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# Differentiation of Human Adipose Stem Cells into Neural Phenotype by Neuroblastoma- or Olfactory Ensheathing Cells-Conditioned Medium

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Olfactory ensheathing cells (OECs) are known to be capable of continuous neurogenesis throughout lifetime and are a source of multiple trophic factors important in central nervous system regeneration. B104 neuroblastoma cells are recognized to induce differentiation of neural stem cells into oligodendrocyte precursor cells. Therefore, the aim of this study was to verify if conditioned medium (CM) obtained from OECs or B104 cells was capable of inducing differentiation of adipose tissue-derived mesenchymal stem cells (AT-MSCs) to a neuronal phenotype. In order to this goal, immunocytochemical procedures and flow cytometry analysis were used and some neural markers, as nestin, protein gene product 9.5 (PGP 9.5), microtubule-associated protein 2 (MAP2), glial fibrillary acidic protein (GFAP), and neuron cell surface antigen (A2B5) were examined 24 h and 7 days after the treatment. The results showed that both OECs- or B104-CM treated AT-MSCs express markers of progenitor and mature neurons (nestin, PGP 9.5 and MAP2) in time-dependent manner, display morphological features resembling neuronal cells, and result negative for GFAP and A2B5, astrocyte and oligodendrocyte markers, respectively. This study demonstrated that AT-MSCs can be influenced by the environment, indicating that these cells can respond to environmental cues also versus a neuronal phenotype.

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The adipose tissue is very complex and consists of mature adipocytes, precursors of adipocytes, fibroblasts, endothelial cells, lymphocytes, vascular smooth muscle cells. This tissue represents a rich source of multipotent stem cells that can easily be obtained from human adipose tissue isolated from cosmetic liposuctions by non-invasive methods and in larger quantities than bone marrow (Zuk et al., 2002; Rodriguez et al., 2005). Thus, it could be considered promising for cell therapy (Schaffler and Buchler, 2007).

Adipose tissue-derived mesenchymal stem cells (AT-MSCs) show capacity to differentiate into cells of mesenchymal origin, as adipocytes, myocytes, ostocytes, and condrocytes (Lee et al., 2004; Dicker et al., 2005; Musumeci et al., 2011). In addition, they secrete some growth factors, such as insulin-like growth factors 1 (IGF-1), vascular endothelial growth factor (VEGF), and hepatocyte growth factors (HGF) (Wang et al., 2006). AT-MSCs are also able to differentiate into cells of nonmesodermal origin, such as endocrine pancreatic cells, hepatocytes, endothelial cells and cardiomyocytes (Schaffler and Buchler, 2007). Moreover, it was reported that human AT-MSCs are able to develop a neuronal phenotype and result to be positive to glial fibrillary acidic protein (GFAP), nestin, and neuronal nuclei (NeuN) (Safford et al., 2002). In particular, these cells under neuroinductive conditions can express the neuronal differentiation marker type III $\beta$-tubulin (Romanov et al., 2005). Some report showed that fibroblast growth factor (FGF)-2 stimulates the proliferation of AT-SCs (Chiou et al., 2006).

The mammalian olfactory system is one of the few areas of the central nervous system (CNS) capable of continuous neurogenesis throughout lifetime. This peculiarity to regenerate is supported by particular glial cells of the olfactory nerve, called olfactory ensheathing cells (OECs). OECs ensheathe unmyelinated olfactory axons, and share many phenotypic properties with astrocytes and Schwann cells (Ramon-Cueto and Avila, 1998; Mackay-Sim, 2005). Immunocytochemical studies have revealed that OECs are a source of growth factors, including NGF, BDNF, GDNF, NT-3 (Woodhall et al., 2001), CNTF (Wewetzer et al., 2001), and express different markers, such as GFAP, p75 NGFr, vimentin, laminin, S-100 protein, and N-CAM (Pellitteri et al., 2010). The finding that OECs are a source of multiple trophic factors

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became very important as they play a decisive role in CNS regeneration.

Several studies have demonstrated that OECs-CM can promote growth and proliferation of neuronal cells (Pellitteri et al., 2009; Yu et al., 2010). Feng et al. (2008) have reported that OECs-CM protect PC12 cells against the early and late apoptosis induced by 6-hydroxydopamine (6-OHDA). In addition, floating neurospheres including multipotent neural stem cells, isolated from various mammalian CNS regions, can differentiate to cells with peripheral glia phenotype, as judged by their expression of mRNA and protein markers, such as GFAP, vimentin, nestin, and S100 proteins (Doncel-Perez et al., 2009). A recent paper has showed that OECs-CM can induce NSCs to form neurons, and electrophysiological characterization demonstrated that the derived neurons presented active electrophysiological properties, which are essential for nervous excitation (Duan et al., 2011).

Some studies described that CM obtained from B104 neuroblastoma cells (B104-CM) provides to obtain oligodendrocyte precursor cells from neural stem cells (Zhang et al., 1998). Moreover, Fu et al. (2005) have showed that neural stem cells derived from the embryonic rat spinal cord could be induced to differentiate into oligodendrocyte precursor cells in the presence of B104-CM.

Considering as described, in this work, we studied the effect of B104- or OECs-CM on cultured adipose tissue-derived mesenchymal stem cells (AT-MSCs). The final aim was to verify if these media were capable of inducing differentiation of AT-MSCs to a neuronal phenotype. In order to, immunocytochemical procedures and flow cytometry analysis were used.

## Materials and Methods

### Patients

Adipose tissue was gathered from 10 donors, 5 men, and 5 women (from 22 to 30 years of age and mean body mass index of $27 \pm 3.8$) undergoing abdominal liposuction procedures. Lipoaspirates were obtained under an approved Institutional Review Board protocol and after informed consent had been obtained from the patients at the Cannizzaro Hospital, Catania (Italy). The patients were not smokers and occasionally taking non-steroidal anti-inflammatory drugs (NSAIDs). The women did not take estrogens replacement therapy.

