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Enhanced neuro-therapeutic potential of Wharton's Jelly–derived mesenchymal stem cells in comparison with bone marrow mesenchymal stem cells culture

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Abstract

Substantial inconsistencies in mesenchymal stem (stromal) cell (MSC) therapy reported in early translational and clinical studies may indicate need for selection of the proper cell population for any particular therapeutic purpose. In the present study we have examined stromal stem cells derived either from umbilical cord Wharton's Jelly (WJ-MSC) or bone marrow (BM-MSC) of adult, healthy donors. The cells characterized in accordance with the International Society for Cellular Therapy (ISCT) indications as well as other phenotypic and functional parameters have been compared under strictly controlled culture conditions. WJ-MSC, in comparison with BM-MSC, exhibited a higher proliferation rate, a greater expansion capability being additionally stimulated under low-oxygen atmosphere, enhanced neurotrophic factors gene expression and spontaneous tendency toward a neural lineage differentiation commitment confirmed by protein and gene marker induction. Our data suggest that WJ-MSC may represent an example of immature-type "pre-MSC," where a substantial cellular component is embryonic-like, pluripotent derivatives with the default neural-like differentiation. These cells may contribute in different extents to nearly all classical MSC populations adversely correlated with the age of cell donors. Our data suggest that neuro-epithelial markers, like nestin, stage specific embryonic antigens-4 or α-smooth muscle actin expressions, may serve as useful indicators of MSC culture neuro-regeneration–associated potency.

Key Words: *Comparative neuroprotective potential, Default neural differentiation, Bone marrow mesenchymal stem (stromal) cells, Wharton's Jelly mesenchymal stem (stromal) cells*

Introduction

The substantial progress in stem cell research and its clinical translation is based on the biotechnical exploitation of at least two categories of human putative regenerating cells: Embryonic Stem or Pluripotency-induced Cells (ESC/iPS) and mesenchymal stem (stromal) cells (MSC).

The cell populations referred to as MSCs are in fact only *in vitro* products of still poorly understood and loosely controlled tissue derivation procedures. The term "mesenchymal" defines an adhesive progenitor cell with fusiform shape able to actively move but it is still difficult to ensure their precise lineage. The growing body of experimental and preclinical/clinical data suggests that only non-manipulated MSCs isolated under strictly controlled procedures may be considered a safe source of stem cells for transplantation purpose. This is because of persistent cellular heterogeneity resulting from the lack of standardized methods for prospective MSC isolation, similar to those used in selection of hematopoietic stem cells (HSCs). A unique surface marker identifying MSCs has not yet been characterized, necessitating the application of a panel of antigens for their characterization.

The accumulating data currently suggests that at least two separate classes of MSCs differ substantially in terms of their ontogenetic origin and essential biological features [1]. The first one being a dominating component of varied fetus-associated stem cell niches in perinatal tissues (umbilical cord, amnion, placenta, umbilical cord blood, etc), whereas the second, isolated from nearly all adult tissues, constitutes a classic

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type of adult MSC. The origin and properties of the first class is connected with the early stages of embryogenesis and comprised cells with broader, embryonic-like spectrum of differentiation able to cross the germ layers [2]. In addition, the recent data indicate that these primitive cells may exert exceptionally low immunogenicity but with high resting immunomodulatory, immunosuppressive and neuroprotective potential [3]. The ontogenic origin of these cells has been elucidated in mice by the inspiring work of Takashima et al [4], where the use of Cre-recombinase mediated lineage tracing analysis has shown that the primitive class of immature somatic progenitors with pluripotent potency and preferences to neural differentiation may originate from an embryonic neuro-epithelium of neural crest (NC). These cells after passing the first developmental epithelial mesenchymal transition (EMT) form a cohort of the primitive mesenchymal cells (pre-MSCs) that transiently populate all fetal tissue niches and then are gradually replaced by the mesoderm-recruited, post-gastrulation, adult MSCs. These primitive MSCs originating from NC (and probably also from additional extra-embryonic tissues) temporary are dominating over typical adult MSCs [5,6].

Until recently research activity of our group has been focused on the fetal stem cells isolated from the adherent mononuclear fraction of the human umbilical cord blood [7]. The developmental immature position of the cord blood [8] may explain the presence of primitive cells with flexible phenotype and ability of neural differentiation. In 2002–2006 the first descriptions of such cord blood–derived preMSC properties able to differentiate toward functional neurons and macroglia, were published by our group [9–11]. These cells transiently express ABCG2 transporter and have the ability to efflux Hoechst dye as a typical property of undifferentiated and highly proliferative progenitors called "side population" [12]. These cells expressed also pluripotency-specific markers and under the "crowd stress" or serum-free conditions lose their adherence and stayed as embryonic-like floating population [11] or formed neurosphere-like structures [13]. Later on, this type of cord blood–derived neural stem/progenitor was used for the first reported intraventricular therapeutic cell transplantation to a critically injured child with magnetic resonance imaging (MRI) monitoring of relative long cell survival and subsequent 5 years of patient clinical observation [14,15].

In recent experiments we have found that Wharton's Jelly (WJ)-derived MSCs display distinct pro-neural properties and immunologic characteristic similar to those previously observed in cord blood–derived mononuclear cells (MNCs) and suggesting their usefulness for neuro-transplantation [16]. In addition, this type of cell, when cultured under lowered to 5% oxygen atmosphere, switched back toward undifferentiated, embryonic-like phenotype and expressed a full panel of pluripotency-connected genes [17]. Later on we have found that adult BM-MSC culture is completely resistant to such neurogenic type of differentiation and did not express any pluripotency-specific genes and proteins either in 21% or in lowered to 5% oxygen culture. Herein we try to explain these and other phenotypic differences between WJ- and BM-derived MSC by their distinct developmental history connected either with the NC neuro-ectoderm or the post-gastrulation's mesoderm origin. Phenotypic and functional specificity of each type of MSC cultures observed after identical derivation conditions may ensure their specification toward defined but different therapeutic challenges.

Materials and methods

All experiments carried out were approved by the Ethics Committee of the Medical University of Warsaw. Informed consent was obtained from six females with full-term pregnancy or patients prepared for bone marrow (BM) aspiration. Three BM samples derived from generally healthy orthopedic patients aged 25, 40, and 50 were processed in two separate BM-MSC cultures for further experimental purpose.

Cells isolation and culture conditions

WJ-MSC

Samples of human umbilical cords were collected from six deliveries and were processed for MSC isolation using mechanical fragmentation techniques. Umbilical cords (~15 cm long) were briefly immersed in sterile phosphate-buffered saline (PBS; Gibco) supplemented with penicillin-streptomycin (1:100; Gibco) and then immediately transferred to the laboratory. The umbilical cords were cut with a sharp, sterile blade into the 2- to 3-mm pieces. Using a biopsy punch (Miltex, GmbH), small cylindrical fragments (2-mm diameter) were removed from the mucous connective tissue (WJ matrix), avoiding blood vessels and amniotic epithelium and transferred to culture dishes. WJ fragments were incubated in human MSC growth medium (MSCGM BulletKit, Lonza) at 37°C in a humidified incubator under 21% O_2 and 5% CO_2 atmosphere. After 7–10 days of culture, the first colonies of WJ-MSC were observed. Then the cells were pursued until subconfluence, nonadherent cells were removed and stromal cells after detachment (0.05% Trypsin-ethylenediaminetetraacetic acid [EDTA], Gibco) were transferred to 25-cm^2 flasks at initial density of $5 \times 10^3/cm^2$ and cultured up to 70–80% confluence before collection for subsequent passages.

