Plasticity of Human Mesenchymal Stem Cell Phenotype and Expression Profile under Neurogenic Conditions

A. V. Shakhbazov, N. V. Goncharova*, S. M. Kosmacheva*, N. A. Kartel', and M. P. Potapnev*

Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 2, pp. 77-80, 2009
Original article submitted June 25, 2008

Human bone marrow MSC cultured in neurogenic medium containing EGF and FGFb demonstrated alteration of the phenotype and expression of neuronal precursor/early neuron markers nestin and NSE. Signals of expression of neuronal and oligodendroglial markers MAP-2, dm-20, and MBP were detected after prolongation of incubation in neurogenic medium to 2 weeks. Cells with neuronal morphology were immunopositive to early neuronal marker β-III-tubulin. Replacement of neurogenic medium for α-MEM with 10% fetal calf serum induced reversion of the phenotype to that typical for human MSC. This indicates high plasticity of the phenotype and expression profile of neuronal markers in MSC cultured under neurogenic conditions or possibility of dedifferentiation of MSC reaching the stage of neuronal precursors/early neurons.

Key Words: *mesenchymal stem cells; neuronal differentiation*

MATERIALS AND METHODS

In order to isolate MSC, aspirated bone marrow was centrifuged in Ficoll density gradient, the mononuclear fraction was collected, washed twice in PBS, and inoculated in α-MEM with 10% FBS (HyClone) [1]. MSC selected by adhesion to plastic were immunotyped as $CD90^+$, $CD105^+$, $CD45^-$, $CD34^-$. The cells were cultured in a CO_2 incubator (5% CO_2) at 37°C; the medium was replaced every other day. In experiments with neurogenic differentiation, MSC were cultured in NeuroCult proliferative medium (Stemcell Tech) with 20 ng/ml EGF, 10 ng/ml FGFb, and 10 ng/ml heparin.

RNA was isolated from the cells using TRIreagent (Sigma) according to the instruction. Complementary DNA (cDNA) was synthesized using RevertAid kit (Fermentas). The expression of neuronal markers was analyzed using combinations of primers [2-6] (Table 1).

The PCR was carried out in buffer containing 40 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 6% sucrose with 0.02 mmol/liter of each dNTP, 1 μmol/liter of each primer, 1 μg cDNA, and 1 U Taq-polymerase (Primetech). Amplification protocol was as follows: 5 min at 95°C, (30 sec at 95°C, 30 sec at 55-60°C, 1 min at 72°C)×35, and 5 min at 72°C. Amplification products were separated in 1.5% agarose gel with ethidium bromide (stain). The results were recorded using a GelDoc 2000 system (BioRad).

For immunocytochemical analysis, MSC were fixed for 30 min in 4% paraformaldehyde in PBS, washed 3 times in PBS, and permeabilized (10 min) in 0.3% Triton X-100 in PBS, after which the cells were washed in PBS and incubated for 30 min in blocking solution. Then primary antibodies to β-III-

Institute of Cytology and Genetics, National Academy of Sciences of Belarus; *Center of Hematology and Transfusiology, Minsk, Belarus. ***Address for correspondence:*** shakhbazau@gmail.com. A. V. Shakhbazov

0007-4888/09/1474-0513

tubulin (1:1000) were added and the mixture was incubated for 12 h at 4°C. After incubation, MSC were washed in PBS and incubated with the second antibodies labeled with Texas Red (1:100). Fluorescence was detected in a Carl Zeiss Axio Imager M1 microscope. The percentage of β-III-tubulin-positive cells was evaluated on a FACScan flow cytometer (BD).

RESULTS

After 2-3 passages of culturing of bone marrow MSC from 5 donors under neurogenic conditions, a part of cell population acquired morphological features of early neurons (Fig. 1). Immunocytochemical analysis showed that cells with neuronal morphology interacted with antibodies to β-III-tubulin (early neuronal marker; Fig. 1). Screening of the populations by flow cytometry showed that the content of β-III-tubulin-positive cells was 18.3±6.5%.