### Cultures of human MSCs from adipose tissue

As previously described (Musumeci et al., 2011) the raw lipoaspirate (50–100 ml) was washed with sterile phosphate-buffered saline (PBS; Invitrogen, Milan, Italy) to remove red blood cells and debris, and incubated for 3 h at 37°C with an equal volume of serum-free Dulbecco's modified Eagle's medium (DMEM)-low glucose (DMEM-1g; PAA Laboratories, Pasching, Austria) containing 0.075% of type I collagenase (Invitrogen). Collagenase activity was then inactivated by an equal volume of DMEM-1g containing 10% of heat-inactivated fetal bovine serum (FBS; Invitrogen). Successively, the digested lipoaspirate was centrifuged at 1,200 rpm for 10 min. The pellets were re-suspended in PBS (plus penicillin/streptomycin 1%) and filtered through a 100-mm nylon cell strainer (Falcon BD Biosciences, Milan, Italy). The filtered cells were again centrifuged at 1,200 rpm for 10 min, plated in T-75 culture flasks (Falcon BD Biosciences) with DMEM-1g (10% FBS, penicillin/streptomycin 1%) containing 1% of MSC growth medium (MSCGS; ScienCell Research Laboratories, Milan, Italy) and incubated at 37°C with 5% $CO_2$. Twenty-four hours after the initial plating, non-adherent cells were removed by intensely washing the plates.

### Determination of MSCs markers

In order to identify MSCs derived from lipoaspirate, immunocytochemical procedures were carried out using several cell surface markers. Particular care was taken to distinguish MSCs type from hematopoietic stem cells.

After reaching confluence (80% of total flask surface), all subpopulations were trypsinized (Sigma–Aldrich, Milan, Italy) and subcultured in 12-well culture dishes for 2 days. For immunocytochemistry, cells were first washed with PBS, then fixed with 4% paraformaldehyde (PFA) in PBS for 30 min and incubated for 30 min with a 5% solution of normal goat serum (NGS; Sigma–Aldrich). They were subsequently incubated overnight at 4°C with primary antibodies (Millipore, Milan, Italy): CD44, 1:200 dilution; CD90, 1:100; CD105, 1:100; CD14, 1:200; CD34, 1:200; CD45, 1:200. The following day, cells were washed with PBS and incubated for 1 h at room temperature with secondary antibody conjugated to different fluorochromes. They were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or Cyanine (Cy)3-conjugated goat anti-mouse secondary antibodies (Millipore). As a control, the specificity of immunostaining was verified by omitting the primary or secondary antibody. As a rule, cell nuclei were counterstained with DAPI for 10 min.

Digital images were acquired using a Leica DMRB fluorescence microscope (Leica Microsystems Srl, Milan, Italy) equipped with a computer-assisted Nikon digital camera (Nital SpA, Turin, Italy). The excitation wavelength was 554 nm for Cy3, 488 nm for FITC and 350 nm for DAPI. Immunostaining was evaluated taking into account the signal-to-noise ratio of immunofluorescence.

### OECs cultures and preparation of OECs-CM

As previously described (Pellitteri et al., 2009), OECs were isolated from 2-day old rat pups (P2) olfactory bulbs. Briefly, pups were decapitated and the bulbs were removed and dissected out in cold (4°C) Leibowitz L-15 medium (Sigma–Aldrich). Subsequently, they were digested in Medium Essential Medium-H (MEM-H, Sigma–Aldrich) containing collagenase and trypsin. Trypsinization was stopped by adding DMEM supplemented with 10% fetal bovine serum (FBS, Sigma–Aldrich). Cells were resuspended, planted in flasks and fed with fresh complete medium DMEM supplemented with 10% FBS. The antimitotic agent, cytosine arabinoside ($10^{-5}$ M), was added 24 h after initial plating to reduce the number of dividing fibroblasts. A method to further purify the samples was adopted following the method by Chuah and Teague (1999): OECs cultures were processed to an additional step, transferring cells from one flask to a new one. This step reduces contaminating cells, because they adhere more readily to plastic than OECs. In the last passage, OECs were plated on 25 $cm^2$ flasks and cultured in DMEM/FBS supplemented with bovine pituitary extract. Cells were incubated at 37°C in fresh complete medium and were fed twice a week. OECs were characterized by immunocytochemistry using S-100 as a marker (Pellitteri et al., 2009).

OECs were cultured to confluence and the medium was collected after approximately 24–48 h. OECs-CM was filtrated (0.45 $\mu$m filter) to remove detached cells, aliquoted, and stored at −20°C until further use.

### B104 cultures and preparation of B104-CM

The B104 neuroblastoma cell line was maintained in logarithmic phase of growth in DMEM supplemented with 10% heat-inactivated FBS, 2 mM of glutamine and 100 $\mu$g/ml penicillin/streptomycin. For B104-CM preparation, B104 cultures (density: 100–150 cells/$mm^2$) were washed twice in PBS and incubated in serum-free DMEM containing 1× N1 supplement (N6530; Sigma–Aldrich), 2 mM of glutamine, and 100 $\mu$g/ml penicillin/streptomycin. After 3 days, the medium was collected, filtered (0.45 $\mu$m), aliquoted, and stored at −80°C.

### MSCs treatments

After reaching confluence (80% of total flask surface), all subpopulations were trypsinized (Trypsin-EDTA; Sigma–Aldrich) and subcultured in six multi-well plates for 24 h. The medium was removed and replaced with OECs-CM and/or B104-CM. Some wells were used as control and incubated with DMEM supplemented with 10% FBS. Cells were then incubated for 1 and 7 days. Some samples were used for immunocytochemistry and others for flow cytometry.

### Effects of OECs-CM or B104-CM treatment

To order to verify if OECs-CM or B104-CM was capable of inducing differentiation of AT-MSCs to a neuronal or glial phenotype, some specific markers, such as nestin, protein gene product 9.5 (PGP 9.5), microtubule-associated protein 2 (MAP2), GFAP and neuron cell surface antigen (A2B5) were examined by immunostaining and flow cytometry 24 h and 7 days after the treatment.

Immunocytochemical staining was carried following the same procedures described above. Primary antibodies used were anti-nestin (1:600; Abcam, Prodotti Gianni, Milan, Italy), -PGP 9.5 (1:600; AbD Serotec, Space Import-Export srl, Milan, Italy), -MAP2 (1:200; Covance, c/o Regus Business Centre, Rome, Italy), -GFAP (1:200; Abcam, Prodotti Gianni), and -A2B5 (1:5, Abcam, Prodotti Gianni) overnight. The following day, cells were washed with PBS and incubated for 1 h at room temperature with secondary antibody conjugated to different fluorochromes. They were FITC-conjugated goat anti-rabbit or Cy3-conjugated goat anti-mouse secondary antibodies (Millipore). As a control, the specificity of immunostaining was verified by omitting the primary or secondary antibody. Digital images were acquired using a Leica DMRB fluorescence microscope (Leica Microsystems Srl) equipped with a computer-assisted Nikon digital camera (Nital SpA).