BM-MSC

Samples of BM-MSCs were obtained by needle puncture aspiration of the iliac crest BM cavity in the presence of heparin mixed with PBS. Samples were transferred on Ficoll mixtures (1.077 g/mL; GE Healthcare) and MNCs were isolated by gradient centrifugation at 250*g* for 25 minutes. The pellet was collected, washed with PBS and centrifuged again. Then, the cells were suspended in MSC Lonza medium and kept in a humidified incubator saturated by 21% O_2 and 5% carbon dioxide at 37°C. After 24–48 hours, the floating cells were removed carefully and fresh medium was added. The culture medium was partially replaced every 3 to 4 days. When primary cultures become almost confluent, the culture were treated with 0.05% Trypsin-EDTA and spread into 25-cm^2 flasks in the density of 5×10^3/cm^2 for subsequent experiments. A purified population of MSCs can be obtained after 3 weeks from initiation of culture.

To avoid influence of methodological differences, the further processing and characteristic of WJ and BM-derived MSC cultures, they were performed under identical and parallel experimental conditions.

Proliferation analysis

Population doubling time

To compare the proliferation rate of BM- and WJ-MSC, the population doubling time (PDT) was determined as described previously [17]. Briefly, when cell density reached sub-confluence, cells were re-seeded at an initial density of 2000 cells/cm^2. PDT was calculated based on the total cell number at each passage using the formula $(t - t_0) \times \log 2/(\log N—\log N_0)$, where $t - t_0$ is culture time (days), N is the number of harvested cells and N_0 is the initial number of cells.

Ki67 expression

In addition, the influence of the 5% oxygen atmosphere on proliferation of both cultures was directly compared here by Ki67 immunofluorescence staining. Cells were labelled with a monoclonal antibody against Ki67, a human nuclear protein whose expression is strictly associated with cell cycle activity. Cells were seeded in 24-well culture plates at 4×10^3 densities and fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) after 2–3 days. A minimum of 100 cells was counted in 5 random fields to determine the percentage of Ki67-positive cells scored as the percentage of all stained nuclei.

Colony-forming unit fibroblasts frequency

Furthermore, the cells at passage 3 were seeded in duplicate in 6-well tissue culture plates at a density of 50 cells/cm^2 cell. Cell culture was maintained in 21% of oxygen and 5% oxygen level, 37°C, 90% humidity. After 14 days, cells were washed with PBS, fixed in 4% PFA for 15 min and stained with toluidine blue (1%) for 30 min, then washed/rinsed with distilled water. The colonies of minimum 5-mm diameter, which have appeared exclusively in WJ-MSC, were then counted and expressed as the percent of seeded cells.

Immunocytochemistry

For immunofluorescence staining, the cells were transferred to 24-well plates and cultured for 1–2 days. Using 4% PFA, cells were fixed for 15 min and extensively washed with PBS. Non-specific reactions were blocked by adding 10% Normal Goat Serum (Sigma-Aldrich) in PBS or 5% Bovine Serum Albumin (Sigma-Aldrich) in PBS and left to react for 1 h. To examine the expression of neural and mesenchymal markers the following primary antibodies (Table I) were added, with primary antibody replaced by PBS in the negative control group. Brachyury expression (mesodermal marker) was detected using Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems). After overnight incubation, a secondary antibody (Alexa Fluor 488/546, 1:750, Invitrogen) was added for 1 h at room temperature. After washing in PBS, cell nuclei were counterstained with Hoechst 33258 (1:150, Sigma-Aldrich). Labelled cells were observed under a confocal laser scanning (Zeiss LSM 510) microscope and images were acquired. Regarding morphometry, a minimum of 100 cells was counted

Table I. Primary antibodies used for immunocytochemistry.

Primary antibodies	Isotype, dilutions	Company
Monoclonal anti-CD73 (mouse)	IgG3 1:200	Santa Cruz
Monoclonal anti-CD90 (mouse)	IgG1 1:200	Santa Cruz
Monoclonal anti-Ki67 (mouse)	IgG1 1:400	Novocastra
Monoclonal anti-Nestin (mouse)	IgG1 1:200	Millipore
Monoclonal anti-NF-200 (mouse)	IgG1 1:400	Sigma
Monoclonal anti-NuMa (mouse)	IgM 1:50	Santa Cruz
Monoclonal anti-SSEA-4 (mouse)	IgG3 1:200	Millipore
Monoclonal anti-Vimentin (mouse)	IgG1 1:100	Dako
Monoclonal anti-αSMA (mouse)	IgG2a 1:200	Sigma
Polyclonal anti-GFAP (rabbit)	IgG (H + L) 1:500	Dako
Monoclonal anti-βTubulin III (mouse)	IgG2b 1:1000	Sigma
Monoclonal anti-βTubulin III (TUJ-1) (mouse)	IgG2a 1:750	Covance

in 5 random fields to determine the percentage of Ki67-positive cells scored as the percentage of all stained nuclei.

Cell surface markers detection

WJ-MSC and BM-MSC were characterized by flow cytometry using anti-human fluorochrome-conjugated antibodies against fluorescein isothiocyanate CD90, peridinin chlorophyll protein complex (PerCP)-Cy.5 CD105, allophycocyanin antibody (APC) CD73 and negative MSC cocktail (PE CD45, PE CD34, PE CD11b, PE CD19, PE human leukocyte antigen [HLA]-DR) (Human MSC Analysis Kit, Becton Dickinson). The third passage was digested with Accutase Cell Detachment Solution (Becton Dickinson), washed in PBS, a single-cell suspension in cold BD Pharmingen Stain Buffer and 1×10^6 cells/mL were made. Also, 100 µL of the cell suspension were added to each Eppendorf tube. One tube was a negative control and each of the following antibodies were added according to the manufacturer's protocol and incubated for 30 min in the dark at room temperature. The suspended MSCs were washed twice in Stain Buffer and then analyzed with a FACSCalibur II fluorescence-activated cell sorter (Becton Dickinson). For each sample, 10000 events were acquired and analyzed with FACSDiva software. Results were expressed as the percentage of positive cells by comparison with the isotype-matched negative control antibodies on histogram plots.

Multilineage WJ-MSC and BM-MSC differentiation

Osteogenic lineage

BM-MSC and WJ-MSC were seeded in 24-well plates at an initial density of 5×10^3 and culture in standard expansion medium for human MSCs (Lonza). After 24–48 hours, when the cells reach more than 70% confluence, the medium was replaced with Osteogenic Differentiation Medium (Gibco) and cultures were incubated for the next 21 days. The medium was replaced 3 times a week. After that time the positive reaction was tested with Alizarin Red S (Sigma-Aldrich) staining. Briefly, cultured human MSC (hMSCs) were fixed with 4% PFA for 30 min, washed with deionized water and stained with 2% Alizarin Red S solution for 2–3 min. Stained cells were extensively washed and positive red staining indicated the deposition of a calcified matrix on the differentiated hMSCs.

Adipogenic lineage

At the third passage, BM-MSC and WJ-MSC were divided into 24-well plates, seeded at an initial density of 1×10^4 and cultured for 24–48 hours in standard for MSC expansion medium (Lonza). The medium was replaced with Adipogenic Differentiation Medium (Gibco) where the cells grew for 14 days. As a negative control, cells were cultured in a proliferative medium deprived of the differentiation factors.

Adipogenic differentiations effect was verified by Oil Red O (Sigma-Aldrich) staining. The MSCs were fixed with 4% PFA for 30 min and washed with deionized water and 60% isopropanol for 5 min. After that the cells were stained with Oil Red O solution for 5 min and washed with tap water.

Chondrogenic lineage

Micromass culture system was used to induce the cartilage differentiation of both WJ-MSCs and BM-MSCs. Cells at passage 3 were cultured with Chondrogenic Differentiation Medium (Gibco) for 14 days. The production of sulfated glycosaminoglycans was tested using Alcian Blue staining. Fixed cultures were washed with PBS and then stained at room temperature with 1% Alcian Blue (Sigma-Aldrich) solution in 0.1 N HCl. After 30 min incubation, the solution was discarded and the samples were rinsed three times with 0.1 N HCl and once with deionized water to neutralize the acidity.

