Analysis of Differentiation of Stem/Progenitor Cells in Tissue Culture of Neocortical Primordium of Mouse Brain

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Differentiation of neural stem/progenitor cells from neocortical primordium of the brain from 14-day mouse embryos was studied by immunohistochemical methods during their culturing. Non-differentiated cells expressing nestin and vimentin persisted in freely floating neurospheres throughout the experiment. Glioblasts, neuroblasts, and differentiated neurons were found in neurospheres cultured in differentiating medium. However, neurons disappeared with increasing the number of passages, the formation of neuroblasts was terminated, and only astrocytes and nestin-positive cells were seen in the culture. It was found that cells of mouse embryonic neocortex lose the capacity for spontaneous multipotent differentiation during culturing.

Key Words: *neural stem and progenitor cells; immunohistochemistry; neurospheres*

Stem cells (SC) occupy special place in neurobiology. The progress in the study of SC biology is associated with the development of methods of their isolation and culturing. SC actively proliferate in serum-free media in the presence of growth factors and form freely floating colonies, neurospheres [15]. The culture of neurospheres is used for the growth of SC and progenitor cells isolated from embryonic or adult mammalian brain. These cultures are important for fundamental studies in the field of cell differentiation and further clinical applications [5,7].

Neurospheres are interesting and convenient model for the study of early stages of neuroontogenesis; however, we should understand general regularities of this biological system. Cultures of neurospheres were studied by various methods: cytometry, immunohistochemistry, genetic methods, and electron microscopy [3,10,11,17-19]. These studies showed that cell composition of neurospheres is heterogeneous: they contain SC and progenitor cells expressing nestin and vimentin and cells with signs of neuronal and glial differentiation. During spontaneous differentiation, SC in neurospheres form neuroblasts, the greater part of their population is presented by glutamate decarboxylase-immunopositive cells, the minor part is presented by tyrosine hydroxylase-positive cells.

Only few works studied freely floating neurospheres as a biological system [20]. An important question is how long cells in neurospheres retain the capacity to multipotent differentiation. Known studies have considerable methodical differences and inaccuracies impeding unambiguous interpretation of the results. For instance, cell differentiation into neurons, oligodendrocytes, and astrocytes only after single passage are often reported [12]. Only few studies evaluated the dynamics of the expression of differentiation markers over several passages [13,16]. All authors reported that the ratio of neurons to glial precursors decreased in each pas-

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sage. Another problem is regional specificity of SC differentiation.

Here we studied differentiation and behavior of cultures of neuronal SC/progenitor cells isolated from the cortical part of mouse embryonic brain, compared the results of cell differentiation in culture with differentiation of cells in native neocortex, and chose optimum conditions for the growth of neurospheres.

MATERIALS AND METHODS

Neural SC/progenitor cells were isolated from cortical parts of the brain of 14-day-old mouse embryos [14]. The embryos obtained after laparotomy were placed into Perti dish with culture medium, the cortical fragments were surgically prepared, minced, placed in accutase, and pipetted. The cells collected after centrifugation were placed into culture flasks with growth medium: DMEM/F12 (1:1 mixture; Gibco) supplemented with epidermal growth factor (20 ng/ml Calbiochem) and basic fibroblast growth factor (20 ng/ml Calbiochem), HEPES (5 mM, Gibco), L-glutamine (2 mM), N2 (Gibco), and heparin (5 mg/ml, Sigma). In some experiments, leukemia inhibitory factor (10 ng/ml, Sigma) was added. The neurospheres were subcultured every 3-4 days. To this end they were collected, placed in accutase for 10-15 min, gently pipetted, and the suspension was placed into fresh medium. For differentiation induction, whole neurospheres in DMEM/F12 mixture containing 10 or 1% fetal calf serum (differentiating medium) were placed onto clean or laminin-coated coverslips (Gibco).

