speculate that this population is induced by the applied isolation procedure itself. In fact, enzymatic isolation has been reported to potentially alter the immunophenotype and functions of the cell [25,55], as well as preselect for endothelial progenitors [56]. In concordance with other studies [57,58], we show that the Wharton's jelly explant method yields lower variability and better growth rates than enzymatic isolation, here performed as collagenase type I digestion. The latter was previously reported to enrich for MSCs [35]. In our hands, a heterogeneous stem cell isolate containing MSC was initially isolated, but expression of MSC markers declined with increasing culture time (passage 5). Similar morphological and marker expression changes were previously reported for senescent BM-MSCs [59] or long term cultured BM- and amnion-derived MSC

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3 [60]. Although enzymatic digestion is the fastest way of obtaining cells from the cord matrix
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5 [52], explant or whole cord cultures are considered highly enriching for mesenchymal stromal
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7 cells, since spontaneous migration out of the tissue needs to occur [25,33,61].
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10 Our further experiments were performed with explant-derived Wharton's jelly stromal cells.
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12 Similar to previous reports [26,62], the cells exhibited MSC-like properties based on their
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14 culture morphology, ability to differentiate into the classical mesenchymal cell types (bone,
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16 fat, cartilage), and their surface marker expression profile. However, we observed subtle
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18 differences in expression of immune-related and adhesion molecules. In summary, these
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20 findings indicate that explant-derived WJ-MSC express the previously defined mesenchymal
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22 phenotype [4], but differ from BM-MSC regarding their cell surface molecules and growth
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24 characteristics.
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27 Extra-embryonic tissue-derived stem cells are attributed extended therapeutic properties over
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29 adult stem cells [63-65]. To further find evidence for that, we performed a whole genome
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31 transcriptional comparison of WJ-MSC to both lineage-restricted (classical BM-MSC) and to
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33 non-restricted MAPC. In this way WJ-MSC were compared to adult cells derived from the
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35 same tissue but with different stem cell potency (multipotent BM vs pluripotent MAPC).
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37 MAPC, which are derived from bone marrow under defined culture conditions, yield a unique
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39 population of stem cells, which have the ability to differentiate into cells from all three germ
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41 layers, express markers similar to embryonic stem cells, and show higher replicative potential
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43 compared to standard MSCs [66-69].
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47 Here, microarray analysis revealed clear transcriptional differences between stem cells
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49 derived from Wharton's jelly and bone marrow. Our data indicate that WJ cells overexpress a
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51 multitude of factors with pro-regenerative functions related to cell-cell adhesion, immune
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53 modulation, and neurotrophic support. It does not imply that in this comparison the bone
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55 marrow-derived stem cell populations do not express these factors. Ample in vitro and
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3 preclinical data is available on their phenotype and regenerative properties [67,70-72].

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5 Moreover, both types of bone marrow-derived cell populations showed a distinct
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7 transcriptional profile. Specifically for WJ-MSC, an upregulated expression of anti-
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9 inflammatory molecules such as e.g. CD200, PD-L1 and non-classical HLAs, along with
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11 adhesion molecules such as ICAM1 and VCAM1 was observed. Such molecules have been
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13 implemented in the immunosuppressive actions of stem cells both *in vitro* and *in vivo* [73,74].

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15 In this study we confirm the immune modulatory activity *in vivo*, after stem cell
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17 administration near the immunization site in Lewis rat EAE. This setup allows for direct
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19 assessment of T cell modulation, and although the intrinsic immune modulatory profile of the
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21 three cell types was different, we found no differences in their ability to reduce T cell
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23 proliferation. We observed aIn this regard, the reduction in autoantigen-induced T cell
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25 proliferation for WJ-, BM-MSC, and MAPC, is indicative of indicating an active interplay
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27 between these cells and the inflammatory milieu after local application (licensing on the spot),
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29 and The observed reduced LN proliferation in transplanted animals is suggestive of an
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31 effector mechanism involving paracrine signaling, stem cell homing to lymph nodes and
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33 activation of immune modulatory action processes and stem cell homing to lymph nodes after
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35 subcutaneous implantation. Multiple molecules might be involved in suppression of T cells
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37 and they are not necessarily the same for each cell type. We have previously shown that IDO-
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39 1 expression in WJ-MSC is involved in suppression of T cell proliferation, but only appears
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41 after licensing [53]. Here, IDO was not detected in the naïve (unlicensed) stem cell
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43 populations. Moreover, other immune cells in the inflammatory cascade might be affected. It
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45 was previously shown for neural precursor cells that their secretome affected antigen
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47 presenting cell functioning after subcutaneous injection in EAE [75]. Regarding LN homing,
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49 wWe and others previously showed that WJ-MSC possess the proper trafficking potential for
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51 homing to the peripheral lymphoid tissues [53,76]. Furthermore, the majority of intravenously
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3 transplanted cells are trapped in the pulmonary vasculature and spleen. By subcutaneous
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5 administration, cell trapping was avoided.
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8 In addition to the expression of immune signaling molecules, we show constitutive higher
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10 levels of BDNF secretion in normal WJ-MSC cultures compared to BM-MSC. Previously we
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12 demonstrated a similar gene activity pattern in WJ-MSC, together with a beneficial effect on
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14 neuroinflammation and neurodegeneration after transplantation in an animal model of
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16 multiple sclerosis [53]. Expression of immune suppressive and neurotrophic factors is a
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18 crucial feature for triggering endogenous repair pathways [25,74]. Hence, the paracrine
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20 potential of WJ- and BM-MSC to induce neurite outgrowth in human SH-SY5Y
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22 neuroblastoma cells was investigated. Here, an adapted protocol based on sequential RA- and
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24 BDNF-signaling was used [47]. In the experimental setup, RA-treated SH-SY5Y cells were
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26 exposed to CM of WJ-MSC or BM-MSC. In parallel, BDNF-induced maturation was
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28 included as a positive control. This RA stimulation prior to the addition of CM or BDNF is
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30 preferable as it increases the expression of Tyrosine Kinase (TK) A and B receptor, thereby
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32 improving the responsiveness for neurotrophins such as BDNF, which was shown to be
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34 secreted by the stem cell populations that were used in this study [46,77]. The results
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36 described here are in line with Pires *et al.* [78], who reported that the secretome of BM-MSC
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38 and human umbilical cord perivascular cells (HUCPVC) boosts neurite outgrowth of SH-
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40 SY5Y cells.
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46 Due to the absence of direct contacts between BM-MSC, WJ-MSC and SH-SY5Y cells, the
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48 observed effects are solely attributed to the paracrine factors produced by these stem cell
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50 populations. BDNF was one of the neurotrophins identified as increased in the secretome of
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52 our WJ-MSC cultures and is a well described neurite outgrowth inducer [79], thereby likely
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54 responsible for the observed increase in neurite outgrowth. Martins *et al.* recently showed that
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56 BDNF derived from HUCPVC is a significant determinant for axonal outgrowth in rat
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3 embryonic cortical neurons [80]. They observed enhanced axonal growth upon local CM
4 application, which was lost when BDNF was depleted from the CM by TK receptor blocking.
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7 Nevertheless, the neurite outgrowth observed in our setup can also be attributed to other
8 factors than the CM-derived BDNF. In our cultures, the concentration of BDNF secreted by
9 WJ-MSCs and BM-MSCs is less than 400 pg/ml, compared to the positive control containing
10 50 ng/ml BDNF, thereby hinting at the involvement of other effector molecules. This
11 hypothesis is in line with Crigler *et al.* who demonstrated that the effect of BM-MSC-
12 produced BDNF is only partially responsible for the observed effects on SH-SY5Y cells [81].
13
14 The involvement of several distinct molecular categories, including neurotrophic and
15 angiogenic growth factors, cytokines, and microvesicles have been reported[64,74,78,82,83].
16
17 Likewise, a number of *in vivo* studies showed that expression of such cytokines and
18 neuroprotective growth factors by WJ-MSC correlates with a beneficial outcome in rat
19 models of Parkinson's disease [84], ischemic stroke ([85,86]), and spinal cord injury [87].
20
21 Finally, WJ-MSC display a plethora of other molecules for immunological interaction, cell
22 adhesion and homing (reviewed elsewhere [62,88]). Importantly, homing capacity together
23 with a reduced co-stimulatory surface profile and immunomodulatory phenotype are essential
24 for effective cell communication and migration towards sites of injury in both autologous or
25 allogeneic application. Similar to the study by Fong *et al.*, we observed comparable results for
26 expression of HLA class I genes, cytokines and adhesion molecules, but we found no
27 expression of pluripotency markers (e.g. *OCT4*, *SOX2*, *NANOG*) in WJ-MSC [63].
28
29 Although many tissue-derived stem cells are designated as MSCs, most comparative *in vitro*
30 studies of stem cells derived from different tissue sources have indicated functional
31 differences in gene and protein expression, postulating the novel stem cell subtype to be better
32 than the other. Plausible reasons for such differences could be (1) no uniform marker set
33 identifies MSCs from all these sources, (2) variations in isolation technique and culture
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3 medium components [89], or (3) MSCs acquire culture induced differences in cell potency
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5 [89,90]. In addition, the aseptic collection and processing of tissues is critical in preventing
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7 microbial contamination but also stem cell activation through e.g. TLR's [91]. Roobrouck *et*
8
9 *al.* indicated that both phenotype and functional properties of human mesangioblasts, BM-
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11 MSC and MAPC were partially influenced by the culture conditions [90]. Gatta *et al.* reported
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13 genotypical changes after extended in vitro culturing of WJ-MSC (12 passages) [92]. In this
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15 study, both BM-MSC and WJ-MSC were harvested at early passage (passage 3), minimizing
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17 the extent of prolonged culturing effects. In addition, all three cell types were cultured in
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19 different media inherent to their isolation method. We found a similar picture for umbilical
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21 cord MSC as was previously reported by Klingemann *et al.*, who compared the overall
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23 transcriptional profiles of umbilical cord and bone marrow derived MSC cultured under the
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25 same conditions [93]. Nevertheless, to date, it remains to be determined whether the isolation
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27 method used or additional growth factors in culture have a potential impact on the observed
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29 expression profile of the WJ cells. Based on our data and others (described above & [25,94]),
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31 we suggest that the isolation method already selects for a certain cell phenotype, which
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33 depending on the amount of physical stress during the handling (e.g. enzymatic cleaving of
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35 membrane receptors, mechanical stress of forceps or centrifugation) predestines for the
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37 specific gene expression patterns observed later on in cultured cells.
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45 In summary, this study reports the isolation of mesenchymal-like cells from the Wharton's
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47 jelly that express a distinct transcriptional signature compared to lineage-restricted and non-
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49 restricted bone marrow-derived stem cells. Overall, our data support the previous notion that
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51 WJ-MSC are equipped with potent trophic and immunomodulatory properties, related to their
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53 functions in vivo at the fetus-maternal interface [24,27,73]. Furthermore, we specifically
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55 observed an overrepresentation of immune modulatory and neurotrophic growth factors in
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3 WJ-MSK, indicating a potential beneficial role for transplantation in neurodegenerative
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5 diseases like spinal cord injury, stroke, multiple sclerosis, Parkinson's disease or amyotrophic
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7 lateral sclerosis, for which there is currently no cure.
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The authors declare that no competing financial interests exist.

