Unique molecular signatures influencing the biological function and fate of post-natal stem cells isolated from different sources

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Abstract

The discovery of mesenchymal stem cells (MSCs) from a myriad of tissues has triggered the initiative of establishing tailor-made stem cells for disease-specific therapy. Nevertheless, lack of understanding on the inherent differential propensities of these cells may restrict their clinical outcome. Therefore, a comprehensive study was done to compare the proliferation, differentiation, expression of cell surface markers and gene profiling of stem cells isolated from different sources, viz. bone marrow, Wharton’s jelly, adipose tissue and dental pulp. We found that although all MSCs were phenotypically similar to each other, Wharton’s jelly (WJ) MSCs and dental pulp stem cells (DPSCs) were highly proliferative as compared to bone marrow (BM) MSCs and adipose tissue (AD) MSCs. Moreover, indistinguishable cell surface characteristics and differentiation capacity were confirmed to be similar among all cell types. Based on gene expression profiling, we postulate that BM-MSCs constitutively expressed genes related to inflammation and immunodulation, whereas genes implicated in tissue development were highly expressed in AD-MSCs. Furthermore, the transcriptome profiling of WJ-MSCs and DPSCs revealed an inherent bias towards the neuro-ectoderm lineage. Based on our findings, we believe that there is no unique master mesenchymal stem cell that is appropriate to treat all target diseases. More precisely, MSCs from different sources exhibit distinct and unique gene expression signatures that make them competent to give rise to specific lineages rather than others. Therefore, stem cells should be subjected to rigorous characterization and utmost vigilance needs to be adopted in order to choose the best cellular source for a particular disease. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords  mesenchymal stem cells; bone marrow; dental pulp; adipose; Wharton’s jelly; gene expression

1. Introduction

Mesenchymal stem cells (MSCs) were first identified by Friedenstein and his colleagues as fibroblast-like cells with a clonogenic potential residing in rat bone marrow (BM) (Friedenstein et al., 1966). Subsequently, they were isolated from human and other organisms and, to date, bone marrow mesenchymal stem cells (BM-MSCs) are among the best-characterized adult stem cells and have provided most information on MSCs. This paved the way for the prospective application of BM-MSCs in almost all ongoing human clinical trials. In concurrence with their enormous potential, the benefits of BM-MSC transplantations have been shown to be multi-faceted, ranging from improved recovery from stroke (Dharmasaroja, 2009).
and cardiac ischaemia (Duan et al., 2003; Wen et al., 2011) to wounds and burns (Rasulov et al., 2005; Bey et al., 2010). However, processing and harvesting MSCs from BM have certain limitations. Bone marrow stem cell isolation has always been an invasive procedure for donors (Ishigene et al., 2009). Moreover, reports suggest that there is significant age-related decrease in the frequency and differentiation potentials of BM-derived MSCs (Mueller and Glowacki, 2001; Stenderup et al., 2003). These drawbacks of BM-MSCs have acted as a stimulus to the discovery of many alternative sources of MSCs.

It has been reported that MSCs are virtually present throughout the human body, reside within the perivascular niche of an organ and are involved in tissue turnover homeostasis ( Mimeault et al., 2007). Nevertheless, researches on MSCs are more focused on adipose-derived MSCs (AD-MSCs), fetal origin MSCs, i.e. from Wharton’s jelly (WJ-MSCs), and neural crest origin MSCs in dental pulp (DPSCs). This is primarily due to their higher proliferation capacity (Miura et al., 2003; Nekanti et al., 2010a; Venugopal et al., 2011) and in the non-invasive procedure of extraction (Peng et al., 2011; Govindasamy et al., 2011a), easy isolation, limited ethical issues and fewer controversies as compared to other sources of MSCs (Miura et al., 2003; Chen et al., 2009a, 2009b; Rojphisan et al., 2009; Fong et al., 2010; Rodriguez-Lozano et al., 2011). Recently, many studies have reported the ability of these cells to differentiate into a myriad of cell types (Nakashima et al., 2002; Wang et al., 2004; Chen et al., 2009a, 2009b; Kadar et al., 2009; Wu et al., 2009; Huang et al., 2010; Inanc and Elicin, 2011; Govindasamy et al., 2011a, Nam et al., 2011) and this has been further supported substantially by successful preclinical studies (Conconi et al., 2006; Zheng et al., 2009; Lin et al., 2010; Yamada et al., 2010). Hence, it is not surprising that these alternative MSCs sources emerge as potent surrogates of BM-MSCs for human clinical trials. However, before drawing any conclusions, several questions need to be addressed in order to ensure maximum efficacy in targeted disease therapies. One of the most important factors to be considered among them is the intrinsic molecular propensity of the cells as it governs their fate. In this context, gene expression profiling is a reliable methods and has been commonly used with the aim of understanding the biology and the ontogeny of the cells (Nagoshi et al., 2008; Secco, et al., 2009; Hill et al., 2010). Furthermore, recent investigations have demonstrated that non-coding RNAs (microRNAs), in addition to protein-coding genes, encompass several functions related to stem cell biology, including the maintenance of pluripotency and the induction of cellular differentiation programmes (Chen and Carmichael, 2010; Mallanna and Rizzino, 2010). Despite the abundance of gene profiling data delineating the molecular and biological processes of cells, very little information is available on the relevance of these mechanisms in clinical application. Lack of understanding of this correlation is perhaps the main reason for the underlying failures in certain clinical trials.

