

Plasticity of Cultured Mesenchymal Stem Cells: Switch from Nestin-Positive to Excitable Neuron-Like Phenotype

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ABSTRACT

Bone marrow mesenchymal stem cells (MSCs) can differentiate into several types of mesenchymal cells, including osteocytes, chondrocytes, and adipocytes, but, under appropriate experimental conditions, can also differentiate into nonmesenchymal cells—for instance, neural cells. These observations have raised interest in the possible use of MSCs in cell therapy strategies for various neurological disorders. In the study reported here, we addressed the question of *in vitro* differentiation of MSCs into functional neurons. First, we demonstrate that when they are co-cultured with cerebellar granule neurons, adult MSCs can express neuronal markers. Two factors are needed for the emergence of neuronal differentiation of the MSCs: the first one is nestin expression by MSCs (nestin is a marker for the responsive character of MSCs to extrinsic signals), and the second one is a direct cell–cell interaction between neural cells and MSCs that allows the integration

of these extrinsic signals. Three different approaches suggest that neural phenotypes arise from MSCs by a differentiation rather than a cell fusion process, although this last phenomenon can also coexist. The expression of several genes—including *sox*, *pax*, *notch*, *delta*, *frizzled*, and *erbB*—was analyzed by quantitative reverse transcription polymerase chain reaction (RT-PCR) in order to further characterize the nestin-positive phenotype compared to the nestin-negative one. An overexpression of *sox2*, *sox10*, *pax6*, *fzd*, *erbB2*, and *erbB4* is found in nestin-positive MSCs. Finally, electrophysiological analyses demonstrate that MSC-derived neuron-like cells can fire single-action potentials and respond to several neurotransmitters such as GABA, glycine, and glutamate. We conclude that nestin-positive MSCs can differentiate *in vitro* into excitable neuron-like cells. STEM CELLS 2005;23:392–402

INTRODUCTION

A stem cell is an unspecialized cell with the ability to renew itself indefinitely; under appropriate conditions, stem cells can give rise to a wide range of mature cell types. Two types of stem cells can be distinguished according to their origin

and their potential of differentiation: embryonic stem cells (ESCs) and somatic stem cells (SSCs) [1]. ESCs are derived from the early blastocyst and the inner cell mass of the embryo and are able to differentiate into the three germ layer cell types (pluripotentiality) [2, 3, 4]. SSCs are isolated from

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fetal (after gastrulation) or adult tissues, but classically they are programmed to produce only the cell types that belong to the tissue from which they originate. However, recent studies suggest that SSCs might be able to exhibit more plasticity than previously thought, as they seem able to differentiate into many cell types. These observations were mainly reported when SSCs were grafted in a damaged tissue [5]. This phenomenon, also known as phenotypic plasticity of SSCs, has been described among others for mesenchymal stem cells (MSCs), which under appropriate in vivo or in vitro conditions can adopt a neural fate [6–8].

Over the past few years in parallel to stem cell research, there has been a growing interest in the molecular mechanisms involved in cell type determination and differentiation during development. The discovery of intrinsic and extrinsic factors leading to the differentiation into ectoderm, mesoderm, and endoderm during the early stages of development; and the identification of factors that are responsible for further differentiation into more restricted cell types later in development, would allow a better understanding of the mechanisms that control the phenotypic plasticity of stem cells.

Recently, we demonstrated that cultured MSCs from adult rat bone marrow are able to express nestin, an intermediate filament protein that is predominantly expressed during neural development [9]. Two factors seem essential for this nestin expression to occur: the first one is the absence of serum-derived components in the culture medium, and the second one is an in vitro maturation of the MSCs. Indeed, a minimum of 25 population doublings are required for nestin to be expressed in 75% of cultured MSCs. In this study, we demonstrated that nestin-positive MSCs, when co-cultured with mouse cerebellar granule (mCG) neurons, are able to differentiate into excitable neuron-like cells. By using quantitative reverse transcription polymerase chain reaction (RT-PCR), we demonstrated that *sox* and *pax* transcription factors, as well as the *frizzled* (wnt receptor) and *erbB* (neuregulin receptors) receptors, are upregulated in nestin-positive MSCs compared with nestin-negative MSCs (nnMSCs), suggesting a possible role for these genes in the acquisition of a responsiveness to neural fate-inducing signals in a nestin-positive MSC population. Electrophysiological recordings of npMSC-derived neuron-like (MDN) cells show that in co-culture with CG neurons, MDN cells first express functional voltage-gated K⁺ channels followed after 8 days of co-culture by voltage-gated Na⁺ channels, allowing firing of action potentials. Finally, several experiments strongly suggest that this neuronal fate in the nestin-positive MSCs is a consequence of a “transdifferentiation” process, although not completely ruling out that cell fusion processes could also contribute to the appearance of neuronal-like cells.

MATERIALS AND METHODS

Preparation and Culture of Rat Mesenchymal Stem Cells (rMSCs)

Adult (8–15-week-old) rat (Wistar Institute, University of Pennsylvania, State College, <http://www.wistar.upenn.edu/>) (housing and breeding are taken care of in intramural animal facilities) bone marrow was obtained from femoral and tibial bones by aspiration and was resuspended into 5 ml of Delbecco's modified Eagle's medium (DMEM; Invitrogen, Merelbeke, Belgium, <http://www.invitrogen.com>) [10]. Between 100 and 200 × 10⁶ marrow cells were plated on 175-cm² tissue culture flasks in DMEM added with 10% (v/v) fetal bovine serum (Invitrogen). After 24 hours, the nonadherent cells were removed. When the MSCs became confluent, they were resuspended with 0.25% trypsin (w/v; Sigma-Aldrich Company Ltd, Dorset, U.K., <http://www.sigmaaldrich.com>), in 1 mM EDTA (Fluka brand, Sigma; <http://www.sigmaaldrich.com/Brands/Fluka>) and then subcultured. Characterization of MSCs and induction of nestin expression are described in our previous paper [9].

