Induction-dependent neural marker expression and electrophysiological characteristics of bone marrow mesenchymal stem cells that naturally express high levels of nestin

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Under certain experimental conditions, bone marrow mesenchymal stem cells (MSCs) express neuronal phenotypes and neuronal markers, which suggests that they could be used to treat various neurological diseases. In the present study, MSCs were isolated from adult rat bone marrow, cultivated, and evaluated for neurotrophin expression profiles, as well as the potential to differentiate into functional neuronal-like cells in vitro. MSCs from passage 5 were pre-induced with DMEM/F12 medium containing 10% fetal bovine serum (FBS) and 10 ng/mL bFGF (fibroblast growth factor-2). Subsequently, a chemical inductor containing Dimethyl Sulphoxide (DMSO), Butylated Hydroxyanisole (BHA) and forskolin were used to induce neural expression of MSCs. Expression patterns of nestin, NF-200, and GFAP at time points before and after induction were detected by immunofluorescence. Nerve Growth Factor (NGF), brain-derived neurotrophic factor (BDNF) expressions in MSCs were evaluated by RT-PCR. The whole-cell patch clamp technique was utilized to elucidate the electrical behavior of MSC before and after 24-h differentiation induction. Immunofluorescence analysis revealed that MSCs expressed nestin (57.1% ± 6.9%), but not NF-200 or GFAP. Following neural induction, the cells exhibited a neuronal-like appearance. Nestin and NF-200 expression was positive in the neuronal-like cells, but GFAP expression was negative. After 6-, 12- and 24-h induction, the ratio of nestin-positive cells was 96.5% ± 1.9%, 88.1% ± 5.4%, and 33.5% ± 5.4%. NF-200 positive cells were 90.1% ± 2.9%, 97.5% ± 1.3%, and 98.1% ± 1.6%, respectively. However, prior to induction, MSCs already expressed NGF and BDNF. With a stimulus impulse of 40 mV, the density of the transient outward K current was (9.95 ± 4.85) pA/pF (n = 9) and (328.50 ± 30.62) pA/pF (n = 9) before and after induction, and the density of transient calcium ion currents was (–0.059 ± 0.027) pA/pF (n = 7) and (–6.66 ± 0.50) pA/pF (n = 7), respectively. Transient outward potassium currents and calcium ions currents gradually increased following induction. In addition, MSCs isolated from bone marrow exhibited characteristics of neuronal progenitor cells and expressed neurotrophins. These cells exhibited the capacity to differentiate into functional neuronal-like cells in vitro. These results suggested that MSCs express high levels of nestin and could be utilized for therapeutic strategies to treat nervous system diseases.

mesenchymal stem cells; neuronal differentiation; nestin; electrophysiology

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Mesenchymal stem cells (MSCs) are tissue-specific stem cells that can self-renew, proliferate, and differentiate into mature lineages of bone, cartilage, adipose tissue, tendon, stroma and cornea [1–3]. Recent studies suggest that MSCs can overcome the barriers of germ layer commitment and differentiate into skeletal and cardiac muscle cells, hepatocytes, and neural cells under specific experimental conditions. In particular, MSCs have been reported to differentiate into neural cells in vitro [4–8] through the use of various chemical inductors, as well as in vivo following implanta-
tion into the brain and spinal cord [9–14]. However, differentiation under chemical induction, or in combination with cytokines, should be reconsidered. Morphological changes and expression of neural markers could be caused by cellular toxicity, cell shrinkage, and cytoskeletal changes [15–17]. Moreover, it is not clear whether these neuronal-like cells possess functional activities similar to neurons and glial cells. In addition, studies have suggested that in vitro differentiation of MSCs into neurons and glial cells could be simply cell fusion [18] or an artifact caused by transfer of donor cell markers [19]. Nevertheless, MSCs have been transplanted into rodent stroke and spinal cord injury models [20,21], as well as monkey models of disease and injury [22], demonstrating that grafted cells survive, proliferate, and migrate into damaged tissue to improve functional recovery. To further determine the differentiation potential of MSCs, rat primary MSCs (rMSCs) were tested in vitro using a combined induction method to analyze morphological, immunoochemical, and electrophysiological changes.