For flow cytometry, cells were trypsinized, fixed with 2% PFA for 20 min at 4°C and permeabilized with 1× Triton (Sigma–Aldrich) for 5 min at 4°C. Afterward, cells were washed once with PBS/BSA 1% and incubated with anti-nestin (1:600; Abcam), -PGP 9.5 (1:600; AbD Serotech), MAP2 (1:200; Covance), -GFAP (1:200; Abcam), and -A2B5 (1:5; Abcam) antibodies for 60 min at room temperature. Cells were then washed once with PBS/BSA 1% and incubated with goat anti-mouse or goat anti-rabbit conjugated with FITC (1:200; Millipore) for 60 min at room temperature in the dark. Samples were analyzed using a Coulter Epics Elite ESP flow cytometer (Coulter, Miami, FL). A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at $\lambda = 488$ nm and fluorescence was monitored at $\lambda = 525$ nm. Fluorescence was detected using logarithmic amplification. Mean fluorescence intensity (MFI) values were calculated and recorded automatically.

### Statistical analysis

Each experiment was repeated at least three times in triplicate and the mean ± SEM for each value was calculated. Statistical analysis of results [Student's *t*-test for paired and unpaired data; variance analysis (ANOVA)] was performed using the statistical software package SYSTAT, version 11 (Systat Inc., Evanston IL). A difference was considered significant at $P < 0.05$.

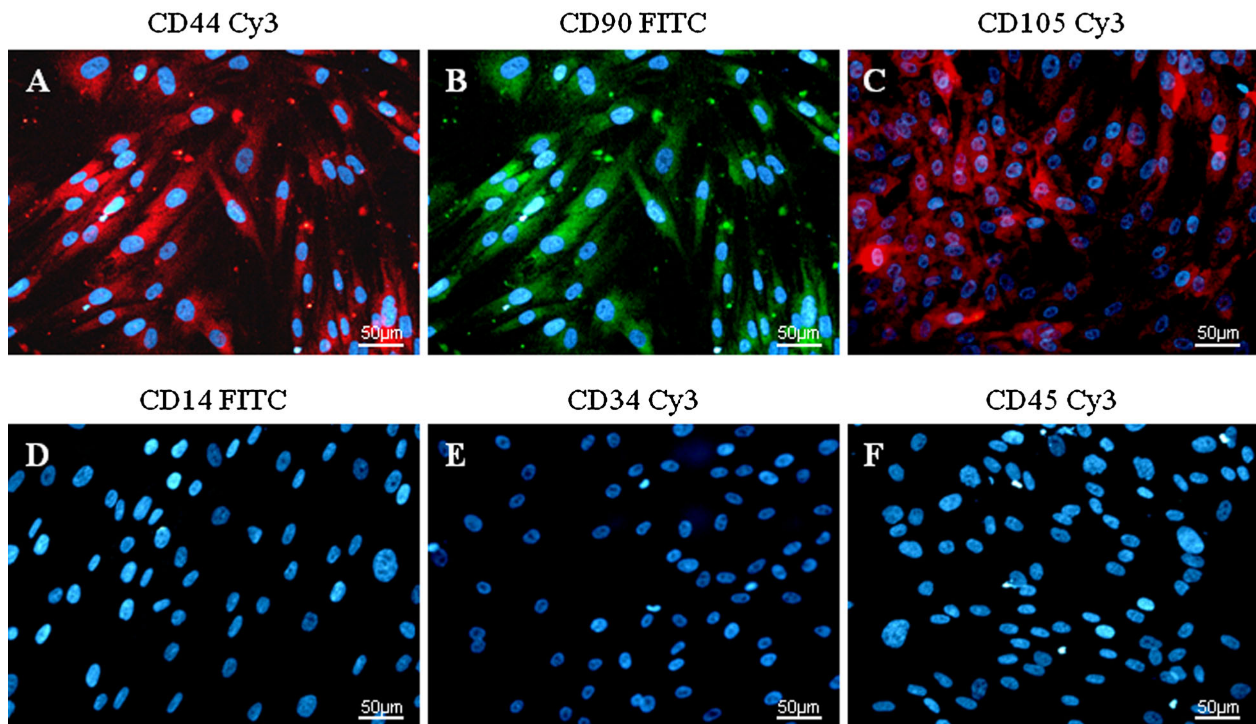


**Fig. 1. Immunophenotypical characterization: CD44 (A), CD90 (B), CD105 (C) (positive markers), CD14 (D), CD34 (E), and CD45 (F) (negative markers) mesenchymal stem cells (MSCs) markers expression by immunocytochemistry and using a Leica DMRB fluorescence microscope equipped with a computer assisted Nikon digital camera. Panels AF: Magnification 40×; Scale bars: 50 μm. DAPI stained MSCs were superimposed to show cell nuclei.**