Preparation and Culture of Mouse Cerebellar Granule Neurons

Mouse cerebellar granule (mCG) neuron cultures were prepared from 3-day-old green C57BL/6 mice (Jackson ImmunoResearch Laboratories, West Grove, PA, <http://www.jacksonimmuno.com>), according to Lefebvre et al. [11]. Green mouse express green fluorescent protein (GFP) under control of the β-actin promoter [12]. Briefly, cerebella were removed and freed of meninges. They were then minced into small fragments and incubated at 37°C for 25 minutes in 0.25% trypsin and 0.01% DNase (w/v, in a cation-free solution). Fragments were then washed with minimum essential medium (Invitrogen) supplemented with glucose (final concentration 6 g l⁻¹), insulin (Sigma; 5 μg ml⁻¹) and pyruvate (Invitrogen; 1 mM). The potassium concentration was increased to 25 mM, while the sodium concentration was decreased in an equimolar amount (MEM-25HS). The dissociation was achieved mechanically by up-and-down aspirations in a 5-ml plastic pipette. The resulting cell suspension was then filtered on a 15-μm nylon sieve (pore size = 15 μm). Cells were then counted and diluted to a final concentration of 2.5 × 10⁶ ml⁻¹. The cell suspension was finally plated on a substrate previously coated with polyornithine (0.1 mg ml⁻¹). The cells were cultured for 24 hours before any other experimental procedure was performed.

Culture of Nestin-Positive MSCs on Paraformaldehyde-Fixed mCG Neurons

First, mCG neurons were isolated from GFP⁺ mice as

described above and cultivated for 5 days before fixation with 4% (v/v) sterile paraformaldehyde. Then the fixed cells were treated with gelatine (1% w/v, in Tris-buffered saline [TBS]; Bio-Rad Laboratories, Hercules, CA, <http://www.bio-rad.com>) for 1 hour at 37°C, under sterile conditions. Nestin-positive MSCs were transferred on these fixed cultures, and the co-cultures were grown in mCG neuron-conditioned medium for 5 days.

Immunocytochemistry

Nestin-positive MSCs were trypsinized and placed with mCG neurons on polyornithine-coated dishes, in guanine and cytosine (GC) medium, for 5–15 days and were processed for immunocytochemistry as already described [9]. Briefly, the cultures were fixed with 4% (v/v) paraformaldehyde for 15 minutes at room temperature and washed three times in TBS. They were then permeabilized in 1% triton-X100 (v/v) for 15 minutes and washed three times in TBS. Nonspecific binding was blocked by a 1 hour treatment in TBST (TBS plus 0.1% w/v Tween) containing defatted milk powder (30 mg ml⁻¹). The cells were then incubated for 1 hour at room temperature with one of the following primary antibodies (diluted in blocking buffer):

- anti-gliab fibrillary acidic protein (GFAP [Dako, Glostrup, Denmark, <http://www.ump.com/dako.html>] mouse immunoglobulin [IgG], dilution 1:500)
- anti-GLAST [13] (rabbit IgG, 1:4000)
- anti-Tuj1 ([Eurogentec, Southampton, Hampshire, UK, <http://uk.eurogentec.com/>] mouse IgG, dilution 1:1,500)
- anti-NeuN ([Chemicon International, Temecula, CA, <http://www.chemicon.com>] mouse IgG, dilution 1:50)
- anti-SMI31 ([Chemicon] mouse IgG, 1:400)
- anti-MAP2ab ([Sigma] mouse IgG, dilution 1:400)
- anti-synaptophysin ([Chemicon] mouse IgG, dilution 1:200)
- anti-M2 ([Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA, <http://www.uiowa.edu/~dshbwww>] rat IgG, dilution 1:500)
- anti-M6 ([Developmental Studies Hybridoma Bank] rat IgG, dilution 1:300)

After three washes in TBS, cells were incubated in fluorescein isothiocyanate (FITC)-conjugated, rhodamine-conjugated, or Cy5-conjugated anti-mouse, anti-rat, or anti-rabbit IgGs (dilution 1:500; Jackson ImmunoResearch) for 1 hour at room temperature and in the dark. The nuclei were stained with ethidium homodimer (0.2 µM; Sigma). Omission of primary antibodies served as negative control and resulted in no detectable staining. The preparations were then mounted in Fluoprep (bioMérieux-Pierre Fabre, Boulogne, France, <http://www.biomerieux.com/>) and observed using a Bio-Rad MRC1024 laser scanning confocal microscope (Bio-Rad Laboratories). The fraction of positive cells was determined by counting 10

nonoverlapping microscopic fields (± 30 MSC-derived cells per field) for each coverslip in at least three separate experiments (*n* then corresponds to the number of coverslips).

FACS Analysis

Fluorescence-activated cell sorting (FACS) analysis of co-cultured rMSCs and CG neurons were performed with anti-GFAP and anti-Tuj1 antibodies. Briefly, after 5 days of co-culture, the cells were trypsinized and suspended in phosphate-buffered solution (PBS) containing 1% (v/v) fetal calf serum. After centrifugation, the pellet was resuspended (750,000 cells per ml) in FACS permeabilization solution (Becton, Dickinson; BD Biosciences, Oxford, U.K., <http://www.bd.com>), for 30 minutes at room temperature. The cells were centrifuged and washed three times in PBS containing 1% (v/v) fetal calf serum before the incubation with anti-GFAP ([Dako] mouse IgG, dilution 1:500) or anti-Tuj1 ([Pharmingen; BD Biosciences; <http://www.bdbiosciences.com/pharmingen>], mouse IgG, dilution 1:400) antibodies, for 1 hour at room temperature. The co-culture cells were then washed three times in PBS containing 1% (v/v) fetal calf serum and incubated with anti-mouse IgG (1:500) or anti-rabbit IgG (1:500) secondary antibodies coupled to rhodamine (Jackson ImmunoResearch) for 1 hour at room temperature and in the dark. The analysis was performed with a FACSort instrument (Becton, Dickinson), and the results were analyzed using the Cellquest program (Becton, Dickinson).

DNA Ploidy

DNA content per cell was determined by FACS analysis after staining the cells with propidium iodide. After 5 days of co-culture, MSCs and mCG neurons were trypsinized and fixed into 70% ethanol, at 4°C for 16 hours. The cells were stained with propidium iodide (400 mg ml⁻¹; Sigma) just before FACS analysis.

