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CELL BIOLOGY

# Proliferation and Differentiation of Mouse Embryonic Stem Cells Modified by the Neural Growth Factor (NGF) Gene

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**Abstract**—The effect of the nerve growth factor (NGF) on the proliferation and differentiation of mouse embryonic stem cells (ESCs) during long-term culturing in vitro was studied using genetically modified cells with stable expression of *ngf* and *gfp* (green fluorescent protein) genes (clone $R_1^{NGF}$). It was shown that, under standard conditions in vitro with the addition of mouse cytokine LIF, $R_1^{NGF}$ cells lose their adhesive properties and acquire the ability to form cellular conglomerates or embryoid bodies (EBs) spontaneously. It is noted that, after attachment to the surface of the culture on days 3–5, EBs differentiated towards the neuroectoderm, as evidenced by the appearance of markers of early (nestin) and late (vimentin) neural differentiation. During long-term cultivation up to 22 days, the production of endogenous NGF protein promotes predominant selection and survival of neuroectodermal derivatives.

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## INTRODUCTION

Human and animal embryonic stem cells (ESCs) differentiated towards the neuroectoderm serve as a model for studying early neurogenesis and a source of cells and tissues for regenerative therapy of the central and peripheral nervous systems. The differentiation of ESCs through the formation of multicellular structures, embryoid bodies (EBs), makes it possible to obtain derivatives of all three germ layers (ectoderm, mesoderm, and endoderm) in vitro (Yamanaka et al., 2008). Neural stem and progenitor cells originate from the ectoderm, which is laid first in the course of embryonic development and/or is formed spontaneously during differentiation of ESCs (Dhara and Stice, 2008; Germain et al., 2010). However, as a result of spontaneous differentiation of ESCs, a heterogeneous population consisting of not only neurogenic progenitors is usually formed, which limits the use of ESCs for transplantation in the treatment of neurodegenerative diseases. To increase the efficiency of neurogenic differentiation, exogenous inducers such as retinoic acid (RA) and fibroblast growth factor (FGF) are used. Treatment with RA triggers the transition to the neuroepithelial phenotype, whereas its combination with FGF makes it possible to obtain specialized neurons.

Among the exogenous regulators, of great interest is the nerve growth factor (NGF), which belongs to the neurotrophin family (Schuldiner et al., 2001; Inanç et al., 2008). This peptide is synthesized during embryogenesis. It regulates the innervation of internal organs and induces differentiation and survival of motor and sensory neurons and the growth of axons and blood vessels in transplanted grafts (Sofroniew et al., 2001; Wyatt et al., 2011). It was found that neurotrophins are involved in the processes of survival, proliferation, and differentiation of neural stem and progenitor cells (Li et al., 2009; Lin et al., 2012). Despite this, the role of NGF in the induction of neural differentiation of ESCs is not quite clear (Schuldiner et al., 2001; Kim et al., 2011). On the one hand, it was noted that a possible factor in improving the culturing efficiency of human ESCs in the presence of neurotrophins is the inhibition of cell death in vitro (Pyle et al., 2006). On the other hand, it was shown that effective neural differentiation of human ESCs is induced only when NGF is combined with RA (Schuldiner et al., 2001). The yield of the neuroectodermal cell and neurons per se in the 3D substrate system also increases significantly when ESCs are treated with several neurotrophins (Kim et al., 2011). Currently, a promising direction in solving the problems of neural differentiation of stem cells is the creation of experimental models of genetically modified ESCs expressing the *ngf* gene (Antonov et al., 2016).

The purpose of this study was to investigate the role of the NGF protein as an endogenous regulator of the early stages of differentiation of mouse ESCs towards the neuroectoderm using a lineage of genetically modified stem cells with an inserted *ngf* gene and NGF production to assess the effect of this factor on the

proliferation and survival of neural progenitors in an in vitro system.

## MATERIALS AND METHODS

Embryonic stem cells of mouse strain $R_1$ and genetically modified $R_1^{NGF}$ cells expressing the growth factor nerve *ngf*$^+$ and green fluorescent protein *gfp*$^+$ genes were kindly provided by I.A. Grivennikov (Somatic Cell Genetics Laboratory, Institute of Molecular Genetics, Russian Academy of Sciences, Moscow).