1 Materials and methods

1.1 Cell culture and identification analysis of rMSCs

Rat primary MSCs (rMSCs) were isolated according to previously described methods [23]. Briefly, 8- to 10-week-old Sprague Dawley (SD) rats were sacrificed, and femurs were removed. MSC culture media (5 mL) consisting of DMEM/F12 (Invitrogen, California, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Israel) and 100 U penicillin/100 U streptomycin was injected into the central canal of the bone to extrude marrow. Whole marrow cells were extracted, cultured at a density of $5 \times 10^5$ to $10^6$ cells/cm² in MSC culture media, and incubated at 37°C with 5% humidified CO₂. Nonadherent cells were removed after 48 h by media replacement. Media was replaced every other day, and when cells were 80% confluent, the MSCs were passaged at a ratio of 1:2 using 0.25% trypsin and 1 mmol/L EDTA, for a total of 4 times. The cells were then extracted, cultured at a density of 5 × 10⁵–10 × 10⁵ cells/cm² in DMEM/F12 supplemented with 2% DMSO (Sigma, USA), 10 μmol/L forskolin (Sigma, USA), 25 mmol/L KCl, 2 mmol/L valproicacid, and 5 μg/mL insulin. The cells were continuously monitored following neuronal induction and were fixed for immunofluorescence staining at specific time points. A non-induced culture dish was also analyzed with each experiment and served as the control.

1.3 Immunofluorescence

Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature and were washed 3 times in TBS buffer. The cells were then permeabilized in 0.3% Triton-X 100 for 2 min and washed 3 times in TBS buffer. Non-specific binding was blocked with 3% normal goat serum (NGS) in PBS, and the cells were incubated with the following primary antibodies: anti-nestin (1:400), NF-200 (1:400) and GFAP (1:200) (Chemicon, USA) overnight at 4°C, followed by fluorescent-labeled goat anti-rats secondary antibodies (C3, Chemicon, USA) for 1 h at room temperature. Nuclear counterstaining (Hoechst33342, Sigma, USA) was used to quantify total cell number. The cells were mounted with Fluorsavek (Calbiochem, La Jolla, USA) and were viewed under observed under an Olympus BX51 fluorescence microscope. Images were captured with a CCD camera connected directly to the system (Leica Microsystems, Heerbrugg, Switzerland).

1.4 RT-PCR Analysis

Total RNA was extracted using TRIzol method according to the manufacture recommendations (Invitrogen, USA) and as previously described [25]. A total of 1 μg total RNA was used as a template in a 20-μL cDNA synthesis reaction containing 0.2 μg random hexamer primer. cDNA was synthesized using 200 U RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) according to manufacture instructions. Two separate negative control reactions, without RNA or without M-MuLV, accompanied each reaction. PCR was performed using 1 μL of synthesized cDNA with 1.25 U Taq polymerase (Fermentas), 200 μmol/L dNTPs, 1.0 μmol/L each primer, 2.0 mmol/L MgCl₂, buffer supplied by the company, and deionized distilled water in a 50-μL total reaction volume. All reaction components were added to the master mix and aliquoted into tubes. Cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 32 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min, and a final extension of 72°C for 10 min. Each experiment was repeated at least 3 times to ensure reproducibility. Primers were designed as follows (5’→3’): NGFb (NGF), GCCCATGCGACTAAACTTCAAGC (Forward) and CCCTGACTGTGGTCTTTATCTTC (Reverse); BDNF, GGT- CACAGTCCTGGAGAAAG (Forward) and GTCTATCC- CACAGTCCTGGAGAAAG (Reverse); VEGF: GGACCGGTGC- TTTACTGCTGTACC (Forward) and TCACCGCCTTGGC-CACAGTCCTGGAGAAAG (Reverse); and TACCCGCTTGGC-
TTGTCACA (Reverse); β-actin: GAACCCTAAGGCCAACCC (Forward) and TGTCACGCACGATTTC (Reverse).