RT-PCR and Quantitative RT-PCR Analysis

Total RNA from nestin-positive MSCs (15 passages in serum-free media) and nestin-negative MSCs (15 passages in serum condition) were prepared using the RNeasy total RNA purification kit (Qiagen, Westburg, U.K., <http://www1.qiagen.com>). For cDNA synthesis, random hexamer primers (Invitrogen) were used to prime RT reactions. The cDNA synthesis was carried out using Moloney-murine leukemia virus (M-MLV) Superscript II reverse transcriptase (Invitrogen) following the manufacturer's instructions. The PCR mix contained 2.5 mM MgCl₂, PCR nucleotide mix of dNTP at 0.2 mM each, 0.5 : M primers (Table.1), 5 U/1 Taq DNA polymerase (Gibco), 10 ng DNA template, and nuclease-free water to a final volume of 50 µl. Cycling parameters were as follows: denaturation at 94°C for 30 seconds, annealing (see Table 1) for 30 seconds,

and elongation at 72°C for 30 seconds, for 35 cycles on the PTC200 DNA Engine (MJ Research, Waltham, MA, <http://www.mjr.com/>). To exclude the possibility of the presence of genomic DNA, we performed control reactions without the RT step. The fzd RT-PCR products were purified using a commercial kit (Qiagen) and subcloned using the TOPO TA cloning kit (Invitrogen). Positive clones were sequenced (Eurogentec), and genes were identified by BLAST search. Quantitative PCR was carried out using standard protocols with Quantitec SYBR Green PCR Kit (Qiagen). The PCR mix contained SYBR Green Mix, 0.5 µM primers (Table 1), 1 ng DNA template, and nuclease-free water to reach a final volume of 25 µl. Quantitative PCR was performed on the Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia, <http://www.corbettresearch.com/>) and analyzed with Rotorgene Software (Corbett Research). The percentage of gene expression by nestin-positive MSCs was normalized in function of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression and compared to the gene expression level of nestin-negative MSCs that was considered as 100%.

Solutions and Drugs for

Electrophysiological Analyses

Nestin-positive MSCs were stained with DiD Vybrant cell-labeling solution (red; Molecular Probes, Invitrogen, Pais-

ley, U.K., <http://www.probes.com/>) and co-cultivated with GFP-positive mCG neurons in order to easily identify the two types of cells. This procedure allowed us to be sure that the cells from which we recorded were not CG neurons, but prevented us from labeling a particular recorded cell for post hoc immunocytochemical characterization, which would have involved quadruple immunostainings. For whole-cell recordings, coverslips with co-cultured nestin-positive MSCs and CG neurons were transferred to the stage of a Zeiss interferential contrast microscope equipped with fluorescence and perfused (3 ml per minute) with a solution containing NaCl (137 mM), KCl (5.4 mM), CaCl₂ (2 mM), H₂O (1.8 mM), D-glucose (22.2 mM), and HEPES; the pH was adjusted to 7.2 with NaOH. The sodium and potassium channel blockers were, respectively, tetrodotoxin (TTX; Sigma, <http://www.fisherscientific.com/>; 1 µM) and tetraethyl ammonium (TEA; 10 µM; Janssen Chemica, Geel, Belgium). The neurotransmitters used were GABA (1 mM), glycine (100 µM), and glutamate (100 µM), and the inhibitors of neurotransmitters were, respectively, gabazine (10 µM), strychnine (1 µM), and a mix of AP-5 (1 µM; Tocris Cookson, Avonmouth, Bristol, UK; <http://www.tocris.com/>) and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; 1 µM; Tocris). All drugs were applied by a microperfusion system (SPS-8, List Medical, Darmstadt, Germany).

Table 1. Primers used for reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR experiments

Gene	Forward	Reverse	Annealing temperature (°) (°)
Sox1	AACCCCAAGATGCACAACCTC	TAGCCCAGCCGTTGACAT	54
Sox2	TAGCACTTGTGGCCAGAACG	AAGCCGCTCTTCTCTTTTCC	54
Sox3	AGAACCCAAGATGCACAAC	CTGCACGAGCGAGTAGGC	54
Sox4	AAGCATATGGCTGACTACCC	GGAGTCCGCACCTGTAGAC	54
Sox9	TGAAGAAGGAGAGCGAGGAA	GGGGCTGGTACTTGTAATCG	54
Sox10	AGCCCAGGTGAAGACAGAGA	CCCCTCTAAGGTCGGGATAG	54
Sox11	TCATGTTTCGACCTGGCTTG	CCCCACCATCCTCTTTATCC	54
Pax3	GCCAATCAACTGATGGCTTT	CATTCTGAAGGAATGGTGCTT	53
Pax6	AGTTCTTCGCAACCTGGCTA	GGAGCTGATGGAGTTGGTGT	58
Notch1	TTACAGCCACCATCACAGCCACACC	ATGCCCTCGGACCAATCAGA	51
Notch2	AACCCGTGTCTGAACCAAGG	CCATTGTTTCATACACGGCTTG	51
Notch3	ACACTGGGAGTTCTCTGT	AGTAGTTCAGGTCTTGTTGCAGAA	48
Delta1	TGTTCTAACGGTGCCAAGTG	CTGCAGTTCCTGCCTGTGTA	48
ErbB2	GAGGTGGTGAGCTGACACT	CCTCTGATTGGTTCACATACT	53
ErbB3	AGATCTGCACCATTGACGTC	TAGGTCTAGGTCCAGTTCTG	52
ErbB4	GCTAGAGACCCTCAAAGATAACC	GCATGGGCATTCTTGTGGTGT	51
FZD	NNNGAATTCTAYCCNGARMGNCCNAT	NNNAAGCTTNGCNGCNARRAACCA	55
NF-L	TACTCTCAGAGCTCGCAGGTCTT	ACCATGCACAGGCTGGCAGCAA	55
GFAP	ACATCGAGATCGCCACCTAC	ACATCACATCCTTGTGCTCC	52
GAPDH	GCCGCATCTTCTTGTGCAGT	GCAACAATCTCCACTTTGCCA	48–56

Electrophysiological Recordings

Borosilicate recording electrodes (5–10 M Ω) were made using a Flaming–Brown microelectrode puller (P97, Sutter Instrument Co., Novato, CA, <http://www.sutter.com/>). Micropipettes were filled with an intracellular-like solution containing KCl (130 mM), CaCl₂ (2 mM), H₂O (1 mM), D-glucose (11.1 mM), EGTA (10 mM), Na₂-ATP (2.5 mM), Mg-ATP (2.5 mM), HEPES (10 mM) at pH 7.4. Electrophysiological recordings were performed with a patch-clamp amplifier (RK400; Bio-Logic, Claix, France, <http://www.bio-logic.info/>) using the whole-cell configuration of the patch-clamp recording technique. Series resistances (10–20 M Ω) were electronically compensated (up to 80%), and current traces were filtered at 3 kHz, acquired and digitized at 0.5 kHz for voltage-clamp recordings and at 20 kHz for current-clamp recordings, and stored on a personal computer system. Data recording and measures were achieved using the TIDA software (HEKA Elektronik Lambrecht/Pfalz, Germany, <http://www.heka.com>). Graphing and statistical analyses were performed with the aid of the GraphPad Prism software (version 4.0; GraphPad, San Diego, <http://www.graphpad.com>).

