

Research article

HUMAN MESENCHYMAL STEM CELLS EXPRESS NEURONAL MARKERS AFTER OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION

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Abstract: Mesenchymal stem cells (MSCs) are multipotent cells that are able to differentiate into mesodermal lineages (osteogenic, adipogenic, chondrogenic), but also towards non-mesodermal derivatives (e.g. neural cells). Recent *in vitro* studies revealed that, in the absence of any kind of differentiation stimuli, undifferentiated MSCs express neural differentiation markers, but the literature data do not all concur. Considering their promising therapeutic potential for neurodegenerative diseases, it is very important to expand our knowledge about this particular biological property of MSCs. In this study, we confirmed the spontaneous expression of neural markers (neuronal, glial and progenitor markers) by undifferentiated human MSCs (hMSCs) and in particular, we demonstrated that the neuronal markers β III-tubulin and NeuN are expressed by a very high percentage of hMSCs, regardless of the number of culture passages and the culture conditions. Moreover, the neuronal markers β III-tubulin and NeuN are still expressed by hMSCs after *in vitro* osteogenic and adipogenic

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Abbreviations used: AIM – adipogenic induction medium; AMM – adipogenic maintenance medium; BSA – bovine serum albumin; cAMP – cyclic adenosine monophosphate; DMEM – Dulbecco's modified Eagle's medium; FACS – fluorescence-activated cell sorting; FBS – fetal bovine serum; DRG – dorsal root ganglia; FSC – forward light scatter; hMSCs – human mesenchymal stem cells; IBMX – 3-isobutyl-1-methylxanthine; NC – neural crest; NF – neurofilament; NGF – nerve growth factor; NPBM – neural progenitor basal medium; NRSF – neuron restrictive silencer factor; OCN – osteocalcin; OPN – osteopontin; OS – osteogenic; P – passage; SSC – side scatter

differentiation. On the other hand, chondrogenically differentiated hMSCs are negative for these markers. Our findings suggest that the expression of neuronal markers could be common to a wide range of cellular types and not exclusive for neuronal lineages. Therefore, the expression of neuronal markers alone is not sufficient to demonstrate the differentiation of MSCs towards the neuronal phenotype. Functional properties analysis is also required.

Key words: Mesenchymal stem cells, Neural markers, β III-tubulin, NeuN, Osteogenic differentiation, Adipogenic differentiation, Chondrogenic differentiation, Neuronal differentiation

INTRODUCTION

It has been demonstrated that in the absence of any differentiation agent, undifferentiated mesenchymal stem cells (MSCs) are able to express neural markers (neuronal, glial and progenitor markers) [1-6]. This finding has been considered evidence of the predisposition of MSCs to differentiate toward a neuronal lineage, thus opening up the possibility of MSCs being used in transplant therapy to correct neuronal loss in neurodegenerative diseases. Although various neuronal differentiation protocols have been proposed, there has been no complete demonstration of the formation of fully developed and functionally active neuronal cells by MSCs [7]. This raises the question of the real biological meaning of the spontaneous expression of neuronal markers by MSCs. In this study, we carried out an extensive investigation of the expression of the most common neuronal, glial and neural progenitor markers by undifferentiated human MSCs (hMSCs; Table 1) under various conditions, both during early and late culture passages and in the presence or absence of serum in the culture medium. In addition, for the first time in this field of research, we examined the expression of neuronal markers in hMSCs that had differentiated toward osteogenic, adipogenic and chondrogenic lineages in order to assess whether the expression of these markers was specific for neuronal differentiation or common to a wide range of cellular types.

MATERIALS AND METHODS

hMSC isolation and cell culture

hMSCs were prepared from aliquots of heparinized bone marrow obtained in excess from 7 healthy individuals undergoing marrow harvests for allogeneic transplantation at San Gerardo Hospital (Monza, Italy). Donor's agreement was obtained. In order to isolate the hMSCs, mononuclear cells were centrifuged in a Ficoll-Hypaque gradient, suspended in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium) containing 10% defined fetal bovine serum (FBS; Hyclone, Logan, UT) and seeded in culture flasks at a concentration of 2×10^3 cells/cm². At this time point, the cells were considered to be at passage 0 (P0).

hMSC cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. After 48 h the non-adherent cells were removed and the cells attaching to the culture flasks were cultured in DMEM plus 10% defined FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 250 µg/ml fungizone (Lonza, Verviers, Belgium) with a change of medium every 3-4 days. When cultures reached 70-80% confluence, cells were passaged by detaching them using 0.05% trypsin/EDTA (Lonza, Verviers, Belgium) and replating them (1/3) in 75-cm² culture flasks (to give passage 1; P1). After about 2 weeks, cells reached confluence and were detached, increasing the passage number. However, the time necessary for reaching confluence varied depending on the donor. Rat dorsal root ganglia (DRG) primary cultures were performed as previously reported [8].

hMSC immunological characterization

The immunological characterization of hMSCs was performed at early and late culture passages by flow-cytometric analysis using specific antibodies for the membrane antigens CD33, CD34, CD45, CD73, CD90, CD105, HLA-DR and HLA-ABC [9].

hMSC clonal expansion

hMSCs growing and adhering in culture flasks were detached with trypsin and counted by using a Burker's chamber and trypan blue staining. To obtain single cell-derived hMSC clones, the detached cells were serially diluted and plated onto 96-well plates in an expansion medium at a final density of 30 cells per 96-well plate. After 24 h, the cells were observed with an optical microscope and the wells containing one cell were selected and maintained in culture medium for cell expansion. Colonies were expanded and tested for neuronal marker expression in immunofluorescence experiments.

hMSC differentiation

hMSCs were analyzed for their capacity to differentiate towards osteogenic, adipogenic, chondrogenic and neuronal lineages using specific protocols. hMSCs grown in culture medium without any differentiation agent were used as a control.

Osteogenic differentiation. Cells were seeded at approximately 4,000 cells/cm² on dishes in a culture medium composed of DMEM supplemented with 10% defined FBS until subconfluence occurred. After this period, cells were grown in culture medium alone or in osteogenic medium (OS medium) consisting of the same culture medium with the addition of the following supplements (Sigma-Aldrich, St. Louis, MO): 100 nM dexamethasone, 10 mM β-glycerophosphate and 0.05 mM ascorbic-2-phosphate acid. Osteogenic differentiation was evaluated using Alizarin red S staining, which visualizes calcium deposits.