In this study, we compared for the first time MSCs isolated from the four sources aforementioned, under identical in vitro conditions, with respect to their proliferation, multilineage differentiation capacity, immunophenotype and, most importantly, their biological functions, through protein-coding gene analysis. Interestingly, although all MSCs were outwardly similar to each other, we found significant differences in the global protein-coding gene expression patterns of MSCs derived from BM, AD, WJ and DP samples, suggesting tissue specificity.

Since, it is clearly known that there is no single master stem cell that can be used to treat all diseases, our findings provide a new insight and valuable information on the choice of the best stem cells for a specific disease.

2. Materials and methods

2.1. Tissue collection and isolation of cells

This study was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya [Medical Ethics Clearance Number: DF CO1107/0066(L)] and all the study subjects provided informed consent.

BM-MSC cultures were established from three donors, aged 24–35 years, as previously described (Pal et al., 2009). Briefly, 60 ml BM was aspirated aseptically from the iliac crest of each patient under deep sedation. The BM was diluted (1:1) with knockout Dulbecco’s modified Eagle’s medium (KO-DMEM; Invitrogen, Carlsbad, CA, USA) and centrifuged at 1800 rpm for 10 min to remove anticoagulants. The supernatant was discarded and the BM washed once with culture medium. Mononuclear cells (MNCs) were isolated by layering onto a lymphoprep density gradient (1:2; Axis-Shield PoC AS). The MNCs present in the buffy coat were washed again with culture medium. The mononuclear fractions that also contained MSCs were plated onto culture flasks.

Human umbilical cords (n = 3) from both sexes were collected from full-term births after either Caesarean section or normal vaginal delivery, as previously described (Nekanti et al., 2010a). After enzymatic treatment, the cells were suspended in 10% fetal bovine serum (FBS; Hyclone, Australia) and plated into tissue culture flasks.

Dental pulp stem cell (DPSC) cultures were established from sound intact third molars of adults aged 24–35 years, as previously described (Govindasamy et al., 2010a). Briefly, the root surfaces were cleaned with povidone-iodine (Sigma-Aldrich, St. Louis, MO, USA) and the pulps were extripated within 2 h post-extraction and processed. The pulp tissue was minced into small fragments prior to digestion in a solution of 3 mg/ml collagenase type I (Gibco, Grand Island, NY, USA) for 40 min at 37 °C. After neutralization with 10% FBS, the cells were centrifuged and seeded in culture flasks.

Human subcutaneous cells were obtained from healthy donors (n = 3; age 25–35 years) undergoing elective procedures of fat removal for aesthetic purposes. The adipose tissue was digested using collagenase (300 U/ml in a Dulbecco’s phosphate-buffered saline (DPBS; Invitrogen),
2% bovine serum albumin (BSA), pH 7.4, for 45 min under constant shaking. Following removal of the floating mature adipocytes, the lower layers containing the stroma vascular fraction (SVF) was centrifuged (200 rpm, 10 min) and the pellet was resuspended in an erythrocyte lysis buffer (155 mM NH₄Cl, 5.7 mM K₂HPO₄, 0.1 mM EDTA, pH 7.3) for 10 min. After successive filtrations through 100 and 70 μm sieves, the cells were resuspended in DPBS/2% FBS and centrifuged to remove the supernatant. The cultures were resuspended and plated into culture flasks.

All cells were cultured under identical culture conditions, i.e. in T25 cm² culture flasks (BD Pharmingen, San Diego, CA, USA) with culture medium containing 0.5% KO-DMEM and 10 000 μg/ml penicillin/streptomycin (Invitrogen), 1% 1× Glutamax (Invitrogen) and 10% FBS, with a humidified atmosphere of 95% air and 5% CO₂ at 37°C, and with a cell-seeding density of 1000 cells/cm². Non-adherent cells were removed 48 h after the initial plating. The medium was replaced every 3 days until the cells reached 80–90% confluence.

