

## Stability of human mesenchymal stem cells during *in vitro* culture: considerations for cell therapy

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### Abstract

*Ex vivo* expansion and manipulation of human mesenchymal stem cells are important approaches to immunoregulatory and regenerative cell therapies. Although these cells show great potential for use, issues relating to their overall nature emerge as problems in the field. The need for extensive cell quantity amplification *in vitro* to obtain sufficient cell numbers for use, poses a risk of accumulating genetic and epigenetic abnormalities that could lead to sporadic malignant cell transformation. In this study, we have examined human mesenchymal stem cells derived from bone marrow, over extended culture time, using cytogenetic analyses, mixed lymphocyte reactions, proteomics and gene expression assays to determine whether the cultures would retain their potential for use in subsequent passages. Results indicate that *in vitro* cultures of these cells demonstrated chromosome variability after passage 4, but their immunomodulatory functions and differentiation capacity were maintained. At the molecular level, changes were observed from passage 5 on, indicating initiation of differentiation. Together, these results lead to the hypothesis that human mesenchymal stem cells cultures can be used successfully in cell therapy up to passage 4. However, use of cells from higher passages would have to be analysed case by case.

### Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate *in vitro* into a variety of mesoderm cell lineages, including osteocytes, chondrocytes and adipo-

cytes. Human MSCs (hMSCs) can be expanded and manipulated *ex vivo* and demonstrate immunomodulatory functions *in vitro* and *in vivo*; thus, they represent promising tools for use in immunoregulatory and regenerative cell therapies. Clinical use of hMSCs is an emerging field for treatment of several diseases, including non-healing bone defects and fractures, inflammatory arthritis (1), inherited bone and cartilage disorders (2), cardiovascular disorders (3), graft-versus-host diseases (4), and for enhancement of haematopoietic stem-cell transplantation (5). Although hMSCs have shown great potential for therapeutic application, little is known of their specific molecular mechanisms or impact of their culture expansion methods (6). Indeed, issues related to their nature have emerged as potential problems in this field.

Approximately  $2 \times 10^6$  cells/kg are required for clinical application of hMSCs. Thus, extensive amplification *in vitro* without affecting cells' genomic characteristics and differentiation properties is necessary. However, such expansion could generate genetic and epigenetic instability, including chromosome alterations, and could also initiate cell senescence. Accumulation of changes during extended culture could lead to spontaneous malignant cell transformation (7–9).

Although it has been shown that hMSCs derived from bone marrow and adipose tissue can be continuously cultured *in vitro* for approximately 20 passages (10), it is unclear how many passages can be performed before the cells acquire chromosomal instability or lose multipotency; karyotypic aberrations have been observed in MSC cultures derived from mice and rats (11,12). Some attempts have been made to examine such defects using different sources of hMSCs, different culture conditions and different passage densities; however, results of the studies are controversial. While some groups have shown retention of genetic stability up to passage three or four, others have shown that hMSC cultures maintain normal karyotype right up to passage 20 (10, 13–16).

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In the study described here, we have analysed karyotype and multipotency of bone marrow-derived hMSCs at passages 1–9. Our results identified in most cases, appearance of random chromosome alterations from passage 5 on, and also showed changes in cell proliferation rate, although the cells did not lose their differentiation capacity. Thus, we investigated whether the chromosome alterations would inhibit use of hMSCs cells in regenerative medicine.

Different approaches have been attempted for use of hMSCs in cell therapy. In some cases, cells are locally injected into the recipient organ, for example, in treatment of bone defects (17,18); here, cells directly participate in the regeneration process. Furthermore, locally injected hMSCs secreted factors that stimulated cell population growth and differentiation of the stem cells, through paracrine effects or to counteract inflammation through immunomodulatory effects (19,20). Alternatively, hMSCs can be intravenously infused to mediate repair of a microenvironment and facilitate treatment or prevention of graft-*versus*-host disease (GVHD) after haematopoietic stem-cell transplantation (21). In this case, the immunomodulatory effect of hMSCs was exerted in peripheral blood, and the regenerative effect was expressed in the bone marrow. While intact hMSC genome is crucial for safety of cell therapy, injected cells do not normally survive over long periods of time in a recipient.

To further evaluate potential use of cultured hMSCs in cell therapy, we examined ability of hMSCs to inhibit *in vitro* lymphocyte proliferation. We showed that the capacity to inhibit this is maintained after a number of passages, even when hMSCs revealed chromosome instability. We used proteomic and transcriptomic approaches to verify molecular profiles and to examine potential modifications in multipotency of hMSCs from different culture passages (for example from passage 1 (P1) to passage 8 (P8)). Our results have indicated that cultured hMSCs develop molecular changes after passage 5.

## Materials and methods

### *Isolation and culture of human bone marrow-derived MSC*

Bone marrow (BM)-derived samples were obtained from healthy donors registered at the Bone Marrow Transplantation Unit, National Cancer Institute, Rio de Janeiro, Brazil, in accordance with guidelines of the local Ethics Committee and Helsinki Declaration. BM aspirates were obtained from 4 male donors (mean age 40.3, range 30–59) and 5 female donors (mean age 40.4, range 32–47). Mononuclear cells (MNC) were isolated from BM sam-

ples (5–10 ml) using density-gradient centrifugation (Ficoll 1.077 g/ml; GE Healthcare Life Sciences, Piscataway, NJ, USA). Cells were plated in non-coated 75-cm<sup>2</sup> polystyrene culture flasks (Techno Plastic Products AG, Trasadingen, Switzerland) at 500 000 cells/cm<sup>2</sup> density, in low-glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 15% foetal bovine calf serum (Hyclone, Thermo Scientific, Waltham, MA, USA), 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen). After 48 h, non-adherent cells were removed, and hMSCs were cultured until 80% confluence was obtained. Subsequently, cells were removed from plates using 0.05% trypsin (Invitrogen) treatment for 5 min at 37 °C, and replated at 2000 cells/cm<sup>2</sup> density (passage 1) in a fresh culture flask. When 80% confluency was obtained (approximately 7 days culture), cells were trypsinized and replated in fresh culture flasks (passage 2). These processes were repeated up to passage 10, for all cultures. Population doubling of the cultured hMSCs was calculated at every passage, according to the equation  $\log_2$  (number of harvested cells/number of seeded cells). For 10 passages, mean cumulative population doubling (CPD) was 24.8 and mean population doubling was 2.4 per passage.