Adipogenic differentiation. Cells were seeded at approximately 20,000 cells/cm² onto dishes containing a culture medium composed of DMEM supplemented with 10% defined FBS. After 24 h, cells were induced by treatment with adipogenic induction medium (AIM), consisting of DMEM (glucose 4.5 g/l) plus 10% defined FBS supplemented with 10 µg/ml insulin, 500 µM isobutylmethylxanthine, 100 µM indomethacin and 1 µM dexamethasone (Sigma-Aldrich, St. Louis, MO). After 12 days of treatment with AIM, hMSCs were treated with adipogenic maintenance medium (AMM) consisting of DMEM (4.5 g/l glucose) plus 10% defined FBS supplemented with 10 µg/ml insulin. Adipogenic differentiation was evaluated by examining the accumulation of lipid vacuoles using Nile Red staining (see immunofluorescence experiments).

Chondrogenic differentiation. Chondrogenic differentiation was induced by growing cells as a pellet in 15-ml tubes, at approximately 250,000 cells/tube, in chondrogenic medium for about 6 weeks. The hMSC chondrogenic medium consisted of serum-free DMEM (4.5 g/l glucose) with the addition of ITS+premix (BD Pharmigen, Germany; 1:100), 1 mM pyruvate (Lonza, Verviers, Belgium), 100 nM dexamethasone, 50 µg/ml ascorbic acid 2-phosphate and 10 ng/ml TGF-β₃ (PeproTech, London, UK). ITS+premix was used as a serum substitute and consisted of 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 µg/ml selenic acid, 5.35 µg/ml linoleic acid and 1.25 µg/ml bovine serum albumin (BSA). Sections of paraffin-embedded pellets were stained with hematoxylin-eosin and Safranin O to evaluate the formation of cartilaginous structures and the presence of proteoglycans and glycosaminoglycans.

Neuronal differentiation. Neuronal differentiation was induced by growing cells at approximately 1,500 cells/cm² onto dishes containing a specific neuronal induction medium as described by Tondreau *et al.* [10]. Briefly, cells were cultured in neural progenitor basal medium (NPBM; Lonza, Verviers, Belgium) supplemented with 5 µM cyclic adenosine monophosphate (cAMP; Sigma-Aldrich, St. Louis, MO), 5 µM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, St. Louis, MO), 2.5 µg/ml insulin (Sigma-Aldrich, St. Louis, MO) and 25 ng/ml nerve growth factor (NGF; Invitrogen, Oregon, USA). Half of the differentiation medium was changed twice a week. After 10 and 21 days of neuronal induction, cells were processed for immunofluorescence experiments.

Immunofluorescence experiments

The expressions of differentiation markers were determined in three different paradigms:

1) hMSCs (P1, P2, P4, P8, P16) were seeded at approximately 10⁴ cells/dish on glass slides in 35-mm diameter dishes using a culture medium composed of DMEM plus 10% defined FBS.

2) hMSCs (P4, P8, P16) were plated onto glass slides in dishes and maintained in DMEM medium plus 10% defined FBS for 24 h. After this time, the medium was replaced with a serum-free one.

3) hMSCs were seeded onto glass slides in dishes and treated for osteogenic or adipogenic differentiation as described above. For chondrogenic differentiation, cells were processed as a pellet (see above).

Immunofluorescence experiments were performed for each passage described above on different days from plating or from the induction of differentiation. Cells were fixed with 4% paraformaldehyde for 10 min, washed with PBS and treated for 10 min with 0.1 M glycine (Sigma-Aldrich, St. Louis, MO) to quench autofluorescence. Then cells were incubated for 1 h at room temperature with a blocking solution (5% BSA, 0.6% Triton X-100 in PBS) and subsequently for 30 min at 37°C with 1 mg/ml RNase (Sigma-Aldrich, St. Louis, MO) in blocking solution. Incubation with the following primary antibodies (diluted in blocking solution) was performed overnight at 4°C: mouse anti-human-nestin (MAB5326, Chemicon, Temecula, CA; 1:50); rabbit anti-human-nestin (AB5922, Chemicon, Temecula, CA; 1:200); anti- β III-tubulin (PRB-435P, Covance, Berkeley, CA; 1:100); anti-NeuN (MAB377, Chemicon, Temecula, CA; 1:50); anti-Neurofilament (M0762, DakoCytomation, Glostrup, Denmark; 1:100); anti-MAP2 (MAB3418, Chemicon, Temecula, CA; 1:100); anti-GFAP (G9269, Sigma-Aldrich, St. Louis, MO; 1:100); anti-S100 (MAB079-1, Chemicon, Temecula, CA; 1:100); mouse anti-osteopontin (sc-21742, Santa Cruz Biotechnology, Inc; 1:100); rabbit anti-osteopontin (ab8448, Abcam, Cambridge, UK; 1:100); anti-osteocalcin (ab13418-50, Abcam, Cambridge, UK; 1:100); and anti-PPAR γ 2 (19481-200, Abcam, Cambridge, UK; 1:500).

The following day, cells were rinsed with washing buffer (PBS plus 0.3% Triton X-100) and incubated at room temperature for 1 h in the dark with appropriate fluorochrome-conjugated secondary antibodies (Alexa Fluor 488, 555, 647 anti-mouse and anti-rabbit; Invitrogen, Oregon, USA; 1:200). The markers used were propidium iodide (Sigma-Aldrich, St. Louis, MO; 2.5 μ g/ml), as a nuclear marker, or Alexa Fluor 546- or 647-conjugated phalloidin (Invitrogen, Oregon, USA; 1:200), as a cytoskeleton filamentous actin marker. To visualize lipid drops, Nile Red staining was used, adding 1-5 μ l of Nile Red stock solution (500 μ g/ml in acetone; Sigma-Aldrich, St. Louis, MO) in 75% of glycerol. After incubation with appropriate fluorochrome-conjugated secondary antibodies, cells were washed with PBS (6 washes of 5 min each) and mounted with polyvinyl alcohol. Microscope analysis was performed with a laser confocal microscope (Radiance 2100; Biorad Laboratories, Hercules, CA, USA). Noise reduction was achieved by Kalman filtering during acquisition.

Cell lysates and immunoblotting analysis

hMSCs were washed twice with ice-cold PBS and total cellular extracts were prepared as previously described [11]. To obtain nuclear protein extracts, the protocol described by Ronca *et al.* was performed [12]. The protein

concentration was determined with the Bradford assay using a Coomassie Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) and aliquots were solubilized in Laemmli buffer 5x, boiled for 5 min, and run onto 13% SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose filters and immunoblotting analysis was performed. Membrane blocking, washing and antibody incubation were carried out according to the manufacturer's instructions. Antibodies against β III-tubulin (1:3000) and NeuN (1:200) were used. Anti-actin (sc-1616, Santa Cruz, Temecula, CA, USA; 1:1000) immunoblotting analysis was performed as a loading control. After incubation with primary antibodies, the membrane was washed and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000): anti-mouse (Chemicon, Temecula, CA) and anti-rabbit (PerkinElmer, Boston, MA). The immunoreactive proteins were visualized using an ECL chemiluminescence system (Amersham, Arlington Heights, IL, USA). DRG neuron and glial cell total protein extracts were prepared as previously described [11].