Embryonic stem cells were cultured in DMEM with a high glucose concentration (4.5 g/L) (Biolot, Russia), which was supplemented with 2 mM L-glutamine (Helicon, Russia), 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol (Gibco, United States), 1 mM of nonessential amino acids (PanEco, Russia), antibiotics, antimycotics, and 10% fetal bovine serum (HyClone, United States). To maintain ESCs in a pluripotent state, the culture medium was supplemented with 10 ng/mL recombinant mouse LIF protein (Sigma, United States). Culturing was performed in a $CO_2$ incubator (Sanyo, Japan) at 37°C and 5% $CO_2$ in humidified air.

To construct growth curves for the $R_1$ and $R_1^{NGF}$ lines, cells were seeded at a concentration of $10^5$ cells/mL in 35-mm Petri dishes (Nunc, Denmark), which were preliminarily coated with 0.1% gelatin, and cultured for 72 h. The cells were counted in a standard hemocytometer chamber every 24 h. When ESCs grew in the form of an EB suspension, cell conglomerates were resuspended by pipetting, after which the number of cells was counted. The doubling time ($T$) of the cell population was calculated using the formula

$$T = \log_{(x/x0)} 2\Delta t,$$

where $x_0$ is the initial cell concentration, $x$ is the final cell concentration, and $\Delta t$ is the culturing time.

The pluripotency of stem cells was assessed by their ability to grow and reproduce in colonies on 0.1% gelatin coating, the endogenous alkaline phosphatase (EAP) activity, the ability to differentiate into EBs after the removal of cytokine LIF from the culture medium, and the activity of the Nanog pluripotency transcription factor.

Colonies and precipitated EBs were fixed in cold acetone for several seconds, air dried, and then incubated for 1 h in a $CO_2$ incubator in a solution containing α-naphthol-AS-BI-phosphate (Sigma) and fast blue BB dye (Sigma). The appearance of blue-colored cells indicated EAP activity.

After culturing for 72 h, EBs were transferred from the suspension to sterile coverslips preliminarily coated with 0.1% of gelatin (Sigma). EBs were cultured in DMEM supplemented with all additives for 5, 8, 14, and 22 days. At each stage of culturing, the morphology of cells and colonies was assessed and the expression of differentiation markers was analyzed using antibodies to nestin, BMP2/4, and vimentin proteins (Santa Cruz Biotechnology, United States). Morphological analysis was performed with an Axiovert 40 CFL inverted microscope (Zeiss, Germany) at a lens magnifications of ×10 and ×20. The fluorescence intensity of the green fluorescent protein GFP in the genetically modified $R_1^{NGF}$ cells was evaluated using an integrated filter for Axiovert 40 CFL with $\lambda$ = 488 nm.

Colonies were fixed on coverslips in 2.5% glutaraldehyde at 37°C for 60 min. The preparations were washed three times in phosphate-buffered saline (PBS) and postfixed for 30 min in absolute ethanol cooled to –20°C. Postfixed preparations were washed thoroughly in PBS and coated with the blocking solution (5% bovine serum albumin in phosphate buffer supplemented with the fluorescent dye Hoechst33258 in 1 : 1000 dilution). Monoclonal rabbit antibodies against NGF and the tissue-specific markers Nanog, nestin, BMP2/4, and vimentin at a dilution of 1 : 500 (Santa Cruz Biotechnology) were used as the primary antibodies. Goat antibodies against rabbit immunoglobulins conjugated with the fluorescent dye FITC (1 : 2000) (Jackson Immunoresearch Laboratories, United States) were used as secondary antibodies. After each procedure, cells were washed in PBS three times for 5 min. The cells labeled with the antibodies were detected with a Leica DM6000B fluorescence microscope using lasers with wavelengths of 494–518 nm for FITC and 353–365 nm for Hoechst33258.

The counting of cells for immunofluorescence analysis and the determination of the size of EBs and colonies were performed using the ImageJ software ver. 1.48a. The mean values were compared using ANOVA.

## RESULTS AND DISCUSSION

It was found that, in contrast to $R_1$ cells (Fig. 1a), their genetically modified descendants expressing *ngf*$^+$ and *gfp*$^+$ plasmid genes ($R_1^{NGF}$ line) grew in DMEM supplemented with 10% fetal bovine serum and 10 ng/mL recombinant protein LIF in the form of a suspension (Fig. 1b). $R_1^{NGF}$ cells aggregated to form EBs, which adhered to the culture dish surface, spread over it, and formed colonies only on days 3–4 of culturing (Fig. 1d). As part of the EBs, $R_1^{NGF}$ cells showed a high EAP activity (Fig. 1c) and were involved in the NGF protein production (Fig. 1d). Thus, an excess of endogenous NGF protein affected the growth characteristics of $R_1^{NGF}$ cells in vitro, primarily their adhesive properties and the ability to grow as monolayer colonies, and hindered the effect of the LIF cytokine aimed at suppressing the formation of EBs. In the sus-