For immunohistochemical analysis, neurospheres (freely floating and differentiated) from the primary cell suspension (zero passage) and cultures after three subsequent passages were fixed. Spheres incubated in differentiating medium were fixed after different time intervals. Mouse embryos, neurospheres, or cells on coverslips were fixed with 4% formaldehyde (Sigma). Cryostat sections of the brain were mounted on gelatin-coated slides. The sections and cells were washed with 0.01 M PBS (pH 7.4) and incubated with primary antibodies (overnight at 4°C). We used primary antibodies to mouse nestin (1:100-1:200; Chemicon), vimentin (1:6; Chemicon), β-tubulin-III (1:200; Chemicon), glial fibrillary acidic protein (GFAP, 1:200-1:300; Chemicon), calbindin (1:200; Chemicon), neurofilament proteins (1:10; ICN), GAD (glutamic acid decarboxylase; 1:200; Chemicon), NeuN (1:1000; Chemicon), N-cadherin (1:100; Sigma), connexin-43 (1:200; Sigma), and tyrosine hydroxylase (1:200; Sigma). On the next day the preparations were washed and incubated with second antibodies for 2-3 h at room temperature, then washed again and embedded in glycerin. Goat polyclonal antibodies to rabbit and mouse immunoglobulins labeled with fluorochromes Cy2 and Texas Red (dilution 1:100, Jackson) were used as second antibodies. Nuclei were stained with Hoechst 33342.

RESULTS

The dynamics of differentiation of neocortical cells in tissue culture was compared with cell differentiation during the development of intact brain cortex in mouse embryos. On day 14 of embryonic development, the neocortex primordium was presented by ventricular, subventricular, and intermediate zones. Practically all cells in these structures were vimentin-immunopositive. Nestin-positive cells with long processes directed towards subventricular and intermediate zones were located near the cerebral ventricles (Fig. 1, *a*). Neuroblasts actively expressed β-tubulin-III. Tight junctions stained with antibodies to N-cadherin were detected in all cells, while connexin-43 was expressed only in meningeal cells.

GFAP-positive astrocytes were not found on brain sections, because they appeared at later terms of embryonic development. No markers of mature neurons (GAD- and NeuN-positive cells, neurofilament fibers, calbinding-immunopositive cells) were found in forebrain. Fibers of tyrosine-hydroxylase-positive cells were clearly seen (their bodies were located in the brainstem).

The cells isolated from presumptive neocortex of 14-day mouse embryos formed floating neurospheres after 1-2-day culturing in a medium with epidermal growth factor and fibroblast growth factor. Cell proliferation in neurospheres leads to their enlargement and, if neurospheres are not passaged, central cells start to die. Neurospheres, freely floating structures, sometimes attach to the bottom of the culture flask and some cells migrate from them. In our experiment, neurospheres were passaged when they attained a diameter of 0.9-1.6 mm. Some floating neurospheres were fixed, some were transferred into differentiating medium and then fixed, and others were disintegrated and passaged.

Immunochemical staining showed that floating neurospheres included cells at different stages of differentiation. They primarily expressed nestin and vimentin, proteins of SC and progenitor cells (Fig. 1, *b*), irrespective of the time of culturing and the number of passages. Cells differentiating into neurons and glia and expressing β-tubulin-III, calbinding, and GFAP were found in some neurospheres,