Flow cytometry analysis

hMSCs were detached by trypsinization, collected in fluorescence-activated cell sorting (FACS) tubes and centrifuged at 500 x g for 5 min. Then, cells were washed with PBS and fixed in 2% paraformaldehyde for 15 min followed by permeabilization with 0.5% saponin (for β III-tubulin staining) or fixed and permeabilized with cold methanol/acetone (3:1) for 30 min (for NeuN). After fixation, cells were washed twice with PBS/BSA and incubated with anti- β III-tubulin (1:100) or anti-NeuN (1:50) for 30 min at 4°C. After incubation with primary antibodies, cells were washed and then incubated with appropriate secondary antibodies for 30 min at room temperature. Negative controls were obtained by incubating cells only with the appropriate secondary antibody without adding the primary one. After incubation, cells were washed twice with PBS and then at least 20,000 events were acquired with a cytometer (BD FACScanto FlowCytometer, BD Biosciences, San Jose, CA, USA) after the establishment of gating windows for forward light scatter (FSC) and side scatter (SSC). Data were analyzed using FACS Diva software.

Statistical analysis

Differences in the number (%) of cells expressing a specific differentiation marker in different passages were analyzed by using one-way analysis of variance (ANOVA). For each marker, an average value of positive cells after 14 days of culture was calculated from the results of at least 4 experiments. Data were expressed as means \pm SD. Comparisons of mean values for the passages were analyzed using Tukey's multiple comparison test. A five per cent probability ($p < 0.05$) was used as the level of significance.

RESULTS

hMSC isolation and culture

According to the criteria established by the International Society for Cellular Therapy [13], the hMSCs isolated from human bone marrow and used in our experiments were:

- a) Plastic-adherent and capable of extensive proliferation when maintained under standard culture conditions;
- b) Positive for the specific antigens CD 73, CD 90, CD 105 and HLA-ABC, and negative for CD33, CD34, CD45 and HLA-DR; and
- c) Able to differentiate into osteogenic, adipogenic and chondrogenic lineages under specific *in vitro* conditions.

Under our culture conditions (culture medium in the presence of serum and in the absence of any differentiation agent), the hMSCs maintained their capacity to actively divide until P14, but for the cells from one donor, this capacity was extended further (P18). From P1 to P10, undifferentiated hMSCs presented a fibroblastic morphology characterized by a spindle shape showing a tendency to become larger in forward passages. From P14 onwards, intracellular granules and cellular detritus were observed.

hMSC genomic stability during the culture period was assessed by monitoring the chromosomal status at several passages *in vitro*. No abnormalities were observed [14]. The hMSC immunophenotype remained unchanged in early and late culture passages.

Expression of mesodermal and neural markers by undifferentiated hMSCs

At different culture passages (P1, P2, P4, P8 and P16) and for each passage at different times (10, 14 and 21 days calculated from the beginning of each passage) we evaluated the ability of the hMSCs to express specific differentiation markers in the presence of serum and in the absence of any differentiation agents. For this purpose, we carried out immunofluorescence experiments to examine the expression of the osteogenic, adipogenic and neural markers listed in Table 1.

Table 1. Markers evaluated in immunofluorescence experiments.

Markers	Type	Localization	References
Nestin	Neural progenitors	Cytoplasmic	[15]
β III-tubulin	Neuronal	Cytoplasmic	[16]
NeuN	Neuronal	Nuclear	[17]
Neurofilament (NF)	Neuronal	Cytoplasmic	[18]
GFAP	Glial	Cytoplasmic	[19]
S100	Glial	Cytoplasmic	[20]
Osteopontin (OPN)	Osteogenic	Cytoplasmic	[21]
Osteocalcin (OCN)	Osteogenic	Cytoplasmic	[22]
PPAR γ 2	Adipogenic	Nuclear	[23]

Table 2. Undifferentiated hMSC expression of differentiation markers at several passages after 14 days of culture. The number of positive cells for each marker is expressed as the percentage \pm SD. - = no positive cells. nsd = no significant differences.

Passage Marker	P1	P2	P4	P8	P16	Significant differences (ANOVA)
Nestin	4.88 \pm 0.43	3.85 \pm 0.44	2.3 \pm 0.68	3 \pm 0.71	0.93 \pm 0.05	p value < 0.001 P1 vs. P4, P16; P2 vs. P16; P8 vs. P16. p value < 0.01 P1 vs. P8; P2 vs. P4. p value < 0.05 P4 vs. P16.
β III- tubulin	90.75 \pm 0.96	90.23 \pm 0.52	90.63 \pm 0.42	91.5 \pm 0.48	90 \pm 0.08	nsd
NeuN	65.5 \pm 4.2	55.5 \pm 4.2	66.25 \pm 4.79	57.25 \pm 2.22	56 \pm 4.55	p value < 0.05 P1 vs. P2, P16; P2 vs. P4; P4 vs. P8, P16.
NF	-	0.43 \pm 0.32	0.12 \pm 0.03	0.22 \pm 0.1	-	p value < 0.01 P1 vs. P2; P2 vs. P16.
GFAP	-	1.23 \pm 0.32	4.75 \pm 2.06	4.13 \pm 2.17	5 \pm 3.16	p value < 0.05 P1 vs. P4, P16.
S100	-	0.75 \pm 0.35	0.6 \pm 0.42	0.9 \pm 0.14	-	p value < 0.01 P1 vs. P2, P8, P16; P8 vs. P16. p value < 0.05 P1 vs. P4; P4 vs. P16.
PPAR γ 2	-	-	7.67 \pm 3.21	3.33 \pm 1.53	0.45 \pm 0.35	p value < 0.001 P1 vs. P4; P2 vs. P4; P4 vs. P16. p value < 0.05 P4 vs. P8.
OPN	-	-	-	-	-	
OC	-	-	-	-	-	

For each marker, the percentage of positive cells was obtained by averaging experimental results using cells from 4 healthy donors. In each experiment, positive cells were counted and averaged in ten randomly chosen microscopic fields.

The data in Table 2 are average values (mean \pm SD) from at least 4 experiments for each marker after 14 days of culture. For each culture passage, the results remained relatively unchanged for the other time points evaluated (10 and 21 days). In all of the experiments, the markers retained their proper cellular localization.