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Figure legends

Figure 1: Culture characteristics of explant-derived WJ-MSC. (A) phase contrast image of an attached explant with cellular outgrowth; (B) WJ-MSC cells displaying heterogeneous morphology of fibroblast-like cells ($\pm 100 \mu\text{m}$, white arrow) and small, often triangular, cells ($\pm 20 \mu\text{m}$, black arrow); (C) colony formation in dense cultures at 100% confluency (large image) and after seeding in low cell numbers at 50 c/cm^2 for 14 days (inset: crystal violet colony stain); (D) CPD of parallel WJ-MSC cultures ($n=5$) isolated using explant culture and collagenase digestion. Culture were grown in DMEM-LG for 80 ± 5 days. (E) Flow cytometry analysis of MSC marker expression in explant and collagenase isolated WJ-MSC ($n=3$) at passage 3, 5, and 7. Collagenase isolated cells showed reduced CD73 and CD90 expression with increasing passage; (F) CPD of WJ-MSC ($n=10$) and BM-MSC ($n=7$) after 70 ± 5 days in culture. For this comparison, WJ-MSC were cultured in KO-DMEM/F12. Scale bars: A&C = $200 \mu\text{m}$; B = $100 \mu\text{m}$. Data are presented as mean with SD; * P value < 0.05 , ** P value < 0.01 .

Figure 2: Hierarchical clustering based on differential gene expression profile of WJ-MSC, BM-MSC and MAPC. (A) Principal component analysis based on the total gene expression matrix. (B) Heatmap displaying differential gene expression patterns and clustering of the stem cell type in separate groups. Variations in expression across samples were identified with a coefficient of variance cutoff value set at 0.95. Yellow marks high and orange-red marks low expression. Inset: Venn diagram plot showing the number of differentially regulated genes in WJ-MSC compared to both BM-MSC and MAPC.

Figure 3: BDNF secretion by WJ-MSC and BM-MSC. BDNF concentration was measured in supernatant of WJ-MSC ($n=10$) and BM-MSC ($n=7$) using sandwich ELISA. Data are shown as mean absolute concentration (pg/ml) with SD. *** P < 0.001 .

Figure 4: Neurite outgrowth of SH-SY5Y cells following exposure to CM BM-MSC and CM WJ-MSC. Control SH-SY5Y cells (A) were stimulated with RA for 5 days (B) after which RA administration was continued (C, prolonged RA exposure) or SH-SY5Y cells were either exposed to CM BM-MSC (D), CM WJ-MSC (E) or BDNF (F) to stimulate SH-SY5Y maturation. Quantitative analysis (G) of the mean neurite length showed that CM WJ-MSC ($n=5$), CM BM-MSC ($n=4$) and BDNF ($n=3$) were able to stimulate neurite outgrowth of SH-SY5Y cells after RA induction compared to control ($n=3$), RA-induced ($n=3$) and RA-

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3 matured SH-SY5Y cells (n=3). RA maturation did not stimulate neurite outgrowth. CM WJ-MSC matured
4 cells had significantly longer neurites than CM BM-MSC matured cells. No difference in mean neurite length
5 could be observed between BDNF- and CM WJ-MSC matured SH-SY5Y cells. Isotype control for staining
6 specificity is presented as insert in (A). Scale bars A-F: 100 μ m. Data are expressed as mean with SD. P values
7 are depicted as follows: #: P < 0.001 compared to control. ~: P < 0.001 compared to RA induced and RA
8 matured conditions. **: P < 0.01; ***: P < 0.001.
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17 **Figure 5: Subcutaneous stem cell transplantations exert local immune modulatory effects in Lewis rat**
18 **EAE.** Stem cells were subcutaneously transplanted 1 day before disease induction, and on day 3 & 6 after EAE
19 induction. Leukocyte cultures derived 9 days post immunization from the popliteal lymph nodes (LN) of WJ-
20 MSC, BM-MSC, and MAPC treated (n=3 each) and control animals (n=3) were restimulated with MBP.
21 Cultures without MBP served as baseline proliferation controls (dotted red line). The stimulation index is
22 presented as mean \pm SD. * P < 0.05 and ** P < 0.01 versus restimulated cultures of saline treated animals.
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Supplemental Figure legends

Figure S1: Proliferation of WJ-MSC in different culture conditions and comparison to BM-MSC. (A) Cumulative population doublings (CPD) of cells cultured in KO-DMEM/F12 (n=8) and DMEM-LG (n=5, culture duration: 75±8 days). (B) Phase contrast image of collagenase isolated cells. The cultures contained large flat cells displaying stress fibers and having low replicative potential at early passage (p3-p4). Scale bar = 50 µm. (C) Flow cytometry analysis of pluripotency associated transcription factors (PPM) and (D) Agarose gel of PCR products after PPM analysis in WJ-MSC (n=5; [donor d1-d5](#)) with positive control cell line NT2/D1. [A description of this procedure can be found in the Supplemental materials and methods.](#) Data are presented as mean with SD; * P value < 0.05.

Figure S2: Mesenchymal differentiation potential of WJ-MSC and BM-MSC. Multilineage differentiation towards adipocytes, osteoblasts and chondrocytes was validated using histochemical staining with Oil Red O (lipid droplets within the cell cytoplasm), Alizarin Red S (diffuse calcium deposits within the extracellular matrix) and Alcian Blue (glycosaminoglycan matrix within the cartilage pellet) respectively. Scale bars: adipo = 50 µm, osteo = 100 µm, chondro = 200 µm.

Figure S3: Differential gene expression of WJ-MSC, BM-MSC and MAPC. Volcano plot showing differentially expressed genes for all three stem cell comparisons (WJ-MSC compared to BM-MSC, WJ-MSC compared to MAPC and BM-MSC compared to MAPC). Log2 fold differences are given, the red line represents a Benjamini & Hochberg (BH)-corrected P value of 0.05.