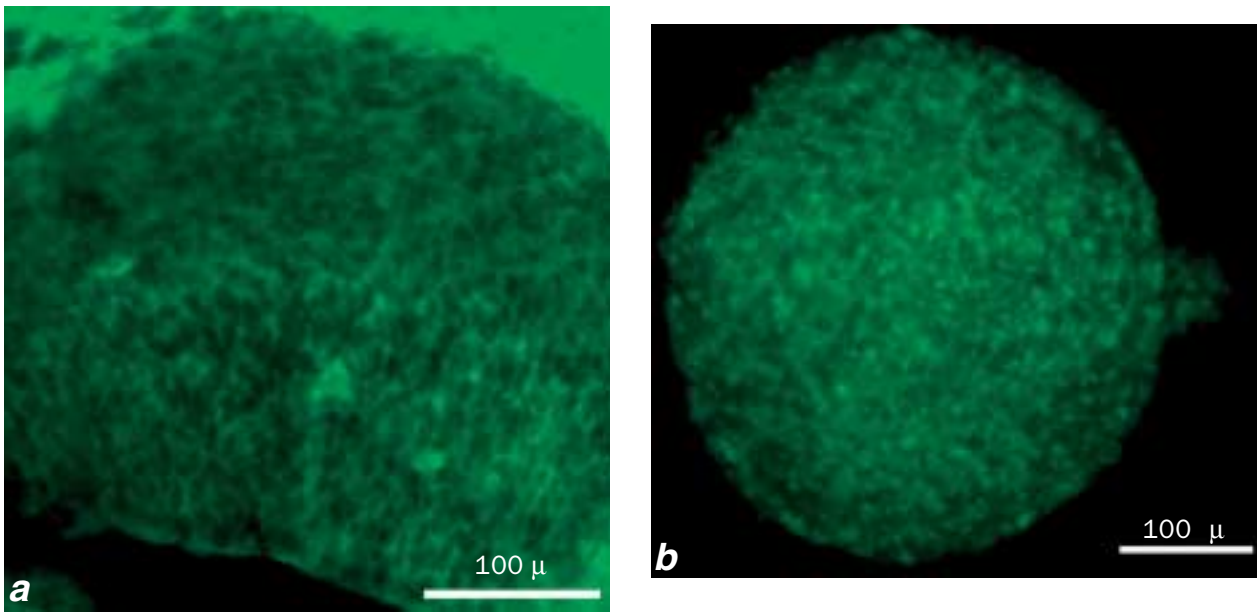

Fig. 1. Expression of nestin in native brain and in neurospheres. *a*) section of mouse neocortical primordium on day 14 of embryonic development; *b*) neurospheres in a medium with growth factors, passage 1.

but expression of these markers correlated with sphere diameter and number of passages. In large neurospheres (2.5-5.1 mm) of zero and first passages, networks of GFAP-positive and clusters of β-tubulin-III-positive cells were seen, in medium-diameter neurospheres (0.9-1.6 mm) only solitary GFAP-positive and β-tubulin-III-positive cells were seen, and in small neurospheres (0.2-0.7 mm) these cells were practically absent and these neurospheres contained only nestin- and vimentin-expressing cells. Markers of mature neurons (NeuN, neurofilament proteins, GAD, tyrosine hydroxylase) were not detected in floating neurospheres.

The profile of cell differentiation in floating neurospheres changed with increasing the number of passages. Most cells expressed nestin and vimentin, intermediate filament proteins, throughout the culturing period, whereas β-tubulin-III and GFAP-positive cells were detected after zero and especially after the first passages, but then expression of β-tubulin-III and GFAP disappeared, and only undifferentiated nestin-positive cells were detected (Fig. 2).

The differentiation pattern of cells in neurospheres sharply changed when the cells were placed in a serum-free medium containing no growth factors. The neurospheres sank and migration and differentiation of cells started; under these conditions GFAP, astrocytes marker, was most actively expressed. As soon as after 2 days, almost all migrating cells were stained with antibodies to GFAP.

In parallel with GFAP, the cells at the initial stages of their migration from neurospheres actively expressed nestin and on day 2 of culturing in differentiating medium many cells were nestin-positive. However, expression of this marker decreased with time and it was detected primarily in processes of cells in the middle of the migration wave and in the bodies of peripheral cells.

At the initial stages, β-tubulin-III-positive cells (neuroblasts) were detected only in settled spheres, while migrating population contained only few β-tubulin-III-positive cells above GFAP-positive cells. The number of neuroblasts in differentiating medium increased only after 6 days. Clusters of these cells were seen on the surface of glioblasts. Only on day 9, neuroblasts formed an extensive network in the layer of migrating cells.

During this period, more differentiated neurons were also detected in the culture. NeuN-immunopositive cells formed large clusters in dense part of neurospheres and small groups in the monolayer. GAD-positive neurons had long processes forming a network on the surface of underlying cells. Antibodies to neurofilament proteins stained long cell processes collected in bundles, which were seen in sites where cells slid down from neurospheres attached to the substrate. Calbindin-positive cells were located inside the spheres starting from the initial terms of fixation of the material and on day 9 they appeared among migrating cells and were presented by solitary cells with long processes. Neurons expressing tyrosine hydroxylase were not found in attached neurospheres.

Hence, cells of neurospheres in the presence of factors of differentiating medium differentiated into neuroblasts and then into neurons with time inter-

vals similar to those in the native brain. Gliogenesis was associated with the expression of GFAP and was considerably accelerated.

For evaluation of cell—cell interaction in settled spheres we used antibodies to connexin-43 and N-cadherin, cell contract proteins. In contrast to organization of cells in native brain, adherent cultures contained more gap junctions (staining for connexin-43), while N-cadherin (tight junction proteins) was expressed by only few cells forming clusters primarily at the periphery.