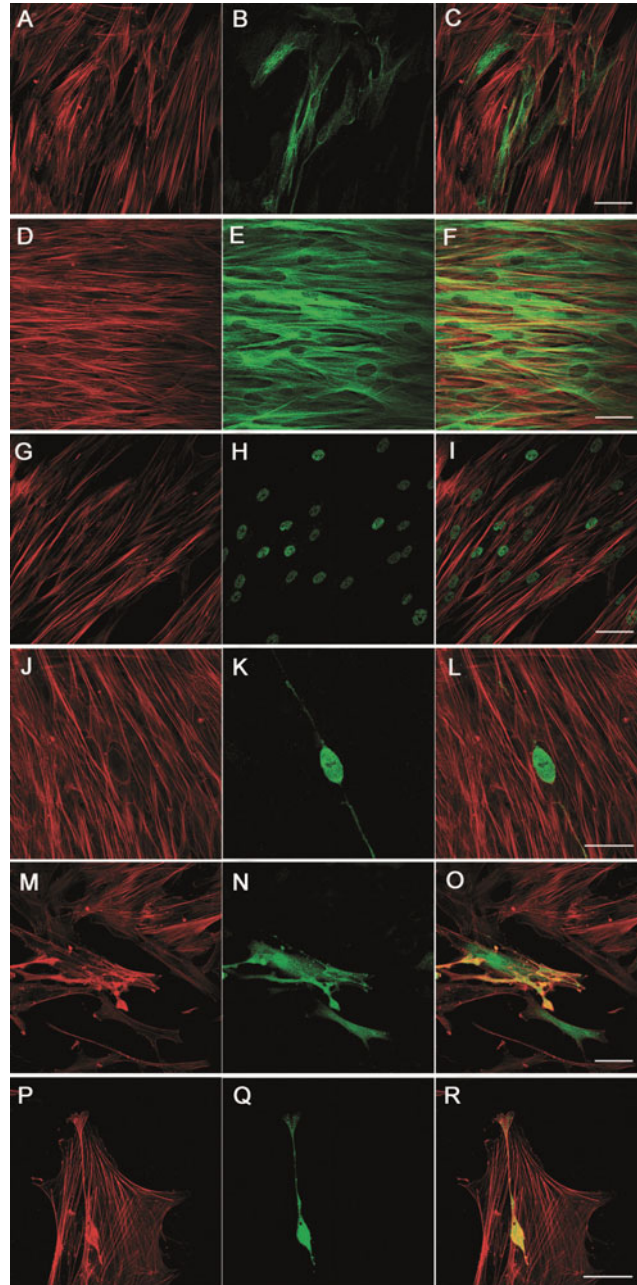


Fig. 1. Spontaneous expression of neural markers by undifferentiated hMSCs (P4) after 14 days of culture. Phalloidin staining labeled the actin filaments in red (A, D, G, J, M, P) and the neural markers were labeled in green. Few cells were nestin positive (B). Most of cells were β III-tubulin positive (E). Numerous cells were NeuN positive with a nuclear localization for the marker (H). Very few cells were positive for NF (K), GFAP (N) and S100 (Q). Merged images: C, F, I, L, O and R. Bars: 50 μ m.

We did not observe variability between hMSCs from different donors with respect to the expression of nestin, β III-tubulin, NeuN, osteopontin (OPN) and osteocalcin (OCN). Neurofilament (NF), GFAP, S100 and PPAR γ 2 expression presented slight variability between donors, but the trend of the expression of these markers was confirmed.

A small number of undifferentiated hMSCs expressed the neuroprogenitor marker nestin (Fig. 1B). The expression of nestin was limited to about 3-5% of cells at P1 and P2, and decreased to 2-3% at P4 and P8, while at P16 it was limited to a very few cells (\leq 1%; Table 2). In general, nestin expression by hMSCs did not extend to the entire cytoplasm, but was often restricted to certain zones.

As shown in Fig. 1, undifferentiated hMSCs expressed early and late neuronal markers. At all passages and times examined, more than 90% of undifferentiated hMSCs that were well-oriented in arranged bundles were positive for β III-tubulin (an early neuronal marker) and showed a characteristic filamentous structure (Fig. 1E), while NeuN (a late neuronal marker) was localized in the nucleus (Fig. 1H) and expressed by about 60% of cells (Table 2). The control was DRG primary cultures containing neurons and glial cells. As shown in Fig. 2, only the neurons were positive for β III-tubulin and NeuN, and only the glial cells were GFAP-positive.

The expression of the late neuronal marker NF was not observed at P1 and P16, while at P2, P4 and P8 it was negligible, limited to less than 1% of cells (Fig. 1K and Table 2). The expression of the glial markers GFAP and S100 was observed in fewer cells than that of the neuronal markers (Fig. 1N and Q). GFAP was not expressed by undifferentiated hMSCs at P1, and from P2 on, its expression was limited to 1-5% of cells (Table 2). GFAP expression was not often equally distributed in the cytoplasm, and zones with different label intensities were observed. Undifferentiated hMSCs were negative for S100 at P1 and P16, while at P2, P4 and P8, its expression was limited to less than 1% of cells (Table 2).

Regarding the expression of mesodermal markers, undifferentiated hMSCs did not express OPN (an early osteogenic marker) or OCN (a late osteogenic marker) at any passage or culture time examined. The adipogenic marker PPAR γ 2 was not expressed at P1 and P2, while at P4 and P8 1-5% of cells were positive, and at P16 its expression was reduced further, becoming limited to a very few cells (\leq 1%; Table 2).

Both the early neuronal marker β III-tubulin and the late neuronal marker NeuN were the most expressed markers by undifferentiated hMSCs from different donors at all passages and times examined. The expression of NeuN and β III-tubulin was also confirmed in hMSCs that were clonally expanded (data not shown). Moreover, double immunolabeling studies revealed that undifferentiated hMSCs that were positive for NeuN also expressed β III-tubulin, while some β III-tubulin positive cells were NeuN negative (Fig. 3G-I). The percentage of

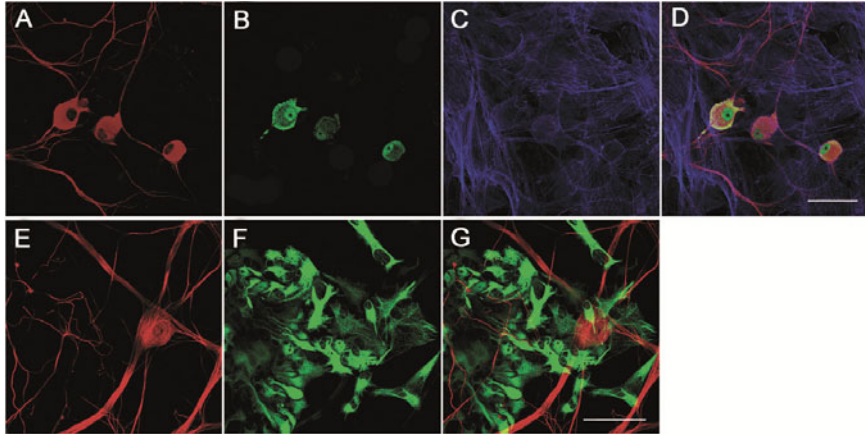


Fig. 2. Control cultures represented by DRG primary cultures containing neurons and glial cells were present. Phalloidin staining labeled actin filaments in blue (C). Only neurons were positive for β III-tubulin (A and E, red) and NeuN (B, green) and only glial cells were GFAP positive (F, green). Merged images: D and G. Bars: 50 μ m.