RESULTS

Neuronal and Astroglial Marker Expression by Nestin-Positive MSCs

MSCs were isolated from femoral and tibial bones of adult rats, propagated in culture, and immunologically and functionally characterized [9]. As we already demonstrated, a nestin-expressing stage is necessary to observe a GFAP⁺ cell differentiation of MSCs co-cultured with neural stem cells [9]. To observe a possible neuronal differentiation of MSCs, we co-cultured nestin-positive MSCs for 5 days with GFP⁺ mCG neurons obtained from 3-day-old green mice. After 5 days of co-culture, immunological labelings revealed that MSCs began to express differentiation markers: 40.17% \pm 2.95% expressed GFAP (Fig. 1A–C), 19.00% \pm 1.58% expressed Tuj1 (Fig. 1D–F), and 19.30% \pm 1.38% expressed NeuN (Fig. 1G) ($n = 12$ for each condition). These results were confirmed by FACS analyses (Fig. 1I, J) as we measured 44.57% \pm 3.59% GFAP⁺ and 18% \pm 4.1% Tuj1⁺ cells, data which are not statistically different from that obtained by eye-counting immunolabeled cells. The FACS analyses were performed on GFP⁺ cell populations corresponding to the first spike of cells in Figure 1K. Moreover, the absence of GFAP and NeuN double-labeled cells suggests that nestin-positive MSCs are specifically oriented toward either an astroglial or a neuronal fate (Fig. 1H). Two conditions are needed before MSCs begin to express neuronal markers: first, for the appearance of GFAP⁺ cells in co-cultures with neural stem cells, MSCs have to express nestin; second, a direct cell–cell contact between nestin-positive

MSCs and CG neurons is needed. Indeed, when nnMSCs are co-cultured with CG neurons or when nestin-positive MSCs and CG neurons are co-cultured with a physical separation (millicell device), no neuronal markers could be detected (data not shown).

Characterization of Nestin-Positive MSCs

The nestin expression by MSCs coincides with the acquisition by these cells of the ability to respond to extrinsic signals and cues driving their neural differentiation [9]. To get some clue into the molecular mechanism(s) involved in this differentiation, we decided to compare the expression in nestin-positive and nestin-negative MSCs of several factors known to have important roles during normal nervous system development. Using RT-PCR and quantitative RT-PCR, the nestin-positive MSC and nnMSC gene expression profiles of the following factors were compared, both qualitatively and quantitatively:

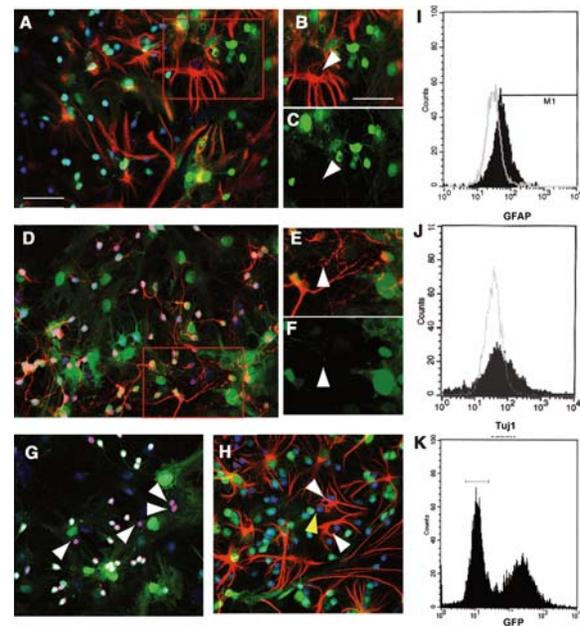


Figure 1. Neuronal and astroglial markers expression by nestin-positive mesenchymal stem cells (MSCs). Nestin-positive MSCs were co-cultured for 5 days with green fluorescent protein (GFP)-positive cerebellar granule (CG) neurons (green). Immunocytochemical labeling and fluorescence-activated cell sorting (FACS) analysis showed that some nestin-positive MSCs expressed neural markers. GFAP (red, A–C) was expressed by about 40% of the nestin-positive MSCs, and Tuj1 (red, D–F) was expressed by about 20% of the nestin-positive MSCs, as was the NeuN marker (red, G). Those results were confirmed by FACS analysis (GFAP, I; Tuj1, J) on a GFP⁺ population (K). A double labeling directed against GFAP (red) and NeuN (blue) (H) showed that nestin-positive MSCs specifically oriented toward an astroglial-like or a neuronal-like fate. Nuclei were counterstained with EtD1 (blue or red, depending on the color used for the secondary antibody). Arrowheads show neural expression markers by nestin-positive MSCs. Scale bars = 40 μ m (A, D, and G–K) and 30 μ m (B–C and E–F).

the *sox* and *pax* transcription factors, as well as frizzled (*wnt* receptors), *erbB* (neuregulin receptors) and the notch-delta signaling pathway. Figure 2 shows that MSCs express *sox2*, *sox9*, *sox10*, *sox11*, *pax6*, *notch1*, *notch2*, *delta1*, *erbB2*, *erbB4*, and *fzd* and do not express *sox1*, *sox3*, *pax3*, *delta2*, and *erbB3*. In the analyses of *fzd* genes, we used degenerated primers that hybridize with all *fzd* genes [14]. The resulting products were cloned using the TA cloning procedure, and random clones were analyzed by sequencing. A BLAST search allowed us to determine the identity of each clone: 80% of *fzd* receptors in nnMSCs were *fzd2*, and 20% were *fzd1*; likewise, in nestin-positive MSCs, we obtained 75% of *fzd2*, 20% of *fzd1*, and 5% of *fzd5* (data not shown). Finally, quantitative RT-PCR demonstrated that nestin-positive MSCs overexpress

sox2, *sox10*, *pax6*, *fzd*, *erbB2*, and *erbB4* when compared with the nnMSCs. We also observed that nestin-positive MSCs overexpress the *GFAP* gene compared with nnMSCs, but none of them express the neurofilament-light gene.