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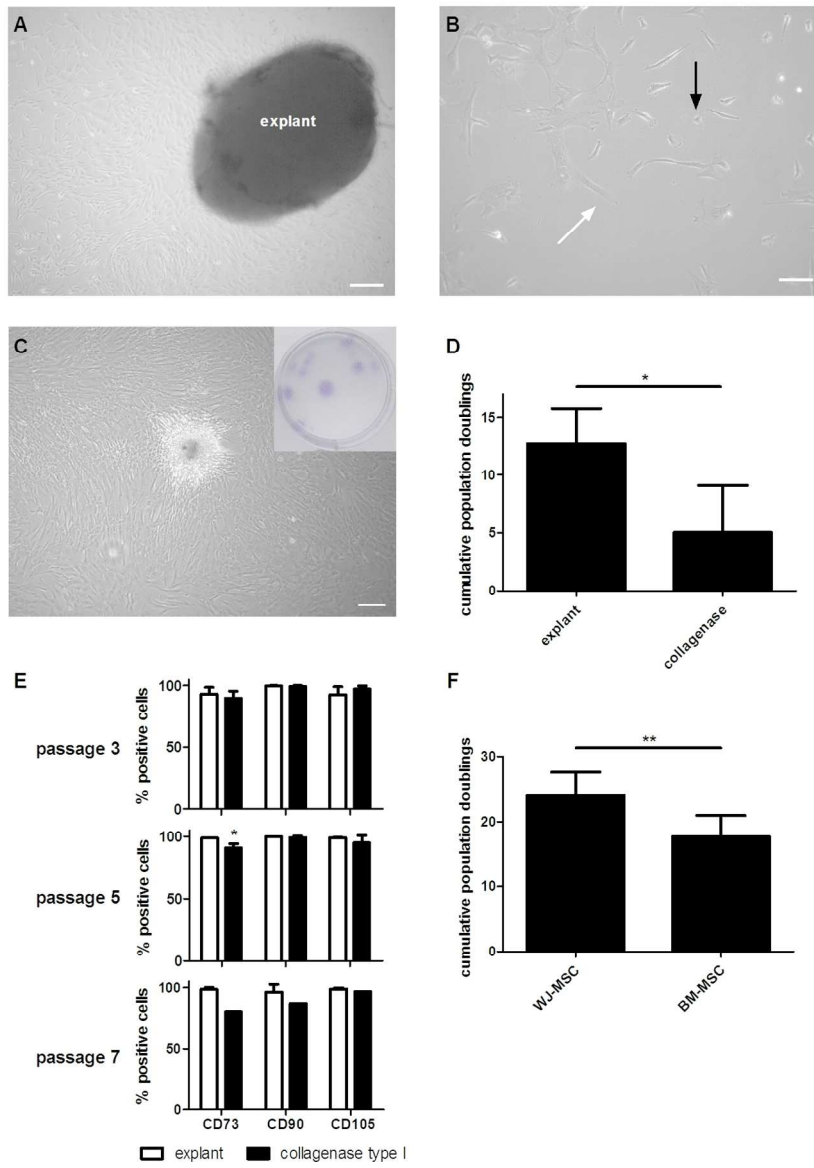


Figure 1 revised

119x168mm (300 x 300 DPI)

tribution

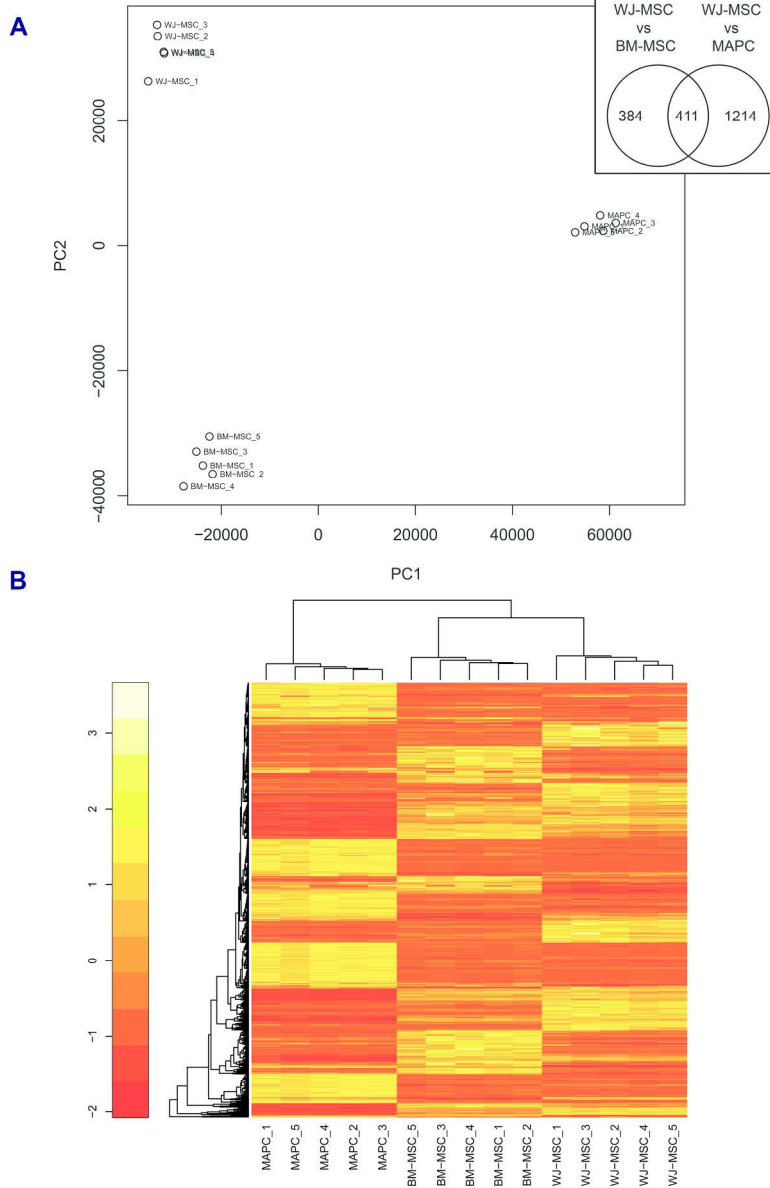


Figure 2 revised

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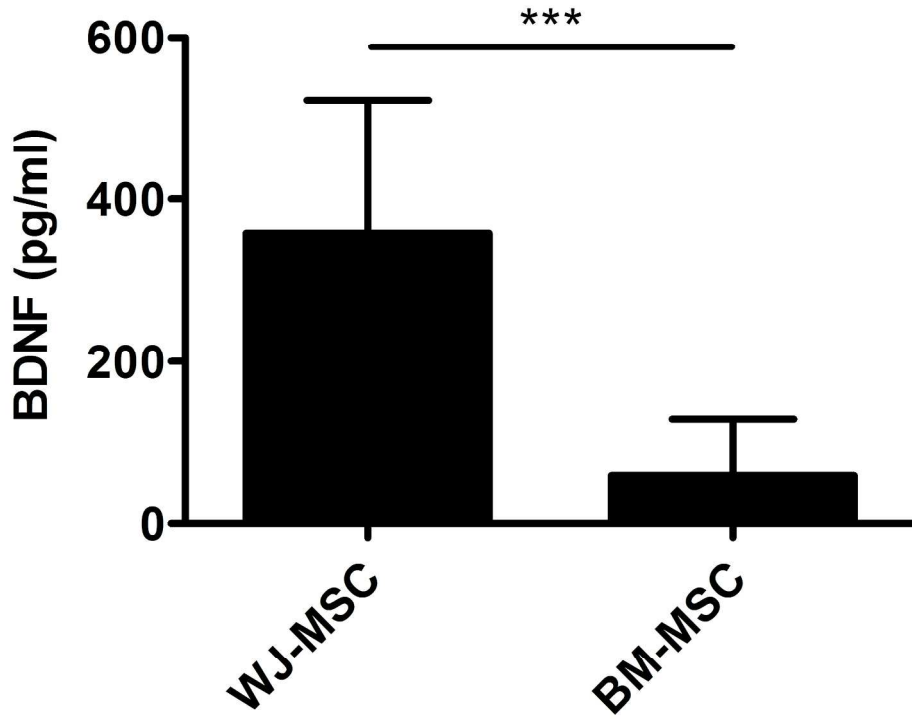


Figure 3 renamed

223x182mm (300 x 300 DPI)