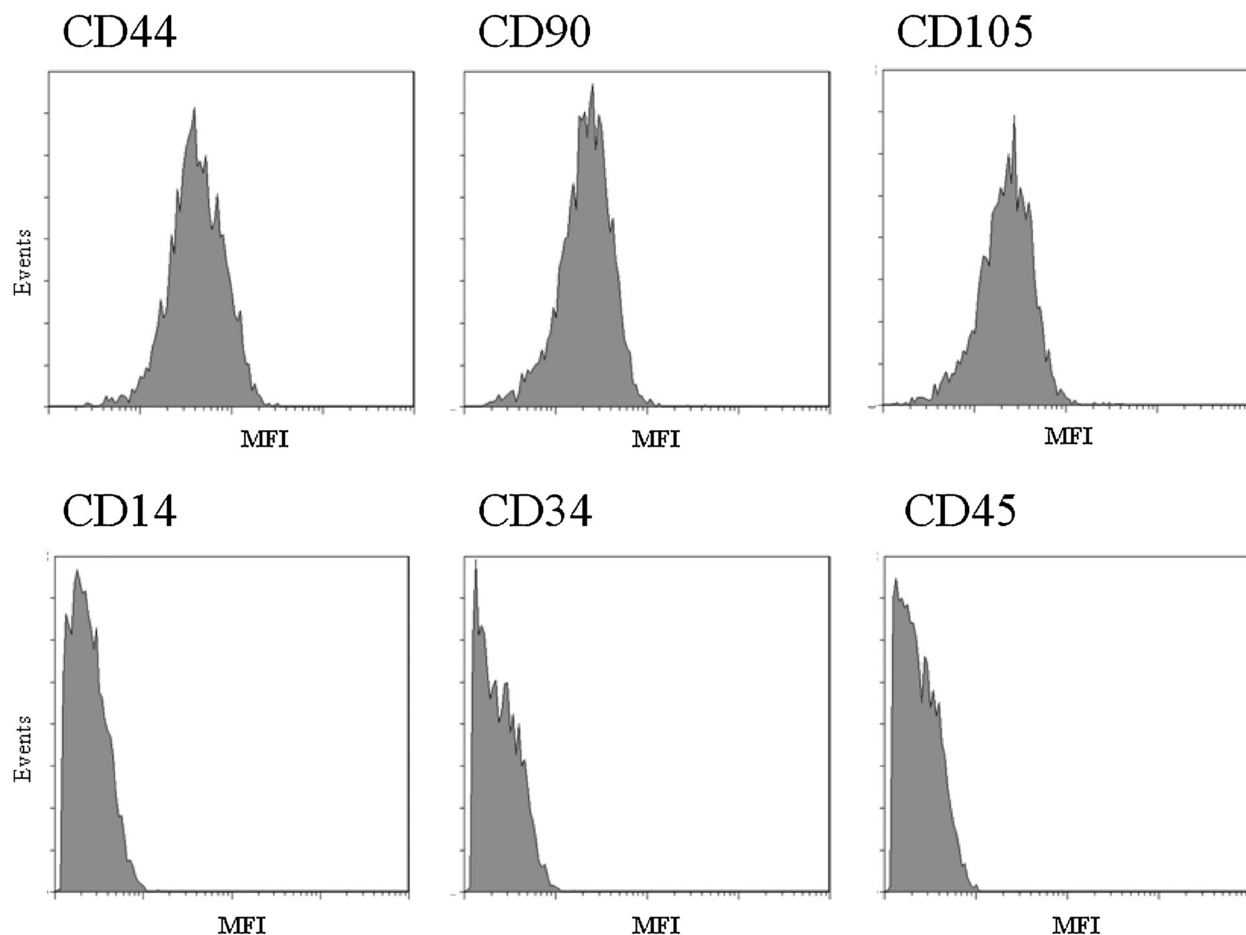


**Fig. 2. CD44, CD90, CD105 (positive markers), CD14, CD34, and CD45 (negative markers) mesenchymal stem cells (MSCs) markers expression by flow cytometry. A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at $\lambda = 488$ nm and fluorescence was monitored at $\lambda = 525$ nm. Fluorescence was detected using logarithmic amplification. MFI values were calculated and recorded automatically.**

## Results

### Identification of MSCs markers

In order to identify MSCs derived from lipoaspirate, immunocytochemical and flow cytometry procedures were carried out using several cell surface markers. Immunostaining demonstrated that the cells belonging to the isolated and plated population were positive for MSCs markers. In the immunocytochemistry analysis performed after the first passage, MSCs did not show labeling for the hematopoietic line, tested by antibodies for CD14, CD34, and CD45. On the contrary, they were positive for the adhesion molecules specific for MSCs, such as CD44 (H-CAM), CD90 (Thy 1), and CD105 (Endoglin) (Fig. 1).

The results obtained by flow cytometry confirmed that cells were positive for CD44, CD90, and CD105, and negative for CD14, CD34, and CD45 (Fig. 2).

### Effects of conditioned media

Cell morphology of control, OECs-CM or B104-CM treated AT-MSCs was analyzed and some markers indicating their potential differentiation in neurons or glial cells were tested by immunostaining and flow cytometry. In particular, the expression of nestin, protein gene product 9.5 (PGP 9.5), microtubule-associated protein 2 (MAP2), GFAP, and neuron cell surface antigen (A2B5) induced by OECs-CM or B104-CM was examined at 24 h and 7 days of culture.

### Cells morphology

The morphology of control cells appeared quite similar at 24 h and 7 days, whereas marked differences were observed using conditioned media and at longer period of culture (Fig. 3A,B, respectively). Data indicating smallest size and lowest complexity, close to the origin of diagrams, were likely to be referred to debris and were not considered.

At 24 h, MSCs cultured in control medium demonstrated typical stromal cell morphology with a characteristic spindle-like shape with elongated projections. At this time, control cells appeared of large dimension and not very complex in shape, while the cells treated with OECs-CM were of smaller size, more elongated and complex. Cells treated with B104-CM were similar to the control (Fig. 3A).

Following 7 days, the number of control AT-MSCs cells was increased, but their morphology was similar to cells at 24 h. At