Electrophysiological Analysis of Mesenchymal-Derived Neuron-Like (MDN) Cells

Using fluorescent microscopy, we selected for cells that presented a neuron-like shape (rounded cell body with extended processes, hereafter termed MDN cells), that had incorporated DiD Vybrant (red) and that were clearly GFP⁻ (see Methods). Whole-cell patch-clamp recordings were obtained from 234 MDN cells that had been co-cultured with CG neurons for 4–15 days. Based on the appearance of different voltage-gated current profiles (see next paragraph), three culture time periods were distinguished that, moreover, showed significantly (ANOVA; $p < .001$) different resting membrane potentials (V_{rest}): -37.6 ± 3.0 mV for MDNs co-cultured with CGs for 4–6 days in vitro (hereafter noted 5DIV; $n = 61$), -50.3 ± 2.0 mV for 7–9 days (8DIV; $n = 76$), and -55.7 ± 2.3 mV for 10–15 days (12.5DIV; $n = 97$).

Excitability

The activation of voltage-gated channels at the three developmental stages (5, 8, and 12.5DIV) was assessed by applying to whole-cell patch-clamped MDN cells hyperpolarizing and depolarizing voltage steps from a holding potential (V_H) of -80 mV (-80 to -125 mV and -80 to $+30$ mV in 15-mV increments; Fig. 3A–D). At 5DIV, depolarizing steps positive to -40 mV induced outward currents in 95% of MDN cells that showed little desensitization and were blocked by $10 \mu\text{M}$ TEA, indicating the activation of potassium voltage-gated channels. Potassium current densities were stable throughout the culture time period (7.25 ± 1.16 pA.pF⁻¹). At 5DIV, no inward currents were observed. Conversely, depolarizing voltage steps positive to -30 mV induced inward currents in 85% of 8DIV MDN cells and in 95% of 12.5DIV MDN cells that rapidly activate and inactivate and that were abolished by a perfusion of $1 \mu\text{M}$ TTX, showing that they are mediated by voltage-activated sodium channels. Between 8DIV and 12.5DIV, no significant differences were found regarding the sodium current densities (8.29 ± 0.87 pA.pF⁻¹ ($n = 51$) at 8DIV compared with 10.15 ± 1.04 pA.pF⁻¹ ($n = 59$) at 12.5DIV). However—and interestingly—the sodium current densities in MDN cells were two times lower (18.63 ± 2.45 pA.pF⁻¹; $n = 21$) than the one recorded in co-cultured CG neurons. Consistent with the occurrence of sodium and potassium voltage-gated currents, an injection of 600 nA current into whole-cell patch-clamped and current-clamped MDN cells elicited action potentials in 21.5% of 8DIV MDN cells ($n = 34$) (Fig. 4A) and in 25% of 12.5DIV MDN cells. These action potentials were always

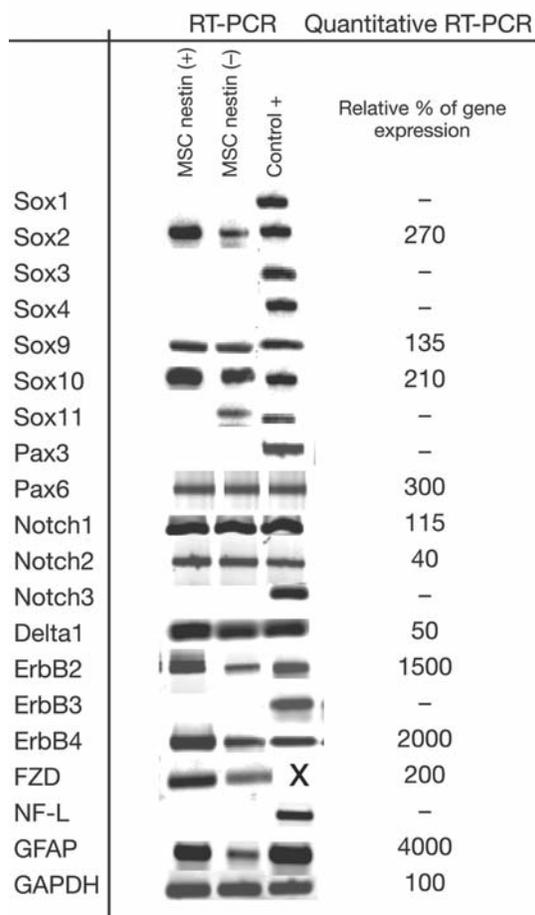


Figure 2. Molecular characterization of nestin-positive mesenchymal stem cells (MSCs). Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR were performed to better characterize the molecular profile of nestin-positive MSCs compared with nestin-negative MSCs (nnMSCs) regarding some genes known to have a role during neuro-ontogenesis. In this case, we observed that nestin-positive MSCs overexpressed *sox2*, *sox10*, *pax6*, *erbB2*, *erbB4*, *fzd*, and *GFAP*. The results of quantitative RT-PCR are expressed as percentage of gene expression in nestin-positive MSCs compared with the same gene expressed in nnMSCs after normalization with the *GAPDH* housekeeping gene.

present as single spikes and were reversibly inhibited by a 1- μ M TTX application (Fig. 4).

Neurotransmitter Effects

Responses of whole-cell voltage-clamped MDNs ($V_H = -80$ mV) to microperfusion-applied neurotransmitters (1