2.2. Growth kinetics

The proliferation rate was determined by plating 5000 cells/cm² of each MSC type into a T25 cm² culture flask (BD Pharmingen). Three replicates were performed for each passage. The cells were detached by trypsinization after reaching confluence of 90%, and were counted and assessed for viability by means of trypan blue dye exclusion before the next passage. The cells were replated for subsequent passages and a total of five passages were studied in this experiment. Growth kinetics were analysed by calculating the population-doubling time (PDT), which was obtained using the formula:

\[ PDT = \frac{\text{t}_{\text{plg}}}{\ln(NH) - \ln NI} \]

where \( NI \) is the inoculum cell number, \( NH \) is the cell harvest number and \( t \) is the time of the culture (in h).

2.3. Flow-cytometric analysis

Immunophenotyping was done using flow cytometry at passage 5. On reaching 90% confluence, the cells were harvested with 0.05% trypsin (Invitrogen) and resuspended in PBS at a cell density of 1.5 × 10⁶ cells/ml. An amount of 200 μl cell suspension (1 × 10⁵ cells) was incubated with the labelled antibodies in the dark for 1 h at 37°C. The following antibodies were used to mark the cell surface epitopes: CD90-phycocerythrin (PE), CD44-PE, CD73-PE, CD166-PE and CD34-PE, CD45-fluoro-isothiocyanate (FITC), and HLA-DR-FITC (all from BD Pharmingen). All analyses were standardized against negative control cells incubated with isotype-specific IgG1-PE and IgG1-FITC (BD Pharmingen). At least 10 000 events were acquired on a Guava Technologies flow cytometer and the results were analysed using Cytosoft v 5.2 (Guava Technologies).

2.4. Differentiation assay

The cultures were initiated at a density of 1000 cells/cm² in six-well plates and were grown to confluence and subjected to differentiation into adipogenic, chondrogenic and osteogenic lineages, according to the method described previously (Pal et al., 2009).

The adipogenic lineage was initiated by inducing the cells with 10% FBS, 200 μM indomethacin, 0.5 mM 3-isobutyl-1-methylenxantine (IMX), 10 μg/ml insulin and 1 μM dexamethasone (all from Sigma-Aldrich). Lipid droplets were visualized by staining with oil red staining (Sigma-Aldrich).

For chondrogenesis differentiation, cells were cultured in medium supplemented with ITS + 1 (Sigma-Aldrich), 50 μM l-ascorbic acid-2-phosphate, 55 μM sodium pyruvate (Invitrogen), 25 μM l-proline (Sigma-Aldrich) and 10 ng/ml transformation growth factor-β (TGFβ; Sigma-Aldrich). Assessment of proteoglycans accumulation was visualized by Alcian blue staining (Sigma-Aldrich).

The osteogenic differentiation was stimulated in a 3 week culture period in medium supplemented with 10% FBS, 10⁻⁷ M dexamethasone, 10 mM glycerol phosphate (Fluka, Buchs, Switzerland) and 100 μM l-ascorbic acid-2-phosphate. The assessment of calcium accumulation was visualized using von Kossa staining (Sigma-Aldrich). Quantitative amplifications of osteogenic gene expression, such as osteocalcin and osterix, were carried out in duplicate, using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). PCR reactions were run on an ABI 7900HT RT–PCR system (Applied Biosystems) and SDS v 2.1 software was used to analyse the results. All measurements were normalized to 18s rRNA. The sense and anti-sense primers used for each gene are shown in Table 1.

2.5. Pluripotent gene array

To analyse the expression of a focused panel of pluripotent and stem cell markers, the Human Stem Cell Pluripotency Array (TLDA; Applied Biosystems), containing a well-defined set of validated gene expression markers to characterize embryonic stem cell (ESC) identity, was used. The 384 wells of each TLDA card were preloaded with fluorogenic probes and primers (Applied Biosystems). Total RNA was extracted from three-donor pooled samples from each MSCs source at passage 5, using a mirVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer’s protocol. The RNA was subjected to DNase I treatment (Ambion) to eliminate any traces of contaminating DNA. After quantifying the yield of DNase I-treated RNA using a spectrophotometer (Agilent, NanoDrop Technologies Inc.), complementary DNA (cDNA) was prepared using a high-capacity cDNA reverse-transcriptase factor (Applied Biosystems). The cDNAs were loaded on the microfluidic cards for thermal cycling on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Expression values for target genes were normalized to the expression of 18s rRNA. Transcriptional analysis was...
performed for BM-MSCs, WJ-MSCs, AD-MSCs and DPSCs. For data analysis, the ABI PRISM 7900HT sequence detection system (SDS) software calculated the levels of target gene expression in samples relative to the level of expression in the calibrator (BM-MSCs), using a comparative Ct method. For estimation of the fold change by TLDAtion in the calibrator (BM-MSCs), using a comparative Ct method. For estimation of the fold change by TLDA when the initial transcript levels were undetectable, the initial expression would lead to a possible underestimation of the actual fold change.