1.5 Patch-clamp recordings

For potassium channels, patch-clamp recordings of MSCs were performed before, 12 h, and 24 h after differentiation induction according to a previously described protocol [26]. Extracellular solution contained 136 mmol/L NaCl, 5.4 mmol/L KCl, 2 mmol/L CaCl2, 1 mmol/L MgCl2, 10 mmol/L HEPES, and 10 mmol/L glucose (pH 7.2, adjusted with NaOH). Electrodes with 8-mV resistance contained 140 mmol/L KCl, 10 mmol/L HEPES, 10 mmol/L EDTA, and 5 mmol/L Na2ATP (pH 7.2, adjusted with KOH). Current was recorded with an Axopatch 2B amplifier (Axon), and data acquisition and pulse sequence analysis were performed with pCLAMP software suite (Version 8.0, Axon). Data acquisition was initiated when the whole-cell patch was formed and stable; the holding potential was set to –80 mV and different depolarizing potentials (with 10-mV steps and 6-s intervals, each lasting for 100 ms) were attempted (each lasting for 20 ms) to evoke K+ ion channel opening. For calcium channels, patch-clamp recordings of MSCs were performed before, 12 h, and 24 h after differentiation induction. Extracellular solution contained 136 mmol/L NaCl, 5.4 mmol/L KCl, 2 mmol/L CaCl2, 1 mmol/L MgCl2, 10 mmol/L HEPES, and 10 mmol/L glucose (pH 7.2, adjusted with NaOH). Electrodes with 8-mV resistance contained 140 mmol/L KCl, 10 mmol/L HEPES, 10 mmol/L EDTA, and 5 mmol/L Na2ATP (pH 7.2, adjusted with HCL). Electrodes with 8-mV resistance contained 100 mmol/L CsCl, 10 mmol/L HEPES, 10 mmol/L EDTA, and 5 mmol/L Na2ATP (pH 7.2, adjusted with Tris). Data acquisition was initiated when the whole-cell patch was formed and stable; holding potential was set to –80 and –40 mv. A 40-ms impulse was used to inactivate the Na ion channel, and depolarizing potentials evoked Ca2+ ion channel opening.

1.6 Statistical analysis

Data were exported to Microsoft Excel or SigmaPlot software for statistical analysis. All results are presented as mean ± SE. Statistical tests were performed using either Student’s t-test or Mann Whitney rank sum test as appropriate.

2 Results

2.1 MSC culture and characterization

rMSCs were isolated from bone marrow and were allowed to adhere to plastic culture plates. Primary cultures reached 90% confluency in 9–12 d and were passaged. After 5 passages, a stable fibroblast-like MSC morphology was observed (Figure 1(a)). Flow cytometry analysis revealed that BMSCs from 5–7 passages strongly expressed CD29 and CD44, but did not express CD34 or CD45 (Figure 2).

2.2 Neural differentiation and morphological changes

Neural differentiation of rMSCs was induced from passage 5 onward. After exposure to NIM for 30 min, MSCs exhibited the expected morphological changes into typical neurons. The cell body became smaller, the cytoplasm shrank and the shape changed from a spindle to a round form, the refractive index increased, and the cells contained process-like extensions. At 24 h after NIM treatment, 95% cells exhibited typical neuronal morphology (Figure 1(b)).

2.3 Nestin expression changes following induction

Nestin expression, as a primary marker suggesting neuronal potential, was analyzed in untreated and NIM-treated MSCs. Immunofluorescence analysis demonstrated that 58.1% ± 9.4% of untreated cells expressed nestin (Figure 3(a)), accounting for 90.5% ± 1.9%, 83.1% ± 5.4%, and 33.5% ± 5.4% nestin-positive cells after 6-, 12-, and 24-h induction, respectively (Figure 3(b)–(d))

2.4 Neuronal differentiation was induced in expression of the neuronal marker neurofilament 200 kDa in MSCs

Differential induction is equal to method 1.2 In vitro neuronal induction. Morphological changes and nestin expression could be coincidental and not necessarily correlate with a neural phenotype. Therefore, these results were verified by quantifying expression of neuronal markers. Neuronal cells are classically characterized by expression of cytoskeletal proteins, such as neurofilament 200 kDa (NF-200). Immunofluorescence analysis showed that the rate of NF-200-positive cells increased in the MSC population to 90.1 ± 2.9%, 97.5 ± 1.3%, and 98.5% ± 2.8% in treated cultures after 6, 12, and 24 h (Figure 4). NF-200 proteins were detected in dotted structures around the nucleus in most cells or throughout the entire cell body, including neurite extensions. However, astrocytic (GFAP) and oligodendrocytic markers (MBP) were not expressed.

2.5 RT-PCR analysis

The expression profile of NTs and VEGF were further characterized in undifferentiated MSCs. RT-PCR analysis results revealed that MSCs express NGF, BDNF, and VEGF prior to neural induction (Figure 5).

2.6 Electrophysiology

Using the patch-clamp technique, the density of transient outward K current at 40 mV was (9.95 ± 4.85) pA/pF (n = 9) and (328.50 ± 30.62) pA/pF (n = 9) before and after induction (Figure 6). In addition, density of transient calcium ion currents at 40 mV was (–0.059 ± 0.027) pA/pF (n = 7)
and \((-6.66 \pm 0.50\) pA/pF \((n = 7)\), respectively (Figure 7). According to time, transient outward potassium and calcium ions currents gradually increased, which suggested that MSCs could become functional neurons following induction with DMSO, BHA, and forskolin.