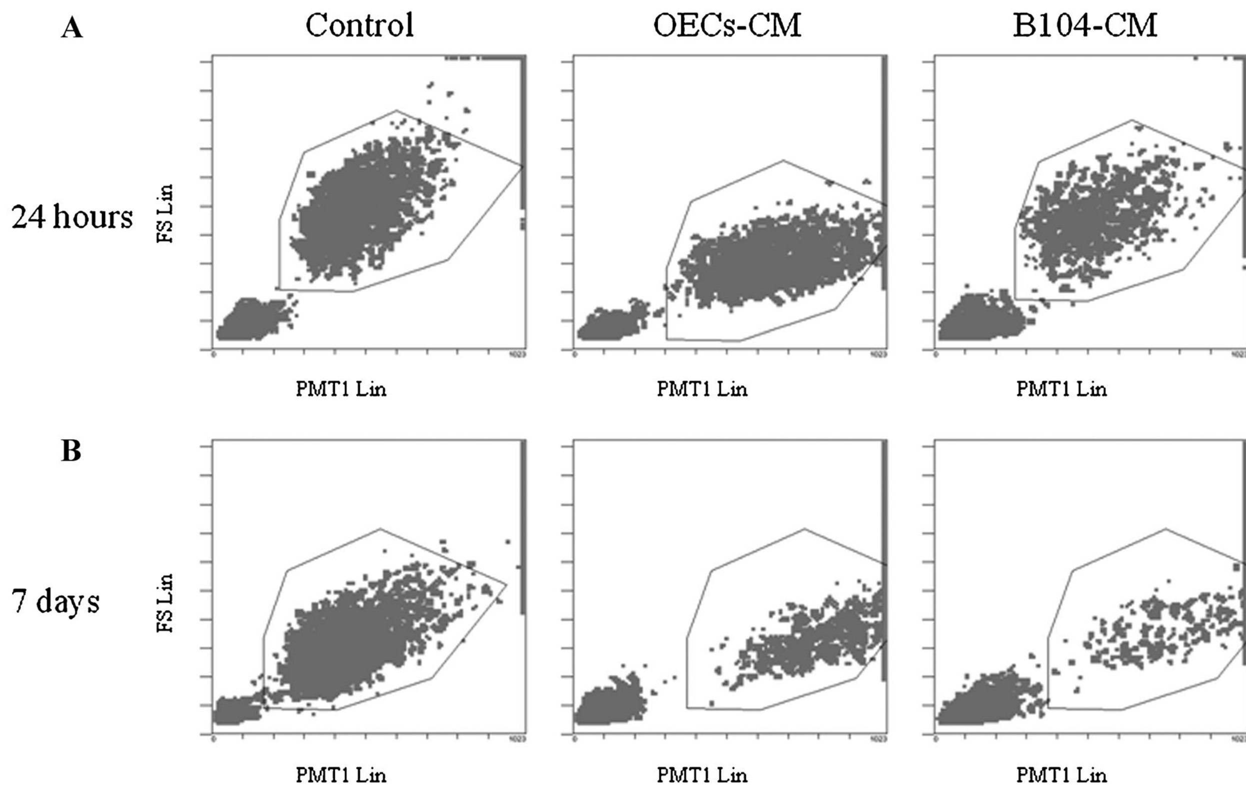


**Fig. 3. Cell morphology analysis of control and OECs-CM or B104-CM treated adipose tissue (AT)-MSCs by flow cytometry 24 h (A) and 7 days (B) after the treatment.**

7 days the morphology of cells treated with either OEC-CM or B104-CM were of smaller size and with greater complexity. A reduction of number of cells was also detected (Fig. 3B). Exposition to OECs-CM or B104-CM induced marked differences. Cells were significantly fewer and smaller, and feature a much higher complexity.

### Immunostaining

At 24 h, MSCs cultured in control medium were clearly immunopositive for nestin and PGP 9.5, at a more low level for MAP2, but very low fluorescence was detected for GFAP and A2B5 (Fig. 4). Compared to control MSCs, cells cultured in OECs-CM or B104-CM appeared more positive for nestin and PGP 9.5, more weakly stained for MAP2 and very few cells were immunopositive for GFAP and A2B5 (Fig. 4).

Following 7 days, exposition to OECs-CM or B104-CM induced marked differences (Fig. 5). Immunostaining for all markers, especially for PGP 9.5, was more pronounced with respect to controls and to correspondent cultures at 24 h.

### Flow cytometry analysis

On the whole, data obtained by flow cytometry were in keeping with observations at the light microscope for immunostaining experiments.

The expression of experimental markers was evaluated by MFI and percentage of positive cells. Results obtained for the different markers, using different media at each time point were summarized in Figs. 6–10 and Table 1.

#### Nestin

At 24 h, percentage of positive cells for nestin was considerably high either in control cultures or using conditioned media (above 90%). It was still high at 7 days. MFI was similar at 24 h in all cultures with values slightly higher in those OECs-CM or B104-CM treated, and increased at 7 days in all samples, especially in cells treated with OECs-CM or B104-CM.

#### PGP 9.5

Percentage of positive cells for PGP 9.5 was invariably high (above 97%) at any time and in all samples, either in controls or in conditioned media. MFI at 24 h was quite strong, particularly in OECs-CM and B104-CM cultures. At 7 days, it increased in B104-CM and markedly in OECs-CM cells, whereas in controls it showed values similar to 24 h.

#### MAP2

Percentage of positive cells for MAP2 was very high at 24 h (about 95%) in all samples. MFI at 24 h showed comparable values in all cultures. At 7 days, a marked increase was observed particularly for OECs-CM cultures, but in any case, signal was higher for both conditioned cultures.

#### GFAP

Percentage of positive cells for GFAP was high at 24 h (about 65%) in control cultures and in cells treated with B104-CM, but

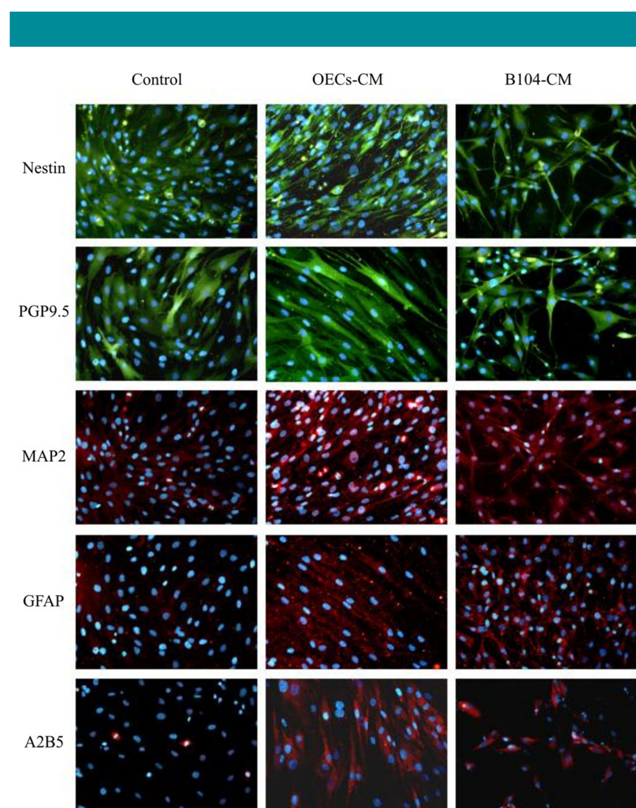


**Fig. 4. Neuronal markers expression by immunocytochemistry of control and OECs-CM or B104-CM treated AT-MSCs 24 h after the treatment.**

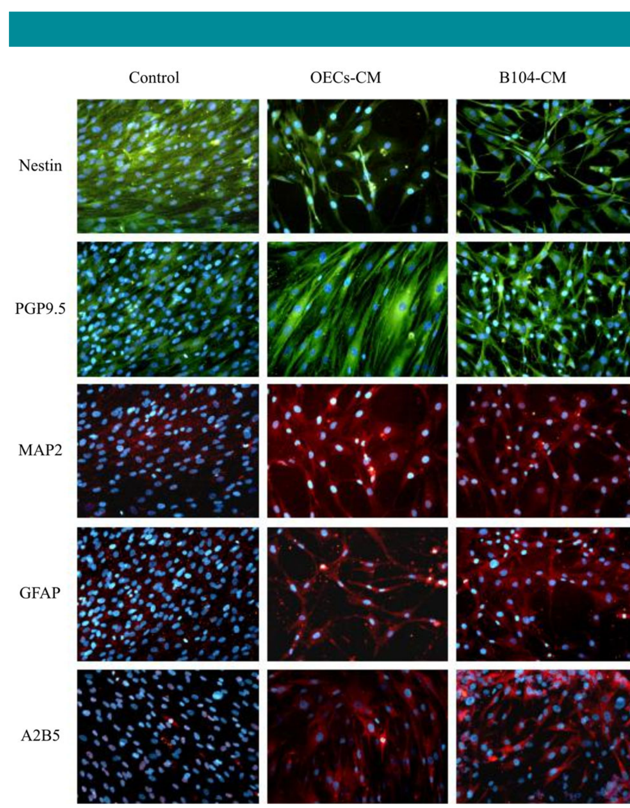


**Fig. 5. Neuronal markers expression by immunocytochemistry of control and OECs-CM or B104-CM treated AT-MSCs 7 days after the treatment.**