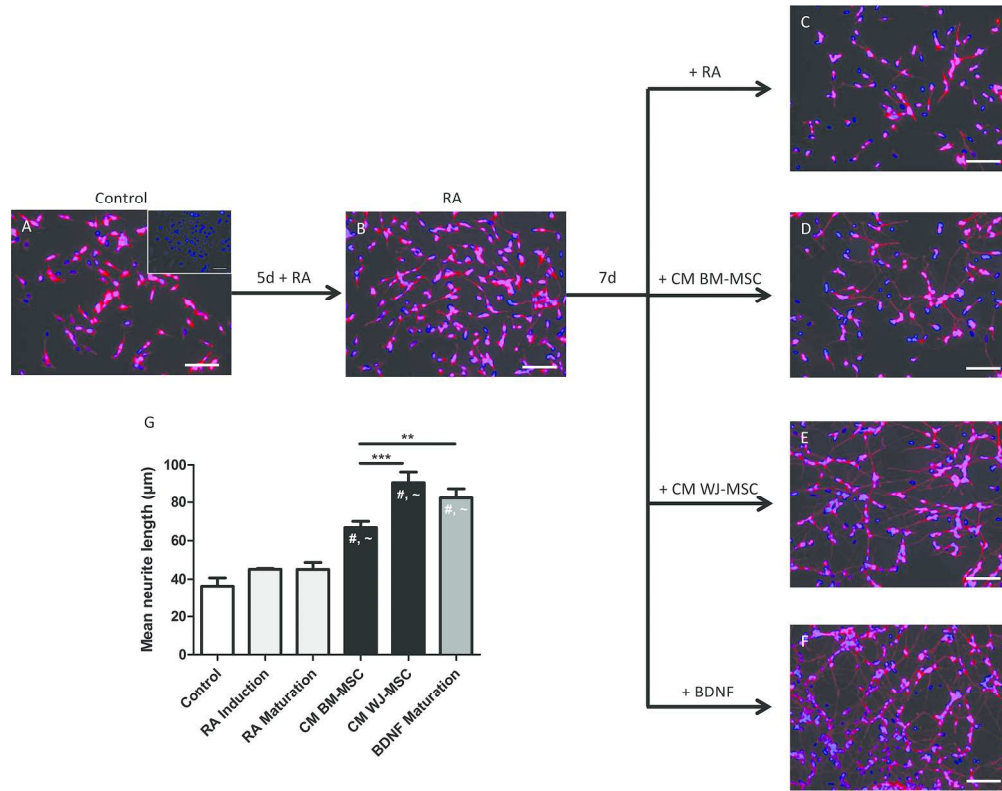


Figure 4 newly added

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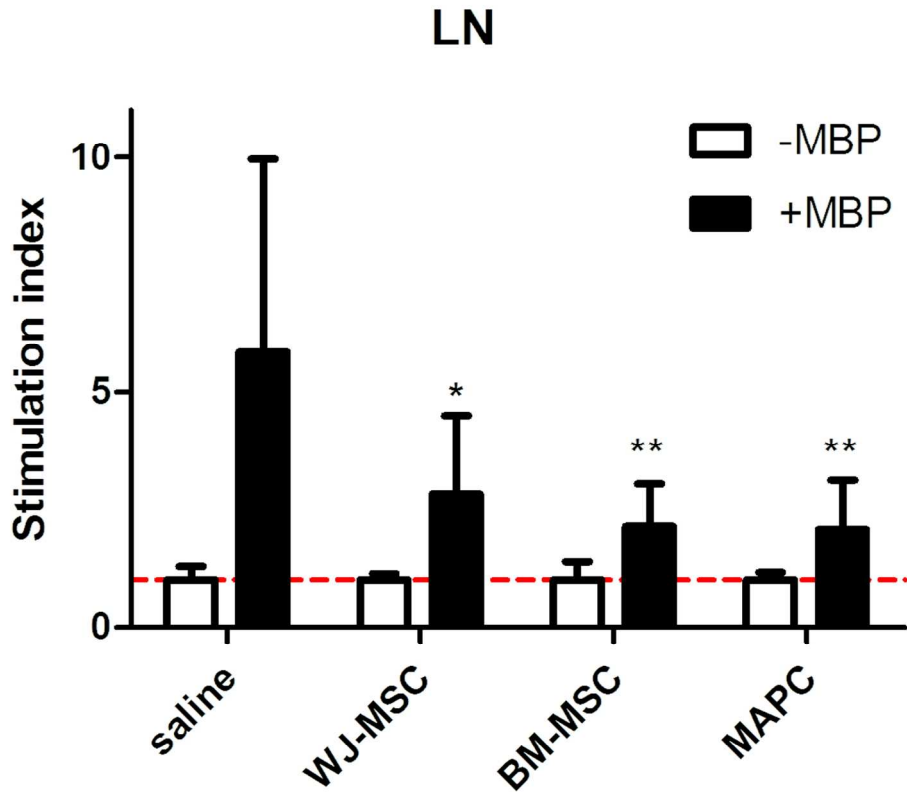


Figure 5 newly added

98x89mm (300 x 300 DPI)

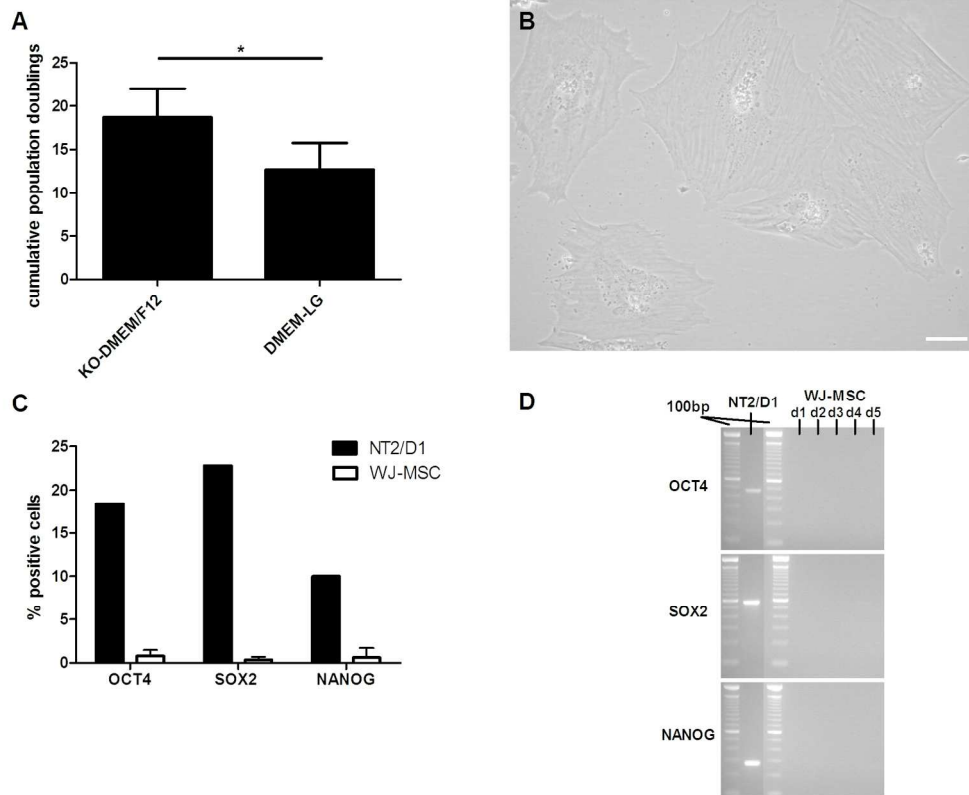
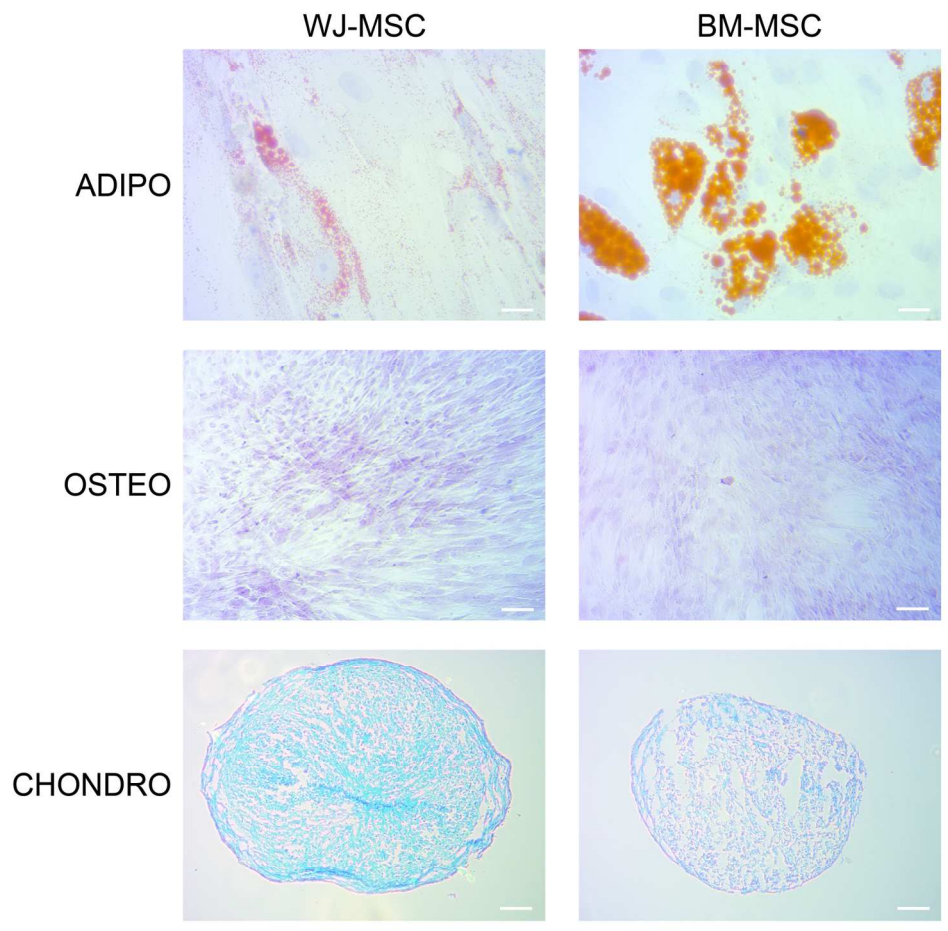