Molecular genetic analysis of MSC after one-week incubation in NeuroCult medium with EGF and FGFb showed activation of the expression of nestin and NSE, markers of neuronal precursors and early neurons, as well as of the early oligodendroglial marker MBP (Fig. 2). After a longer incubation (2 weeks), slight signals of MAP-2 (postmitotic neuron marker) and dm-20 (oligodendrocytic marker) were detected. The expression of Olig2, plp, TH, AADC, GFAP, and NFM (mature neuron markers) was not detected under these experimental conditions (PCR data not presented), because these conditions do not provide terminal maturation of neurons, astrocytes, and oligodendrocytes. Resident glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as the marker of cDNA presence.

The data of RT-PCR and immunocytochemical analysis indicate that bone marrow MSC, cultured under neurogenic conditions, can acquire signs of early neuronal differentiation. However, replacement of the neurogenic medium with αMEM with 10% FBS resulted in a rapid (3-5 days) reversion of the phenotype and restoration of the common human MSC phenotype. The expression of early and progenitor markers nestin, NSE, and MBP in this case persisted for at least 10 days. No expression of MAP-2, dm-20, or other above-mentioned markers was detected in reversed MSC. Immunocytochemical analysis of reversed cells showed slight residual reaction to β-III-tubulin (Fig. 1).

The findings of different studies indicate the possibility of obtaining neuronal precursors with subsequent differentiation into various nervous tissue subtypes (neurons, astrocytes, oligodendrocytes) [2,7,8]. On the other hand, some authors indicate potential causes of false-positive results in attempts at neurogenic differentiation of MSC [9,