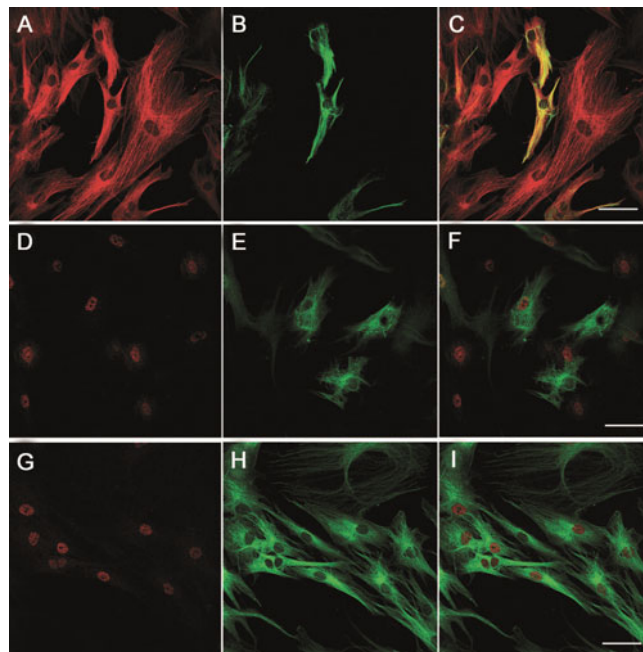


Fig. 3. Spontaneous co-expression of neural markers by undifferentiated hMSCs after 14 days of culture. Most of the cells were β III-tubulin positive (A, red) and the few nestin-positive cells (B, green) were always β III-tubulin positive, as shown in the merged image (C). Numerous cells were NeuN positive (D, red), and the few nestin-positive cells (E, green) were also positive for NeuN as shown in the merged image (F). NeuN-positive cells (G, red) were always β III-tubulin positive (H, green) as shown in the merged image (I). Bars: 50 μ m.

nestin-positive cells was very limited but these cells were always β III-tubulin positive (Fig. 3A-C) and, in some cases, NeuN positive (Fig. 3D-F). We did not observe any nestin-positive hMSCs that co-expressed GFAP or PPAR γ 2.

In the absence of serum, the percentages of hMSCs expressing the neuronal markers β III-tubulin and NeuN were comparable to those observed in hMSCs in the presence of serum. Conversely, the number of nestin-positive hMSCs was reduced further while almost all the cells were positive for GFAP (data not shown).

Evaluation of β III-tubulin and NeuN expression by immunoblotting and flow cytometry

β III-tubulin and NeuN expression in undifferentiated hMSCs were evaluated through immunoblotting analysis. Concerning β III-tubulin, a band corresponding to a predicted 50 kDa molecular weight was evident in all the donors' total extracts (Fig. 4A). Immunoblotting of NeuN (nuclear extracts) showed that all the donors expressed the two major NeuN species at 45-50 kDa and additional reactive bands at \sim 66 kDa and between 70 and 90 kDa, as per the literature data [24] (Fig. 4B). The positive result for the presence of β III-tubulin and NeuN was confirmed by means of flow cytometric analysis, as shown in Fig. 5.

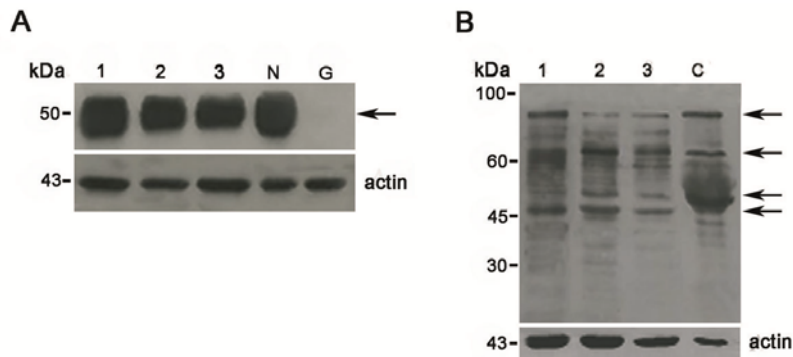


Fig. 4. Undifferentiated hMSC expression of β III-tubulin and NeuN, assessed by immunoblotting. A – Total protein extracts (40 μ g) from undifferentiated hMSCs of three different donors (1, 2, 3) and from a neuron culture (N) and glial cell culture (G) were separated by 13% SDS-PAGE and transferred to a nitrocellulose membrane that was blotted with anti- β III-tubulin antibody. A 50-kDa molecular weight band was evident in all of the donors' total extracts and in the neuron protein extracts, but not in the glial ones. Actin was used as loading control. B – Nuclear protein extracts (12 μ g) from hMSCs of three different donors (1, 2, 3) and DRG culture protein extracts as a control (C) were separated using 13% SDS-PAGE and transferred to a nitrocellulose membrane that was blotted with anti-NeuN antibody. All the donors express the two major NeuN species at 45-50 kDa, additional reactive bands at \sim 66 kDa and between 70 and 90 kDa, with an expression profile similar to the control (C). Actin was used as the loading control.

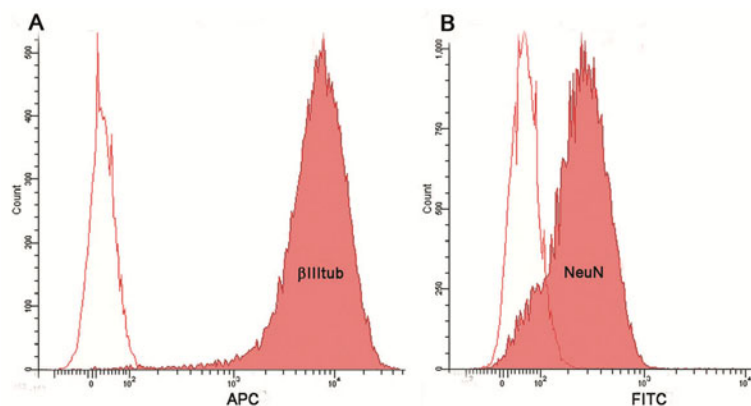


Fig. 5. Undifferentiated hMSC expression of β III-tubulin and NeuN by flow cytometry analysis. A – Histograms of β III-tubulin staining and the negative control represented by undifferentiated hMSCs incubated with the appropriate APC-conjugated secondary antibody without the addition of the primary one (white). B – Histograms of NeuN staining and the negative control represented by hMSCs incubated with the appropriate FITC-conjugated secondary antibody without the addition of the primary one (white). The positive result for the presence of β III-tubulin and NeuN was confirmed by means of flow cytometric analysis.