Figure S1 adapted

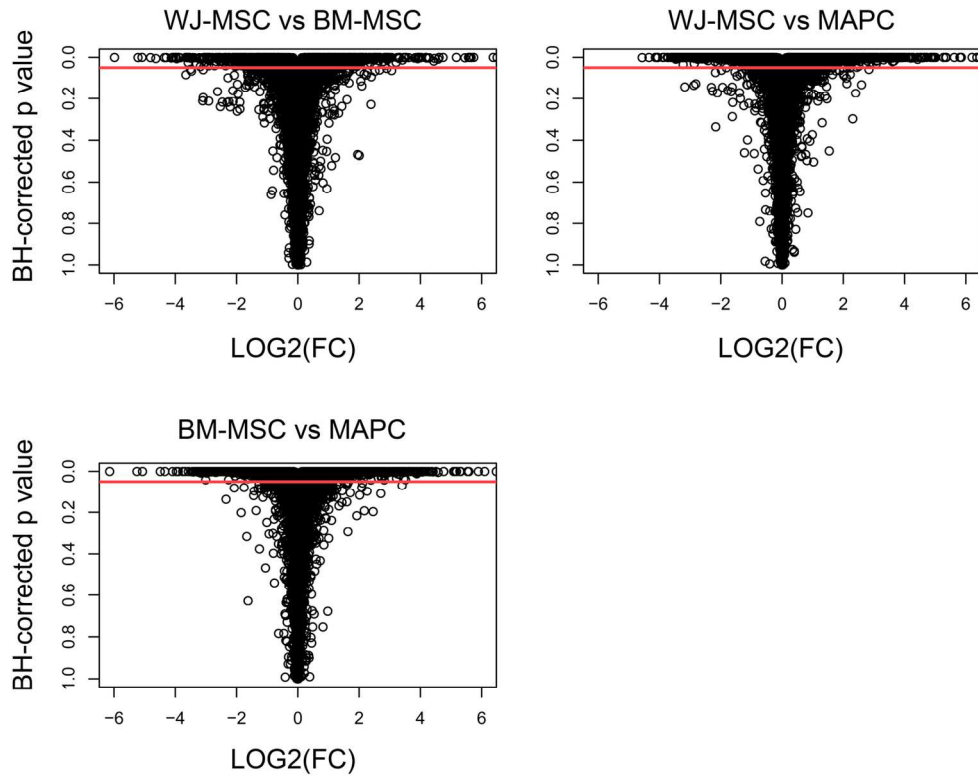
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Distribution



145x115mm (300 x 300 DPI)

Table 1: WJ-MSC and BM-MSC surface marker expression by flow cytometry

| marker | % expression | | marker | % expression | |
|--|--------------|-------------|-----------------|--------------|-------------|
| | WJ-MSC | BM-MSC | | WJ-MSC | BM-MSC |
| <u>MSC markers</u> | | | | | |
| CD105 | 95.8 ± 1.1 | 96.5 ± 0.9 | CD10*** | 73.7 ± 5.2 | 25.3 ± 6.8 |
| CD90 | 97.3 ± 0.9 | 96.1 ± 1.3 | CD13** | 58.4 ± 4.4 | 93.1 ± 3 |
| CD73 | 95.9 ± 1 | 95.6 ± 1.8 | CD44 | 95.9 ± 1 | 98.8 ± 0.3 |
| CD45 | 2.9 ± 1.1 | 4.4 ± 4 | CD146*** | 68.2 ± 5.3 | 19.3 ± 6.9 |
| CD34 | 2.2 ± 0.7 | 1.4 ± 1 | CD117 | 7.1 ± 2.4 | 7 ± 2.6 |
| CD19 | 0.9 ± 0.2 | 0.1 ± 0.1 | CD271 | 27.5 ± 4.1 | 24.1 ± 7.8 |
| CD14 | 2.5 ± 0.7 | 2.4 ± 1.1 | CD133 | 1.8 ± 0.9 | 16.4 ± 13.4 |
| <u>Adhesion molecules</u> | | | | | |
| Integrin-β1 | 96.6 ± 1.3 | 96.9 ± 1.1 | L-selectin | 1.8 ± 0.5 | 1.6 ± 0.7 |
| PECAM-1 | 0.7 ± 0.2 | 1.1 ± 0.4 | VCAM-1 | 17.8 ± 4.4 | 27.5 ± 8.8 |
| Integrin-α4** | 20.9 ± 2.7 | 7.4 ± 1.7 | PSGL-1 | 2.9 ± 1.1 | 3.5 ± 1.2 |
| ICAM-1*** | 80.2 ± 3.6 | 12.2 ± 3.4 | | | |
| <u>Cytokine / Chemokine receptors</u> | | | | | |
| IFN-γR1 | 15.2 ± 0.8 | 10.2 ± 3.1 | CCR2 | 2 ± 0.7 | 1.4 ± 0.2 |
| CXCR3 | 15.1 ± 3.1 | 13.9 ± 7.1 | CCR5 | 2.1 ± 0.6 | 1.8 ± 0.5 |
| CXCR4 | 4.1 ± 1.9 | 1.5 ± 0.4 | CCR7 | 0.3 ± 0.1 | 0.4 ± 0.1 |
| CCR1** | 1.5 ± 0.5 | 9.9 ± 1.1 | CX3CR1 | 0.7 ± 0.1 | 1.5 ± 0.2 |
| <u>Immune stimulatory / inhibitory molecules</u> | | | | | |
| TLR4* | 34.8 ± 9 | 71.4 ± 7.9 | PD-L1** | 76 ± 4.3 | 29.1 ± 7.5 |
| CD80 | 2.8 ± 1.3 | 1.5 ± 0.5 | PD-L2** | 58.6 ± 4.6 | 9.8 ± 1.6 |
| CD86 | 7.6 ± 1 | 6.8 ± 1.1 | HLA-ABC | 80.4 ± 4.9 | 78.2 ± 7.6 |
| CD40** | 8.1 ± 1.2 | 2.3 ± 0.9 | HLA-DR | 2.5 ± 1.6 | 1.4 ± 0.8 |
| CD95 | 40.7 ± 5.7 | 60.3 ± 14.4 | HLA-G | 3.3 ± 0.8 | 2.1 ± 0.6 |
| CD200* | 88.4 ± 4.6 | 50.9 ± 13.4 | HLA-E* | 31.9 ± 7.2 | 9.4 ± 5.1 |

* P < 0.05, ** P < 0.01, *** P < 0.001 (unpaired t-test)