Reverse transcription-polymerase chain reaction analysis

In our studies messenger RNA (mRNA) level was calculated from MSC samples derived from three independent donors and performed in three replicates each.

Total mRNA was isolated from 1×10^5 WJ-MSC and BM-MSC using TRIzol Reagent (Invitrogen). Before reverse transcription (RT), the samples of isolated RNA were treated with 1 U/mL DNase (Ambion) per 1 mg RNA according to the manufacturer's protocol. The RNA purity was then evaluated by reading absorbance in a NanoDrop ND-1000 spectrophotometer and the ratio from 260/280 and 260/230 was determined. For non-contaminating samples, we assumed a 260/230 ratio >1.8. Also, 1 µg RNA was used for complementary DNA (cDNA) preparations using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems). RT-polymerase chain reaction (PCR) was performed according to the Taq PCR Core Kit (Qiagen) using the primers listed in Table II.

For quantitative RT-PCR, an ABI PRISM 7300 Sequence Detection System or 7500 Real Time PCR System (Applied Biosystems) was used. The final reaction contained template cDNA, SYBR Green PCR Master Mix (Life Technologies) and gene-specific primers (Table III). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tubulin alpha-1B chain (TUBA1B) and β-actin were used as an internal control. The cycle threshold values of reference and other specific genes were acquired after PCR. The nor-

Table II. The primers used for RT-PCR.

Gene/protein	Product size (bp)	Primer sequence (5′→3′)
CD 73	241	Forward: CGCAACAATGGCACAATTAC
		Reverse: CTCGACACTTGGTGCAAAGA
CD 90	236	Forward: CTAGTGGACCAGAGCCTTCG
		Reverse: TGGAGTGCACACGTGTAGGT
CD 105	165	Forward: CACTAGCCAGGTCTCGAAGG
		Reverse: CTGAGGACCAGAAGCACCTC
βTubulin III	160	Forward: CTCAGGGGCCTTTGGACATC
		Reverse: CAGGCAGTCGCAGTTTTCAC
NF-200	829	Forward: GAGGAACACCAAGTGCGAGA
		Reverse: CTTTGCTTCCTCCTTCGTTG
GFAP	266	Forward: GCAGAGATGATGGAGCTCAATGACC
		Reverse: GTTTCATCCTGGAGCTTCTGCCTCA
β-actin	122	Forward: TTCTACAATGAGCTGCGTGTG
		Reverse: GGGGTGTTGAAGGTCTCAAA

malized fold expression was obtained using the $2^{-\Delta\Delta Ct}$ algorithm. The results were expressed as the normalized fold expression for each gene.

Statistics

Statistical analysis of the raw data was conducted using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. The values were considered to be significant when $P < 0.05$. Data were presented as mean ± SD.

Results

Phenotypic MSC characteristic of WJ- and BM-derived cell cultures

The cellular composition of WJ- and BM-derived MSCs in primary cultures was highly heterogeneous at the time of their isolation (passage 0). However, the majority of cells derived from umbilical cord punctured mini-slices, due to their high migratory ability, outgrowth as selected, fibroblast-like, spindle-shaped cell population (Figure 1a). The WJ primary culture reached a 90% confluence at 7–10 days, whereas those isolated from the BM aspirate stay heterogeneous much longer, achieve the confluence not earlier than at 15–20 days and then are gradually selected as an adhesive, fibroblast-like culture during subsequent passaging in the appropriate media (Lonza).

To define whether and to what extend BM-MSC share the stem cell properties with WJ-MSCs, we have compared expression of their surface markers, like CD73 and CD90, together with the cytoskeletal marker vimentin expression in the initial cultures (Figure 1d–f). Flow cytometer (FACS) and molecular PCR analyses confirmed that both cell populations are positive for expression of a panel of antigens typical for MSCs: CD73 (Ecto-5-nucleotidase), CD90 (Thy-1) and CD105 (Endoglin) (Figure 1j and k), but negative for CD14, CD19, CD34, CD45, CD79a, or HLA-DR (not shown here), which is in agreement with the International Society for Cellular Therapy (ISCT) established minimal criteria for MSC culture characteristics [18].

The very important feature of MSCs is their ability to expand their colony to large-scale proportions. Comparing the cellular morphology at the early stages of culture, we have found that the majority of cells, independent of their origin (BM or WJ), revealed similar spindle-shaped, fibroblast-like features. The main difference between them was the frequency of the observed proliferation centers like "colony-forming unit fibroblasts" (CFU-F), which in the BM-MSC monolayer stood out only sporadically and looked like "clusters" of dispersed cells with a comparably slow grow rate, low spreading ability and visible aging of the flattened, less contacted and slowly replicating cells. It was in contrast to a monolayer of WJ- where CFU-Fs appeared in the much higher frequency, contained the densely packed, small, round cells laying one on the top of another in the center and producing fast proliferating offspring on their rims (Figure 1b).

As shown on the right-side of the diagrams, frequency of the typical CFU-F colony in WJ-MSCs was stimulated in low oxygen cultures from 2% to 5% of the whole cell population (Figure 1l). In BM culture, similar dense centers of proliferation were hard to identify by the employed method (see Materials and Methods). Proliferation rate monitored by the relative density of Ki67-positive cell nuclei in both types of cell cultures increased significantly in lowered oxygen but again was more abundant in the case of WJ-MSCs than BM-MSCs (Figure 1c and l).

Table III. The primers used for quantitative RT-PCR.

Gene/protein	Product size (bp)	Primer sequence (5′→3′)
VEGFA	136	Forward: GAGCCGCGAGAAGTGCTA
		Reverse: GCCTCACCCGTCCATGAG
NGF	147	Forward: GCAAGCGGTCATCATCCCAT
		Reverse: TGTTGTTAATGTTCACCTCTCCC
GDNF	112	Forward: TGGCAGTGCTTCCTAGAAGAG
		Reverse: AAGACACAACCCCGGTTTTTG
BDNF	168	Forward: GATGCTCAGTAGTCAAGTGCC
		Reverse: GCCGTTACCCACTCACTAATAC
HGF	151	Forward: GCCCTATTTCTCGTTGTGAAGGT
		Reverse: CTGTATCTCAAACTAACCATCCATCCTATG
EGF	85	Forward: GCAGAGGGATACGCCCTAAGT
		Reverse: CAAGAGTACAGCCATGATTCCAAA
NT4	100	Forward: GCGAGGTGGAGGTGTTGG
		Reverse: CCTTCCTCAGCGTTATCAGC
NT3	141	Forward: GGTACGCGGAGCATAAGAGTC
		Reverse: GAGAGTTGCCCGTTTTGATCT
$p75^{NTR}$	109	Forward: CCTACGGCTACTACCAGGATG
		Reverse: CACACGGTGTTCTGCTTGTC
GFRA1	137	Forward: GTACAGGTCGGCGTACATCAC
		Reverse: AGCAGAAGAGCATTCCGTAGC
GFRA2	195	Forward: AGCGCCAAGAGCAACCATT
		Reverse: CATGCGGTAGGTGTACTCGA
TRKA	96	Forward: GTCAGCCACGGTGATGAAATC
		Reverse: CAGCACGTCACGTTCTTCCT
TRKB	131	Forward: CTGGTGCATTCCATTCACTG
		Reverse: CGTGGTACTCCGTGTGATTG
TRKC	138	Forward: TGGCTGGACTATGTGGGCT
		Reverse: CCCATTGCTGTTCCCTGAATC
IGF	68	Forward: TGCTTCCGGAGCTGTGATCTA
		Reverse: GCTGACTTGGCAGGCTTGAG
CNTF	69	Forward: TGTGCGTGCTTGCATGTG
		Reverse: ACCCTGAAGTGGAAGGACGTT
GAPDH	73	Forward: CCACATCGCTCAGACACCAT
		Reverse: TGACCAGGCGCCCAATA
TUBA1B	101	Forward: GCCTCCTAATCCCTAGCCAC
		Reverse: TCCAGGCAGTAGAGCTCCC
Nestin	168	Forward: TGGCTCAGAGGAAGAGTCTG
		Reverse: CCCCCATTCACATGCTGTGA
β-actin	122	Forward: TTCTACAATGAGCTGCGTGTG
		Reverse: GGGGTGTTGAAGGTCTCAAA

The average PDT estimated for the BM-MSC culture displayed significantly higher values. Calculated for 6 passages it was about 5 days in 5% O_2 culture and 10 days in 21% O_2 atmosphere. The same parameters estimated for WJ-MSC culture were 2 and 6 days, respectively.