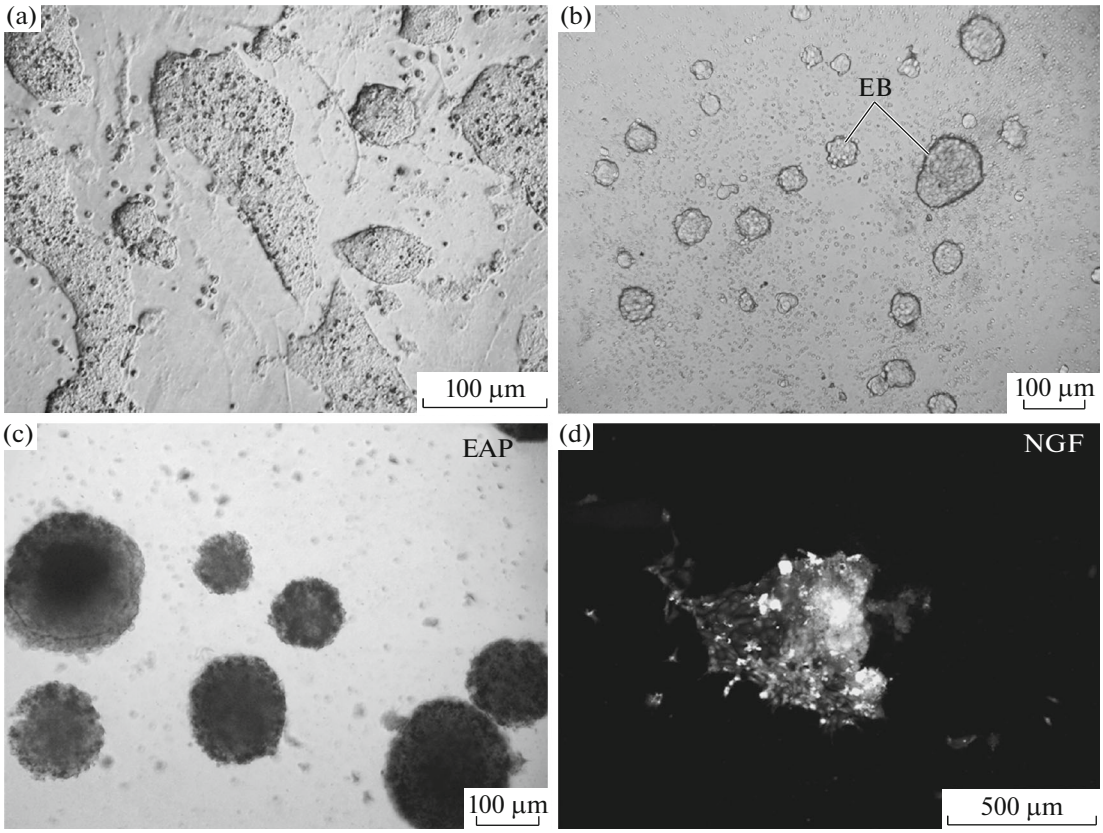


**Fig. 1.** Changes in the morphology of mouse ESCs modified with *ngf*$^+$ and *gfp*$^+$ plasmid genes in culture in vitro: (a) monolayer colonies $R_1$ (day 3 of culturing in the control); (b) embryoid bodies (EBs) formed by $R_1^{NGF}$ cells on day 3 of culturing in the medium supplemented with 10 ng/mL recombinant cytokine LIF (experiment); (c) activity of endogenous alkaline phosphatase (EAP); (d) immunofluorescence analysis of NGF production using the FITC dye (day 4 of culturing). Scale bar: 100 μm (a–c) and 500 μm (d).

pension, $R_1^{NGF}$ cells retain their pluripotent potential and ability to proliferate, which was confirmed by the test for the EAP activity in them (Fig. 1c).