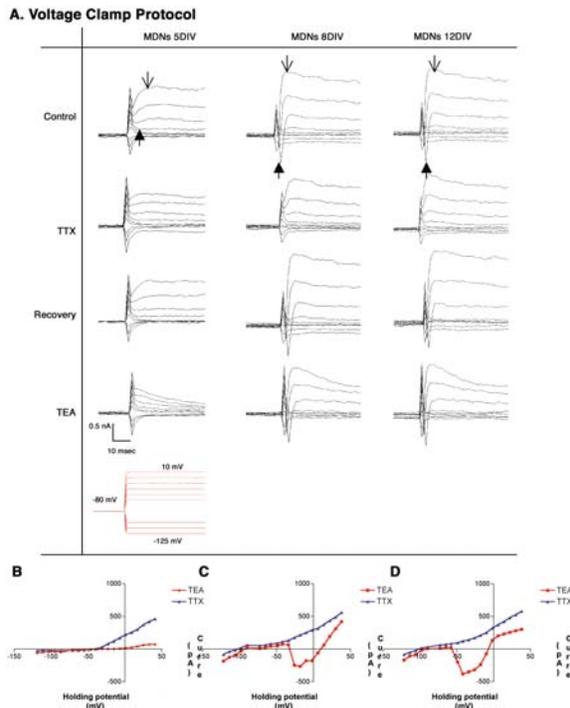


Figure 3. Voltage-gated channel activation in mesenchymal stem cell (MSC)-derived neuron-like (MDN) cells. **(A):** Typical recordings of single whole-cell patch-clamped and voltage-clamped MDN cells at three culture time periods (5, 8, and 12.5 DIV). Hyperpolarizing and depolarizing steps were applied from a holding potential of -80 mV (-80 to -120 mV and -80 to $+30$ mV in 15-mV increments). The first and third lines show recordings in control conditions, and the second in the presence of 1 μ M tetrodotoxin (TTX). The arrows indicate the time where the measures were performed (filled arrows for outward currents, and open arrows for inward currents). **(B–D):** Current-voltage relationships for outward (triangle; recordings obtained in the presence of 1 μ M TTX) and inward (square; recording obtained in the presence of 10 μ M tetraethyl ammonium [TEA]).

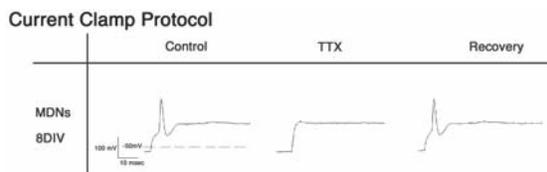


Figure 4. Excitability as shown in a typical recording of a single whole-cell patch-clamped and current-clamped mesenchymal stem cell (MSC) neuron-like (MDN) cell at 8 DIV. A depolarizing 600 nA current injection induced the firing of a single action potential that could be reversibly blocked by 1 μ M tetrodotoxin (TTX).

mM GABA, 100 μ M glycine, and 100 μ M glutamate) were subsequently measured. As soon as at 5 DIV, neurotransmitters elicited inward currents in 98% of MDN cells ($n = 78$, Fig. 5A). Since no significant differences in current densities were observed between the different culture time periods, measures of current densities were pooled for each neurotransmitter, yielding means of 28.2 ± 2.7 pA.pF $^{-1}$ for GABA, 61.4 ± 10.6 pA.pF $^{-1}$ for glycine, and 7.5 ± 2.6 pA.pF $^{-1}$ for glutamate. The responses elicited by GABA, glycine, and glutamate were mediated by their respective ionotropic receptors—that is, GABA $_A$ receptors, glycine receptors, and AMPA/KA/NMDA receptors—since transmitter-evoked currents were reversibly blocked by specific antagonists of these receptors: namely, by gabazine (10 μ M), strychnine (1 μ M), and a mix of CNQX and AP-5 (1 μ M each), respectively. Before the co-culture of nestin-positive MSCs with CG neurons, inward currents elicited by those neurotransmitters were already present, but only in 35% of nestin-positive MSCs ($n = 15$, Fig. 5B) and with much lower mean current densities: 0.06 ± 0.02 pA.pF $^{-1}$ for GABA, 0.07 ± 0.03 pA.pF $^{-1}$ for glycine, and 0.13 ± 0.05 pA.pF $^{-1}$ for glutamate. Current densities of neurotransmitter-evoked responses were also measured in CG neurons with which nestin-positive MSCs were co-cultured. In CG neurons, GABA, glycine, and glutamate elicited currents in 100% of the cells ($n = 15$), with mean current densities of 19.71 ± 3.96 pA.pF $^{-1}$ for GABA, 26.02 ± 5.55 pA.pF $^{-1}$ for glycine, and 12.68 ± 4.54 pA.pF $^{-1}$ for glutamate. Current density comparison between MDN cells and CG neurons yielded a significant difference for glycine responses only (ANOVA; $p < .01$). Finally, we looked for a possible synaptic activation of the neurotransmitter receptors present in MDN cells. Although MDN cells expressed axonal (MAP2ab, Fig. 6A–C), dendritic (SMI31, Fig. 6D–F), and synaptic markers (synaptophysin, Fig. 6G–I), we never observed spontaneous synaptic events in cultured MDN cells.

Mechanisms of Neural Differentiation of Nestin-Positive MSCs

Recent studies have indicated that MSCs are able to fuse with mature neurons [8, 15, 16]. In this study, three types of experiments were performed which strongly suggest that MSCs differentiate into neuron-like cells rather than fuse with existing CG neurons. The first experiment involved the analysis of the DNA content of nestin-positive MSCs and CG neurons before and after the co-culture. In all cases, we did not observe a significant difference in ploidy, which could explain the 60% of neural differentiation (40% into astrocytes and 20% into neurons) in the MSC population (Fig. 7A). Although the sensitivity of this technique does not allow us to completely rule out that some rare polyploid cells could be present, the majority of the observed cells were diploid. In a second set of experiments, we used the M2 and M6 markers, which specifically recog-