Both categories of MSC cultures derived either from WJ or BM tissues revealed typical tri-lineage (cartilage, bone and adipose) differentiation capabilities (Figure 1g–i) but each of them displayed somewhat different preferences to the particular lineages. WJ-MSCs exhibited lower ability of adipogenic differentiation and relatively higher commitment toward osteogenesis. The potential toward chondrogenesis seemed to be on the similarly mild level in both types of cultures.

Spontaneous commitment to differentiation toward neural lineage of WJ-MSCs and lack of this tendency in the BM-MSC cultures

In our previous experiments [16], we showed that WJ-MSCs are spontaneously induced to express typical markers of neural differentiation *in vitro*: Nestin, βTubulin III and glial fibrillary acidic protein (GFAP). Herein we have extended monitoring of the spontaneous neural phenotype appearance also in BM-MSC culture in comparison with WJ-MSC, starting our observation from the very beginning stage of the cells outgrowth from their first isolates (passages 0) up to logarithmically growing third passages (Figure 2, upper diagram).

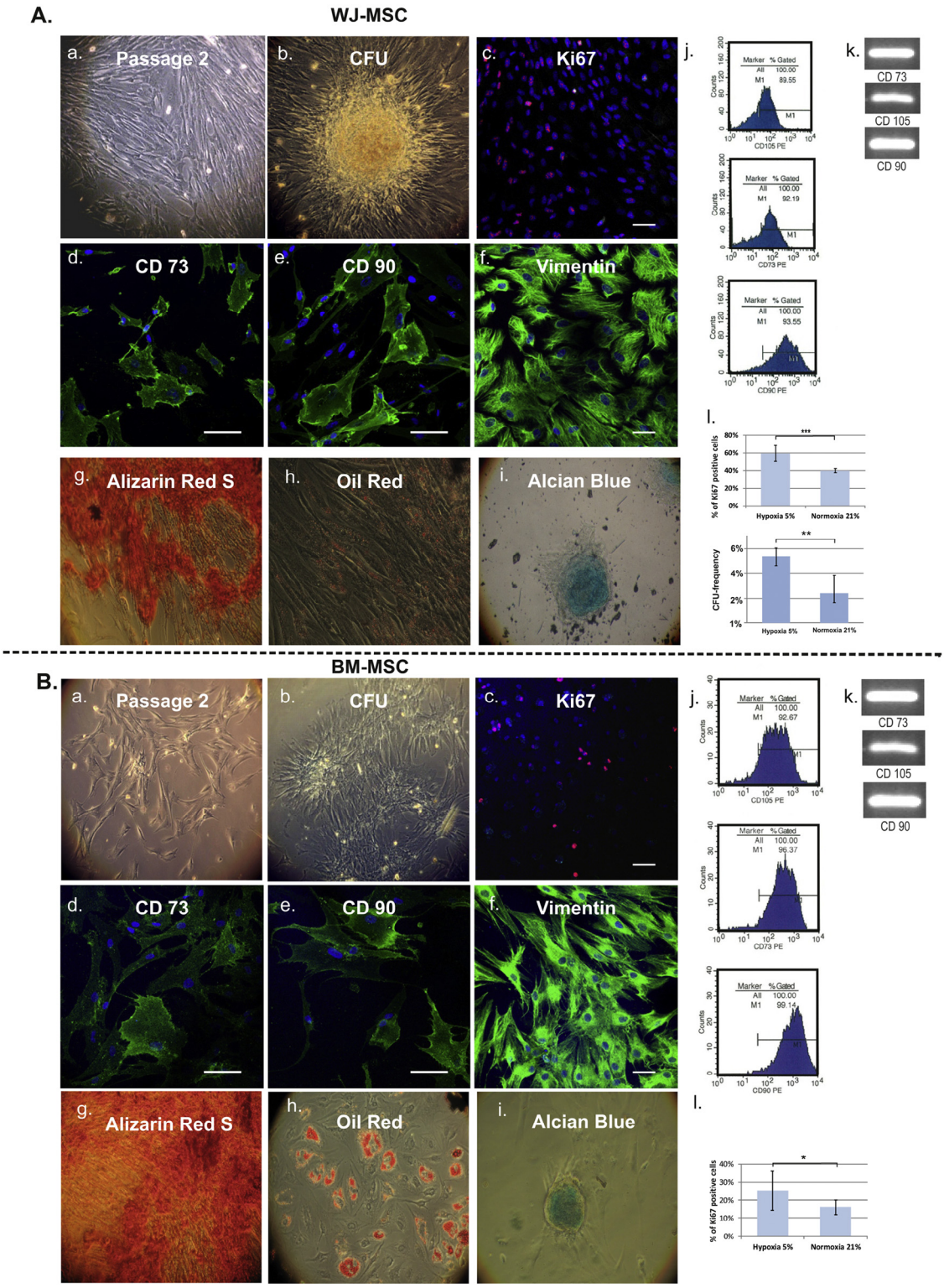

Figure 1. Phenotypic characteristic of hMSC cultures derived from WJ (top panel A) and BM (bottom panel B). Phase contrast imaging in both primary adherent cultures of cells with typical, fibroblast-like morphology (a). Classic CFU-F in WJ-MSCs and dispersed cell clusters in BM-MSCs (b). Immunocytochemical detection of Ki67 (c), mesenchymal surface antigens CD73 (d) and CD90 (e) and cytoskeletal component vimentin (f). Multilineage differentiation: representative photos of MSC differentiated into bone, adipose and cartilage lineages after Alizarin Red S (g), Oil Red O (h) and Alcian Blue (i) staining, respectively. FACS analyses of positive surface mesenchymal markers: CD73, CD90 and CD105 (j). Expression of representative RT-PCR for these antigens (k). Percentage of Ki67-positive cells at the logarithmic phase of growing cultures showed an increased number of dividing cells in 5% oxygen conditions (l). CFU-F frequency has been estimated only in WJ-MSCs and also increased in 5% O_2 (l- lower diagram). The bars represent means of the raw data obtained from three independent isolations estimated twice in parallel cultures ± SD.