More detailed studies using fluorescent techniques showed that only part of the cells in EBs are involved in the production of NGF (Fig. 1d) and GFP (Fig. 2a). After 24 h of culturing, the NGF-producing cells aggregate to form cell conglomerates, EBs. At this time, EBs do not differ in size and have a fuzzy morphology (Fig. 2a). As the duration of culturing increases, EBs increase in size and acquire a spherical shape (Figs. 1b, 2a), and the number of EBs in the suspension increases. The largest number of EBs was observed after 48 h of culturing (Fig. 2c). After 72 h of culturing, the heterogeneity of EBs in size (100–200 μm in diameter) in the in vitro culture was maximum. However, the number of large EBs (>250 μm) was not significant (Fig. 2a). The doubling rate of $R_1^{NGF}$ cells in EBs was higher than that of $R_1$ cells cultured in a monolayer (Figs. 2d, 2e) and strongly depended on the EB density in the culture (Fig. 2c). For example, after reaching the maximum density of EBs after 48 h of culturing (Fig. 2c), the average doubling time for $R_1^{NGF}$ was 10.3 h versus 14 h for $R_1$ cells growing in colonies (Fig. 2e).

The increase in the size of EBs is due to enhanced cell division under the influence of NGF (Fig. 2a) and the fusion of smaller EBs. It is known that EBs ~200 μm in diameter settling on a surface form predominantly a population of neuroectodermal progenitors in vitro (Zhou et al., 2010). Upon reaching the size of 200 μm after 72 h of culturing, EBs settle on the surface of the culture dish and form colonies, which is followed by spontaneous differentiation of cells on the periphery of the colony. The initial stages of differentiation are accompanied by an increase in the heterogeneity of the cell population, as evidenced by the changes in cell morphology and the expression of tissue-specific markers.

It was found that the $R_1^{NGF}$ and $R_1$ cell populations differ from each other not only in the growth characteristics but also in their ability to differentiate into the