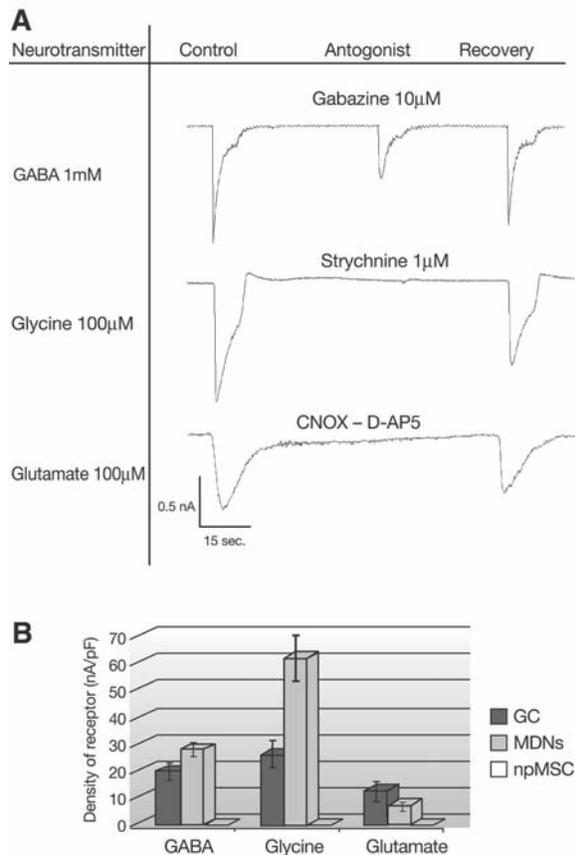


Figure 5. Neurotransmitter sensitivities. **(A):** Typical recording of single whole-cell patch-clamped and voltage-clamped mesenchymal stem cell (MSC) neuron-like (MDN) cell at 8DIV. Either 1 mM GABA, 100 μ M glycine, or 100 μ M glutamate was applied for 10 seconds every 30 seconds, and the resulting current was recorded in the absence or presence of specific antagonists—namely, 10 μ M gabazine, 1 μ M strychnine, and a mix of 1 μ M CNQX and 1 μ M D-AP5, respectively. Antagonists were first applied alone for 10 seconds before being applied for 10 seconds with neurotransmitters. **(B):** Responses to GABA, glycine, and glutamate in mouse cerebellar granule (mCG) neurons, MSC-derived neuron-like (MDN) cells, and nestin-positive MSCs (before co-culturing with mCG neurons) are expressed as current densities—that is, mean peak amplitudes corrected for cell capacitance ($\text{pA}\cdot\text{pF}^{-1}$). $**p < .01$ (one-way ANOVA followed by Dunnett's post-tests)

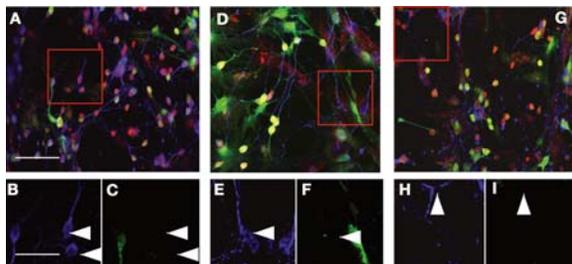


Figure 6. **(A–I):** Expression of axonal (MAP2ab, blue, **A–C**), dendritic (SMI31, blue, **D–F**), and synaptic (synaptophysin, blue, **G–I**) markers by 8DIV cultured MDN cells.

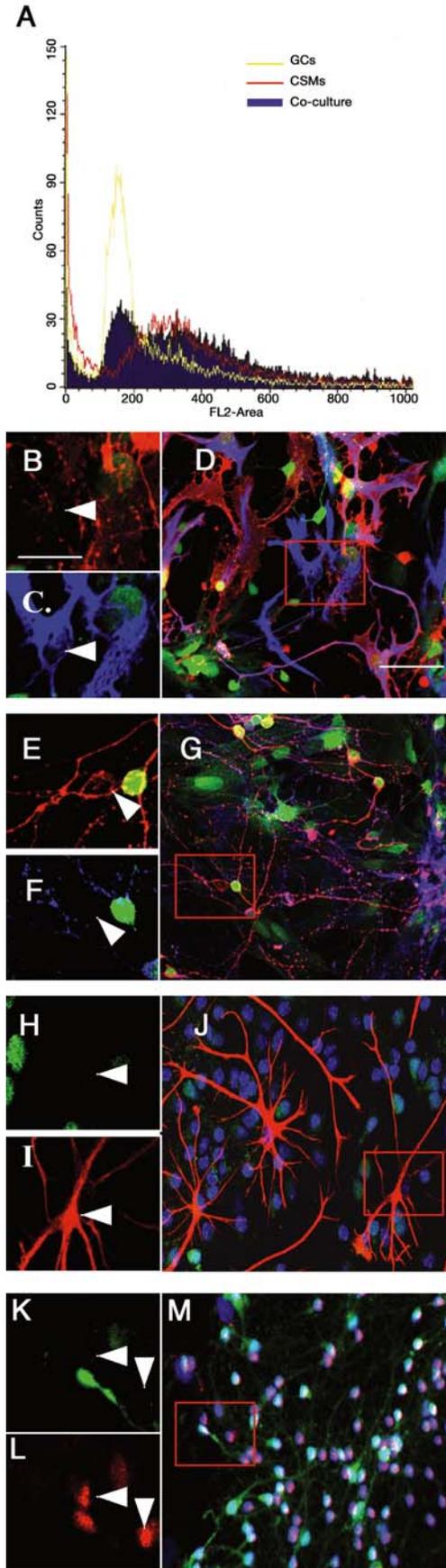
nize respectively astrocytes and neurons from mouse but not from rat [17]. Rat npMSCs were co-cultivated for 5 days with mouse CG neurons. Thereafter, double labeling with GFAP and M2 antibodies, on one hand, and Tuj1 and M6, on the other hand, were performed. We could observe (1) that all GFAP⁺/GFP⁻ and Tuj1⁺/GFP⁻ cells are also negative for M2 and M6, respectively, allowing us to conclude that those cells are of rat origin and thus likely derive from the MSC population (Fig. 7B–G); and (2) that all the cells recognized either by the M2 or by the M6 antibodies are also GFP⁺, ruling out a downregulation of GFP expression during the co-culture period. Finally, nestin-positive MSCs were cultured for 5 days on paraformaldehyde-fixed GFP⁺ CG neurons in the presence of CG-conditioned medium. In such conditions, we observed that $16.1\% \pm 2.6\%$ ($n = 8$) nestin-positive MSC-derived cells were NeuN⁺ (Fig. 7H–J), and $23.1\% \pm 2.1\%$ ($n = 7$) nestin-positive MSC-derived cells were GFAP⁺ (Fig. 7K–M). Note that the level of GFP fluorescence in the fixed cells maintained for 5 days in culture remains stable. Although some rare fusion events cannot be excluded in these experiments, we can conclude that most of the MDN cells result from a true differentiation process of nestin-positive MSCs.

DISCUSSION

Cellular therapies using stem cells are promising approaches for the treatment of several chronic or acute neurological diseases such as Parkinson's [18] and Huntington's diseases [19] and spinal cord injuries [20]. One main problem relates to the origin and the nature of the cells to be used for such procedures. Although somatic stem cells derived from adult tissues seem to be good candidates [21], a better knowledge of the mechanisms underlying the phenotypic plasticity of somatic stem cells is a prerequisite before considering their use in the treatment of neurological diseases. In the study reported here, two major issues were addressed: (1) characterizing some molecular factors that could play a part in directing nestin-positive MSCs into a neural phenotype; and (2) determining the environmental conditions that allow nestin-positive MSCs to differentiate, at least in vitro, into functional neuron-like cells. Furthermore, we have tried to distinguish between true differentiation and cell fusion processes in the in vitro commitment of nestin-positive MSCs to a neural fate.