Expression of neuronal differentiation markers by hMSCs differentiated toward mesodermal lineages

Osteogenic differentiation. The hMSCs induced to osteogenic differentiation formed more aggregates than the control cells. Amorphous material positive for Alizarin red S staining (Fig. 6A) was present in the cultures after 21-28 days of osteogenic induction for one of the donors evaluated and on day 35 for the other donors. Osteogenic-treated hMSCs that were positive for OPN were also positive for β III-tubulin (Fig. 6B and D) and NeuN (Fig. 6E and G). In some cases, OPN was also observed in the extracellular environment. In osteogenic-treated cell cultures, β III-tubulin (Fig. 6C) was characterized by a granular staining and was arranged differently with respect to undifferentiated hMSCs that presented an ordered arrangement with a filamentous β III-tubulin structure (Fig. 1E). The results obtained by immunofluorescence experiments were confirmed by immunoblotting analysis that demonstrated the expression of β III-tubulin and NeuN by osteogenically differentiated hMSC cultures (Fig. 7).

Adipogenic differentiation. Adipogenically differentiated hMSCs lost their fibroblastic morphology, becoming more rounded and being characterized by the presence of Nile Red-positive lipid drops in their cytoplasm, and by a nucleus that was often deformed and positioned in the cell periphery. Nile Red-positive lipid drops were evident in the cytoplasm of hMSCs after 10 days of treatment

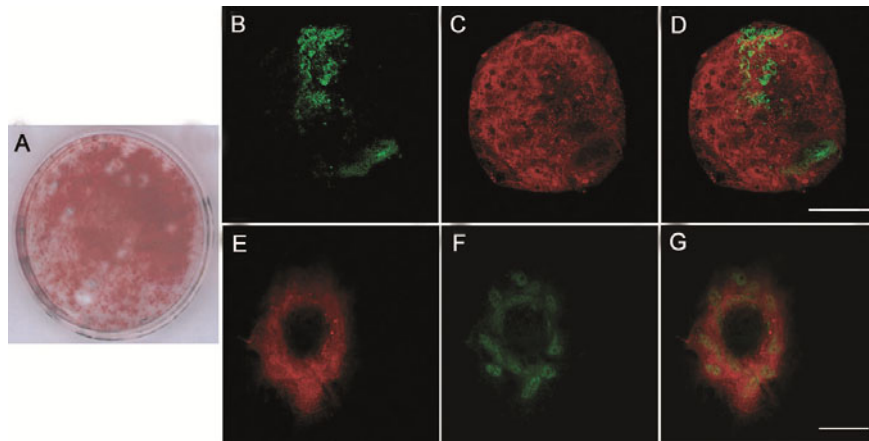


Fig. 6. Expression of neuronal markers by osteogenically differentiated hMSCs. Positive Alizarin red S staining (A). Osteogenic treated hMSCs that were positive for osteopontin (B and E) were also positive for β III-tubulin (C) and NeuN (F) as shown in the merged images (D and G). Bars: 50 μ m.

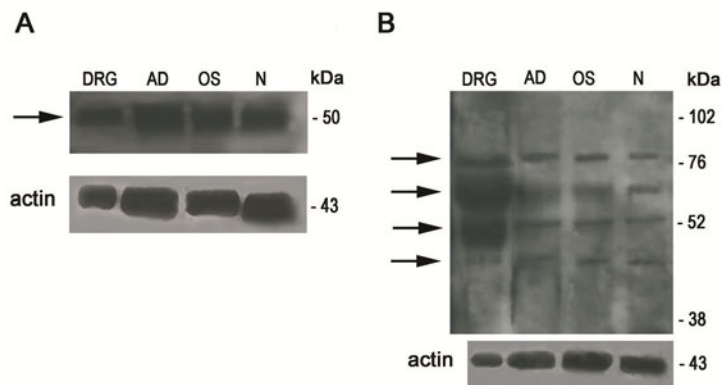


Fig. 7. Differentiated hMSC expression of β III-tubulin and NeuN as detected through immunoblotting. A – Total protein extracts from hMSC cultures treated with adipogenic (AD), osteogenic (OS) and neurogenic (N) induction media were separated by 13% SDS-PAGE and transferred to a nitrocellulose membrane that was blotted with anti- β III-tubulin antibody. DRG protein extracts were used as the positive control. A 50-kDa molecular weight band was evident in all of the differentiated hMSC protein extracts examined, as in the positive control (DRG). Actin was used as a loading control. B – Nuclear protein extracts from DRG culture protein extracts as a control and from hMSC cultures treated with adipogenic (AD), osteogenic (OS) and neurogenic (N) induction media were separated by 13% SDS-PAGE and transferred to a nitrocellulose membrane that was blotted with anti-NeuN antibody. All of the differentiated hMSCs examined express the two major NeuN species at 45-50 kDa, and additional reactive bands at \sim 66 kDa and between 70 and 90 kDa, with an expression profile similar to that of the control. Actin was used as a loading control.

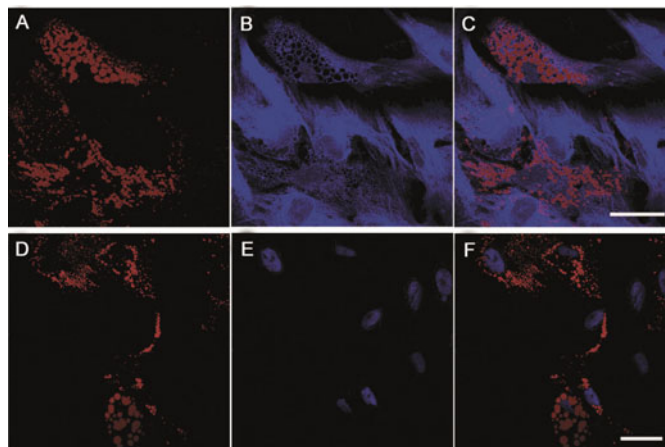


Fig. 8. Expression of neuronal markers by adipogenically differentiated hMSCs. Nile Red-positive hMSCs (A and D, red) were positive for β III-tubulin (B, blue) and NeuN (E, blue) as shown in the merged images (C and F). Bars: 50 μ m.

with adipogenic medium. The number of cells with cytoplasmatic lipid drops (Fig. 8A and D) increased after 14-21 days (to about 20%) and remained constant 35 days post-induction, with the only difference being that the drops became bigger, probably due to a process of drop fusion. hMSCs with fibroblast-like morphology (similar to that of the control cells) were also present in the cell cultures treated with the adipogenic medium. They had no lipid drops and were negative for Nile Red staining.