### 2.6. Array validation

Pluripotent gene array data were evaluated and confirmed by semi-quantitative and quantitative RT–PCR. Briefly, the cell pellets were collected and total RNA was isolated by the TRIZOL method (Invitrogen), according to the manufacturer’s protocol, was quantified using a spectrophotometer (Agilent) and the purity was assessed by the 260:280 nm ratio. The RNA was stored at –80°C. The first-strand cDNA was synthesized using 1 µg RNA treated with RNase-OUT ribonuclease inhibitor and the Superscript II First Strand Synthesis system (Invitrogen) according to the manufacturer’s instructions. PCR was performed in 0.2 ml Eppendorf tubes (Axygen) with a reaction volume of 12.5 µl. cDNA amplification was performed in a thermocycler using Taq polymerase supplied with KCl buffer and 1.5 mM MgCl₂ (Invitrogen) at 94°C for 1 min, 58°C for 30 s and 72°C for 1 min. PCR products were resolved on 1.5% agarose gel run in 1× Tris borate–EDTA buffer. The primer sequences are listed in Table 1.

The expressions of some primers in the semi-quantitative RT–PCR analysis were quantified in duplicate, using SYBR Green Master Mix (Applied Biosystems). PCR reactions were run on an ABI 7900HT RT–PCR system (Applied Biosystems) and SDS v 2.1 software was used to analyse the results. All measurements were normalized to 18s rRNA.

### 2.7. Gene ontology analysis of differentially expressed genes

Lists generated in the pairwise comparisons between the four different stem cell types were used as input for the online Functional Annotation Tool at DAVID Bioinformatics Resources (National Institute of Allergies and Infectious Diseases (NIAID), NIH, Bethesda, MD, USA; http://david.abcc.ncifcrf.gov/). DAVID Bioinformatics Resources consists of an integrated biological knowledge base and analytical tools intended to systematically extract biological meaning from large gene/protein lists. The procedure first requires uploading a gene list containing any number of common gene identifiers, followed by analysis using one or more texts. By following this protocol, users are able to gain an in-depth understanding of the biological themes expressed genes

### 2.8. Karyotype analysis

Cultures were treated with colcemid 2 h before harvest, detached by trysinization, and treated with 0.5 M hypotonic solution (KCl/water) before fixing with Conroy’s solution (3:1 methanol:glacial acetic acid; Sigma-Aldrich). The spreads were treated with 0.05% trypsin, stained with Giemsa (Sigma-Aldrich) and 20–30 separate metaphase spreads were examined for each culture.

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**Table 1. Sets of gene primers used, with forward and reverse sequences**

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene symbol</th>
<th>Forward</th>
<th>Reverse</th>
<th>Base pairs</th>
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<td>2</td>
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<td>CAGCATCCTCAAACAGGATCCTG</td>
<td>282</td>
</tr>
<tr>
<td>3</td>
<td>OCT 3/4 (POUSFI)</td>
<td>CGACACCTCTGCGCCCTTTGAG</td>
<td>CCCGCTTCCCAATCTCTA</td>
<td>572</td>
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<tr>
<td>4</td>
<td>NANO2</td>
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<td>TCGGCGCGGGGGAGATCAT</td>
<td>259</td>
</tr>
<tr>
<td>5</td>
<td>STELLAR</td>
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<tr>
<td>6</td>
<td>DPPAS</td>
<td>ATGGGGAGGCTCCGGAGCAGC</td>
<td>TCACCTCAAGGGGGCCTA</td>
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<td>7</td>
<td>OSTEOLACIN</td>
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<td>AGAGGACAACCCCTTAGAC</td>
<td>314</td>
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<tr>
<td>8</td>
<td>OSTERIX</td>
<td>GCACGTAGAGGGGAGTGTGGT</td>
<td>CGACGCGAGGTGATTCTCTCC</td>
<td>358</td>
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<td>16</td>
<td>HNF-3</td>
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<td></td>
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<td>17</td>
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<tr>
<td>18</td>
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<td>CRB4</td>
<td>CTCCTCAGCGAGGAGGACAGC</td>
<td>GAGATCGGACGCGGCGGCA</td>
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</table>
2.9. Statistical analysis

Data are presented as mean ± standard deviation (SD). Graphical representation was performed using Graph Pad Prism software (Graph Pad, San Diego, CA, USA). Statistical comparisons were made using Student’s t-test and \( p < 0.05 \) was considered significant.