### *Confirmation of multipotentiality*

These experiments were performed in accordance with minimal criteria for defining multipotent mesenchymal stromal cells, as defined by the International Society for Cellular Therapy (22). The cultured plastic-adherent cells, expressing CD73, CD90 and CD105, in the absence of lineage commitment markers such as CD14, CD19, CD34, CD45 and HLA-DR (BD Biosciences, San Jose, CA, USA), were able to differentiate into adipocytes and osteoblasts. Ability of the hMSCs cultures to differentiate into adipocytes and osteoblasts was tested at passages 1, 5 and 8. To induce adipogenic differentiation, the cells were cultured with 10<sup>-8</sup> M dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) and 5 µg/ml insulin (Sigma-Aldrich); oil-red-O (Sigma-Aldrich) staining was used to detect lipid accumulation. Osteogenic differentiation was induced using 10<sup>-8</sup> M dexamethasone (Sigma-Aldrich), 10 mM β-glycerolphosphate (Sigma-Aldrich) and 50 µg/ml ascorbic acid (Sigma-Aldrich). Osteoblasts were identified using alizarine red S (Isifar, Brazil) staining.

### *Cytogenetic analysis*

Nine hMSC cultures were cytogenetically defined at a number of passages. Prior to obtaining confluence, cells

were incubated at 37 °C with colcemid (Sigma-Aldrich) at 1 µg/ml for 2 h. Then they were trypsinized and processed using standard cytogenetic procedures. GTG-banding was performed, and chromosomes were identified and analysed according to the International System of Human Cytogenetic Nomenclature (ISCN, 2009).

#### Quantitative PCR

Quantitative PCR (Q-PCR) analyses were performed using one µg of mRNA, treated with DNase Amplification Grade I (Invitrogen), which was reverse transcribed using Superscript II Reverse Transcriptase® (Invitrogen). Q-PCR reactions contained 1× Power SYBR Green PCR Master Mix® (Applied Biosystems, Carlsbad, CA, USA), 2.5 µl of 100-fold diluted cDNA and 0.1 µM of each primer. Quantitative determination of mRNA levels was performed using Rotor-Gene 6000 Series Software (Corbett, Australia), and reactions were performed in a Rotor-Gene 6000 thermocycler (Corbett, Australia) using the following program: 95 °C for 5 min and 45 cycles at 95 °C for 15 s followed by 60 °C for 1 min. Dissociation curve analysis was used to demonstrate equal amplification efficiency of a specific PCR product for all primers used in these studies. Determination of fold-expression was calculated using the DDCT method according to Livak and Schmittgen (23). Data were analysed statistically using Prisma 4 and ANOVA.

Expression levels were estimated in triplicate, and *β-actin* and *GAPDH* were used as reference genes for normalization. The following primers were used: *CD44* Fw, 5'-AGACCCACTC AGCCAAATCT -3'/ Rev, 5'-CTTTCACAGGA GAGAACAGGTG-3'; *SCF* Fw, 5'-ATGCAAATGGTTGCCTGGTCCC -3'/ Rev, 5'-TGCCAATGTCCTTCTTGGCAGA -3'; *c-Kit* Fw, 5'-ATGGGAAAGAAGACAACG ACACGC -3'/ Rev, 5'-GCA-GAC AGAGCCGATGGTAGG -3'; *BMPR* Fw, 5'-CGA AGTGCT GGACGAAAGCCT -3'/ Rev, 5'-TTGACA-CACACAACCTCACGCA -3'; *β-actin* Fw, 5'-ACCTGA-GAACT CCACTACCCT-3'/ Rev, 5'-GGTCCCACCCATG TTCCAG -3'; *GAPDH* Fw, 5'-ACCACAGTCCATGC-CATCAC-3'/ Rev, 5'-CCACC ACCCTGTTGCTGTA -3'

#### Mixed lymphocyte reaction

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized samples of unrelated healthy volunteers, using density-gradient centrifugation. For the mixed lymphocyte reaction (MLR),  $5 \times 10^5$  carboxy fluorescein diacetate succinimidyl ester (CFSE)-labelled responder PBMC (R) and  $5 \times 10^5$  irradiated (2500 cGy) stimulator PBMC (S)/well were incubated in 24-well

flat-bottom tissue-culture plates containing RPMI-1640 (Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen), 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen) and 10% FCS (Hyclone) in the absence or presence of  $5 \times 10^4$  hMSCs. After seven days, cultures were analysed using flow cytometry in a FACScan instrument (Becton Dickinson, Franklin Lakes, NJ, USA) and data were analysed using CELLQuest software (Becton Dickinson). With Student's *t*-test, *P*-values < 0.05 were considered to be statistically significant. Statistical analyses and graphic representations were performed using GraphPad Prism™ software (GraphPad software, San Diego, CA, USA).

#### Expression chip array data analysis

Total RNA from hMSC cultures at passages 1, 5 and 8 were obtained using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. One µg of total RNA was used to synthesize biotinylated cRNA according to GeneChip Whole-Transcript (WT) Sense Target Labelling Assay (Affymetrix, Santa Clara, CA, USA). Biotinylated cRNA was subsequently hybridized to GeneChip human exon 1.0 ST array (Affymetrix), washed and stained according to the manufacturer's protocols. GeneChip arrays were scanned using GeneChip® Scanner 3000. Affymetrix Expression Console Software Version 1.0 was used to create summarized expression values (CHP-files) and Robust Multichip Analysis (RMA) algorithm was applied. Data were analysed using Partek® software (<http://www.parstek.com>) (24), with differentially expressed genes,  $\geq 5$ -fold change, as criteria to define overexpression or downregulation. Gene ontology analysis was conducted using Ingenuity Pathways Analysis (IPA) software, available at NCBI or <http://www.ingenuity.com>.