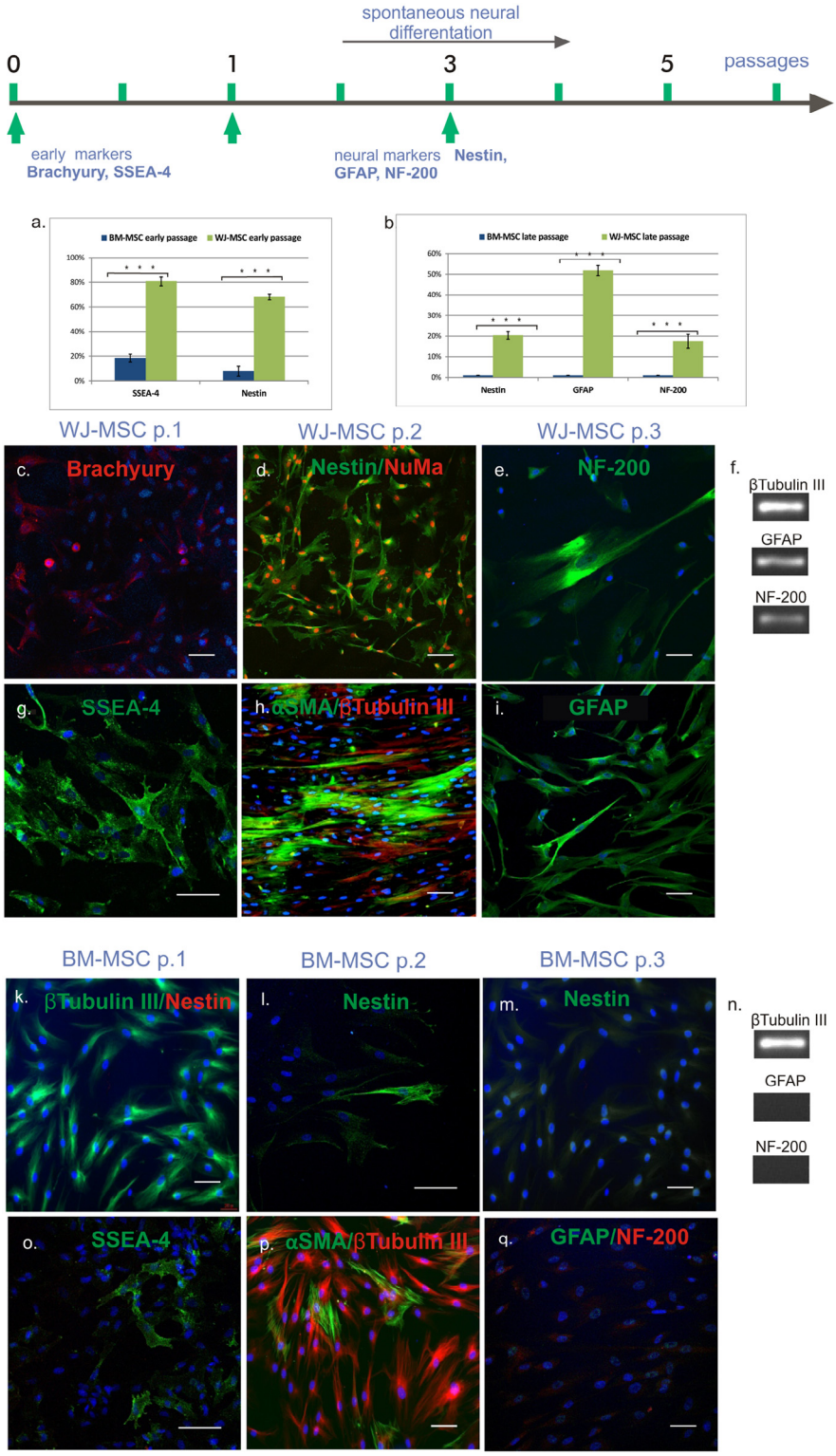

Figure 2. At the top there are diagrams illustrating the timing of the experiment and comparison of percentage of SSEA-4 and nestin (a) and nestin, GFAP and NF200 (b) positive cells at different times in both classes of MSC cultures. The diagrams show the means from three isolations estimated in two parallel growing cultures ± SD. Immunocytochemical visualization of time-dependent differentiation of WJ-MSC (upper panel) and BM-MSC (bottom panel) cultures. Only WJ-MSCs at passage 0–1 express mesodermal marker Brachyury (c) and embryonic marker SSEA-4 (g). Later on, the majority of WJ-MSCs transiently express neural progenitor marker nestin (d, e) and α-SMA (h), which is in opposition to very low immunoreaction in BM-MSCs (l, m, p). Similarly, only WJ-MSCs express neuronal marker NF-200 (j) and glial marker GFAP (i), which never were observed in BM-MSCs (q). Cell nuclei were counterstained with Hoechst 33258 (blue) or NuMa (red). Scale bars are 50 μm. Included are gel images of RT-PCR for neural differentiation gene marker expression in WJ-MSCs (f) and BM-MSCs (n).

These experiments comprise the regions of the migrating fibroblast-like cells outgrowing directly from WJ tissue explants and the primary culture of adherent cells derived directly from BM aspirates. We have found that in this initial phase a high percentage of WJ-MSC can express positive immunoreaction to the neuro-ectodermal specific markers Nestin (68.07%) (Figure 2a) together with accidental immunoreaction to mesoderm-specific antigen Brachyury (Figure 2c and d). In addition, the cells outgrowing from the WJ fragments immune-reacted with Stage Specific Embryonic Antigen (SSEA-4) in 80.79% (Figure 2a and g). The few SSEA-4–positive cells were also found, although with significantly lowered frequency (18.45%) in the first passages of BM-MSC cultures (Figure 2o). Interestingly, SSEA-4, an antigen believed to be one of the most typical stemness markers of undifferentiated pluripotent cells was also proposed as a surface marker for prospective isolation of the purified, self-renewing subpopulation of MSC itself [19].

In later passages, the WJ-derived cells gradually lose abundant Nestin expression but instead acquired reactivity with βTubulin III antibody (Figure 2e and h), the early neuronal marker commonly used for lineage identification. Interestingly, at this intermediary stage of the WJ-MSC culture, a lot of cells reacted, including βTubulin III and α-Smooth Muscle Actin (α-SMA) antibody (Figure 2h). This antigen, initially thought to be specific to vascular smooth muscle cells, was verified too as a marker for NC-derived cells in mouse [20] and human [21] neural-crest stem cells (NCSC) lines *in vitro* and in the migratory pericyte progenitors in the brain infarct penumbra [22,23]. Similarly to SSEA-4, the α-SMA was expressed only incidentally in the earliest phases of BM-MSC culture but at a significantly lower level than in WJ-MSCs (Figure 2p). A low level of Nestin-positive cells was found also on the most early stage of BM-MSC culture (7.84%) but was never followed by appearance of the other (NF-200 and GFAP) neuronal or glial/Schwann cell markers (except of βTubulin III, which is discussed later) (Figure 2k–q). The WJ-derived culture, opposite BM-MSC, at higher passages expressed, in addition to βTubulin III, also the other, more matured neuronal antigens, a heavy neurofilament NF-200 (17.55%) together with extended GFAP immunostaining (51.83%) (Figure 2e and i).

All of these results are consistent with each other and with the related gene transcripts expression (Figure 2f and n). This includes the gel images representing RT- PCR data for NF-200 and GFAP (Figure 2f and n) obtained from early and late passages of WJ but never of BM cell cultures. The only exception was the strong immunoreactivity toward βTubulin III observed in all passages of BM-MSC cultures (Figure 2k and p). This positive reaction was noticed independently of the different screened βTubulin III antibodies (TUJ-1, Covance; βTubulin III, Sigma) derived from recommended commercial sources or even in the Lonza-supplied MSC culture itself (MSCGM BulletKit; Lonza). This unexpected finding was positively verified on a transcriptional level by RT-PCR gene expression where βTubulin III (Figure 2n) was the only neural lineage-connected marker genes strongly expressed in BM-MSC at the level similar to that in WJ-MSC cultures.

Different gene expression of neurotrophins and nestin in neonatal (WJ) versus adult (BM) MSC cultures

The unique paracrine and adjuvant effects of MSCs in repair processes of various damaged tissues are linked either with the ability of these cells to differentiate and replace the function of damaged cells or more probably with the secretion of the numerous immunomodulatory and trophic factors supporting cell survival, growth, migration and many other lineage-specific functions. To address the nature of this "rescue from a distance" phenomena as well as the observed differences between WJ and BM-derived MSC behavior *in vitro*, we have estimated the intrinsic expression of mRNA in the panel of selected neurotrophins (NTs), growth and angiogenic factors (GFs) as well as their receptors playing a crucial role in neural tissue regeneration.