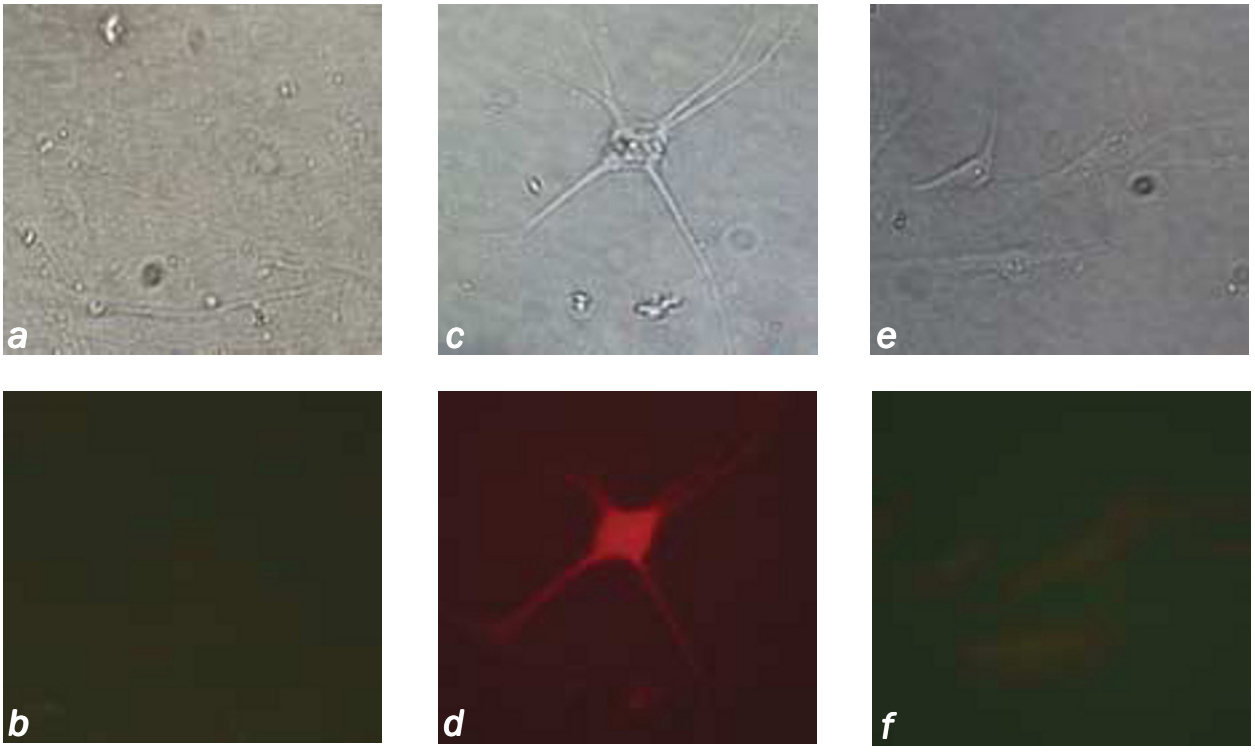

Fig. 1. Phenotype of MSC and staining with antibodies to β-III-tubulin. *a*, *b*) control; *c*, *d*) MSC in neurogenic medium; *e*, *f*) MSC in reversion. *a c*, *e*: visible light; *b*, *d*, *f*: fluorescence.

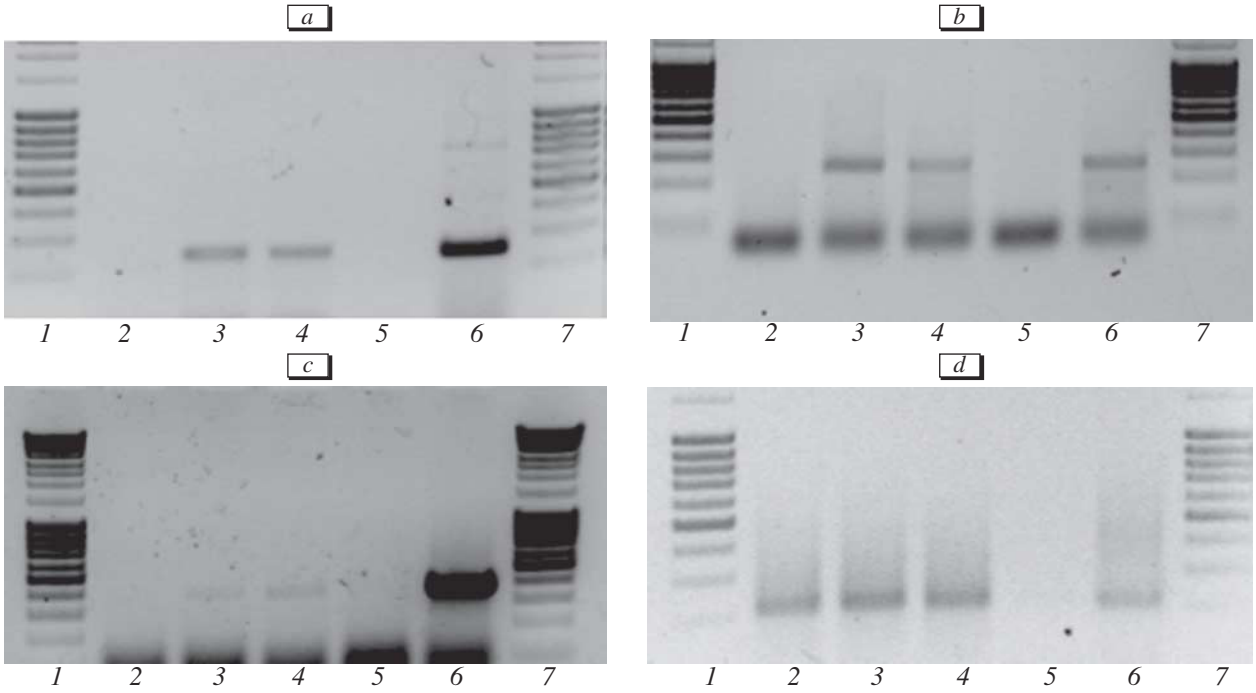

Fig. 2. RT-PCR analysis of expression of nestin (*a*), NSE (*b*), MBP markers (*c*), and resident GAPDH gene (*d*). *1*, *7*) MassRuler molecular weight marker (Fermentas); *2*) control MSC; *3*) MSC in neurogenic medium; *4*) MSC in reversion; *5*) MilliQ water; *6*) positive control (cDNA from human cerebral subventricular zone).