#### Two-dimensional gel electrophoresis

To prepare protein extracts,  $7 \times 10^6$  cells at passages 1, 5 and 8 were washed 3 times in phosphate-buffered saline (PBS), centrifuged and resuspended in cold lysis buffer containing 50 mM Tris pH 7.5, 5 mM ethylene diamine tetraacetic acid (EDTA), 10 mM ethylene glycol tetraacetic acid (EGTA), 50 mM sodium fluoride, 20 mM  $\beta$ -glycerol-phosphate, 250 mM sodium chloride, 0.1% Triton X-100, 20 mM sodium orthovanadate, and protease inhibitor mix (2.4 µg/ml AEBSF, 0.1 µg/ml Bestatin, 0.1 µg/ml Pepstatin, 0.1 µg/ml Leupeptin, and 1.8 µg/ml E-64) (Calbiochem, Ardmore, PA, USA). After incubation for 30 minutes on ice, lysates were spun at 12 000 rpm at 4 °C, for 30 min. Total protein concentration obtained was determined using the



Bradford assay (25). Five hundred  $\mu\text{g}$  of total cell protein extracts was precipitated using a 2D Cleanup Kit (GE Healthcare, Piscataway, NJ, USA) and resuspended in 200  $\mu\text{l}$  of reswelling buffer containing 6 M urea, 2 M thiourea, 15 mM DTT, 2% (w/v) ASB14, 0.5% IPG buffer pH 3 (GE Healthcare, Sweden) and traces of bromophenol blue. Electrophoresis in the first dimension was performed using 11 cm of Immobiline DryStrip, pH 4-7 (GE Healthcare), at 30 550 VhT.

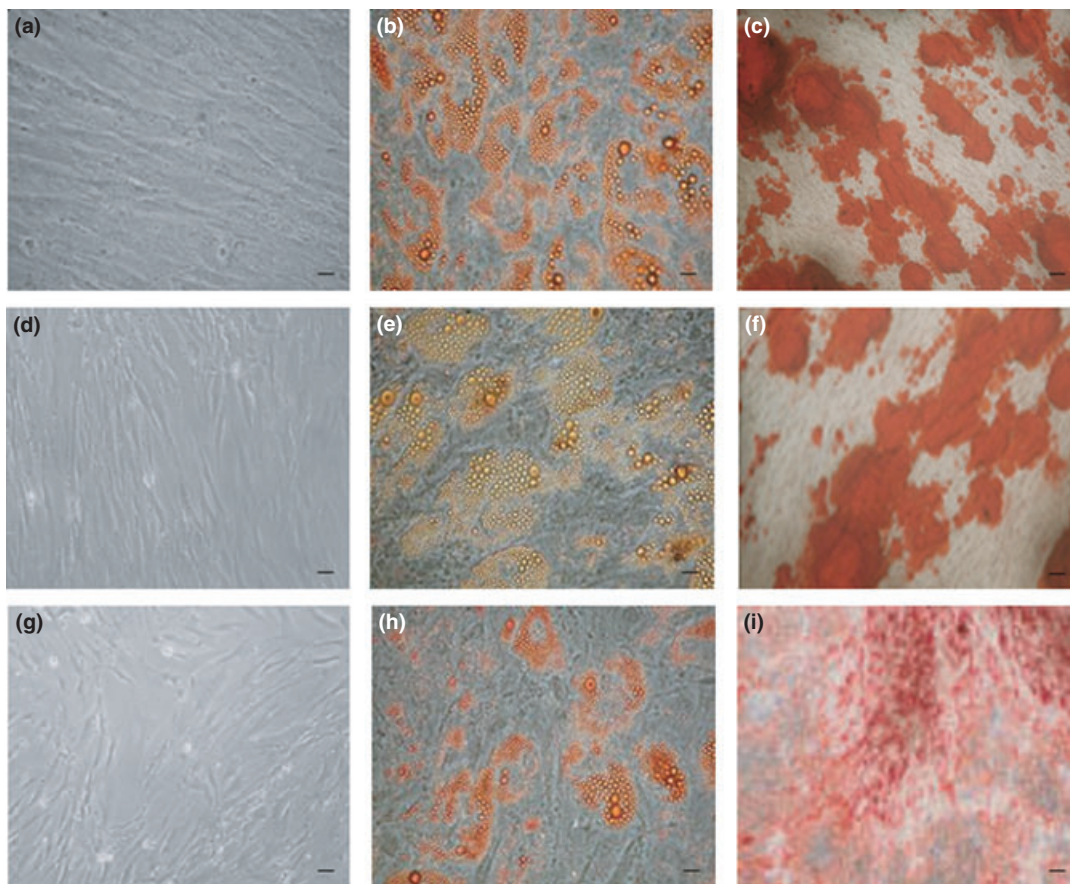
IPG strips were run in ExcelGel SDS 8–18% according to the manufacturer's instructions (GE Healthcare) and ExcelGels were stained with colloidal Coomassie Blue G-250 (Sigma-Aldrich). All gels were scanned on an image scanner using LabScan software (GE Healthcare), analysed and compared using Image Master 2D Platinum 6.0 software (GE Healthcare), followed by additional visual analysis. Gels were analysed and averaged separately. Normalized spot volumes were compared and averaged for all visualized spots in each gel.

After normalization of images, spot volume was calculated to quantify intensity of each spot. Comparison reports of qualitative and quantitative differences between samples were generated, and only spots that showed greater than 2-fold difference between samples, were considered to be differentially expressed.

## Results

### *Differentiation potential of hMSC cultures and maintenance of hMSC multipotency markers*

To determine whether the expanded hMSC cultures maintained multipotency differentiation characteristics, we tested them at passages 1, 5 and 8 for differentiation into adipogenic and osteogenic cells. Our results showed that at all passages, hMSC cultures were able to differentiate into adipogenic and osteogenic cells that maintained their multipotency capacity (Fig. 1).

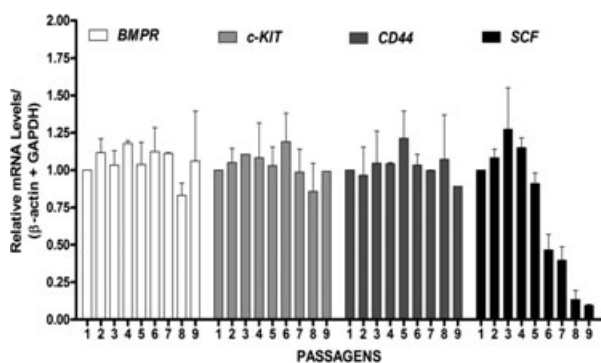


**Figure 1.** hMSC capacity for differentiation. (a), (d) and (g) – undifferentiated hMSCs at passages 1, 5 and 8. (b), (e) and (h) – adipogenic differentiation at passages 1, 5 and 8. Accumulation of neutral lipid vacuoles stained with oil red O indicate differentiation. (c), (f) and (i) – osteogenic differentiation at passages 1, 5 and 8. Calcium deposition stained with alizarin red indicate differentiation. Scale bars - 26  $\mu\text{m}$  (100 $\times$  magnification).