The results have shown that WJ-MSC revealed higher mRNA expression of: vascular endothelial growth factor A (VEGFA), glial cell-derived neurotrophic factor (GDNF), hepatocyte growth factor (HGF) and brain-derived neurotrophic factor (BDNF) (Figure 3A), NT3, NT4 and unspecific p75 neurotrophin receptor ($p75^{NTR}$) receptor (Figure 3B) compared with BM-MSC. The other factors, insulin-like growth factor (IGF), epidermal growth factor (EGF) and ciliary neurotrophic factor (CNTF), did not differ substantially, generally being expressed on an evidently lowered level than the previous group of growth factors in BM-MSCs and WJ-MSCs. Decreasing trends in the expression of TRK (A, B, C), NGF and GFRA1 transcripts were observed in WJ-MSCs compared with BM- and MSC samples. No significant differences were observed in GFRA2 receptor expression (Figure 3B). However, the lack of significance on the mRNA level encoding these receptors was probably caused by the overall low abundance of respective transcription, which prevented reliable quantitative mRNA determination.

As mentioned above, our analyses of the regeneration-associated grow factors and their receptors were limited here to differences in mRNA expression. Certainly in further work the resulting

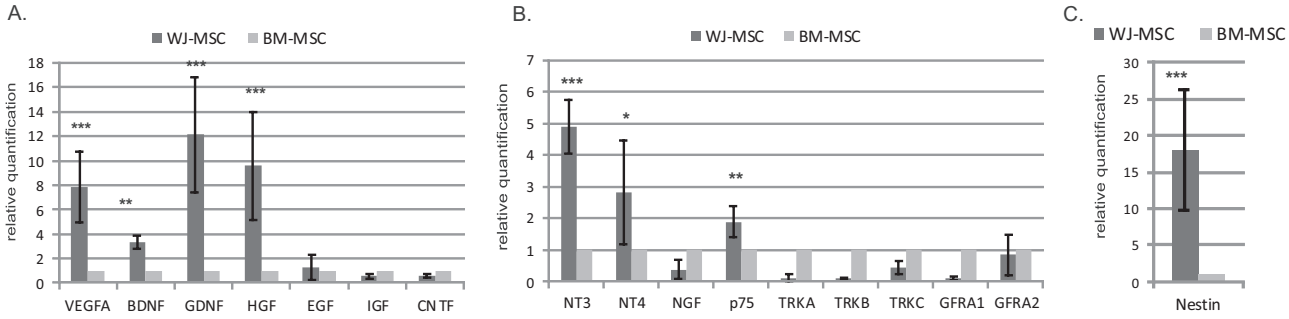

Figure 3. Quantitative RT-PCR mRNA of growth factors (A), neurotrophins and receptors (B) and neuroectodermal marker nestin (C) expressions in WJ-MSC and BM-MSC cultures. The graphs show the results in comparison with mRNA expression obtained by $2^{-\Delta\Delta Ct}$ method normalized to the reference gene TUBA1B value (A) or GAPDH (B, C). The relative gene expression of the WJ-MSC population was compared with BM-MSC as a calibrator group. The results are presented as mean values of three isolations estimated in three parallel samples ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

changes in secretion of the related proteins and mechanisms of their protective functions must be addressed and evaluated separately.

In Figure 3C we have shown quantitative comparison of the nestin expression level in WJ-MSC versus BM-MSC cultures. The expression of this neuroectoderm-specific gene was found definitely much higher in the immature WJ-MSCs in accordance with the uneven protein levels determined using immunocytochemistry (Figure 2a and b).

Discussion

The results obtained in the present study support a growing body of evidence that MSCs derived from WJ or BM tissues represent a distinguished set of progenitors. In line with current data, the cultures may contain a varied proportion of cells originating from disparate ontogeneic sources. The characteristic features presented here by the WJ-MSCs seem to be typical for the whole cohort of immature "preMSC" [2]. They consist of neuro-ectodermal cells that, instead of that of mesoderm-originating adult MSCs, derive from NC (NCSC), an early embryonic structure–generating dominant component of fetal MSCs. This feature may be representative for a whole class of other primitive somatic progenitor populations existing in different perinatal tissues like umbilical cord blood, amnion, placenta, etc.

The early stem/progenitor cells isolated from cord blood are still one of the best explored categories of cells widely used in the hematologic clinic. This has been possible because of early implementation of the highly specific cytometry techniques for prospective selection of homogeneous hematopoietic cell populations [24]. In contrast, MSCs/progenitor cells do not possess any similarly highly specific surface markers or their combinations as well as any other attributes with comparable usefulness for cell selection *in vitro*. The only recently proposed MSC markers are the stage-specific embryonic antigen SSEA-4 [19], platelet-derived growth factor receptors (PDGFR) β [25] or the ganglioside GD2 [26], however, their properties have till now not fully been confirmed. Interestingly, we observed a significant difference in the expression of SSEA-4 antigen similar to nestin in the primary passages between our two types of culture (Figure 2a, g, and o). Our hypothesis on the existence of at least two main categories of fetal/perinatal and adult tissue-specific MSCs in humans is based on differential expression of the panel of specific antigens and growth factor genes or eventually on distinguished cell growth characteristics with different reaction to atmospheric oxygen changes. We and others [17,27–29] have previously found that reducing the oxygen content in the initial atmospheric concentration to values more in-line with the neural stem cell niches [30] can accelerate growth and longevity of MSCs or neural stem cells in culture. However, only WJ-MSC populations could sense a reduction of the oxygen by increasing hypoxia inducible factor (HIF) 1α and 2α transcription with concomitant "rejuvenation" of the cell phenotypes and induction of stemness-related gene transcripts (Oct4, Sox2, Nanog, Rex1) [17]. This response was blocked by Trichostatin A (TSA) in concentrations activating histone acetylation and counteracting HIF induction, with concomitant release of the cell differentiation blockade. On the other hand, BM-MSC cultures have never reacted in a similar manner to lowered culture oxygenation.

It seems that low-oxygen tension in the ranges specific for stem cell niches *in vivo*, which vary from <1%–10% O_2, only in case of WJ-preMSC culture, stabilize or even promote undifferentiated cell phenotypes with reversion of already acquired stage of cell differentiation. Interestingly, we can not exclude that also *in*

vivo, for example, under local ischemia, the differentiation of this remnant cells component, derived primary from preMSC lineage, would be suppressed by local hypo-oxygenation, which facilitates their proliferation, dedifferentiation and migration toward site of injury [22,31].

The WJ-MSCs may demonstrate a strong neuroregenerative paracrine potential, presumably due to the high level of diffusible growth and neurotrophic factors being released by transplanted cells in the proximity of compromised tissues [32]. It is not a new observation, however, our data again confirms transcriptional activity of these regeneration-associated neurotrophic factors being expressed significantly higher in WJ-MSC than in BM-MSC cultures. The enhanced transcription was noticed for factors like BDNF, GDNF, HGF, VEGF, NT3 and NT4, all being confirmed as a source of the strong pro-survival signals for nervous tissue [31–35]. Convincing is also our finding that from various neurotrophic receptors, exclusively $p75^{NTR}$, previously reported as highly specific for NC stem cells [36], was significantly elevated in WJ-derived MSC culture. Actual observation confirms that WJ-MSC cultures in comparison with BM-MSC secrete preferentially a panel of factors being correlated with neuroprotection, neurogenesis and angiogenesis [32] This notion might be supported also by other data showing exceptionally high production of diffusible neurotrophic factors *in vivo* achieved after transplantation of WJ-MSCs into vitreous for rescue of eye photoreceptors in a rodent model of retina disease [37]. This unique pattern and high paracrine neurotrophic potential of WJ-derived MSCs was found to be similar to the recently explored purified populations of the umbilical cord blood neural/progenitor cells [38]. These results once again support a common developmental origin and regenerative properties of different cell populations isolated from perinatal tissues. However, incomparable easiness in isolation and expansion of WJ stem cell derivatives should be stressed.