was greater for OECs-CM cultures (about 88%). At 7 days percentages were low in OECs-CM and B104-CM. MFI at 24 h was similar in controls and in B104-CM cultures, whereas it was higher in OECs-CM cultures. At 7 days, a progressive decrease was detected for all samples considered.

### A2B5

Percentage of positive cells for A2B5 was low at 24 h (about 6%) in all cultures. At 7 days percentage was higher in controls, but showed a very marked increase in B104-CM cultures (about 60%) and, especially, in OECs-CM cultures (72%). MFI at 24 h showed comparable values in all cultures considered, with a slightly higher value for B104-CM cultures. At 7 days, no significant differences were detected for all samples.

## Discussion

Recent advances in human stem cell biology and the optimization of protocols for in vitro differentiation of stem cells into different cell lineages have opened new possibilities for generating cellular models. The remarkable advances in the ability to obtain stem cells from different tissues, including adipose (Zuk et al., 2002; De Ugarte et al., 2003), which are able to differentiate into several cell lineages, offer the possibility to generate cells with neuronal characteristics from easily accessible sources.

The aim of this study was to develop a human neuronal model by inducing neuronal differentiation of mesenchymal stem cells isolated from adipose tissue and treated with B104- or OECs-CM. The results confirmed that it is possible to isolate clonogenic adult stem cells expressing mesenchymal and pluripotent state associated markers from adipose tissue.

Moreover, AT-MSCs treated with B104- or OECs-CM differentiated to neuronal fate, expressed markers of progenitor and mature neurons (nestin, PGP 9.5, and MAP2) and displayed morphological features resembling neuronal cells.

Nestin has traditionally been noted for its importance as a neural stem cell marker. It is required for brain development and for survival, renewal, and mitogen-stimulated proliferation of neural progenitor cells. It promotes the disassembly of phosphorylated vimentin intermediate filaments (IF) during mitosis and plays a role in the trafficking and distribution of IF proteins and other cellular factors to daughter cells during progenitor cell division. Therefore, nestin is expressed both in embryonic and adult CNS stem cells. Nestin expression in the embryonic CNS is spatially and temporally linked closely to the proliferating cell state, which makes it a commonly used marker for this cell type. Similarly, nestin expression is a hallmark of adult CNS stem cells, both in vivo and when the adult CNS stem cells are cultured in vitro under non-differentiating conditions. Nestin expression is downregulated when CNS stem cells differentiate and other members of the intermediate filament gene family are expressed, primarily neurofilament in neurons and GFAP in astrocytes (Lendahl et al., 1990). The down-regulation of nestin protein and its mRNA correlates with multipotential cells leaving the proliferative state and differentiating into astrocytes, which express GFAP, or neurons, which express neurofilament proteins (Dahlstrand et al., 1995; Xu et al., 2008). In the present study, the expression of the neural stem cell marker nestin

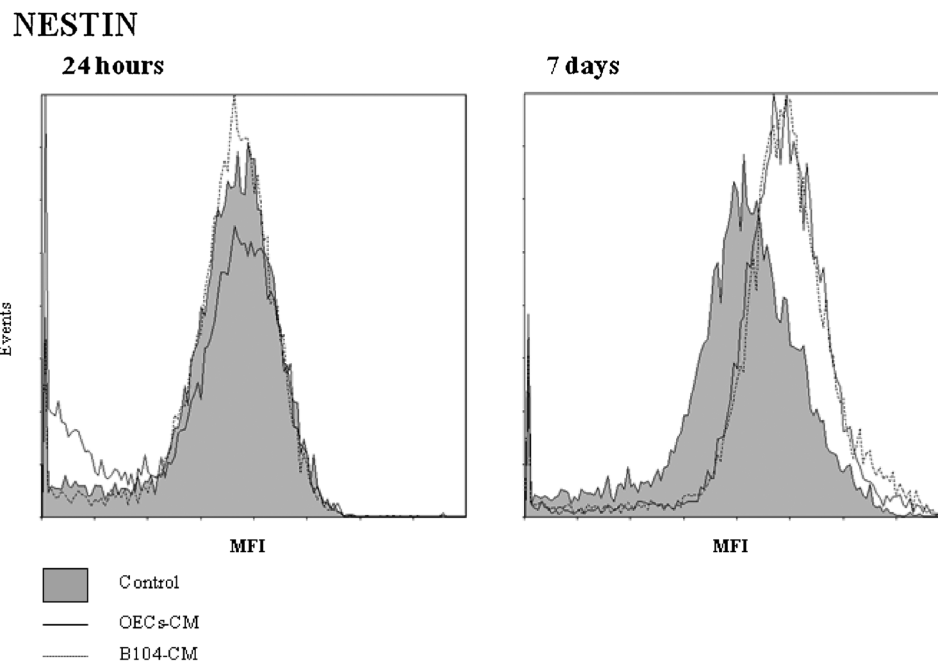


**Fig. 6. Nestin (neural stem cell marker) expression by flow cytometry of control and OECs-CM or B104-CM treated AT-MSCs 24 h and 7 days after the treatment. A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at $\lambda = 488$ nm and fluorescence was monitored at $\lambda = 525$ nm. Fluorescence was detected using logarithmic amplification. MFI values were calculated and recorded automatically.**

was detected in AT-MSCs treated with B104- or OECs-CM. Furthermore, immunofluorescent and cytofluorimetric analysis revealed that the treated AT-MSCs had the ability to differentiate into neurons. The expression of the mature neuronal markers PGP 9.5 and MAP2, even if in presence of expression of nestin probably due at the early time of differentiation (24 h and 7 days), indicated that the AT-MSCs had been differentiated into more mature neuronal cells.