Passaging of neurospheres changed differentiation capacity of cells in the serum-supplemented medium. Zero-passage neurospheres contained cells at different stages of differentiation: nestin- and β-tubulin-III-positive cells and mature neurons expressing NeuN, GAD, neurofilaments, and calbindin. Mature neurons disappeared after passage 1 and neuroblasts predominated, but their number considerably decreased after passage 2. After zero and first passages, groups and solitary neuroblasts were presented by small bipolar cells forming networks in the adherent culture. After passage 2, only solitary cells with long branching processes were seen in the culture.

The number of glioblasts (GFAP-positive cells) increased with increasing the number of passages (in contrast to β-tubulin-III-positive neuroblasts). During passages 2 and 3, cells expressing GFAP predominated in cultures, nestin-positive cells were still present, but neuroblasts were practically absent. These data suggest that SC of the neocortical primordium lose the capacity for spontaneous neuronal differentiation during culturing (Fig. 3).

In our experiments we chose optimum conditions and parameters of nutrient medium for culturing of SC and progenitor cells of mouse neocortex and their further differentiation. For evaluation of changes we compared the rates of differentiation and profiles of expression of the major early differentiation antigens nestin, β-tubulin-III, and GFAP.

We analyzed the behavior of cells in neurospheres in media with different serum concentration (10 and 1%). To this end, neurospheres after passage 1 were transferred onto laminin-coated slides and fixed after 2 days. In both cases, the majority of cells inside neurospheres and in migrating layer expressed nestin. In the medium with high serum concentration, cell migration and formation of neuroblasts were more rapid: we observed a large pool of cells inside neurospheres and many cells with long processes in the layer. In the medium with low serum concentration, all β-tubulin-III-expressing cells were primarily located in neurospheres, they had neuronal morphology and their long processes stretched far beyond the settles spheres. The layer contained solitary neuroblasts with short nonbranched processes.

Serum concentration in the medium considerably modulated the behavior of cells in the migra-

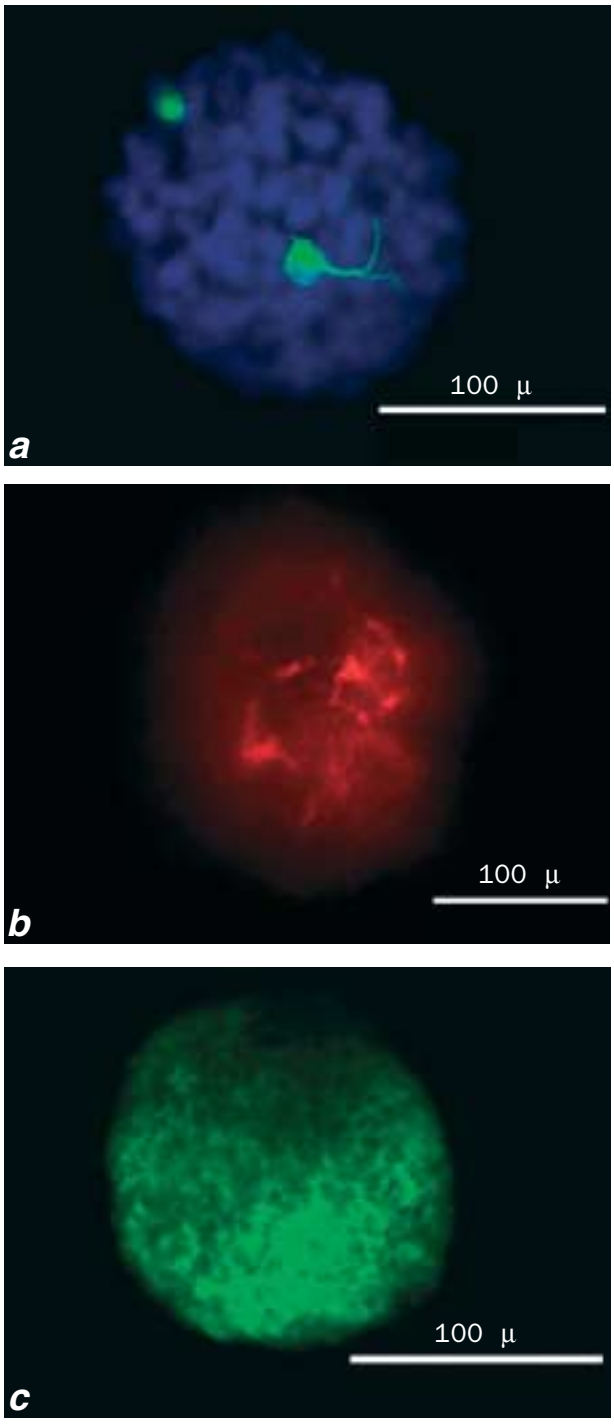

Fig. 2. Expression of differentiation markers in floating neurospheres in different passages. *a*) passage 1: neuroblasts stained with antibodies to β-tubulin-III, nuclei are poststained with Hoechst 33342, *b*) passage 1: astrocytes stained with antibodies to GFAP; *c*) passage 3: staining with antibodies to nestin.