It is worth it to note that, similar to the other published data, our results concerning BM-MSC have shown rather high variability. This could be linked either with different age of the donors as well as with additional uncontrolled events that may occur at time of processing of a particular sample. To minimize these types of variabilities we have duplicated each donor-derived culture and, as in the case of quantitative RT-PCR, also repeated twice each performed sampling procedure. As a result, most of the related neurotrophic factor transcriptional activities, despite substantial variations, were confirmed to be significantly higher for WJ-MSC samples than for BM-MSC cultures.

In addition to the observed differences in the possible neurotrophic support between the two examined (WJ and BM) MSC categories, their uneven potential to neural differentiation may contribute to higher restorative function of cells derived from immature sources [12,39–43]. The result presented in Figure 2 supports well the notion about the primary neuroectodermal or pluripotent character of umbilical cord MSC derivatives. The exceptionally high SSEA-4, α-SMA or Nestin expression in WJ-MSCs correlates well with a later appearance of the stage-related neural markers, NF-200 and GFAP.

As we already mentioned in the results, and not consistent with the above conclusion is the high expression of βTubulin III (Figure 2k and n) observed, unlike the other neural markers, not only in WJ-MSC cultures but also in all examined BM-MSC passages. Recent publications have discussed the unexpected hyper-reactivity of this neurofilament protein in various types of adult MSC cultures [44–46]. The weak correlation of βTubulin III expression with other neural differentiation–specific antigens may call into question the nature and function of this protein in adult stem cell biology. Meaningfully, unspecific βTubulin III expression was never observed for the duration of our previous work with human umbilical cord blood neural stem/progenitors as well as in recently investigated WJ-MSC cultures [16,17]. The discussed above unique pro-neural activities of immature type MSCs have been additionally proven by the expression (not shown here) of other neural marker genes like Pax6 and Sox10 and NG2, never found in the case of BM-MSC probes. In addition, in previous work, we have demonstrated a panel of functional and epigenetic data confirming genuine neural properties being achieved by the similar type of immature preMSCs derived from umbilical cord blood [11,16,47–49].

In summary, our data strongly suggests that WJ-derived cells, like the whole group of other preMSCs, should be recommended for cell therapy of neurological diseases. This is due to their distinguished spontaneous commitment toward neurogenic differentiation as well as the restorative impact of the whole spectrum of highly expressed neurotrophic and regeneration-associated genes. Therefore, WJ-MSCs should be more than BM-MSCs considered to the treatment of degenerative brain [31,35], retinal [37] or neuro-immunological type of central nervous system diseases [3,50,51]. Finally, as we have shown in our recent data [52], the human WJ-MSC can be easily induced to endothelial differentiation, confirmed *in vivo* in the rodent brain ischemic model [51]. The transplantation of exceptionally low immunogenic WJ-MSCs would be much safer than adult BM-MSCs for allotransplant application [53]. The above properties might be shared with the whole class of perinatal tissue–originating preMSCs, due to their unique developmental history leading to enrichment of the

immature, embryonic-like component, displaying strong potency to default neural differentiation. The contribution of this unique cell population to any particular MSC culture can be primary quantified by the level of neuro-ectodermal markers of expression, like SSEA-4, α-SMA or nestin. This type of assay could be easily included in the standard isolation procedures of cells with the enhanced neuro-therapeutic characteristic.

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References

[1] Troyer DL, Weiss ML. Wharton's jelly-derived cells are a primitive stromal cell population. Stem Cells 2008;26(3):591–9.
[2] Trento C, Dazzi F. Mesenchymal stem cells and innate tolerance: biology and clinical applications. Swiss Med Wkly 2010;140(26):w13121.
[3] Li X, Bai J, Ji X, Li R, Xuan Y, Wang Y. Comprehensive characterization of four different populations of human mesenchymal stem cells as regards their immune properties, proliferation and differentiation. Int J Mol Med 2014;34(3):695–704.
[4] Takashima Y, Era T, Nakao K, Kondo S, Kasuga M, Smith AG, et al. Neuroepithelial cells supply an initial transient wave of MSC differentiation. Cell 2007;129(7):1377–88.
[5] Sadler SE, Jacobs ND. Stimulation of Xenopus laevis oocyte maturation by methyl-beta-cyclodextrin. Biol Reprod 2004;70(6):1685–92.
[6] Ogawa M, Oshima M, Imamura A, Sekine Y, Ishida K, Yamashita K, et al. Functional salivary gland regeneration by transplantation of a bioengineered organ germ. Nat Commun 2013;4:2498.
[7] Stachowiak EK, Tzanakakis ES. Stem cells, from mechanisms to technologies. Word Sci Publ 2012;303–33.
[8] Sadler TW. Langmann medical embryology. Philadelphia: Williams & Wilkins; 2004. p. 5164.
[9] Buzańska L, Machaj EK, Zabłocka B, Pojda Z, Domańska-Janik K. Human cord blood-derived cells attain neuronal and glial features in vitro. J Cell Sci 2002;115(10):2131–8.
[10] Buzańska L, Jurga M, Stachowiak EK, Stachowiak MK, Domańska-Janik K. Neural stem-like cell line derived from a nonhematopoietic population of human umbilical cord blood. Stem Cells Dev 2006;15(3):391–406.
[11] Jurga M, Markiewicz I, Sarnowska A, Habich A, Kozlowska H, Lukomska B, et al. Neurogenic potential of human umbilical cord blood: neural-like stem cells depend on previous long-term culture conditions. J Neurosci Res 2006;83(4):627–37.
[12] Habich A, Jurga M, Markiewicz I, Lukomska B, Bany-Laszewicz U, Domanska-Janik K. Early appearance of stem/progenitor cells with neural-like characteristics in human cord blood mononuclear fraction cultured in vitro. Exp Hematol 2006;34(7):914–25.
[13] Habich A, Domanska-Janik K. Aggregation-promoted expansion of neuraly committed human umbilical cord blood progenitors in vitro. Acta Neurobiol Exp (Wars) 2011;71(1):1–11.
[14] Jozwiak S, Habich A, Kotulska K, Sarnowska A, Kropiwnicki T, et al. Intracerebrobentricular transplantation of cord blood-derived neural progenitors in a child with severe global brain ischemic injury. Cell Med 2010;1:71–80.
[15] Janowski M, Walczak P, Kropiwnicki T, Jurkiewicz E, Domanska-Janik K, Bulte JW, et al. Long-term MRI cell tracking after intraventricular delivery in a patient with global cerebral ischemia and prospects for magnetic navigation of stem cells within the CSF. PLoS ONE 2014;9(2):e97631.
[16] Sarnowska A, Jablonska A, Jurga M, Dainiak M, Strojek L, Drela K, et al. Encapsulation of mesenchymal stem cells by bioscaffolds protects cell survival and attenuates neuroinflammatory reaction in injured brain tissue after transplantation. Cell Transplant 2013;22(Suppl. 1):S67–82.
[17] Drela K, Sarnowska A, Siedlecka P, Szablowska-Gadomska I, Wielgos M, Jurga M, et al. Low oxygen atmosphere facilitates proliferation and maintains undifferentiated state of umbilical cord mesenchymal stem cells in an hypoxia inducible factor-dependent manner. Cytotherapy 2014;16(7):881–92.
[18] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8(4):315–17.
[19] Gang EJ, Bosnakovski D, Figueiredo CA, Visser JW, Perlingeiro RC. SSEA-4 identifies mesenchymal stem cells from bone marrow. Blood 2007;109(4):1743–51.
[20] Ishii M, Arias AC, Liu L, Chen YB, Bronner ME, Maxson RE. A stable cranial neural crest cell line from mouse. Stem Cells Dev 2012;21(17):3069–80.
[21] Thomas S, Thomas M, Wincker P, Babarit C, Xu P, Speer MC, et al. Human neural crest cells display molecular and phenotypic hallmarks of stem cells. Hum Mol Genet 2008;17(21):3411–25.
[22] Sharma V, Ling TW, Rewell SS, Hare DL, Howells DW, Kourakis A, et al. A novel population of α-smooth muscle actin-positive cells activated in a rat model of stroke: an analysis of the spatio-temporal distribution in response to ischemia. J Cereb Blood Flow Metab 2012;32(11):2055–65.
[23] Etchevers HC, Vincent C, Le Douarin NM, Couly GF. The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. Development 2001;128(7):1059–68.
[24] Kucia M, Wysoczynski M, Ratajczak J, Ratajczak MZ. Identification of very small embryonic like (VSEL) stem cells in bone marrow. Cell Tissue Res 2008;331(1):125–34.
[25] Bühring HJ, Battula VL, Treml S, Schewe B, Kanz L, Vogel W. Novel markers for the prospective isolation of human MSC. Ann NY Acad Sci 2007;1106:262–71.
[26] Martinez C, Hofmann TJ, Marino R, Dominici M, Horwitz EM. Human bone marrow mesenchymal stromal cells express the neural ganglioside GD2: a novel surface marker for the identification of MSCs. Blood 2007;109(10):4245–8.
[27] Storch A, Paul G, Csete M, Boehm BO, Carvey PM, Kupsch A, et al. Long-term proliferation and dopaminergic differentiation of human mesencephalic neural precursor cells. Exp Neurol 2001;170(2):317–25.
[28] Braunschweig L, Meyer AK, Wagenführ L, Storch A. Oxygen regulates proliferation of neural stem cells through Wnt/β-catenin signalling. Mol Cell Neurosci 2015;67:84–92.