TABLE 1. Combinations of Primers

Marker	Primer sequences	Fragment
Nestin	5'-AGGATGTGGAGGTAGTGAGA-3' 5'-TGGAGATCTCAGTGGCTCTT-3'	251 n. p.
NSE	5'-CCCACTGATCCTTCCCGATACAT-3' 5'-CCGATCTGGTTGACCTTGAGCA-3'	254 n. p.
MBP	5'-TTAGCTGAATTCGCGTGTGG-3' 5'-GAGGAAGTGAATGAGCCGGTTA-3'	379 n. p.
NF-M	5'-GAGCGCAAAGACTACCTGAAGA-3' 5'-CAGCGATTTCTATATCCAGAGCC-3'	430 n. p.
MAP-2	5'-TCAGAGGCAATGACCTTACC-3' 5'-GTGGTAGGCTCTTGGTCTTT-3'	321 n. p.
Olig2	5'-GCTGTGGAAACAGTTTGGGT-3' 5'-AAGGGTGTTACACGGCAGAC-3'	291 n. p.
TH	5'-TGTCAGAGCTGGACAAGTGT-3' 5'-GATATTGTCTTCCCGGTAGC-3'	298 n. p.
Plp, *dm-20*	5'-CCATGCCTTCCAGTATGTCATC- 3' 5'-GTGGTCCAGGTGTTGAAGTAAATGT- 3'	*plp* 354 n. p. *dm-20* 249 n. p.
GFAP	5'-GATCAACTCACCGCCAACAGC-3' 5'-CTCCTCCTCCAGCGACTCAATCT-3'	207 n. p.
AADC	5'-CTCGGACCAAAGTGATCCAT-3' 5'-GGGTGGCAACCATAAAGAAA-3'	252 n. p.
GAPDH	5'-CTGCTTTTAACTCTGGTAAAGT-3' 5'-GCGCCAGCATCGCCCCA-3'	197 n. p.

10]. Our data indicate high plasticity of the phenotype and expression profile of neuronal markers in MSC cultured under neurogenic conditions and suggest the probability of dedifferentiation of MSC reaching the stage of neuronal precursors/early neurons after removal of the neurogenic factors.

The importance of evaluation of the neuronal phenotype stability is explained by the fact that MSC differentiation to the stage of neuronal precursors/early neurons is more attractive from the viewpoint of possible therapeutic application, while induction of *in vitro* terminal maturing of neurons can negatively affect their capacity to transplantation and subsequent viability. The development and introduction of methods of neurogenic differentiation will make it possible to produce populations of neuronal precursors and later mature neurons and glia cells from patient's bone marrow MSC, which will essentially extend potentialities of cell therapy of neurodegenerative diseases and traumas of the nervous system.

REFERENCES

1. T. Ben-Hur, M. Idelson, H. Klaner, *et al.*, *Stem Cells*, **22**, No. 7, 1246-1255 (2004).
2. C. Benzinga, M. Segschneidera, A. Leinhaasa, *et al.*, *Neuroreport*, **17**, No. 16, 1675-1681 (2006).
3. N. Bertani, P. Malatesta, G. Volpi, *et al.*, *J. Cell Science*, **118**, Pt. 17, 3925-3936 (2005).
4. Y. Chen, F. Y. Teng, and B. L. Tang, *Cell Mol. Life Sci.*, **63**, No. 14, 1649-1657 (2006).
5. R. I. Freshney *et al.*, *Culture of Human Stem Cells*, Hoboken, USA (2007).
6. A. Hermann, R. Gastl, S. Liebau, *et al.*, *J. Cell Sci.*, **117**, Pt. 19, 4411-4422 (2004).
7. S. Kim, O. Honmou, K. Kato, *et al.*, *Brain Res.*, **1123**, No. 1, 27-33 (2006).
8. V. G. Kukekov, E. D. Laywell, O. Suslov, *et al.*, *Exp. Neurol.*, **156**, No. 2, 333-344 (1999).
9. X. Long, M. Olszewski, W. Huang, and M. Kletzel, *Stem Cells and Development*, **14**, 65-69 (2005).
10. A. L. Vescovi, E. A. Parati, A. Gritti, *et al.*, *Exp. Neurol.*, **156**, No. 1, 71-83 (1999).