tion front. In the medium with high serum concentration, cell formed broad lamellae yielding a regular front at the boundary with cell-free space, while in the medium with low serum concentration migration was irregular. A similar picture was observed after sliding of cells from spheres. In the medium with 10% serum, migration of GFAP-positive cells is an ordered process; the cells were bound to the neurosphere via a long process. Then they detached from the sphere and acquired a fibroblast-like shape. In the medium with 1% serum, the cells retained their processes and migrated chaotically.

When studying the effect of substrate on cell migration and differentiation we found that cells from neurospheres slowly migrated on clean slide, expression of nestin was discontinued (though initially all cells in suspended neurospheres were nestin-immunopositive), while expression of GFAP started. β-Tubulin-III-positive cells formed small clusters in settled spheres, solitary cells were sometimes seen in the layer of migrating cells. Cells from neurospheres more rapidly migrated on laminin-coated slides, which agreed with previous findings [8]. The number of β-tubulin-III-positive cells increased under these conditions; they were located in spheres and formed a net in the layer of migrating GFAP-positive and nestin-positive cells. Most cells under these conditions were nestin-positive, whereas cells cultured without laminin coating lost this property. It can be hypothesized that laminin is a factor maintaining cells in undifferentiated state.

Analysis of growth dynamics of floating neurospheres in the medium containing different growth factors showed that the combination of epidermal growth factor and basic fibroblast growth factor is required for culturing of neocortical primordium, which agrees with previous findings [3]; the cells died and even medium-size neurospheres were not formed in the presence of only one factor. Culturing of neurospheres in the presence of both growth factors and leukemia inhibitory factor provided no advantages. Thus, the presence of two growth factors and 10% serum in the differentiating medium,