Furthermore, in adipogenic-treated cultures, cells with adipocytic morphology (with lipid drops) were fluorochrome-conjugated phalloidin negative (phalloidin being a toxin that binds to filamentous actin), while cells with fibroblast-like morphology (without lipid drops) were phalloidin positive. hMSCs cultured in the absence of any differentiation agent (control cells) were also phalloidin positive.

The evaluation of neuronal markers in cell cultures treated with adipogenic medium revealed that hMSCs with adipocytic morphology were β III-tubulin (Fig. 8B) and NeuN (Fig. 8E) positive at all the examined times. In these cells, NeuN maintained its nuclear localization, even if the nucleus was deformed and positioned in the cell periphery (Fig. 8F), while β III-tubulin lost its typical filamentous structure and was characterized by a granular staining in the cytoplasm surrounding lipid drops (Fig. 8C). hMSCs with fibroblast morphology were also positive for both neuronal markers (Fig. 8C and F). The results obtained in immunofluorescence experiments were confirmed by immunoblotting analyses that demonstrated the expression of β III-tubulin and NeuN by adipogenically differentiated hMSC cultures (Fig. 7).

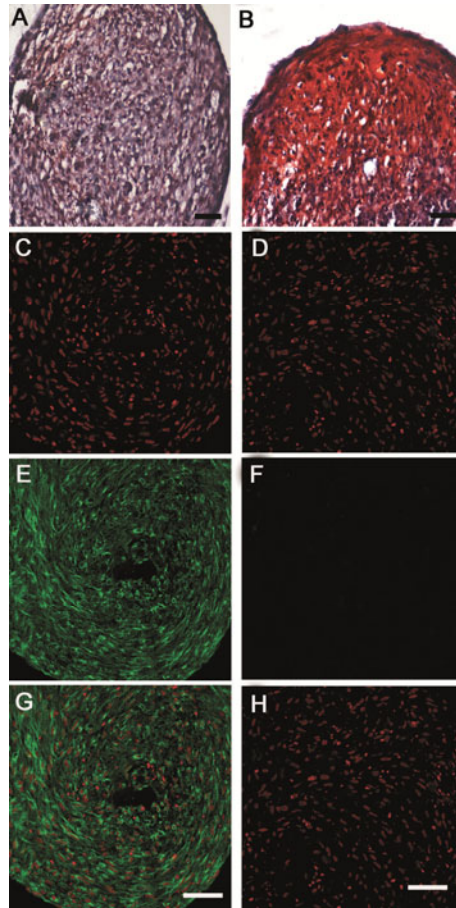


Fig. 9. Expression of neuronal markers by chondrogenically differentiated hMSCs. Paraffin-embedded pellet sections stained with hematoxylin-eosin and Safranin O of undifferentiated (control, A) and chondrogenically differentiated (B) hMSCs. Nuclei of control (C) and chondrogenically differentiated (D) cells were visualized by propidium iodide staining (red). β III-tubulin expression (green) was observed in the control cells (E) but not in the chondrogenically differentiated cells (F). Merged images: G and H. Bars: 50 μ m.

Chondrogenic differentiation. After 3-6 weeks of treatment with the chondrogenic medium, hMSC chondrogenic differentiation had not occurred, as demonstrated by the lack of Safranin O staining of pellet sections. β III-tubulin-positive cells were observed all over the sections and no NeuN-positive cells were present. After 7 weeks of chondrogenic induction, paraffin-embedded pellet sections stained with hematoxylin-eosin and Safranin O showed the presence of large oval or polygonal cellular structures with abundant cytoplasm in a few pellet areas. At 8 weeks post induction, these cellular structures had extended all over the pellet section and were surrounded by a Safranin O-positive matrix (Fig. 9B). Immunofluorescence experiments demonstrated that after 7-8 weeks of treatment

with chondrogenic medium, pellet sections were negative for both β III-tubulin (Fig. 9F) and NeuN. hMSCs were enclosed in small areas comparable to cartilaginous lacunae containing more than one cell, similar to an isogen group of cells derived from a unique progenitor cell. These cellular structures were not evident in the control pellet sections that did not display Safranin O staining (Fig. 9A). The control pellets were β III-tubulin positive cells, observed all over the section (Fig. 9E), whereas the cells were negative for NeuN. The control and chondrogenic-induced pellet sections were negative for phalloidin staining.

hMSC neuronal differentiation

After 10 days of neurogenic induction, most of the hMSCs maintained their fibroblast-like morphology with a neuronal marker expression profile comparable to that of undifferentiated hMSCs. More than 90% were β III-tubulin positive, about 40-50% were NeuN positive, and about 5% were GFAP positive. However, they were negative for the neural progenitor marker nestin and for the late neuronal markers NF and MAP2. The expression of β III-tubulin and NeuN by hMSC cultures treated with neurogenic induction medium for 10 days was confirmed by immunoblotting analysis (Fig. 7).

After 21 days of culture in neurogenic medium, about 15% of the cells were morphologically characterized by a bipolar structure. These cells were β III-tubulin positive (Fig. 10A) and GFAP positive (Fig. 10D), but tested negative for the mature neuronal markers NeuN, NF and MAP2.

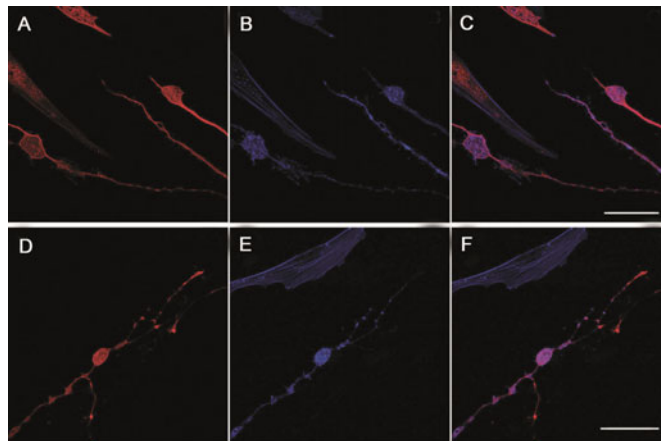


Fig. 10. hMSCs after 21 days of culture in neurogenic medium. Cells that were morphologically characterized by a bipolar structure were β III-tubulin positive (A, red) and GFAP positive (D, red). Phalloidin staining labeled the actin filaments in blue (B and E). Merged images: C and F. Bars: 50 μ m.