Table 2: Top 30 differentially expressed genes of WJ-MSCs compared to BM-MSC

| probe ID | gene name | gene symbol | P value | fold change | probe ID | gene name | gene symbol | P value | fold change |
|------------------------------|--|-------------------|----------|-------------|--------------------------------|--|----------------|----------|-------------|
| upregulated vs BM-MSC | | | | | downregulated vs BM-MSC | | | | |
| 8054712 | interleukin 1, alpha | <i>IL1A</i> | 3.73E-04 | 90.71 | 8152522 | ectonucleotide pyrophosphatase/phosphodiesterase 2 | <i>ENPP2</i> | 1.66E-04 | -50 |
| 8054722 | interleukin 1, beta | <i>IL1B</i> | 2.88E-05 | 83.16 | 8104758 | natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C) | <i>NPR3</i> | 3.35E-04 | -33.33 |
| 8020779 | desmoglein 2 | <i>DSG2</i> | 3.24E-07 | 53.46 | 8123104 | fibronectin type III domain containing 1 | <i>FNDC1</i> | 3.24E-07 | -33.33 |
| 8022692 | desmocollin 3 | <i>DSC3</i> | 2.98E-04 | 50.43 | 8104746 | natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C) | <i>NPR3</i> | 4.82E-04 | -25 |
| 8180266 | ST6 (alpha-N-acetylneuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5 | <i>ST6GALNAC5</i> | 8.11E-07 | 49.42 | 8051583 | cytochrome P450, family 1, subfamily B, polypeptide 1 | <i>CYP1B1</i> | 2.87E-03 | -25 |
| 8005048 | myocardin | <i>MYOCD</i> | 8.38E-06 | 48.37 | 7916493 | phosphatidic acid phosphatase type 2B | <i>PPAP2B</i> | 6.08E-04 | -20 |
| 8144786 | solute carrier family 7 (cationic amino acid transporter, y+ system), member 2 | <i>SLC7A2</i> | 1.15E-04 | 47.57 | 7933194 | chemokine (C-X-C motif) ligand 12 | <i>CXCL12</i> | 3.99E-04 | -20 |
| 8042788 | actin, gamma 2, smooth muscle, enteric | <i>ACTG2</i> | 1.04E-03 | 43.81 | 8148070 | collagen, type XIV, alpha 1 | <i>COL14A1</i> | 1.54E-03 | -16.66 |
| 7902441 | ST6 (alpha-N-acetylneuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5 | <i>ST6GALNAC5</i> | 8.11E-07 | 36.13 | 8123739 | neurtin 1 | <i>NRN1</i> | 3.35E-04 | -16.66 |
| 7970763 | fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) | <i>FLT1</i> | 1.49E-04 | 26.72 | 8138888 | phosphodiesterase 1C, calmodulin-dependent 70kDa | <i>PDE1C</i> | 8.38E-06 | -16.66 |
| 7921916 | regulator of G-protein signaling 5 | <i>RGS5</i> | 1.17E-04 | 24.44 | 8131844 | glycoprotein (transmembrane) nmb | <i>GPNMB</i> | 1.05E-04 | -16.66 |
| 8100808 | sulfotransferase family 1E, estrogen-preferring, member 1 | <i>SULT1E1</i> | 4.48E-03 | 23.89 | 7937039 | early B-cell factor 3 | <i>EBF3</i> | 1.07E-06 | -16.66 |
| 8141016 | tissue factor pathway inhibitor 2 | <i>TFPI2</i> | 2.46E-03 | 22.11 | 7920165 | filaggrin | <i>FLG</i> | 3.28E-03 | -16.66 |
| 8161755 | aldehyde dehydrogenase 1 family, member A1 | <i>ALDH1A1</i> | 9.68E-03 | 21.68 | 8143127 | family with sequence similarity 180, member A | <i>FAM180A</i> | 2.88E-05 | -14.29 |
| 8046536 | homeobox D10 | <i>HOXD10</i> | 9.41E-04 | 19.54 | 7909730 | potassium channel, subfamily K, member 2 | <i>KCNK2</i> | 8.76E-04 | -14.29 |
| 7912520 | natriuretic peptide B | <i>NPPB</i> | 1.66E-03 | 19.34 | 8115543 | early B-cell factor 1 | <i>EBF1</i> | 2.99E-05 | -12.5 |
| 8095680 | interleukin 8 | <i>IL8</i> | 1.55E-03 | 18.37 | 7951077 | sestrin 3 | <i>SESN3</i> | 7.88E-04 | -12.5 |
| 8047788 | ADAM metallopeptidase domain 23 | <i>ADAM23</i> | 9.25E-04 | 18.19 | 7924071 | potassium voltage-gated channel, subfamily H (eag-related), member 1 | <i>KCNHI</i> | 4.58E-06 | -12.5 |
| 7963567 | keratin 8 | <i>KRT8</i> | 2.88E-05 | 18.15 | 7984813 | immunoglobulin superfamily containing leucine-rich repeat | <i>ISLR</i> | 7.56E-05 | -11.11 |
| 8086517 | CUB domain containing protein 1 | <i>CDCP1</i> | 1.90E-04 | 16.39 | 7975779 | FBJ murine osteosarcoma viral oncogene homolog | <i>FOS</i> | 5.76E-04 | -11.11 |
| 8138997 | T-box 20 | <i>TBX20</i> | 1.01E-04 | 15.79 | 7966122 | transmembrane protein 119 | <i>TMEM119</i> | 2.28E-03 | -11.11 |
| 8092726 | claudin 1 | <i>CLDN1</i> | 3.61E-04 | 15.66 | 8102792 | protocadherin 18 | <i>PCDH18</i> | 1.54E-03 | -11.11 |
| 8056222 | dipeptidyl-peptidase 4 | <i>DPP4</i> | 1.35E-03 | 15.59 | 8055952 | nuclear receptor subfamily 4, group A, member 2 | <i>NR4A2</i> | 4.49E-04 | -11.11 |
| 8005043 | hypothetical protein FLJ34690 | <i>FLJ34690</i> | 7.45E-06 | 15.46 | 7965410 | decorin | <i>DCN</i> | 7.91E-04 | -10 |
| 8131944 | nuclear factor (erythroid-derived 2)-like 3 | <i>NFE2L3</i> | 1.90E-04 | 15.36 | 8014063 | ecotropic viral integration site 2B | <i>EVI2B</i> | 2.74E-03 | -9.09 |
| 8025601 | intercellular adhesion molecule 1 | <i>ICAM1</i> | 4.22E-04 | 15.29 | 7919028 | T-box 15 | <i>TBX15</i> | 7.99E-04 | -9.09 |
| 8081657 | CD200 molecule | <i>CD200</i> | 3.16E-03 | 15.07 | 8157524 | toll-like receptor 4 | <i>TLR4</i> | 3.38E-03 | -9.09 |
| 7985493 | transmembrane 6 superfamily member 1 | <i>TM6SF1</i> | 7.56E-05 | 15.07 | 7919815 | cathepsin K | <i>CTSK</i> | 6.77E-04 | -8.33 |
| 8105267 | integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2) | <i>ITGA2</i> | 6.59E-04 | 14.41 | 8122099 | ectonucleotide pyrophosphatase/phosphodiesterase 1 | <i>ENPP1</i> | 1.79E-03 | -8.33 |
| 7976012 | neurexin 3 | <i>NRXN3</i> | 5.91E-04 | 14.38 | 8150962 | thymocyte selection-associated high mobility group box | <i>TOX</i> | 2.90E-03 | -8.33 |

Table 3: Top 30 differentially expressed genes of WJ-MSCs compared to MAPC

| probe ID | gene name | gene symbol | P value | fold change | probe ID | gene name | gene symbol | P value | fold change |
|----------------------------|--|-------------------|----------|-------------|------------------------------|---|------------------|----------|-------------|
| upregulated vs MAPC | | | | | downregulated vs MAPC | | | | |
| 8146863 | sulfatase 1 | <i>SULF1</i> | 6.22E-07 | 116.57 | 8157524 | toll-like receptor 4 | <i>TLR4</i> | 5.85E-04 | -25 |
| 8054712 | interleukin 1, alpha | <i>IL1A</i> | 9.20E-05 | 96.39 | 8138527 | STEAP family protein MGC87042 | <i>MGC87042</i> | 2.13E-04 | -20 |
| 8042788 | actin, gamma 2, smooth muscle, enteric | <i>ACTG2</i> | 2.02E-05 | 87.83 | 8095744 | amphiregulin | <i>AREG</i> | 1.50E-05 | -16.66 |
| 8054722 | interleukin 1, beta | <i>IL1B</i> | 3.19E-06 | 82.09 | 8143127 | family with sequence similarity 180, member A | <i>FAM180A</i> | 2.01E-05 | -16.66 |
| 8081657 | CD200 molecule | <i>CD200</i> | 2.03E-06 | 73.72 | 7916493 | phosphatidic acid phosphatase type 2B | <i>PPAP2B</i> | 4.03E-04 | -16.66 |
| 8005048 | myocardin | <i>MYOCD</i> | 8.99E-07 | 57.73 | 8150962 | thymocyte selection-associated high mobility group box | <i>TOX</i> | 4.95E-05 | -16.66 |
| 8020779 | desmoglein 2 | <i>DSG2</i> | 1.40E-06 | 56.04 | 8059580 | delta/notch-like EGF repeat containing | <i>DNER</i> | 9.81E-05 | -14.29 |
| 8022692 | desmocollin 3 | <i>DSC3</i> | 8.22E-05 | 49.25 | 8149955 | PDZ binding kinase | <i>PBK</i> | 9.35E-06 | -14.29 |
| 8006433 | chemokine (C-C motif) ligand 2 | <i>CCL2</i> | 2.03E-06 | 41.05 | 7989647 | KIAA0101 | <i>KIAA0101</i> | 4.13E-06 | -14.29 |
| 8180266 | ST6 (alpha-N-acetyl-neuraminy-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5 | <i>ST6GALNAC5</i> | 1.84E-07 | 40.47 | 7914878 | claspin | <i>CLSPN</i> | 1.39E-05 | -14.29 |
| 7922976 | prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) | <i>PTGS2</i> | 1.16E-04 | 38.64 | 8001133 | SHC SH2-domain binding protein 1 | <i>SHCBP1</i> | 2.07E-04 | -12.5 |
| 7921916 | regulator of G-protein signaling 5 | <i>RGS5</i> | 1.72E-04 | 32.31 | 7896709 | | | 1.43E-04 | -12.5 |
| 8136200 | carboxypeptidase A4 | <i>CPA4</i> | 1.45E-05 | 31.33 | 8161964 | FERM domain containing 3 | <i>FRMD3</i> | 1.10E-04 | -12.5 |
| 8056222 | dipeptidyl-peptidase 4 | <i>DPP4</i> | 3.64E-05 | 30.65 | 7906930 | NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae) | <i>NUF2</i> | 7.19E-05 | -12.5 |
| 7902441 | ST6 (alpha-N-acetyl-neuraminy-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5 | <i>ST6GALNAC5</i> | 2.29E-07 | 30.55 | 7970513 | spindle and kinetochore associated complex subunit 3 | <i>SKA3</i> | 5.22E-06 | -12.5 |
| 8092726 | claudin 1 | <i>CLDN1</i> | 2.19E-04 | 30.49 | 7974404 | cyclin-dependent kinase inhibitor 3 | <i>CDKN3</i> | 2.07E-04 | -12.5 |
| 8157487 | pregnancy-associated plasma protein A, pappalysin 1 | <i>PAPPA</i> | 6.78E-06 | 29.82 | 8077899 | peroxisome proliferator-activated receptor gamma | <i>PPARG</i> | 8.39E-06 | -12.5 |
| 8047788 | ADAM metalloproteinase domain 23 | <i>ADAM23</i> | 1.45E-06 | 28.84 | 7978846 | polymerase (DNA directed), epsilon 2 (p59 subunit) | <i>POLE2</i> | 5.72E-05 | -11.11 |
| 7976012 | neurexin 3 | <i>NRXN3</i> | 5.94E-06 | 26.39 | 7982757 | cancer susceptibility candidate 5 | <i>CASC5</i> | 2.61E-04 | -11.11 |
| 8136248 | mesoderm specific transcript homolog (mouse) | <i>MEST</i> | 7.18E-05 | 25.05 | 8157650 | prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase) | <i>PTGS1</i> | 6.84E-06 | -11.11 |
| 8131803 | interleukin 6 (interferon, beta 2) | <i>IL6</i> | 9.59E-04 | 24.14 | 8117594 | histone cluster 1, H2bm | <i>HIST1H2BM</i> | 5.52E-06 | -11.11 |
| 8025601 | intercellular adhesion molecule 1 | <i>ICAM1</i> | 7.22E-07 | 23.65 | 8124394 | histone cluster 1, H2bb | <i>HIST1H2BB</i> | 1.07E-05 | -11.11 |
| 7970763 | fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) | <i>FLT1</i> | 4.42E-06 | 23.52 | 7962058 | transmembrane and tetrapeptide repeat containing 1 | <i>TMTC1</i> | 1.83E-03 | -11.11 |
| 7960919 | microfibrillar associated protein 5 | <i>MFAP5</i> | 9.35E-06 | 23.45 | 8097356 | polo-like kinase 4 | <i>PLK4</i> | 1.56E-04 | -11.11 |
| 8121838 | tumor protein D52-like 1 | <i>TPD52L1</i> | 1.69E-06 | 22.94 | 7916898 | DEP domain containing 1 | <i>DEPDC1</i> | 4.55E-05 | -11.11 |
| 7965403 | lumican | <i>LUM</i> | 2.16E-04 | 22.93 | 7940147 | family with sequence similarity 111, member B | <i>FAM111B</i> | 1.14E-05 | -11.11 |
| 8161755 | aldehyde dehydrogenase 1 family, member A1 | <i>ALDH1A1</i> | 5.21E-03 | 21.49 | 7927710 | cyclin-dependent kinase 1 | <i>CDK1</i> | 3.22E-04 | -11.11 |
| 8102532 | phosphodiesterase 5A, cGMP-specific | <i>PDE5A</i> | 2.47E-06 | 21.37 | 8097628 | hedgehog interacting protein | <i>HHIP</i> | 4.89E-03 | -11.11 |
| 8123246 | solute carrier family 22 (extraneuronal monoamine transporter), member 3 | <i>SLC22A3</i> | 5.88E-05 | 19.43 | 7960340 | forkhead box M1 | <i>FOXM1</i> | 7.31E-05 | -11.11 |
| 8046536 | homeobox D10 | <i>HOXD10</i> | 1.12E-03 | 19.25 | 8151871 | cyclin E2 | <i>CCNE2</i> | 2.03E-06 | -11.11 |