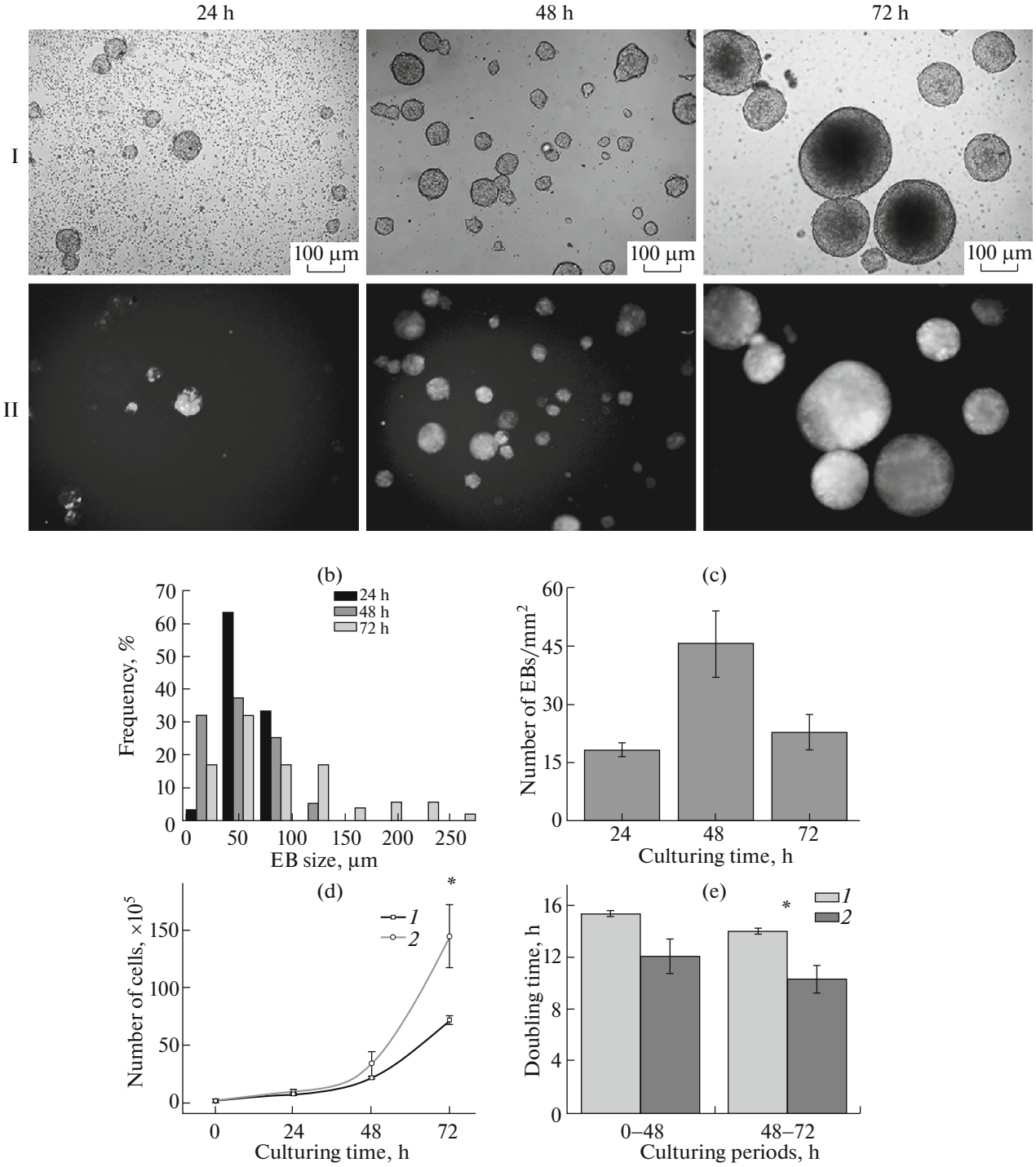


**Fig. 2.** Morphology of EBs and the growth characteristics of genetically modified $R_1^{NGF}$ cells: (a) changes in morphology and the size of EBs after 24–72 h culturing in vitro (I, transmitted light; II, GFP fluorescence at a wavelength of 488 nm (for Figs. 2, 3)); (b) distribution of EBs in suspension by size during culturing; (c) number of EBs per 1 $mm^2$ of the surface of the culture dish; (d, e) comparison of growth characteristics and population doubling time, respectively (*1*, $R_1$ cells; *2*, $R_1^{NGF}$ cells). * Significant differences between the comparison groups ($p < 0.05$). Scale: 100 μm.

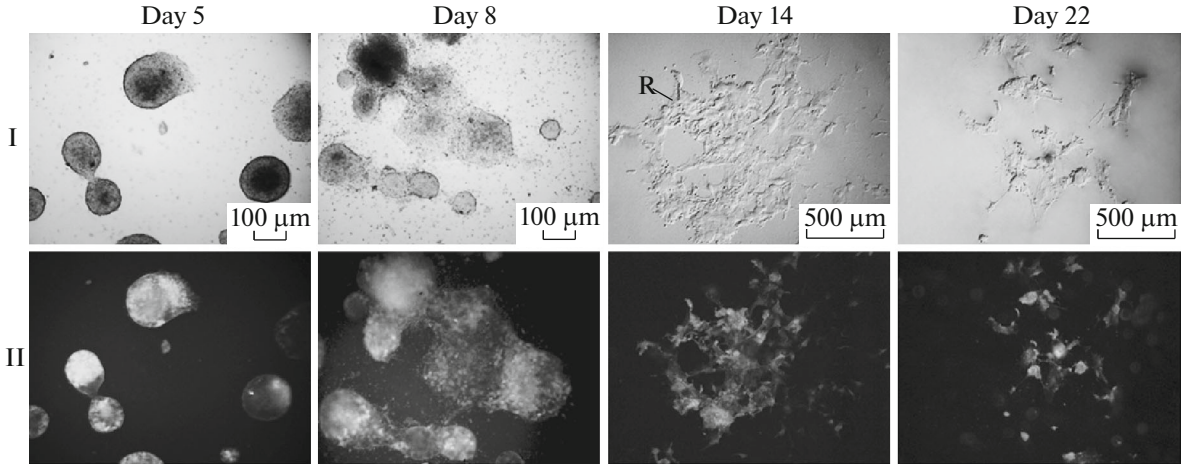


**Fig. 3.** Morphological changes in $R_1^{NGF}$ cells at different stages of EB differentiation. Designations: R, rosettelike colony consisting of morphologically homogeneous cells with voids emerging in colonies.

neuroectoderm, as evidenced by the pattern of changes in the $R_1^{NGF}$ morphology after the attachment of EBs on day 5 of culturing (Fig. 3, Table 1). By this time, actively proliferating fibroblastlike cells were detected on the periphery of the colony. They quickly grew to form a continuous monolayer. A similar pattern was observed at the early stages of differentiation of $R_1$ cells. However, their further growth required the use of a selection medium supplemented with growth factors; otherwise, the cells died. On day 14 of culturing, the number of colonies dramatically decreased and the monolayer included cells with different morphology, as well as rosettelike structures containing voids due to the death of a significant part of the cells (Fig. 3). This pattern is typical for the early stages of neuroectodermal differentiation, which are associated with the epithelial–mesenchymal transition of cells on the periphery of the colony and apoptosis (Cimadamore et al., 2011).