DISCUSSION

The reporting of undifferentiated hMSCs spontaneously expressing neuronal, glial and progenitor markers is not novel, but no extensive studies with a panel of markers and culture passages such as ours have been published before. A perusal of our data shows that the early neuronal marker β III-tubulin and the late neuronal marker NeuN, unlike other markers evaluated, are expressed by a very high percentage of hMSCs at all the passages examined, and their expression is serum-independent. Undifferentiated hMSCs expressing β III-tubulin and NeuN do not have a neuron-like cell shape and retain a mesenchymal morphology and immunophenotype, excluding a spontaneous hMSC neuronal differentiation. Furthermore, following treatment of undifferentiated hMSCs expressing β III-tubulin and NeuN with a protocol for neuronal differentiation, cells do not adopt a mature neuronal phenotype or express mature differentiation markers. This does not exclude the possibility that neuronal differentiation occurs using different induction protocols but suggests that the expression of neuronal markers is not by itself indicative of neuronal differentiation.

The spontaneous expression of neuronal markers by undifferentiated MSCs may depend on many factors. Recently, it has been demonstrated that during embryogenesis, MSCs are generated from the neuroepithelium including the neural crest (NC) [25], and multipotent progenitors of NC origin are still present in the adult in some organs and tissues, including the bone marrow [25-27]. However, NC origin concerns only a small MSC subpopulation [25] so the expression of neuronal markers by undifferentiated MSCs should be limited to a low percentage of cells, which was not the case with the spontaneously β III-tubulin and NeuN-positive cells in our cultures. The spontaneous expression of neuronal markers by hMSCs could also be due to the cellular stress caused in response to removing cells from their niche and to their growth in abnormal environmental conditions, as occurs *in vitro* [28]. However, in such a case, not only would β III-tubulin and NeuN be expressed by a high percentage of hMSCs, but so would the other markers evaluated, including the mesodermal ones. Furthermore, serum removal, which causes cellular stress, does not change the number of cells expressing β III-tubulin and NeuN and even induces a decrease in nestin expression. These two hypotheses, NC origin and cellular stress, are not mutually exclusive. An alternative explanation has been proposed [29]. It has been hypothesized that neuronal gene expression in MSCs could be due to the dysregulation of neuron-restrictive silencer factor (NRSF), a transcriptional regulator factor involved in the repression of neuron-specific genes in non-neuronal cells [30]. This hypothesis could account for the spontaneous expression of neuronal markers in hMSCs, but it remains to be demonstrated. Whatever the explanation for the spontaneous expression of neuronal markers by hMSCs, nothing suggests a link between the expression of neuronal markers and the propensity of hMSCs to be committed toward the neuronal lineage.

In this study, for the first time it has been reported that the neuronal markers β III-tubulin and NeuN are expressed in hMSCs after *in vitro* osteogenic and adipogenic differentiation. β III-tubulin and NeuN are considered specific markers for proving neuronal differentiation, and their time-dependent expression is used to monitor the different phases of differentiation [31]. β III-tubulin, a neuron-specific tubulin isotype, is a constituent of neuronal microtubules [16] and it is required in axon growth and guidance and in normal brain development [32]. NeuN is an intrinsic component of the neuronal nuclear matrix that is only expressed in post-mitotic neurons. Recently, NeuN has been identified as Fox3, a member of the RNA-binding protein Fox-1 gene family [33, 34]. The specific role played by these proteins in neurons does not exclude the possibility that they may also perform important functions in mature mesodermal cells. Therefore, in osteogenically and adipocytically differentiated hMSCs, β III-tubulin and NeuN could be involved in the differentiation process, respectively as components of the cytoskeleton and as a regulatory protein. Under the view of a more generalized function of β III-tubulin and NeuN, the persistence of the expression of these markers in hMSCs after osteogenic and adipogenic differentiation becomes conceivable, but again suggests that there is no tight linkage between neuronal marker expression and neuronal differentiation of hMSCs.

Unlike the osteogenically and adipocytically differentiated hMSCs, chondrogenically differentiated hMSCs do not express β III-tubulin and NeuN. This may be simply due to technical issues connected with the different ways in which the cells are processed. Osteogenically and adipogenically differentiated hMSCs are arranged in monolayers, while chondrogenically differentiated hMSCs form pellets, and it is possible that antibodies have accessibility problems in reaching the antigens. However, it is more probable that the absence of the expression of β III-tubulin and NeuN in chondrogenically differentiated hMSCs compared to adipogenically and osteogenically differentiated hMSCs is due to different biological properties of the differentiated cells. There is considerable evidence that suggests a large degree of plasticity and a potential for reciprocal interconversion between osteoblasts and adipocytes [35, 36]. Furthermore, cross-talk between complex signaling pathways regulating osteogenic and adipogenic differentiation programs has been demonstrated [37, 38], and inducers of osteogenic differentiation may inhibit adipogenic differentiation and viceversa [39, 40]. On the contrary, very little evidence has been reported for transdifferentiation of chondrocytes to adipocytes [41].

The finding that hMSCs express both β III-tubulin and NeuN in an undifferentiated state and after osteogenic and adipogenic differentiation suggests that the expression of neuronal markers could be common to a wide range of cellular types and not exclusive of neuronal lineages. Our hypothesis is supported by literature data reporting that neuronal, glial and progenitor markers can be expressed in cells that are other than neural ones. The early neuronal

marker β III-tubulin, abundantly expressed in the central and peripheral nervous system [16], is also present in non-neural tissues, both in embryonic and adult stages [16, 42], in tumor cells [43, 44] and as a component of the mitotic spindles of many cell types [45]. Nestin, considered a major neural progenitor marker [46], has also been found in extra-neural tissues [47] and various types of human solid tumors [48], and has been considered a predictor of poor prognosis in patients with malignant melanoma [49]. Similarly, the glial cell markers S100 and GFAP are expressed in a great variety of normal and pathological tissues [50, 51]. On the other hand, neural cells can express mesodermal-specific markers such as the osteogenic markers osteopontin and osteocalcin and the adipogenic marker PPAR γ [52, 53].

In conclusion, the results of this study appear to warn against using protein expression as the only evidence of MSC differentiation towards a neuronal phenotype. Cellular morphology and functional properties are critical for demonstrating neuronal differentiation of MSCs or other types of stem cells [7].

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