### 3 Discussion

Cellular therapies utilizing stem cells are promising approaches for the treatment of certain central nervous
system injury diseases, such as stroke [9–11] or spinal cord injury [13], and MSCs appear to be good candidates for these procedures. However, before these cells are used to treat neurological disorders, the underlying phenotypic plasticity of MSCs needs to be more fully investigated. Therefore, the present study tested the possibility of inducing MSCs into functional neurons using a combined method. Two major issues were addressed in the study: (i) Neural marker expressions; and (ii) electrophysiological characteristics of undifferentiated and differentiated MSCs. The current methods of MSC induction to differentiated neurons utilize a variety of cytokines, gene transfection, and chemical inducers. However, both cytokines and gene transfection methods have limitations: These methods require a long induction time and the neuron ratio in differentiated MSCs is low [4,5]. The present study used the combination of bFGF, BHA, DMSO, and forskolin to induce MSCs. After 1-h induction, 95% MSCs differentiated into cells with typical neuronal morphology, which was consistent with previous results [6].

Nestin is considered to be a specific marker of neural stem cells (NSCs) and progenitors [27,28], and the presence of nestin expression in untreated MSCs suggested that these cells exhibited the potential to differentiate into neural cells under appropriate induction. Nestin expression decreases during differentiation and maturation of neural stem cells. In the present study, nestin expression was transiently increased after 6-h induction and decreased with further induction, which was similar to NSC differentiation. NF-200 is a marker for mature neurons and, under normal circumstances, it is only expressed in the axon; the cell body contains very little or no NF-200 [29]. The present results demonstrated and increased rate (90%) of NF-200-positive cells in MSCs after 6-h induction, demonstrating that MSCs gradually differentiated to mature cells following induction. GFAP is a specific astrocytic marker, and no GFAP-positive cells were detected in this experiment, which suggested that this induction process induces MSC differentiation into neurons, but not glial cells. It is worth noting the co-existence of nestin and NF-200 starting at 6-h induction, suggesting cross-expression of early and delayed marker proteins in neuronal development. However, the use of one marker could be used to determine whether MSCs were fully differentiated.

Similar to previous studies [30,31], NGF, BDNF and VEGF were expressed by untreated MSCs. NGF and BDNF secretion plays an important role in survival and maturation of transplanted MSCs and also improves functions in host neurons. In addition, NGF and BDNF could be important for the survival of induced neuronal-like cells. Results from the present study demonstrated that some MSCs differentiated into excitable neuronal-like cells. Previous studies have
confirmed that MSCs can be induced to express neural markers and exhibit a neuronal appearance under specific induction conditions [4–8]. However, a normal mature neuron is not only defined by these characteristics, but more importantly by electrophysiological properties and functional activity. In the present study, a weak transient outward K current was recorded in the untreated MSCs, and current significantly increased in differentiated neurons derived from MSCs. From the current-voltage curve, it was possible to determine that transient outward K current was activated at −40 mV with a strong outward rectification, which was similar electrophysiological characteristics of neurons derived from embryonic [32] and neural stem cells [33]. Similar electrophysiology characteristics have been observed in neurons derived from MSCs, although induction procedures were complicated [34] or results were obtained by co-culturing with cerebellar granule neurons [35]. Calcium ions currents in untreated and neural differentiated MSCs were also measured, because Ca2+ signal transduction was thought to play an important role in excitability following MSCs differentiation [36]. L-type calcium ion currents were recorded in MSCs-derived neuronal-like cells at 24 h after induction, suggesting that these cells were excitable. To further evaluate excitability of MSCs-derived neurons, functional neurotransmitter receptors in these cells should be determined in future studies.

In conclusion, these results confirmed the possibility of functional neuronal differentiation in MSCs through the use of a combined induction method. MSCs in culture expressed the neural stem cell marker nestin, as well as the neurotrophins NGF and BDNF prior to induction. Following induction, the transdifferentiated MSCs developed neuronal-like morphology, expressed the mature neuronal marker NF-200, and exhibited excitable electrophysiological characteristics. Although these results should be interpreted with caution and the function of MSC-derived neurons should be further investigated in vivo, this study suggests that this induction method provides a process for obtaining MSC-derived neurons for cell-based therapies for treating nervous system disorders.

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