PGP 9.5 (also known as UCHL1), a 27 kDa ubiquitin-protein hydrolase, is expressed in the cytoplasm of neurons and cells of the diffuse neuroendocrine system. PGP 9.5 hydrolyses a peptide bond at the C-terminal glycine of the proteolysis tagging protein ubiquitin, thereby generating/recycling ubiquitin monomers, essential in the ubiquitin-proteasome cytoplasmic protein degradation system (Day and Thompson, 2010).

Microtubule-associated protein 2 (MAP2) is a major component of the neuronal cytoskeleton and is known to promote the assembly and stabilization of microtubules, functions, which have important implications in neuronal differentiation (Przyborski and Cambray-Deakin, 1995). MAP2 antibody (SMI 52) reacts with microtubule-associated protein 2 (MAP2). SMI 52 recognizes neuronal cell bodies and dendrites in tissue sections and cell cultures.

Moreover, the results demonstrated that AT-MSCs treated with conditioned media result to be negative for GFAP and A2B5.

GFAP, a astrocyte marker and a class-III intermediate filament, is a cell-specific marker that, during the development of the CNS, distinguishes astrocytes from other glial cells.

A2B5 is a cell surface ganglioside epitope expressed in developing thymic epithelial cells, oligodendrocyte progenitors and neuroendocrine cells.

In the present study, we employed growth factors from B104- or OECs-CM for the differentiation of MSCs into neuron-like cells. In contrast, earlier studies that utilized

TABLE 1. Effects of OECs-CM or B104-CM on AT-MSCs determined by flow cytometry

| | | Control | | OECs-CM | | B104-CM | |
|---|---|---|---|---|---|---|---|
| | | 24 h | 7 days | 24 h | 7 days | 24 h | 7 days |
| Nestin | MFI | 6.1 ± 0.2 | 12.2 ± 3 | 8.0 ± 2 | 25.0 ± 4* | 7.5 ± 2 | 28.4 ± 4* |
| | % positive cells | 97 ± 2 | 97 ± 1 | 97 ± 2 | 98 ± 4 | 98 ± 3 | 98 ± 4 |
| PGP9.5 | MFI | 19.4 ± 3 | 18.9 ± 5 | 28.1 ± 2* | 52.0 ± 1* | 27.0 ± 1* | 31.7 ± 1* |
| | % positive cells | 99 ± 1 | 98 ± 5 | 99 ± 2 | 98 ± 2 | 99.6 ± 3 | 98.2 ± 2 |
| MAP2 | MFI | 2.1 ± 0.5 | 2.2 ± 1 | 3.3 ± 0.3* | 5.0 ± 0.2* | 2.5 ± 0.1* | 3.3 ± 0.3* |
| | % positive cells | 96 ± 4 | 94 ± 5 | 98 ± 2 | 97 ± 2 | 96 ± 1 | 94 ± 1 |
| GFAP | MFI | 1.8 ± 0.7 | 1.3 ± 0.2 | 3.0 ± 0.8 | 1.8 ± 0.2 | 2.0 ± 0.5 | 1.9 ± 0.6 |
| | % positive cells | 66 ± 5 | 23 ± 3 | 88 ± 6 | 80 ± 5 | 66 ± 4 | 50 ± 5 |
| A2B5 | MFI | 1.7 ± 0.2 | 1.2 ± 0.5 | 1.7 ± 0.3 | 2.0 ± 0.4 | 2.8 ± 0.5 | 1.4 ± 0.2 |
| | % positive cells | 6 ± 1 | 25 ± 5 | 8 ± 2 | 73 ± 5 | 6 ± 2 | 59 ± 6 |

MFI, mean fluorescence intensity.
*$P < 0.05$ compared to untreated with OECs- CM or B104-CM (Control).

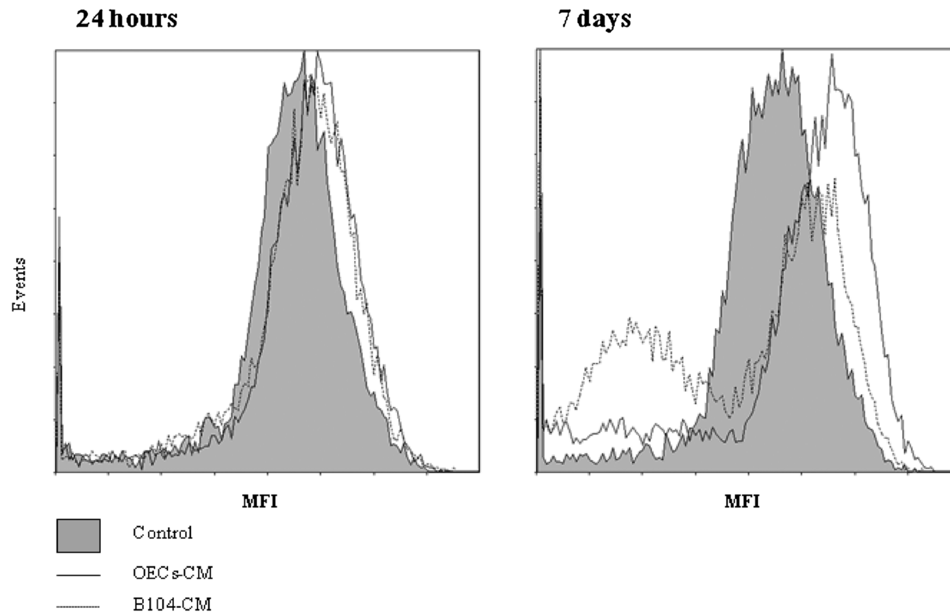


**Fig. 7. PGP 9.5 (neuronal differentiation marker) expression by flow cytometry of control and OECs-CM or B104-CM treated AT-MSCs 24 h and 7 days after the treatment. A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at $\lambda = 488$ nm and fluorescence was monitored at $\lambda = 525$ nm. Fluorescence was detected using logarithmic amplification. MFI values were calculated and recorded automatically.**