3. Results

3.1. BM-MSCs, WJ-MSCs, DPSCs and AD-MSCs are bona fide MSCs

Among all the four sources of MSCs (Figure 1A), WJ-MSCs grew rapidly and maintained almost the same growth rate throughout the five passages, followed by DPSCs, while BM-MSCs were growing slowly after passage 2. Compared with DPSCs and WJ-MSCs, AD-MSCs appeared to be slow but maintained constant growth until the fifth passage (Figure 1B). This result reflected the PDT of the cells. The average PDTs of BM-MSCs, WJ-MSCs, AD-MSCs and DPSCs were \( 51.5 \pm 0.5 \), \( 29.80 \pm 0.45 \), \( 60.5 \pm 0.5 \) and \( 30.5 \pm 0.5 \) h, respectively (Figure 1C). To further characterize these cells, cell surface markers were examined by flow cytometry. MSCs from all four sources were negative for haematopoietic lineage markers CD34 and CD45, whereas > 90% of cells were positive for mesenchymal stem cell markers CD44, CD73, CD90 and CD166 (Table 2 and Figure S1). We investigated the potential of BM-MSCs, WJ-MSCs, AD-MSCs and DPSCs to differentiate to the adipogenic, chondrogenic and osteogenic lineages. Adipogenic differentiation was demonstrated by the accumulation of neutral lipid vacuoles indicated by oil red O stain. Large and compact lipid vacuoles were seen in BM-MSCs, whereas AD-MSCs were small and distributed throughout the flask (Figure 2A). Interestingly, we discovered that both DPSCs and WJ-MSCs failed to differentiate efficiently into adipogenic cells. Next, for chondrogenic differentiation, the positive staining of Alcian blue indicated that all stem cell types could be differentiated efficiently (Figure 2A). Osteogenic differentiation was confirmed by the detection of silver-stained mineralized matrix (Figure 2A). Although all the stem cell types

![Image of morphological and growth kinetics of MSCs](https://example.com/image.jpg)
Table 2. Phenotype characterization of various mesenchymal stem cells source cultured at passage 5

<table>
<thead>
<tr>
<th>Phenotypic characterisation (%)</th>
<th>BM-MSCs</th>
<th>AD-MSCs</th>
<th>WJ-MSCs</th>
<th>DPSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>0</td>
<td>0.02 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0</td>
</tr>
<tr>
<td>CD44</td>
<td>94.21 ± 2.9</td>
<td>95.83 ± 1.8</td>
<td>99.36 ± 0.25</td>
<td>92.82 ± 1.50</td>
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<tr>
<td>CD45</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD73</td>
<td>99.88 ± 3.1</td>
<td>99.45 ± 4.1</td>
<td>99.43 ± 0.69</td>
<td>95.65 ± 6.58</td>
</tr>
<tr>
<td>CD90</td>
<td>93.71 ± 0.9</td>
<td>99.49 ± 0.8</td>
<td>97.57 ± 0.92</td>
<td>99.54 ± 0.28</td>
</tr>
<tr>
<td>CD166</td>
<td>98.11 ± 0.8</td>
<td>79.85 ± 6.8</td>
<td>99.88 ± 3.1</td>
<td>99.45 ± 4.1</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>0</td>
<td>0</td>
<td>0.02 ± 0.01</td>
<td>0.01 ± 0</td>
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</tbody>
</table>

Figure 2. (A) In vitro mesoderm differential potentiality of MSCs. Osteogenesis was confirmed by mineralized matrix deposition stained with von Kossa at day 21 in (a) BM-MSCs, (b) WJ-MSCs, (c) AD-MSCs and (d) DPSCs. Chondrogenesis was detected by the presence of proteoglycans stained with Alcian blue at day 21 in (e) BM-MSCs, (f) WJ-MSCs, (g) AD-MSCs and (h) DPSCs. Adipogenesis was detected by neutral oil droplet formation stained with oil red O at day 21 in (i) BM-MSCs, (j) WJ-MSCs, (k) AD-MSCs and (l) DPSCs. (B) mRNA expression of Osterix and Osteocalcin by real-time RT–PCR, using SYBR green reagent; values were normalized to 18s RNA; all experiments were conducted at passage 5.
Gene expression of mesenchymal stem cells from various sources

were able to secrete calcium into the matrix, reinforcing their differentiation capacity, the mRNA expression of two osteoblast markers, Osteocalcin and Osterix, were found to be higher in DPSCs, followed by BM-MSCs and AD-MSCs. Remarkably, despite the higher cell proliferation, gene expression profiling was lowest in WJ-MSCs, indicating that these cells may not efficiently differentiated into the osteogenic lineage (Figure 2B). No staining was seen in undifferentiated cells (data not shown). Although there were some variations between different stem cell types, the overall results presented here are consistent with the definition of mesenchymal stem cells that was set by the Mesenchymal and Tissue Stem Cells Committee of the International Society for Cellular Therapy (ISCT; Dominici et al., 2006), thus qualifying all these stem cells as bona fide MSCs.