As described in previous studies, a number of genes, including *SCF*, *c-Kit*, *BMPR* and *CD44*, are considered to be hMSCs multipotency markers (26). Although they are not specific to hMSCs, they are expressed in progenitor cells (27–29). To further confirm multipotency during hMSC culture, we quantified expression of these markers during subsequent passages of all hMSC cultures. We performed Q-PCR in nine different cell cultures from passage 1 to 9. Expression of *SCF* mRNA was reduced 2- to 5-fold from passages 5 to 9 in all tested cultures (Fig. 2), although expression of *c-Kit*, *BMPR* and *CD44* was conserved throughout all passages, indicating profile maintenance. *SCF* is a gene related to cell proliferation and not to differentiation (30–32); thus, changes in *SCF* expression suggests that after passage 5, cells had reduced proliferation. These results could also indicate senescence during *in vitro* expansion. Under our culture conditions, typical morphological changes associated with senescence were observed from passage 8 (Fig. 3); cells were enlarged and had irregular shapes (33). These data could explain the delayed growth observed in our experiments. In early passages, cell cultures reached confluence at 7 days; however, after passage 6, confluence was obtained after 12 days here, under the same culture conditions.

#### hMSCs cytogenetic analysis

To verify whether the hMSCs maintained their normal karyotype in culture throughout all passages, the same nine cultures were tested for their genomic stability using conventional cytogenetic analysis. Our results showed



**Figure 2. Maintenance of hMSC markers.** Total RNA was isolated from hMSC cultures at different passages (1 to 9) and examined using Q-PCR to determine changes in mRNA expression levels after normalisation to  $\beta$ -actin and *GAPDH* expression. Analysis of fold changes in expression of *SCF*, *CD44*, *c-Kit* and *BMPR* were performed at the different passages. Data are presented as fold induction relative to control group (passage 1). Mean  $\pm$  SD ( $n = 9$ ). \*Statistical significance was considered at  $P < 0.001$ .

that until passage 4, hMSCs from all donors were of normal diploid karyotype. In subsequent passages, seven of the nine cultures presented random aneuploidy, which is a characteristic of cytogenetic instability (Table 1). However, in most cases, these abnormalities were lost by the next passage. Nevertheless, in one culture, a clonal abnormality was identified and amplified from passage 6 to passage 8 (Table 1, Sample 1).

#### *In vitro* hMSC-mediated T-cell inhibition

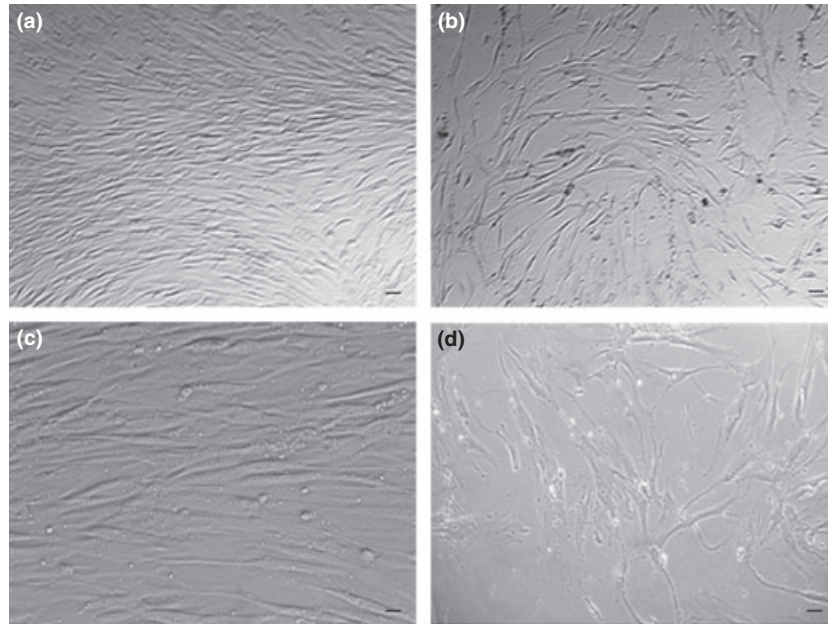
To further determine whether our hMSC cultures maintain their ability to inhibit proliferation of lymphocytes through alloantigen stimulation at different passages, we performed mixed lymphocyte reaction assays (MLR) in the absence or presence of hMSCs. hMSCs from different passages were added to MLR on day 0, and cells were harvested on day 7. Data collected showed that hMSC cultures from the same 9 different donors previously tested for cytogenetics, were capable of inhibiting lymphocyte proliferation by 70%, in response to alloantigen stimulation at different passages (P1 to P9) (Fig. 4). The low peak of data shown in Fig. 4b in early and late passages represents reduced proliferation of the cells as a consequence of hMSC-induced immunosuppression of lymphocyte proliferation. These results confirmed that hMSC cultures, even at subsequent passages, had *in vitro* immunomodulatory properties.

#### Proteomic and transcriptomic profile similarity of hMSCs at different passages

Although hMSCs, even with chromosomal instability, are able to differentiate into various lineages and inhibit lymphocyte proliferation, it is possible that multipotency would not be maintained. Therefore, we tested whether molecular profiles of these cells would be conserved during passaging. For this purpose, we performed comparative expression analysis, using proteomic and transcriptome approaches, using three different hMSC cultures from different donors that showed chromosomal instability at passages 1, 5 and 8. Samples 1, 2 and 5 shown in Table 1 were used in these analyses.

Using proteomic approaches, we compared and analysed the cultures using 2D electrophoresis. One representative gel from one donor culture at passages 1, 5 and 8 is shown in Fig. 5. After image analysis, all gels demonstrated  $195 \pm 3$  spots, a 95% match.

In our previous work, proteins spots were identified, and results demonstrated that hMSCs would have similar protein expression patterns at the first passage (34). Here we observed similar results in all cultures at all passages analysed, suggesting high similarity among



**Figure 3. Morphologic changes indicating senescence.** Using the same confluence stage, representative morphology of hMSCs is shown at early passage (P3) [(a) and (c)] and late passage (P8) [(b) and (d)]. (a) and (b): Scale bars - 65  $\mu\text{m}$  (40 $\times$  magnification). (c) and (d): Scale bars -13  $\mu\text{m}$  (200 $\times$  magnification). Cells were seeded at the same density (2,000 cells/cm<sup>2</sup>), and at P3, the hMSC culture obtained confluence at 7 days, while at P8, confluence was obtained at 12 days.

different ones, as shown in the scatter plot in Fig. 6. These results suggest that protein profile of hMSC cultures derived from different passages and different donors is equivalent, at least based on the results from this approach.