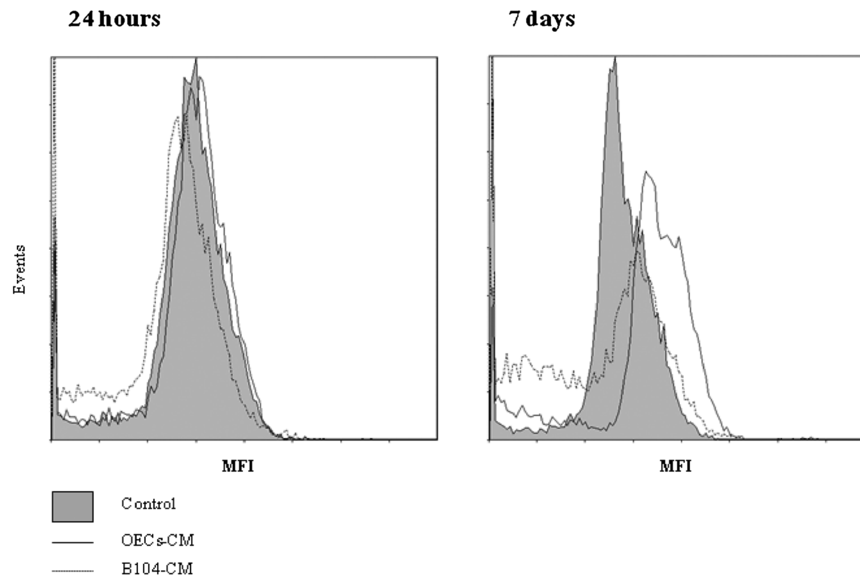


**Fig. 8. MAP2 (neuronal differentiation marker) expression by flow cytometry of control and OECs-CM or B104-CM treated AT-MSCs 24 h and 7 days after the treatment. A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at $\lambda = 488$ nm and fluorescence was monitored at $\lambda = 525$ nm. Fluorescence was detected using logarithmic amplification. MFI values were calculated and recorded automatically.**

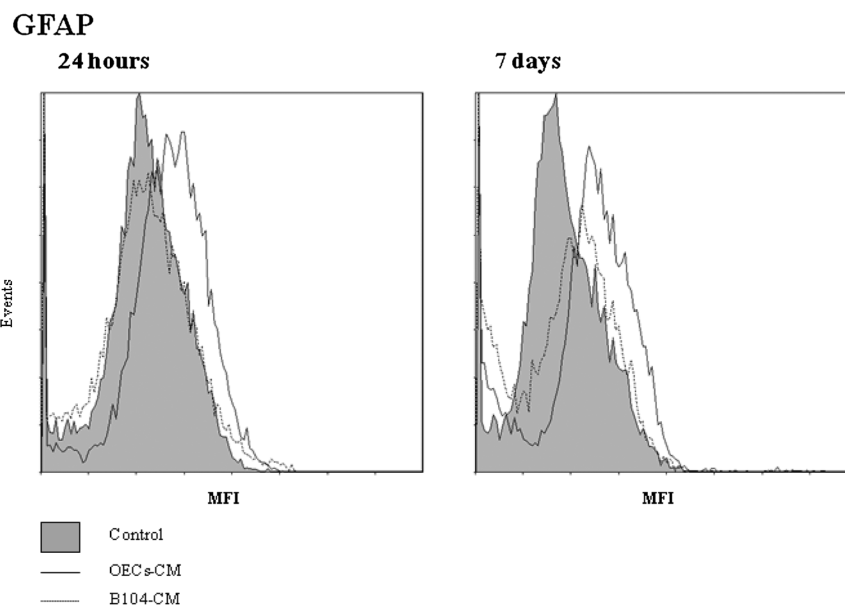


**Fig. 9. GFAP (astrocyte differentiation marker) expression by flow cytometry of control and OECs-CM or B104-CM treated AT-MSCs 24 h and 7 days after the treatment. A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at $\lambda = 488$ nm and fluorescence was monitored at $\lambda = 525$ nm. Fluorescence was detected using logarithmic amplification. MFI values were calculated and recorded automatically.**

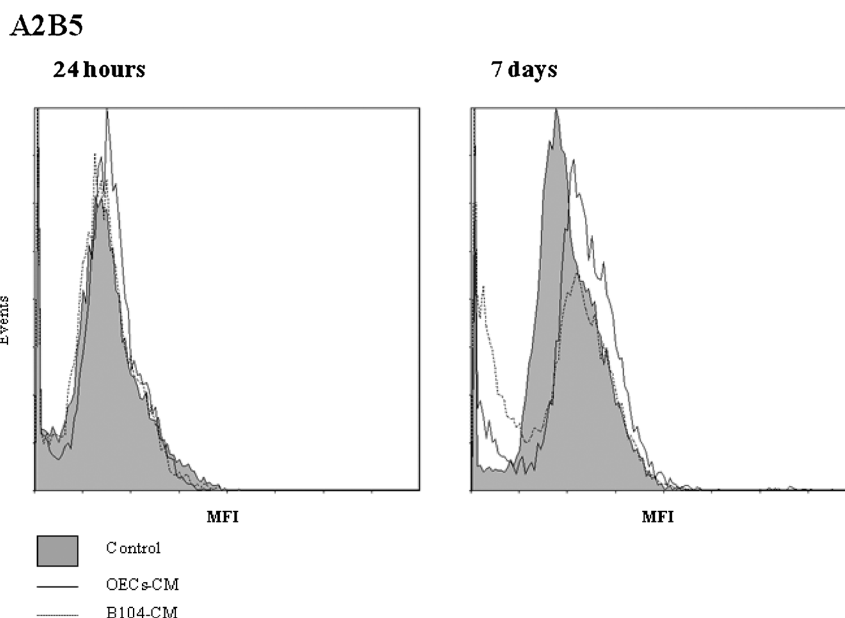


**Fig. 10. A2B5 (oligodendrocyte differentiation marker) expression by flow cytometry of control and OECs-CM or B104-CM treated AT-MSCs 24 h and 7 days after the treatment. A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at $\lambda = 488$ nm and fluorescence was monitored at $\lambda = 525$ nm. Fluorescence was detected using logarithmic amplification. MFI values were calculated and recorded automatically.**

certain chemical agents such as beta-mercaptoethanol (BME), dimethyl sulfoxide (DMSO), and butylated hydroxyanisole (BHA) (Woodbury et al., 2000) reported that MSCs could differentiate into cells of neuronal morphology within a few hours, and therefore, these cells may be feasible for transplantation. However, the reversible morphological nature of the differentiated neuronal cells (Zurita et al., 2008) and the toxicity of the chemical substances employed limit their use in clinical trials (Yang et al., 2008). On the contrary, cells differentiated in the presence of growth factors are safer for clinical use.

The ideal type of cells for CNS transplantation will be an expandable population of cells, which can differentiate into appropriate phenotypes.

This study demonstrated that AT-MSCs can be influenced by the environment, indicating that these cells can respond to environmental cues also versus a neuronal phenotype. As potential material for replacement therapy in CNS degenerative diseases, this culture system may therefore offer many advantages, including the ability to expand, safety test, and bank these cells before transplantation. Other experiments are necessary to confirm this possibility.

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