[29] Ivanović Z, Bartolozzi B, Bernabei PA, Cipolleschi MG, Rovida E, Milenković P, et al. Incubation of murine bone marrow cells in hypoxia ensures the maintenance of marrow-repopulating ability together with the expansion of committed progenitors. Br J Haematol 2000;108(2):424–9.

[30] Silver I, Erecińska M. Oxygen and ion concentrations in normoxic and hypoxic brain cells. Adv Exp Med Biol 1998;454:7–16.

[31] Weiss ML, Medicetty S, Bledsoe AR, Rachakatla RS, Choi M, Merchav S, et al. Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. Stem Cells 2006;24(3):781–92.

[32] Hsieh JY, Wang HW, Chang SJ, Liao KH, Lee IH, Lin WS, et al. Mesenchymal stem cells from human umbilical cord express preferentially secreted factors related to neuroprotection, neurogenesis, and angiogenesis. PLoS ONE 2013;8(8):e72604.

[33] Bothwell M. Neurotrophic factors. Handb Exp Pharm 2014;220:3–15.

[34] Fu YS, Cheng YC, Lin MY, Cheng H, Chu PM, Chou SC, et al. Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopaminergic neurons in vitro: potential therapeutic application for Parkinsonism. Stem Cells 2006;24(1):115–24.

[35] Jomura S, Uy M, Mitchell K, Dallasen R, Bode CJ, Xu Y. Potential treatment of cerebral global ischemia with Oct-4+ umbilical cord matrix cells. Stem Cells 2007;25(1):98–106.

[36] Morrison SJ, White PM, Zock C, Anderson DJ. Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. Cell 1999;96(5):737–49.

[37] Lund RD, Wang S, Lu B, Girman S, Holmes T, Sauvé Y, et al. Cells isolated from umbilical cord tissue rescue photoreceptors and visual functions in a rodent model of retinal disease. Stem Cells 2007;25(3):602–11.

[38] Paczkowska E, Kaczyńska K, Pius-Sadowska E, Rogińska D, Kawa M, Ustianowski P, et al. Humoral activity of cord blood-derived stem/progenitor cells: implications for stem cell-based adjuvant therapy of neurodegenerative disorders. PLoS ONE 2013;8(12):e83833.

[39] Kozlowska H, Jablonka J, Janowski M, Jurga M, Kossut M, Domanska-Janik K. Transplantation of a novel human cord blood-derived neural-like stem cell line in a rat model of cortical infarct. Stem Cells Dev 2007;16(3):481–8.

[40] Sarnowska A, Braun H, Sauerzweig S, Reymann KG. The neuroprotective effect of bone marrow stem cells is not dependent on direct cell contact with hypoxic injured tissue. Exp Neurol 2009;215:317–27.

[41] Guillot PV, Gotherstrom C, Chan J, Kurata H, Fisk NM. Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC. Stem Cells 2007;25(3):646–54.

[42] Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells 2004;22(7):1330–7.

[43] Ma L, Feng XY, Cui BL, Law F, Jiang XW, Yang LY, et al. Human umbilical cord Wharton's Jelly-derived mesenchymal stem cells differentiation into nerve-like cells. Chin Med J 2005;118(23):1987–93.

[44] Kwon EB, Lee JY, Piao S, Kim IG, Ra JC, Lee JY. Comparison of human muscle-derived stem cells and human adipose-derived stem cells in neurogenic trans-differentiation. Korean J Urol. 2011;52(12):852–7.

[45] Foudah D, Redondo J, Caldara C, Carini F, Tredici G, Miloso M. Human mesenchymal stem cells express neuronal markers after osteogenic and adipogenic differentiation. Cell Mol Biol Lett 2013;18(2):163–86.

[46] Foudah D, Monfrini M, Donzelli E, Niada S, Brini AT, Orciani M, et al. Expression of neural markers by undifferentiated mesenchymal-like stem cells from different sources. J Immunol Res 2014;2014:987678.

[47] Jurga M, Dainiak MB, Sarnowska A, Jablonska A, Tripathi A, Plieva FM, et al. The performance of laminin-containing cryogel scaffolds in neural tissue regeneration. Biomaterials 2011;32(13):3423–34.

[48] Jurga M, Lipkowski AW, Lukomska B, Buzanska L, Kurzepa K, Sobanski T, et al. Generation of functional neural artificial tissue from human umbilical cord blood stem cells. Tissue Eng Part C Methods 2009;15(3):365–72.

[49] Sun W, Buzanska L, Domanska-Janik K, Salvi RJ, Stachowiak MK. Voltage-sensitive and ligand-gated channels in differentiating neural stem-like cells derived from the nonhematopoietic fraction of human umbilical cord blood. Stem Cells 2005;23(7):931–45.

[50] Cho PS, Messina DJ, Hirsh EL, Chi N, Goldman SN, Lo DP, et al. Immunogenicity of umbilical cord tissue derived cells. Blood 2008;111(1):430–8.

[51] Wu KH, Zhou B, Lu SH, Feng B, Yang SG, Du WT, et al. In vitro and in vivo differentiation of human umbilical cord derived stem cells into endothelial cells. J Cell Biochem 2007;100(3):608–16.

[52] Obtulowicz P, Lech W, Strojek L, Sarnowska A, Domanska-Janik K. Induction of endothelial phenotype from Warton Jelly-derived MSC and comparison of their vaso- and neuro-protective potential with primary WJ-MSC in CA1 hippocampal region *ex vivo*. Cell Transplant 2015;[Epub ahead of print].

[53] Ankrum JA, Ong JF, Karp JM. Mesenchymal stem cells: immune evasive, not immune privileged. Nat Biotechnol 2014;32(3):252–60.