To determine the global pattern of gene expression in hMSC cultures, we performed a transcriptome comparison analysis using an expression chip array assay. For this study, we used the same donors analysed in the proteomic assay at passages 1, 5 and 8. mRNA in hMSCs from these passages was amplified, followed by labelling and hybridization to the Affymetrix GeneChip human exon 1.0 ST array. After normalization and analysis of the microarray data using Partek<sup>®</sup> software (<http://www.partek.com>), we observed alterations in some genes at passage 5. Using a 5-fold change as a cut-off in expression level, we observed 21 and 91 differentially expressed genes at passages 5 and 8, respectively, compared to passage 1 (Tables 2 and 3).

*In silico* analysis of these results using Ingenuity software (NCBI) showed that overexpressed genes at P5 and P8 were associated with the cell cycle, cell expansion and proliferation, cell-to-cell signalling and their interactions, and cell differentiation, among other functions. Tables 4 and 5 describe biological processes and functional interaction network classes, respectively, which are associated with the genes that were over expressed at P5 and P8. Networks associated with genes

upregulated and downregulated at both P5 and P8 are described in Supplementary Figs 1 and 2.

Interestingly, at passage 5, we observed upregulation of genes such as *PRUNE2*, *DLG1*, and *VANGL1*, which are involved in differentiation in *Drosophila melanogaster*. Indeed, at passage 8, we observed over expression of genes, such as *FGF7* and *COL1A1*, indicative of cell differentiation. We also observed upregulation of *ARHGAP29*, which is associated with cell senescence, at both passages 5 and 8.

Together, these findings show that although results of the proteomic analysis indicated that the same protein profile would be maintained through all passages, levels of gene expression revealed that small but important changes occur from passage 5 on.

## Discussion

Clinical application of hMSCs requires use of a significant number of cells and, considering low numbers of hMSCs *in vivo* (35), their extensive *ex vivo* expansion is required. However, such expansion increases the risk of genetic and epigenetic changes. Cell proliferation *in vitro* could result in occurrence of mutations and chromosomal aberrations, eventually leading to malignant transformation. Thus, cytogenetic and molecular studies must be used to determine appropriate hMSCs to use for cell therapy (36, 37).



**Table 1.** Cytogenetic analysis of hMSCs from different donors and passages

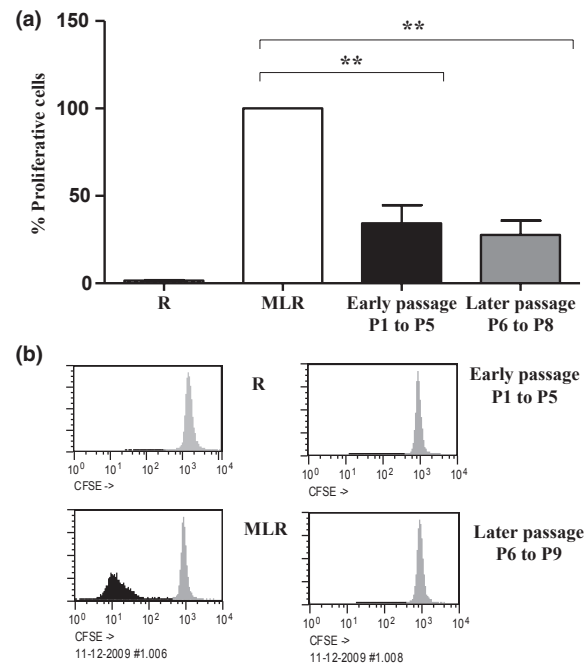
Samples	Culture Passage	Karyotype
1	P1	46,XX[20]
	P3	46,XX[21]
	P5	46,XX[20]
	P6	46,XX[17]/46,X,-X,+16[20] <sup>a</sup> /36~42,XX,-3,-7,-11,-12,-13,-14,-14,-16,-16,-19[1]/5,-8,-11,-14,-20[1]/-4,-7,-10,-13[1]
2	P1	46,XX[20]
	P4	46,XX[22]
3	P1	46,XX[21]
	P4	46,XX[22]
4	P1	46,XX[20]
	P8	46,XX[20]
5	P1	46,XY[23]
	P3	46,XY[25]
	P5	46,XY[20]
	P7	46,XY[20]/42~45,XY,-4,-6,-13,-20[1]/-3,-5,-13,-20[1]/-6,-10,-14,-19[1]/-15,-21[1]/-8,-15[1]/-22[1]
	P9	46,XY[10]/42~45,XY,-4,-6,-9,-15[1]/-8,-13,-18,-21[1]/-9,10,-14,-20[1]/-5,-8,-22[1]/-4,-15[1]/-22[1]
	P1	46,XY[20]
	P3	46,XY[15]
6	P5	46,XY[18]
	P6	45,XY,-21[2]/46,XY[11]
	P8	44,XY,-10,-13[4]/46,XY[7]
	P7	44,XY,-6,-15[2]/46,XY[8]
7	P1	46,XY[20]
	P2	46,XX[18]
	P5	47,XX,+mar[1]/46,XX[7]
	P7	46,XX[6]/45,XX,-4[1]
8	P1	46,XY[10]
	P3	46,XY[15]
	P5	46,XY[8]/43~45,XY,-10,-13,-19[1]/-9,-20,-21[2]/-10[1]
9	P1	46,XX[21]
	P4	46,XX[19]
	P6	46,XX[16]

<sup>a</sup>The bold text indicates the clonal abnormality.

P- Passage.