3.2. Presence of unique signature genes

A Venn diagram illustrating the unique and common genes is shown in Figure 3A. We have arranged the genes considering BM-MSCs as the control source, and hence whatever genes were downregulated by BM-MSCs is considered to be upregulated in other cell lines. In this context, while 18 and 19 upregulated unique genes were found in AD-MSCs and WJ-MSCs, respectively, the number went up to 44 in DPSCs and 104 in BM-MSCs. Nevertheless, many overlapping genes were found in WJ-MSCs and AD-MSCs (21 genes) as compared to other groups (DPSCs and AD-MSCs, five genes; WJ-MSCs and DPSCs, eight genes). Interestingly, only four genes were found to be upregulated in all the stem cell types when compared to BM-MSCs. The upregulated genes in WJ-MSCs, AD-MSCs and DPSCs are illustrated in Figure 3B. To more generally define the gene expression signature in the different stem cell types, we set out to analyse whether differentially expressed genes represent biological distinct profiles. The gene expression data were subjected to GO analysis, using the online functional annotation tool DAVID. To this end, lists of upregulated genes were generated between the different stem cells types and only genes that were significantly upregulated (p < 0.05) were included in this analysis.

3.3. Gene ontology (GO) analysis reveals gene expression signatures specific to each MSC source

In the case of genes that are upregulated in BM-MSCs as compared to other stem cells, it is evident that the genes associated with the regulation of cell differentiation were specifically present in BM-MSCs. The GO class related to cell differentiation was highly expressed in DPSCs, WJ-MSCs and AD-MSCs as compared to BM-MSCs. Another salient feature was the expression of genes related to the GO class of cell migration and cell motion, which were only found in DPSCs, WJ-MSCs and AD-MSCs. WJ-MSCs and DPSCs showed another unique GO class (GO class of regulation of cell proliferation) with the former cells having a higher gene percentage than the latter (Figure 4; see also Supporting information, Tables S1–S4).

3.4. BM-MSCs express many genes involved in inflammation and immune response

Markedly, a high number of genes associated with GO classes involving inflammation and the immune responses, such as the GO classes of response to stress, response to wounding, response to external stimulus, inflammatory response and defence response, were shown by BM-MSCs. Surprisingly, this phenomenon was not observed in any other stem cell type, indicating the specificity of BM-MSCs (Figure 4; see also Supporting information, Tables S1–S4).

3.5. Several genes implicated in tissue development are expressed at higher levels in WJ-MSCs and AD-MSCs

Apart from the genes that are involved in cell regulation, surprisingly both WJ-MSCs and AD-MSCs expressed high number of genes associated with a GO class involving organ and tissue development in comparison with BM-MSCs. In fact, one of the most prevalent classes appeared to be AD-MSCs, where out of the 12 significant classes shown, 10 were directly linked to organ and tissue development (Figure 4; see also Supporting information, Tables S1–S4).

3.6. The transcriptomic profile of WJ-MSCs and DPSCs are biased to differentiate towards the neuro-ectodermal lineage

When comparing WJ-MSCs vs BM-MSCs and DPSCs vs BM-MSCs, both WJ-MSCs and DPSCs posed a strong bias towards GO classes related to the neuro-ectoderm lineage. A breakdown of this class into smaller GO subclasses revealed that these genes are primarily involved in nervous system development and the generation of neurons. Interestingly, DPSCs expressed a distinct GO class related to neuron migration that was not observed in WJ-MSCs (Figure 4; see also Supporting information, Tables S1–S4).

3.7. Confirmation of pluripotent array analysis

The expression of OCT4, SOX2, NANOG, DPPA5, REX1 and STELLAR was significantly upregulated in DPSCs, followed by WJ-MSCs, BM-MSCs and AD-MSCs. Similarly, the expression of some of the neuro-ectoderm markers (NESTIN, βIII-Tub, NF) was upregulated in DPSCs, followed by WJ-MSCs, AD-MSCs and BM-MSCs. In contrast, the expression of early endoderm and mesoderm markers was higher in AD-MSCs and BM-MSCs compared to
WJ-MSCs and DPSCs (Figure 5). These results were consistent with the pluripotent array results.

3.8. Cytogenetic stability of all MSCs

All the cell types showed normal karyotypes at passage 5. A representative ideogram is illustrated in Figure 6.