Table 4: Microarray comparison of different effector molecule categories in WJ-MSC versus BM-MSC and MAPC

| Gene description | Gene symbol | FC WJ/BM | FC WJ/MAPC |
|---|----------------|-------------|---------------|
| <u>Adhesion molecules and receptors</u> | | | |
| intercellular adhesion molecule 1 | <i>ICAM1</i> | 15.29 | 23.65 |
| toll-like receptor 4 | <i>TLR4</i> | -9.09 | -25 |
| vascular cell adhesion molecule 1 | <i>VCAM1</i> | -4.22 | 5.68 |
| major histocompatibility complex, class I | <i>HLA-ABC</i> | 1.36 | 4.36 |
| major histocompatibility complex, class I, G | <i>HLA-G</i> | 1.23 | 3.73 |
| major histocompatibility complex, class I, E | <i>HLA-E</i> | 1.34 | 2.86 |
| major histocompatibility complex, class I, F | <i>HLA-F</i> | 1.34 | 2.93 |
| desmoglein 2 | <i>DSG2</i> | 53.46 | 56.04 |
| desmocollin 3 | <i>DSC3</i> | 50.43 | 49.25 |
| lumican | <i>LUM</i> | -2.58 | 22.93 |
| <u>Immune modulatory molecules</u> | | | |
| CD200 molecule | <i>CD200</i> | 15.07 | 73.72 |
| prostaglandin E synthase | <i>PTGES</i> | -4.40 | 1.58 |
| prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase) | <i>COX-1</i> | 1.1 | -11.11 |
| prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) | <i>COX-2</i> | 1.98 | 38.64 |
| integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor) | <i>CD49B</i> | 14.40 | 4.65 |
| hepatocyte growth factor (hepapoietin A; scatter factor) | <i>HGF</i> | -2.15 | 1.22 |
| Programmed death-ligand 1 (CD274 / B7-H1) | <i>PD-L1</i> | 11.25 | 1.89 |
| Indoleamine 2,3-Dioxygenase 1 | <i>IDO-1</i> | n.d. | n.d. |
| <u>Cytokines and chemokines</u> | | | |
| Interleukin 1, beta | <i>IL1B</i> | 83.16 | 82.09 |
| Interleukin 6 | <i>IL6</i> | 1.11 | 24.14 |
| Interleukin 8 | <i>IL8</i> | 18.37 | 6.29 |
| chemokine (C-C motif) ligand 2 | <i>CCL2</i> | 3.07 | 41.05 |
| chemokine (C-X-C motif) ligand 2 | <i>CXCL2</i> | 2.21 | 1.14 |
| chemokine (C-X-C motif) ligand 12 | <i>CXCL12</i> | -20 | -1.11 |
| <u>Growth factors</u> | | | |
| brain-derived neurotrophic factor | <i>BDNF</i> | 2.99 | 5.15 |
| leukemia inhibitory factor | <i>LIF</i> | 2.41 | 5.82 |
| neurotrophin 3 | <i>NTF3</i> | 3.17 | 3.87 |
| transforming growth factor, beta 2 | <i>TGFB2</i> | 3.57 | 7.68 |
| vascular endothelial growth factor A | <i>VEGFA</i> | 1.13 | 5.08 |
| fibroblast growth factor 2 (basic) | <i>FGF2</i> | 1.83 | 2.37 |
| insulin-like growth factor 1 (somatomedin C) | <i>IGF1</i> | -1.56 | 1.08 |
| glial cell derived neurotrophic factor | <i>GDNF</i> | 1.36 | 1.05 |
| <u>Enzymes and signaling molecules</u> | | | |
| sulfatase 1 | <i>SULF1</i> | 1.31 | 116.57 |
| aldehyde dehydrogenase 1 family, member A1 | <i>ALDH1A1</i> | 21.68 | 21.49 |
| SMAD family member 3 | <i>SMAD3</i> | 2.13 | 4.40 |
| POU class 5 homeobox 1 pseudogene 3 | <i>OCT4</i> | -1.12 | 1.02 |
| SRY (sex determining region Y)-box 2 | <i>SOX2</i> | n.d. | n.d. |
| Nanog homeobox | <i>NANOG</i> | 1.10 | 1.08 |

FC = fold change; n.d. = not detectable

Table 5: Canonical pathways and functions in the WJ-MSC gene pool (n=411 genes) differentially regulated to both BM-MSC and MAPC (IPA)

| category | P value * | # molecules |
|--|---------------------|-------------|
| <i>Canonical pathways</i> | | |
| HMGB1 Signaling | 1.26E-05 | 12/118 |
| Hepatic Fibrosis / Hepatic Stellate Cell Activation | 1.33E-05 | 15/181 |
| Caveolar-mediated Endocytosis Signaling | 1.87E-05 | 9/68 |
| Agranulocyte Adhesion and Diapedesis | 1.01E-04 | 13/168 |
| Granulocyte Adhesion and Diapedesis | 2.20E-04 | 12/158 |
| <i>Molecular and cellular functions</i> | | |
| Cellular Development | 5.97E-05 - 5.33E-18 | 201 |
| Cellular Movement | 6.01E-05 - 7.89E-18 | 139 |
| Cellular Growth and Proliferation | 5.97E-05 - 2.02E-17 | 213 |
| Cell Morphology | 5.39E-05 - 4.10E-15 | 132 |
| Cell Death and Survival | 5.38E-05 - 9.86E-15 | 174 |
| <i>Physiological system development and function</i> | | |
| Embryonic Development | 6.01E-05 - 3.80E-17 | 138 |
| Organismal Development | 5.58E-05 - 3.80E-17 | 189 |
| Organismal Survival | 3.05E-05 - 1.34E-15 | 147 |
| Tissue Morphology | 4.88E-05 - 4.11E-15 | 146 |
| Cardiovascular System Development and Function | 5.58E-05 - 1.05E-14 | 133 |