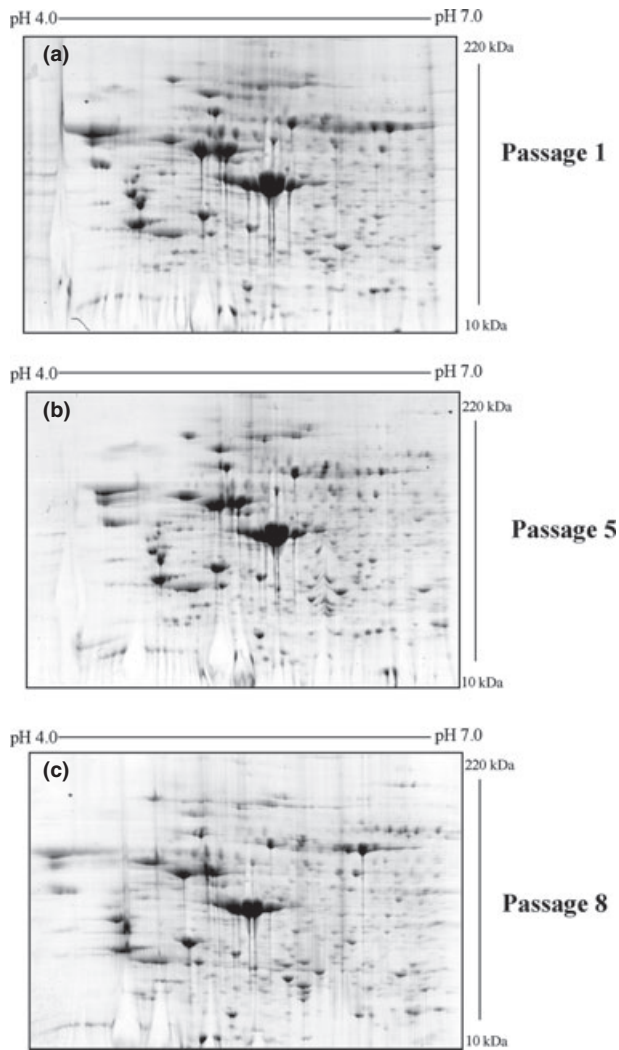
Chromosomes were analysed according to the International System of Human Cytogenetic Nomenclature (ISCN, 2009).

Although it has been shown that hMSCs can be continuously cultured *in vitro*, it is unclear how many passages can be performed before they acquire chromo-



**Figure 4.** hMSC-mediated T cell inhibition. (a) hMSC inhibition of MLR. Lymphocyte proliferation was assessed using MLR in absence (MLR) or presence of 10% hMSCs/well, administered at early (P1 to P5) or late (P6 to P10) passage. Results expressed as mean  $\pm$  sd for independent experiments using 9 hMSC cultures ( $P < 0.01$ ); (b) Representative assay showing MLR in absence (MLR) or presence of 10% MSC/well at early (P1 to P5) or late (P6 to P9) passages. Black represents proliferative cells and grey represents non-proliferative cells. R - responders without stimulators.

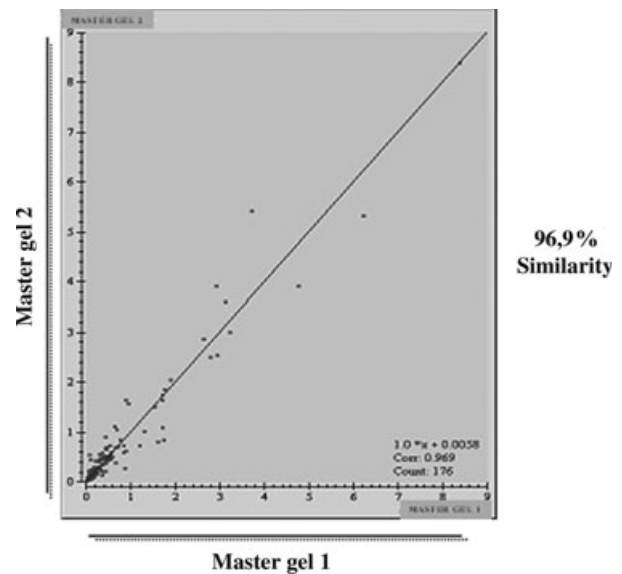
somal instability or lose their multipotency. Using different sources, culture conditions and passage densities, some groups have attempted studies concerning chromosome stability of human MSCs in culture. Zhang and co-workers, using  $10^6$  cells/cm<sup>2</sup> cultured in DMEM/15% FBS (foetal bovine serum) medium, showed that *in vitro* cultured bone marrow MSCs retained normal cytogenetics until passage three, indicating that genetic stability was retained until this passage (12). In addition, Bochkov and co-workers evaluated chromosome variability of hMSCs derived from adipose tissue, cultured in DMEM/F12 10% FCS (foetal calf serum) at  $1.5 \times 10^3$  cells/cm<sup>2</sup> density. This group verified that at early passages (P1 to P5), the cells had normal karyotypes, while at later passage cultures (P6 to P14) they began to show chromosomal aberrations (13). However, Bernado *et al.* revealed that MSCs derived from bone marrow and cultured in Mesencult/10% FCS medium at  $1.6 \times 10^4$  cells/cm<sup>2</sup> density did not undergo spontaneous transformation and thus that these MSCs could be safely expanded *in vitro* without developing chromosomal abnormalities. Moreover, Izadpanah and colleagues



**Figure 5.** hMSC proteomic profile obtained using cells at passages 1, 5 and 8 from the same donor. (a) 2-DE gel - passage 1. (b) 2-DE gel - passage 5. (c) 2-DE gel - passage 8. Values on horizontal axes represent pI, and values on vertical axes represent molecular weight.

verified that hMSC cultures derived from bone marrow and adipose tissue and cultured in  $\alpha$ -MEM /20% FBS medium displayed normal karyotypes up to passage 20 (14,15). Furthermore, Tarte and colleagues showed that occurrence of aneuploidy in bone marrow-derived hMSCs did not persist in culture and was not associated with transformation events (16). Thus, the determinants that define genetic stability remain unknown.

In this study, karyotypic analysis of each culture was performed which showed that different cultures underwent genetic instability after passage 4. Interestingly, most of abnormalities observed using conventional cytogenetic analyses were not clonal, which suggests that the alterations could be lost over subsequent passages. Moreover, during cell therapy, such



**Figure 6.** Bidimensional gel analysis using Image Master 2D Platinum 6.0. Values on horizontal axes represent master gel 1 products from pool of 2-DE gels from passages 1, 5 and 8 from the same donor. Values on vertical axes represent master gel 2 products from pool of 2-DE gels from passages 1, 5 and 8 from the same donor, which were different from those of master gel 1. Scatter plot calculated using results from analysed gels and shows relationship between spot values (% volume), which show 96.9% similarity.

hMSCs would not survive for long in tissues due to their inability to stabilize in this niche; they would, most likely, undergo apoptosis. However, results from our assays showed one culture that acquired clonal abnormalities at passage 6.