4. Discussion

Transplantation of autologous or allogeneic MSCs represents a novel medical innovation, since substantial promises can be seen in the treatment of a number of diseases that have been incurable by conventional medicine. Although BM-MSCs are always considered to be the best source for therapy, the controversial aforementioned
Figure 4. Functional genomics analysis. Enriched GO-terms (biological process), obtained by comparing the GO term distribution in each gene list and the whole set of genes printed onto the array, were used to generate multilevel pie charts. GO classes associated with the immunity are indicated in light to dark red; GO classes associated with the development are indicated in light to dark blue; GO related to neurogenesis are indicated in light to dark green; and GO related to cell growth and proliferation of cells are indicated in white.
issues described earlier in this paper have restricted their usage. Furthermore, the hype and hope that was given to BM-MSCs as ‘master stem cells’ have failed in certain clinical trials (Breitbach et al., 2007). Hence, a new initiative of establishing tailor-made stem cells from the myriad of tissues for disease-specific therapy has been initiated in the hope of obtaining a higher efficacy level for cell transplantation. Nevertheless, lack of understanding of the inherent differential propensities of these cells may restrict their clinical application. Therefore we have undertaken the present work to study four different sources of MSCs, viz. bone marrow (BM), adipose tissue (AD), dental pulp (DP) and Wharton’s jelly (WJ), in terms of their common MSCs characteristics and specific lineage propensity.

The cells isolated from these sources exhibited typical MSC characteristics: fibroblastoid morphology, the formation of CFU-F, multipotent differentiation capability and the expression of typical set of surface proteins. Although
MSCs from different origins share important biological properties, to date it is not clear which types of adult stem cells or MSCs should be selected for the different therapeutic approaches. The origin of MSCs may determine their fate and functional characteristics and perhaps an important question could arise, i.e. whether MSCs from different origins exhibit unique gene expression profiles that could explain their differentiation characteristics. In this scenario, analysing the mRNA expression levels has been adopted as a powerful approach for understanding the propensity and capacity of MSCs from a particular source to differentiate towards a particular lineage or adopt a certain fate.

The growth kinetics results revealed that both WJ-MSCs and DPSCs have higher proliferation rates than BM-MSCs and AD-MSCs. Our results are concurrent with previous reports on the comparison between BM-MSCs and WJ-MSCs (Nekanti et al., 2010c) or between BM-MSCs and DPSCs (Govindasamy et al., 2010a, 2010b). Furthermore, AD-MSCs have been reported to have a lower proliferation rate (Neupane et al., 2008). Interestingly, this observation was well captured with gene signalling pathways, in which the GO related to the cell proliferation rate was only found in WJ-MSCs and DPSCs, where the former showed a higher percentage of genes. This was confirmed by the overexpression of many transcription factors, such as POU5F1 (also known as Oct3/4), SOX2, NANOG and LIN28, that are related to the maintenance of pluripotence in early embryos and embryonic stem cells (ESCs) (Nichols et al., 1998; Avilion et al., 2003; Chambers et al., 2003; Rizzino, 2009). This reconfirms the fact that WJ-MSCs, which are of fetal origin, have more primitive characteristics than adult MSCs (Nekanti et al., 2010c). While we have not included ESCs in our expression profiling analysis, it is tempting to speculate that WJ-MSCs are closely related to ESCs in our gene expression profiling analysis. Similarly, the higher proliferation of DPSCs relates to the fact that they were isolated at an early stage of adult tooth development (Bakopoulou et al., 2011).

Next we demonstrated that all MSC sources were able to differentiate into osteoblasts, adipocytes and chondrocytes, hence complying with the minimum requirement of MSCs (Augello et al., 2010; Lindner et al., 2010). Notwithstanding that, the differentiation efficacy, especially in adipogenic cells, varies among the cell types, with BM-MSCs being the highest and WJ-MSCs and DPSCs the lowest. Higher proliferation and extensive expansion in these cells may, in some clonal populations, have caused loss of adipogenic potential that was originally present in the cells, as has been suggested for BM-MSCs (Muraglia et al., 2000). It can be postulated that both WJ-MSCs and DPSCs clones retained their multipotentiality through high numbers from population doubling, whereas other clones, such as the one in our case, lost the ability to form adipocytes whilst retaining chondro-osteogenic capacity. Another possible reason to single
out from our GO analysis is the presence of a unique class associated with the regulation of cell differentiation, which is only present in BM-MSCs. Surprisingly, GO linked to cell differentiation are expressed in all other MSCs except BM-MSCs. Based on the observation of these GO classes, we suggest that BM-MSCs might have a homogeneous population and are most likely to contain true stem cells, which might play a vital role in regulating the cell differentiation, whereas other MSCs are most likely to be heterogeneous populations in which gene expressions reflecting the cell differentiations are highly expressed. To the best of our knowledge, this is the first study to report the existence of cell populations in the WJ-MSCs that are chondro-osteogenic but unable to differentiate into adipocytes. Nevertheless, there are reports on the inability of DPSCs to differentiate into adipocytes (Balic et al., 2010). As reported with BM-MSCs (Muraglia et al., 2000) and AD-MSCs, we also did not observe cell populations with a differentiation potential restricted to the osteo-adipogenic or to the chondro-adipogenic phenotype.