First, we analyzed the gene expression profile for different factors like *sox*, *pax*, *notch*, *delta*, *frizzled*, and *erbB* and observed that the nestin-positive MSCs express a higher level of *sox2*, *sox10*, *pax6*, *fzd*, *erbB2*, and *erbB4*. The expression of *sox2*, which encodes a high-mobility group (HMG)-box-type transcription factor, has been proposed to act by maintaining a neural progenitor identity [22]. It becomes downregulated in the neural plate when neural crest cells segregate from the dorsal neural tube, and it remains low during neural crest cell

migration. *Sox2* expression is subsequently upregulated in some crest-derived cells in the developing peripheral nervous system and is later restricted to glial sublineages [23]. *Sox10* is expressed in immature glial cells, in both the peripheral and the central nervous systems, where it is linked to Schwann cell [24] and oligodendrocyte [25] differentiation, respectively. Although it has been reported that, in vivo, MSCs are able to differentiate into oligodendrocytes [26], we never observed oligodendroglial differentiation of MSCs in our conditions and have not looked for a possible differentiation into Schwann cells. However, it would be interesting to search for a possible differentiation of *Sox10*⁺ MSCs specifically into GFAP⁺ and GLAST⁺ cells. *Pax6* is a paired-box-containing transcription factor and is required for the development of the embryonic central nervous system, eye, and pancreas [27]. Recently, Talamillo et al. [28] suggested that the primary role of *Pax6* is the regulation of the cell surface properties that are responsible for both cellular identity and radial migration. As only nestin-positive MSCs can grow in suspension culture, it would be interesting to test a possible role of *Pax6* overexpression on these modified cell surface properties. ErbB4 is one of the neuregulin receptors and is expressed with ErbB2 by neural stem cells when they proliferate [29]. Neuregulins have been suggested to stimulate neural stem cell [29] and oligodendroglial progenitor [30] proliferation and survival. Moreover, several reports implicate neuregulins in neuron and oligodendrocyte differentiation [29, 31]. Neuregulins form a large family of proteins: some being secreted, some being membrane-spanning, and some being cleaved or shredded [32]. In the study reported here, we demonstrated that neural differentiation of nestin-positive MSCs requires both a direct cell-to-cell



contact and the presence of conditioned medium. This observation suggests that both membrane proteins and soluble factors play a part in the induction of neural phenotypic plasticity of MSCs. This molecular comparison should be pursued using a more global approach such as a proteomic analysis of both nestin-negative and nestin-positive protein extracts.

One of the most exciting finding of this work is the ability of some nestin-positive MSCs to differentiate into excitable MSC neuron-like (MDN) cells. Those cells were functionally characterized, and three maturation stages were observed. At 4–6 days of co-culture, MDN cells showed some neurotransmitter sensitivities (GABA, glycine, serotonin, and glutamate) and K^+ currents inhibited by TEA. At that culture time period, MDN cells do not express functional sodium voltage-gated channels and have a low membrane potential (V_{rest}) (-37.6 ± 3 , $n = 61$). After 7–9 days to 10–15 days of co-culture, MDN cells displayed Na^+ currents reversely inhibited by TTX and were able to fire single-spike action potentials. In those older co-cultures, the V_{rest} reaches a more negative value, which is closer to the value usually measured in neurons (7–9 days, -50.3 ± 2 , $n = 76$; 10–15 days, -56.7 ± 2.3 , $n = 97$). Those changes in electrophysiological properties demonstrate that nestin-positive MSCs have acquired several, but not all, electrical characteristics of neuronal cells, as we never observed trains of action potentials or synaptic activities in co-cultured nestin-positive MSCs. Nevertheless, it is tempting to compare these modifications of the bioelectrical properties of MDN cells with the functional maturation of neuroblasts in adult animals. As described by Carleton et al. [33], normal maturation of young neurons born in the subventricular zone which migrate toward the olfactory bulb includes three subsequent stages: (1) the cells display neither Na^+ currents nor spikes; they have a low V_{rest} , and they respond to some neurotransmitters; (2) the cells show both Na^+ and K^+ currents and fire single spikes; they also exhibit a more negative V_{rest} ; (3) mature neurons fire trains of spikes and have strong synaptic activities together with a still very negative V_{rest} . We can thus speculate that, when co-cultured with CG neurons, MDN cells can reach the above-mentioned second stage of maturation but fail to achieve their maturation process, due either to a lack of the appropriate stimulating factors in this model or to an intrinsic inability.

During the past 2 years, several studies demonstrated that the acquisition by MSCs of a neural phenotype could actually be the result of a cell fusion process between a donor and

grafted MSC and a host and mature and already differentiated cell [8, 15, 16]. To characterize the mechanism of nestin-positive MSC differentiation when they are co-cultivated with mCG neurons, several sets of experiments were performed: (1) analyses of DNA content of MSCs and mCG neurons before and after the co-culture; (2) use of M2 and M6 markers [17]; and (3) culturing nestin-positive MSCs onto paraformaldehyde-fixed CG neurons and incubating them in CG-conditioned medium. Altogether, these results suggest that the mechanism of astrocyte-like and neuron-like cell appearance from the MSC population co-cultured with CG neurons more likely results in differentiation than in a cell fusion process. In this context, it is also noteworthy that the majority of MSC fusion-reported observations were made in vivo when cells were grafted in lethally irradiated rats. One could thus hypothesize that irradiation could induce genomic modification(s), which, in turn, could modify the membrane properties of the irradiated cells and, hence, favor fusion events.

In conclusion, nestin-positive MSCs should be regarded as a source of stem cells for cellular therapy in neurological diseases, but many more experimental studies are needed before using them in human graft protocols in order to secure a safe issue to the grafted patient. All of the experiments in this study were realized in vitro, and even if we demonstrated that a majority of nestin-positive MSCs differentiate into neural cells without fusion events, the cellular environment in vivo (without irradiation) could still modify the MSC behavior and induce fusion events. A better characterization of the molecular steps leading to a neural phenotypic plasticity of the MSCs is necessary. As human MSCs are easily available [34], obviously our results have to be repeated using those human MSCs.

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