To better understand the consequences of this alteration, we decided to further characterize these cells. We analysed their immunomodulatory and molecular profiles, with and without clonal abnormalities, from different passages. Our results showed that hMSC cultures could be cultured long-term *in vitro* without losing their immunomodulatory capacity and multipotency, even when the analysis included samples with karyotypic abnormalities. However, molecular analysis revealed that some changes could be observed along the passages. Such changes were the same in all analysed samples, but were not related to presence of the abnormality.

The proteomes and transcriptomes of mesenchymal stem cells in undifferentiated and differentiated states have previously been characterized, but these studies were not focused on variations observed in extensively expanded cultures (26). Analysis of molecular profiles of our hMSC cultures revealed that they underwent molecular changes from passage 5 on. Although we did not observe differences in their proteomic profiles using 2D analysis, the expression chip array assay (which had



**Table 2.** Genes upregulated in hMSC culture (fold change > 5)

	Gene Symbol	Description	Fold change-P5	Fold change-P8
1	PRUNE2	Prune homolog 2 (Drosophila)	5.34 up	12.05 up
2	EPGN	Epithelial mitogen homolog (mouse)	—	12.47 up
3	C4orf3	Chromosome 4 open reading frame 3	5.91 up	12.50 up
4	SLC7A11	Solute carrier family 7	5.16 up	12.72 up
5	TUBA1B	Tubulin, alpha 1b	49.14 up	17.39 up
6	ACTC1	Actin, alpha, cardiac muscle 1	—	18.17 up
7	CHORDC1	Cysteine and histidine-rich domain (CHORD)-containing 1	17.76 up	28.48 up
8	FAM36A	Family with sequence similarity 36, member A	—	5.00 up
9	MAPRE3	Microtubule-associated protein, RP/EB family, member 3	—	5.00 up
10	UAP1	UDP-N-acetylglucosamine pyrophosphorylase 1	—	5.01 up
11	PREPL	Prolyl endopeptidase-like	—	5.02 up
12	VGLL3	Vestigial-like 3 (Drosophila)	—	5.05 up
13	PGRMC2	Progesterone receptor membrane component 2	—	5.07 up
14	PLD5	Phospholipase D family, member 5	—	5.08 up
15	AMIGO2	Adhesion molecule with IgG-like domain 2	—	5.10 up
16	ANKRD1	Ankyrin repeat domain 1 (cardiac muscle)	—	5.10 up
17	SC4MOL	Sterol-C4-methyl oxidase-like	—	5.10 up
18	SERPINB2	Serpin peptidase inhibitor, clade B (ovalbumin), member	9.07 up	5.10 up
19	FAM198B	Family with sequence similarity 198, member B	—	5.12 up
20	C4orf18	Chromosome 4 open reading frame 18	—	5.16 up
21	BCAP29	B-cell receptor-associated protein 29	—	5.18 up
22	HAPLN1	Hyaluronan and proteoglycan link protein 1	—	5.18 up
23	LYPD6B	LY6/PLAUR domain containing 6B	—	5.26 up
24	MANBA	Mannosidase, beta A, lysosomal	—	5.27 up
25	LMBR1	Limb region 1 homolog (mouse)	—	5.33 up
26	COL8A1	Collagen, type VIII, alpha 1	—	5.34 up
27	ATP11B	ATPase, class VI, type 11B	—	5.35 up
28	FMN2	Formin 2	—	5.39 up
29	PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	—	5.42 up
30	NEK7	NIMA (never in mitosis gene a)-related kinase 7	—	5.45 up
31	TOMM20	Translocase of outer mitochondrial membrane 20 homolog	—	5.50 up
32	SEPT7	Septin 7	—	5.53 up
33	IVNS1ABP	Influenza virus NS1A binding protein	—	5.56 up
34	ITGA6	Integrin, alpha 6	5.05 up	5.58 up
35	ELTD1	Latrophilin and seven transmembrane domain containing	6.08 up	5.63 up
36	HINT3	Histidine triad nucleotide binding protein 3	—	5.64 up
37	OGFRL1	Opioid growth factor receptor-like 1	—	5.64 up
38	SERPINI1	Serpin peptidase inhibitor, clade I (neuroserpin)	—	5.64 up
39	TOR1AIP2	Torsin A interacting protein 2	—	5.66 up
40	PDE11A	Phosphodiesterase 11A	—	5.69 up
41	RAP1B	RAP1B, member of RAS oncogene family	5.23 up	5.71 up
42	FAM174A	Family with sequence similarity 174, member A	—	5.73 up
43	XRCC4	X-ray repair complementing defective repair in Chinese ham	—	5.73 up
44	SYT14	Synaptotagmin XIV	—	5.78 up
45	EVI5	Ecotropic viral integration site 5	—	5.81 up
46	STC1	Stanniocalcin 1	—	5.87 up
47	ME1	Malic enzyme 1, NADP(+)-dependent, cytosolic	—	6.08 up
48	DLG1	Discs, large homolog 1 (Drosophila)	6.23 up	6.11 up
49	SLITRK1	SLIT and NTRK-like family, member 1	—	6.13 up
50	FAM38B	Family with sequence similarity 38, member B	—	6.32 up
51	MCART1	Mitochondrial carrier triple repeat 1	—	6.32 up
52	PCDH10	Protocadherin 10	—	6.44 up
53	PLCB4	Phospholipase C, beta 4	—	6.46 up
54	FLRT2	Fibronectin leucine-rich transmembrane protein 2	—	6.62 up
55	SCN9A	Sodium channel, voltage-gated, type IX, alpha subunit	—	6.66 up
56	ERV3	Endogenous retroviral sequence 3	—	6.74 up
57	SCIN	Scinderin	—	6.99 up

(continued)

**Table 2** (continued)

	Gene Symbol	Description	Fold change-P5	Fold change-P8
58	CD36	CD36 molecule (thrombospondin receptor)	—	7.07 up
59	FGF7	Fibroblast growth factor 7 (keratinocyte growth factor)	—	7.17 up
60	CPE	Carboxypeptidase E	—	7.19 up
61	VANGL1	Vang-like 1 (van Gogh, <i>Drosophila</i> )	7.50 up	7.20 up
62	PVRL3	Poliovirus receptor-related 3	—	7.27 up
63	EPHA5	EPH receptor A5	—	7.39 up
64	HIST1H2BK	Histone cluster 1, H2bk	5.60 up	7.72 up
65	MAMDC2	MAM domain containing 2	—	7.78 up
66	ATRNL1	Attractin-like 1	—	7.89 up
67	COL11A1	Collagen, type XI, alpha 1	—	7.90 up
68	ARHGAP29	Rho GTPase activating protein 29	9.08 up	8.62 up
69	DPP4	Dipeptidyl-peptidase 4	5.14 up	9.62 up
70	ANKRD36B	Ankyrin repeat domain 36B	7.58	—
71	CCDC99	Coiled-coil domain containing 99	5.03 up	—
72	USP53	Ubiquitin-specific peptidase 53	5.19 up	—

The fold change is related to passage 1.