In the next set of experiments, for all the MSCs sources investigated, the cells expressed the immune phenotype of a list of candidate markers proposed by the Mesenchymal and Tissue Stem Cells Committee of the ISCT. Since, HLA-DR molecules are important to T cells stimulation and immune recognition (Sotiropoulou et al., 2006), we examined the expression level of this marker. Flow cytometry analysis demonstrated that the cells obtained did not significantly express HLA-DR, thus clearly demonstrating that the capacities of all MSCs cells are immune-privileged. Our immune-phenotype results were in agreement with those of many previous reported studies on BM-MSCs (Bittencourt et al., 2006; Menendez et al., 2009; Antoniou et al., 2010; Hanson et al., 2010), WJ-MSCs (Weiss et al., 2010a, 2010b, 2010c; Venugopal et al., 2011), AD-MSCs (de Mattos et al., 2009; Sathishkumar et al., 2011; Shih et al., 2011) and DP-MSCs (Agha-Hosseini et al., 2010; Gronthos et al., 2011; Rodríguez-Lozano et al., 2011); however, our findings of immunogenic studies showing the absence of HLA-DR may not be sufficient to brand them as non-immunogenic. On the other hand, GO analysis clearly shows that BM-MSCs are in the highest rank in terms of expression of genes related to immodulation and inflammation, whereas all other MSCs showed a downregulation for the same set of the genes. Our results are contradictory to the previous research that reported the abundance of gene expressions related to this group in higher frequency in AD-MSCs (Jansen et al., 2010). It should be noted that our data showed the gene expression pattern at the basal level and, as such, we are predicting that most of the genes will not be expressed until they are exposed to a suitable environment.

It is not surprising to see the large amount of GO linked to neuron-ectoderm-related genes being expressed in DPSCs. This is because it is a well-established fact that DPSCs originate from the neural crest (Govindasamy et al., 2012).
Gene expression of mesenchymal stem cells from various sources

et al., 2011a, 2011b; Tirino et al., 2011) and, by default, it contributes to the expression of neuro-ectoderm-related genes. Nevertheless, our data revealed that WJ-MSCs are also prone to neuro-ectoderm lineage characteristics that are novel and unexpected. In contrast, BM-MSCs and AD-MSCs expressed genes are involved in organ and system development at higher levels. In a nutshell, we were able to show that different sources of MSCs showcased unique gene profiling under in vitro environments. Despite the fact that all the tested stem cell sources in the present research were able to qualify to some of the minimum MSCs threshold set by the Mesenchymal and Tissue Stem Cells Committee of the ISCT, their inherent gene expression clearly showed that these cells are in situ-centric while retaining a normal karyotype, as demonstrated in the present study.

BM-MSCs and AD-MSCs express genes that are involved in tissue and organ development that falls under the mesodermal lineage. From our results, we postulate that BM-MSCs are mostly likely to contain homogeneous populations and be similar to the mesenchymal lineage. This is due to the presence of a lineage ‘imprinting’ in each cell compartment that influences the differentiation potential of MSCs (Charbord et al., 2011; Satomura et al., 2000). In the present study it was shown that BM-MSCs inherently have the ability to differentiate into mesodermal lineages and hence seem to strictly follow the mesenchymal stem/stromal cells classification set by ISCT. Conversely, DPSCs are most likely to contain a heterogeneous population and are primed towards neurogenesis. Our findings on WJ-MSCs revealed that, to some extent, they resemble ESCs. Taking these findings into account, we propose several clinical applications proportional to their inherent gene expression characteristics (Figure 7). We speculate that these MSCs may work for the mentioned clinical applications and that they may be disease-specific. Our arguments are consistent with the findings of other groups as well (Al-Nbaheen et al., 2012). Nevertheless, a few questions need to be considered before taking these alternative sources of MSCs on board: (a) will these cells retain their intrinsic lineages after large-scale expansion and also in serum-free culture conditions; (b) is the gene expression profile reflected in the paracrine activities of the cells essential for therapeutics; (c) is the gene variation between donors, ages and genders responsible for any variation in the activities? Answering these questions could further harness the potential of these cells as alternative sources to cure diseases with a tailor-made choice of stem cells for better clinical efficacy.

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Supporting information on the internet

The following supporting information may be found in the online version of this article:

- Figure S1. Immunophenotype analysis of BM-MSCs, AD-MSCs, WJ-MSCs and DPSCs at passage 5
- Table S1. GO analysis of genes upregulated in BM-MSCs
- Table S2. GO analysis of genes upregulated in DPSCs
- Table S3. GO analysis of genes upregulated in WJ-MSCs
- Table S4. GO analysis of genes upregulated in AD-MSCs

Conflict of interest

The authors have declared that there is no conflict of interest.

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