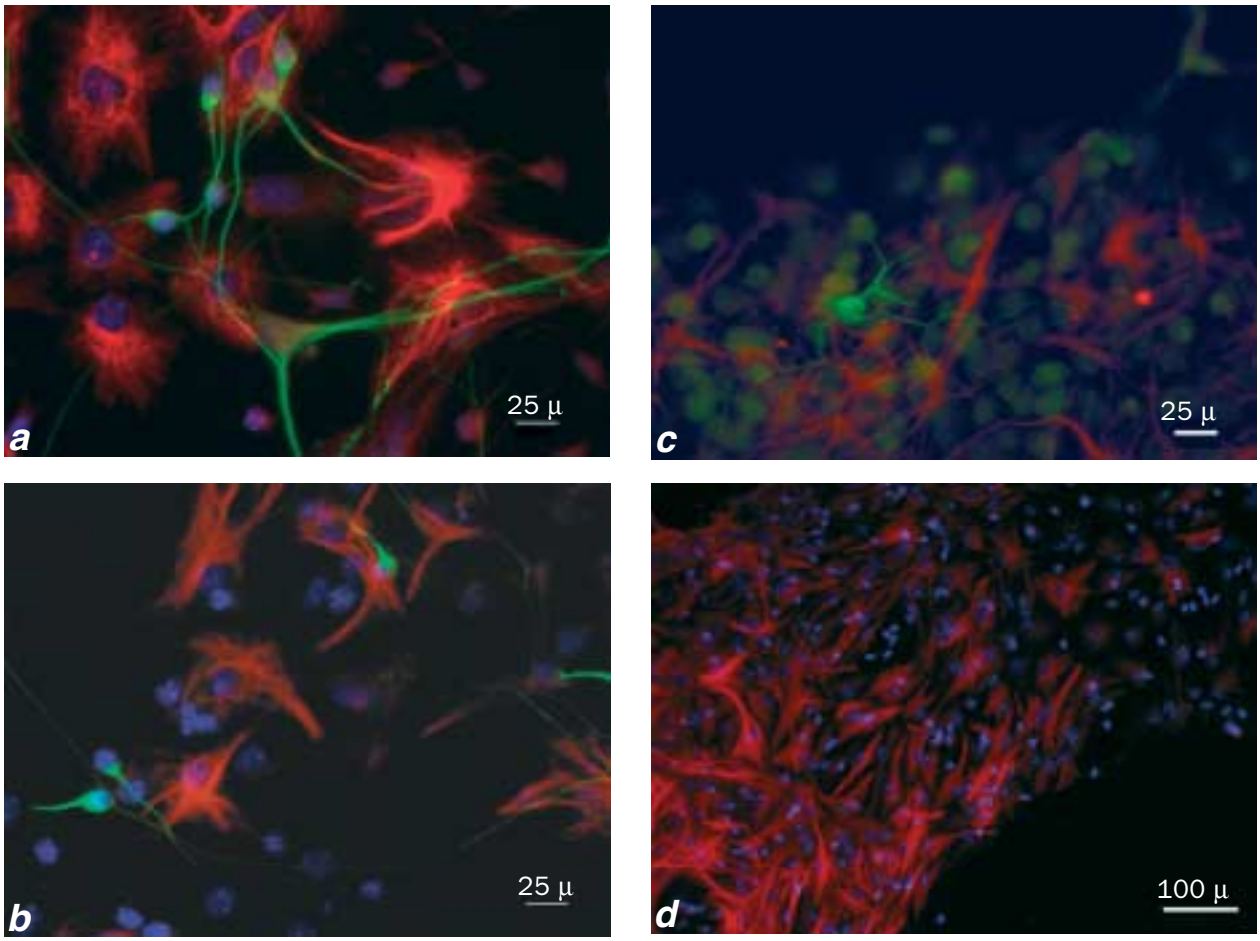

Fig. 3. Differentiation of cells in neurospheres in a medium with serum after several passages. Staining with antibodies to HGAP and β-tubulin-III. Nuclei are poststained with Hoechst 33342. *a*) zero passage; *b*) passage 1; *c*) passage 2; *d*) passage 3.

and the use of laminin as the substrate are optimum conditions for culturing of these cells.

Our findings drove us to the following conclusions: cells of mouse embryonic neocortex cultured in a medium with epidermal growth factor and basic fibroblast growth factor form neurospheres where the population of cells expressing SC/progenitor cell markers is maintained during passaging. In the differentiating medium, cells of neurospheres differentiate into glia, while neuroblasts and mature neurons appear later. During passaging, neurogenesis was almost completely replaced by gliogenesis and after passage 3 neuroblasts and mature neurons were practically absent. These results agree with previous data that the number of neuroblasts decreased below 10% after passage 3 [13,16]. This dynamics of cell differentiation in neurospheres to a certain extent agrees with the fact that neurogenesis gradually decreases, while gliogenesis increases during normal brain development [2,9]. This was confirmed by our findings: the number of neurons was higher in neurospheres isolated from the forebrain of 14-day embryos compared to neurospheres obtained from 19-day embryos, whereas the number of astrocytes was higher in late cultures [4]. Moreover, some authors showed that astrocytes can induce glial differentiation of neuronal SC under conditions of combined culturing [6]. This fact can explain accumulation of glial cells and decrease in the number of neuronal cells in culture with increasing the number of passages. However, it cannot be excluded that neocortical SC/progenitor cells in culture can realize the program of their differentiation (time and type of cells). Although the mechanism of differentiation is not quite clear, it is important that cells capable of neuronal differentiation do not survive in neocortical neurospheres.

Increasing the number of passages was associated with an increase in the number of GFAP-positive cells, but expression of nestin, marker of early neural differentiation, was still detected. This regularity was observed in a previous study [16], where the increase in the number of cells co-expressing both markers with increasing the number of passages was reported, and confirmed in our experiments. We can hypothesize that culturing leads to selection or formation of cells homologous to GFAP-positive neural SC of adult brain [1], which cannot differentiate in a medium containing no essential factors.

Of special importance is the fact that formation of neurospheres and expression of nestin by neural cells from embryonic brain do not prove unambiguously preserved multipotency of SC during culturing. Only testing of neurospheres under differentiating conditions provides real information on cell phenotype and this testing should be carried out for all cultures intended to cell transplantation.

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