(—) Not differentially expressed.

**Table 3.** Genes downregulated in hMSC culture (fold change > 5)

	Gene Symbol	Description	Fold change-P5	Fold change-P8
1	NRN1	Neuritin 1	—	7.67 down
2	IFNA21	Interferon, alpha 21	—	7.23 down
3	KRTAP13-3	Keratin-associated protein 13-3	—	6.64 down
4	HIST1H4L	Histone cluster 1, H4l	—	6.58 down
5	TRHDE	Thyrotropin-releasing hormone degrading enzyme	—	6.32 down
6	MT1IP	Metallothionein 1I (pseudogene)	—	6.23 down
7	PTGER2	Prostaglandin E receptor 2 (subtype EP2), 53 kDa	—	6.13 down
8	PCDH11Y	Protocadherin 11 Y-linked	6.55 down	6.05 down
9	HEPH	Hephaestin	—	5.99 down
10	OSTBETA	Organic solute transporter beta	—	5.90 down
11	FBLN1	Fibulin 1	—	5.83 down
12	OSTbeta	Organic solute transporter beta	—	5.69 down
13	LCE2D	Late cornified envelope 2D	—	5.36 down
14	HGF	Hepatocyte growth factor	—	5.11 down
15	DPPA3	Developmental pluripotency associated 3	—	5.07 down
16	OR5M3	Olfactory receptor, family 5, subfamily M, member 3	—	5.03 down
17	EIF2S2	Eukaryotic translation initiation factor 2, subunit 2 bet	7.36 down	—
18	IFNA17	Interferon, alpha 17	6.44 down	—
19	MS4A4A	Membrane-spanning 4-domains, subfamily A	6.33 down	—

The fold change is related to passage 1.

(—) Not differentially expressed.

5-fold expression level as cut-off), revealed that 21 genes underwent alterations after passage 5, and numbers of genes differentially expressed increased by passage 8. Analysis using Ingenuity software (IPA) showed that these overexpressed genes were associated with cell growth and proliferation, cell-cell signalling interactions, and cell differentiation, suggesting that they had begun differentiation from passage 5.

Notably, some of the altered genes present in passage 5 are homologous to *Drosophila melanogaster*

development genes, such as *PRUNE2*, which plays an important role in regulating differentiation (38), *DLG1*, which is associated with cell proliferation (39), and *VANGL1*, which is associated with neural differentiation (40). Homology of these genes between insect and mammal suggests that important changes are taking place in these cells.

Development genes play fundamental roles in a range of processes, specially those involving cell differentiation, proliferation and apoptosis. The balance of

**Table 4.** Biological processes<sup>a</sup> including molecular and cellular classes associated with genes overexpressed in P5 and P8

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Cell-to cell signalling and interaction  
 Cell cycle  
 Cell morphology  
 Cellular organization  
 Cellular development  
 Molecular transport  
 Carbohydrate metabolism  
 Lipid metabolism

---

<sup>a</sup>Gene Ontology (GO) terms were used to define the biological processes using the Ingenuity Pathways Analysis (Ingenuity® Systems). Processes listed in the table are those statistically most relevant (with the smallest *P* values).

**Table 5.** Functional interaction networks classes<sup>a</sup> associated with genes overexpressed in P5 and P8

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Cell cycle, cell death  
 Cellular growth and proliferation  
 Cell-to-cell signalling and interaction  
 Gene expression  
 Cellular function and maintenance  
 Cellular compromise  
 Cellular development  
 Molecular transport

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<sup>a</sup>Gene Ontology (GO) terms were used to define the biological processes using the Ingenuity Pathways Analysis (Ingenuity® Systems). The processes listed in the table are the most statistically relevant (with the smallest *P* values).

these processes maintains cell homeostasis and preserves genomic stability. Thus, changes in expression of developmental genes after passage 5 are indicative of alterations in balance of cells of the culture, which could be associated with cell instability. This results from chromosomal anomalies or epigenetic changes that alter gene expression.

While these genes are not classified as oncogenes, their expression could lead to malignant transformation. Changes in expression of *PRUNE2*, *DGLI* and *VANGLI* have previously been associated with specific types of cancer (41–43). Moreover, the hMSC cultures might begin differentiation processes at passage 8, as we observed expression of genes, such as *FGF7*, which play regulatory roles in differentiation of adipocytes (44), and *COL11A1I*, a chondrogenic marker gene (45).

Interestingly, *ARHGAP29* was upregulated in both passages 5 and 8. This gene has recently been associated with senescence as a part of the senescence-associated gene expression signature (37). This finding is consistent with changes in *SCF* expression after passage 5 in hMSC culture that we observed in this study, suggesting reduction in proliferation and potential induction of senescence.

Downregulation of *IFNA21* and *IFNA17* after passage 5 could be associated with secretion. It is well known that hMSCs have immunomodulatory properties. However, this function must be activated by a stimulus, mediated by secreted factors. We used hMSCs from different passages in the chip array assay, but all cells were in an inactive state. Thus, our results do not provide evidence for immunomodulatory capacity of hMSCs. Indeed, altered expression of genes, such as *IFNA21* and *IFNA17*, at passages 5 and 8 could not be associated with immunomodulatory properties, or in presence of stimulus-induced changes in expression of all the genes.

Taken together, the results of this work suggest that hMSC cultures were genetically stable up to passage 4. Until then, not only was it possible to obtain clinical grade expansion for clinical applications, but use of the cells would appear to be a safe and effective tool for cell therapy. However, use of cells from more advanced culture passages would seem to have to be analysed on a case-by-case basis.

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## Supporting Information

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

**Fig. S1.** (a) Networks related to genes downregulated in hMSC culture passage 5 (P5), (b) Networks

related to genes overexpressed in hMSC culture passage 5 (P5)

**Fig. S2.** (a) Networks related to genes downregulated in hMSC culture passage 8 (P8) (b) Networks related to genes overexpressed in hMSC culture passage 8 (P8)