P value* = for molecular and cellular functions and physiological system development and function a range is given as the categories are comprised of multiple sub functions

Supplemental tables**Table S1: Antibodies for flow cytometry analysis**

| Marker | Clone | Isotype | Label | Company |
|-------------|-------------|---------|------------------|-------------------|
| CD10 | CB-CALLA | mIgG2b | PE | eBioscience |
| CD13 | WM-15 | mIgG1κ | FITC | eBioscience |
| CD14 | MEM-15 | mIgG1 | PE | Immunotools |
| CD19 | LT19 | mIgG1 | FITC | Immunotools |
| Integrin-β1 | TS2/16 | mIgG1 | FITC | eBioscience |
| PECAM-1 | MEM-05 | mIgG1 | FITC | Immunotools |
| CD34 | 4H11 | mIgG1 | PE | Immunotools |
| CD40 | 5C3 | mIgG1κ | FITC | eBioscience |
| CD44 | IM7 | rIgG2bk | FITC | eBioscience |
| CD45 | MEM-28 | mIgG1 | FITC | Immunotools |
| Integrin-α4 | BU49 | mIgG1 | FITC | Immunotools |
| ICAM-1 | HA58 | mIgG1κ | PE | eBioscience |
| L-selectin | DREG-56 | mIgG1κ | FITC | BD Biosciences |
| CD73 | AD2 | mIgG1κ | PE | BD Biosciences |
| CD80 | L307.4 | mIgG1κ | PE | BD Biosciences |
| CD86 | 2331(FUN-1) | mIgG1κ | FITC | BD Biosciences |
| CD90 | 5E10 | mIgG1κ | FITC | eBioscience |
| CD95 | DX2 | mIgG1κ | FITC | BD Biosciences |
| CD105 | SN6 | mIgG1 | PE | eBioscience |
| VCAM-1 | STA | mIgG1κ | PE | eBioscience |
| CD117 | YB5.B8 | mIgG1κ | PE | eBioscience |
| IFN-γR1 | GIR-208 | mIgG1 | PE | eBioscience |
| CD133 | AC133 | mIgG1 | PE | Miltenyi Biotec |
| CD146 | P1H12 | mIgG1κ | PE | BD Biosciences |
| PSGL-1 | KPL-1 | mIgG1κ | PE | BD Biosciences |
| CXCR3 | 1C6 | mIgG1κ | PE | BD Biosciences |
| CXCR4 | 12G5 | mIgG2ak | PE | BD Biosciences |
| CCR1 | 53504 | mIgG2b | PE | R&D Systems |
| CCR2 | 48607 | mIgG2b | PerCP | R&D Systems |
| CCR5 | R22/7 | mIgG1κ | PE | eBioscience |
| CCR7 | 3D12 | rIgG2ak | PE | eBioscience |
| CD200 | OX104 | mIgG1κ | PE | eBioscience |
| CD271 | ME20.4 | mIgG1κ | FITC | Biolegend |
| CX3CR1 | 2A9-1 | rIgG2ak | PE | MBL International |
| HLA-ABC | W6/32 | mIgG2a | FITC | Immunotools |
| HLA-DR | MEM-12 | mIgG1κ | PE | Immunotools |
| HLA-E | 3D12 | mIgG1 | PE | eBioscience |
| HLA-G | 87G | mIgG2a | PE | eBioscience |
| NANOG | N31-355 | mIgG1κ | PE | BD Biosciences |
| OCT3/4 | 40/Oct-3 | mIgG1κ | PerCP-Cy5.5 | BD Biosciences |
| PD-L1 | MIH1 | mIgG1 | PE | eBioscience |
| PD-L2 | MIH18 | mIgG1κ | PE | eBioscience |
| SOX2 | 245610 | mIgG2ak | Alexa Fluor® 647 | BD Biosciences |
| TLR4 | HTA125 | mIgG2ak | PE | eBioscience |

For isotype: m = mouse; r = rat

Table S2: Overrepresented KEGG pathways and biological functions within the WJ-MSC gene pool (n=411 genes) differentially regulated to both BM-MSC and MAPC (DAVID)

| Category | Term | P-value | Benjamini | |
|---|---|----------------------------------|-----------|----------|
| KEGG | arrhythmogenic right ventricular cardiomyopathy | 4,50E-06 | 5,10E-04 | |
| | ECM-receptor interaction | 8,90E-05 | 5,00E-03 | |
| | focal adhesion | 1,00E-03 | 3,70E-02 | |
| | o-glycan biosynthesis | 1,20E-03 | 3,40E-02 | |
| | hypertrophic cardiomyopathy | 1,90E-03 | 4,20E-02 | |
| | hematopoietic cell lineage | 2,20E-03 | 4,10E-02 | |
| | MAPK signaling pathway | 2,40E-03 | 3,80E-02 | |
| | dilated cardiomyopathy | 3,20E-03 | 4,40E-02 | |
| | complement and coagulation cascades | 1,10E-02 | 1,30E-01 | |
| | cell adhesion molecules | 2,50E-02 | 2,50E-01 | |
| | apoptosis | 3,20E-02 | 2,90E-01 | |
| | GOTERM_BP | cell adhesion | 1,40E-07 | 3,10E-04 |
| | | biological adhesion | 1,50E-07 | 1,60E-04 |
| | | regulation of cell proliferation | 3,00E-06 | 1,10E-03 |
| positive regulation of macromolecule metabolic process | | 2,20E-04 | 1,60E-02 | |
| positive regulation of biosynthetic process | | 1,70E-05 | 3,50E-03 | |
| positive regulation of cellular biosynthetic process | | 3,10E-05 | 4,30E-03 | |
| regulation of apoptosis | | 6,60E-04 | 3,40E-02 | |
| regulation of programmed cell death | | 7,80E-04 | 3,70E-02 | |
| regulation of cell death | | 8,30E-04 | 3,60E-02 | |
| positive regulation of macromolecule biosynthetic process | | 3,10E-05 | 4,10E-03 | |
| response to wounding | | 2,90E-06 | 1,30E-03 | |
| homeostatic process | | 1,70E-03 | 5,50E-02 | |
| immune response | | 3,70E-04 | 2,40E-02 | |
| cell death | | 3,10E-03 | 8,30E-02 | |
| <i>Top 14 out of 145</i> | | | | |

Supplemental materials and methods

Polymerase chain reaction (PCR)

Total RNA from additional WJ stem cell donors (n=5) and the NT2/D1 cell line was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics, Vilvoorde, Belgium) according to manufacturer's instructions. RNA quality was assessed with a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) according to standard procedures. 1µg of RNA was reverse transcribed to cDNA using the Reverse transcription system (Promega, Leiden, The Netherlands) according to manufacturer's protocol. For each test, a 20µl reaction volume was used containing 19µl master mix consisting of nuclease-free water (Promega), 2µl 10x PCR buffer + MgCl₂ (Invitrogen™, ThermoFisher Scientific), 10mM forward (fw) and reverse (rev) primers (Eurogentec, Seraing, Belgium), 0.2µl dNTP mix (20mM, Invitrogen™), 0.16µl *Taq* polymerase (5U/µl; Invitrogen™) and 1µl of sample cDNA. As a negative control 1µl of nuclease-free water was added instead of cDNA. The PCR program consisted of a pre-denaturation step of 1 minute at 95°C followed by 40 cycles of 30 seconds at 95°C (denaturation), 30 seconds at 56 / 60°C (*OCT4-SOX2* / *NANOG* annealing respectively) and 1 minute at 72°C (elongation). Next, a final elongation step was performed for 10 minutes at 72°C after which the samples were held at 4°C until gel electrophoresis. Primers for *POU5F1* (*OCT4*), *SOX2*, and *NANOG* were as follows: *POU5F1* 5'-AGCCCTCATTTCACCAGGCC-3' (fw) and 5'-TGGGACTCCTCCGGGTTTTG-3' (rev); *SOX2* 5'-AGAAGCGGCCGTTTCATCGAC-3' (fw) and 5'-TGCTGATCATGTCCCGGAGGT-3' (rev); *NANOG* 5'-CAGAAGGCCTCAGCACCTAC-3' (fw) and 5'-GAATTGGCTGGAAGTGCAT-3' (rev). The PCR products or a 100 bp DNA ladder (Invitrogen™, ThermoFisher Scientific) were mixed with Orange G (Sigma-Aldrich, St. Louis, MO, USA) and separated by electrophoresis using a 2 % agarose gel in Tris-acetate-EDTA buffer containing ethidium

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bromide (1/10.000; Bio-Rad, Hercules, CA, USA). Gels were imaged using a Gel Doc XR system (Bio-Rad).