It was found that the rosettes (Fig. 3, day 14) are formed primarily by a morphologically homogeneous population of cells with a neural phenotype. Figure 4 shows the location of the NGF-positive cells and the cells expressing the markers of pluripotency (Nanog), neuroectoderm (nestin), mesoderm (BMP2/4), and neuroglia (vimentin) at different stages of differentiation.

During long-term culturing, the number of differentiated cells expressing neural differentiation markers progressively increased (Figs. 4a–4d). The rosettes contained primarily NGF/nestin-positive cells and a small amount of pluripotent cells expressing Nanog (Fig. 4d). Single cells were mostly vimentin-positive.

It should be emphasized that the dynamics of changes in the ratio of pluripotent and differentiated $R_1^{NGF}$ stem cells indicates the prevalence of neuroectodermal derivatives in the in vitro culture (Figs. 5a–5d). Therefore, excessive secretion of the endogenous NGF protein does not prevent the epithelial–mesenchymal transition characteristic of the early stages of laying the neuroectodermal derivatives in early embryogenesis (Cimadamore et al., 2011). As a result of cell selection, the total number of neuroectodermal derivatives progressively increased (to 80.2%) on day 22 of culturing. As the culturing duration increased,

**Table 1.** Changes in the morphology of $R_1^{NGF}$ embryonic stem cells during differentiation

| In vitro differentiation stage, day | Total number of measurements | Number of morphological structures, % | | | |
|---|---|---|---|---|---|
| | | colonies | monolayer | rosettes | individual cells |
| 5 | 27 | 38 | 62 | – | – |
| 8 | 23 | 4.4 | 65.2 | 30.4 | – |
| 14 | 24 | – | – | 41.7 | 58.3 |
| 22 | 22 | – | – | 45.5 | 54.5 |

"–" Designates the absence of morphological structures.

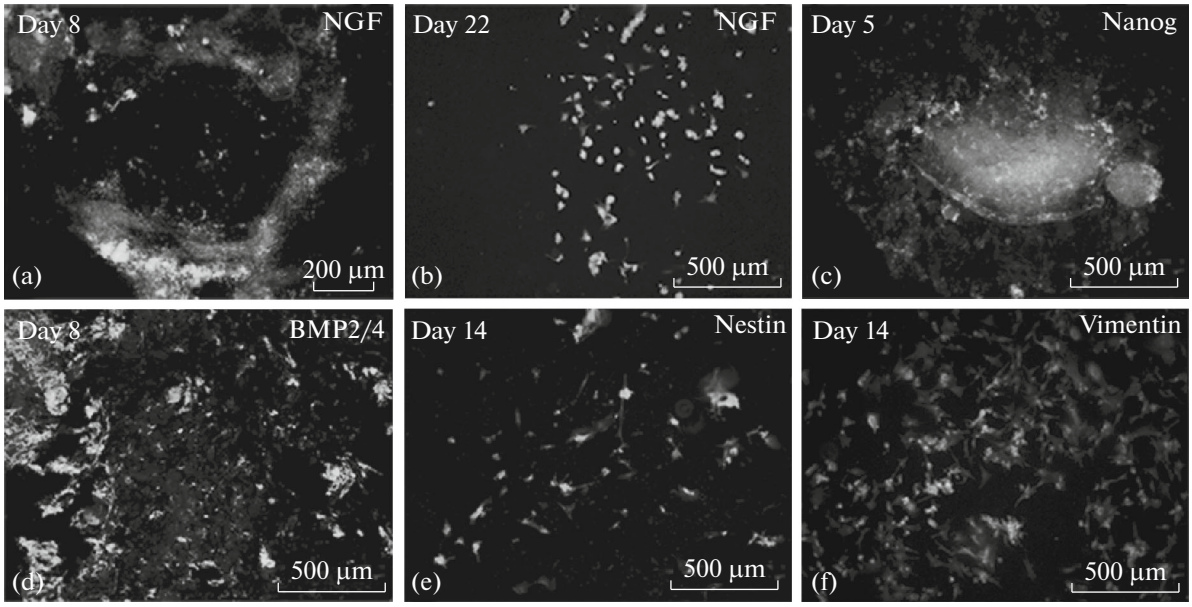


**Fig. 4.** Immunofluorescence analysis of expression of the genes encoding (a, b) NGF and the specific markers (c) Nanog, (d) BMP2/4, (e) nestin, and (f) vimentin at different stages of $R_1^{NGF}$ differentiation. Cells were stained with FITC-labeled antibodies (494–518 nm). Scale bar: 200 μm (a) and 500 μm (b–f).

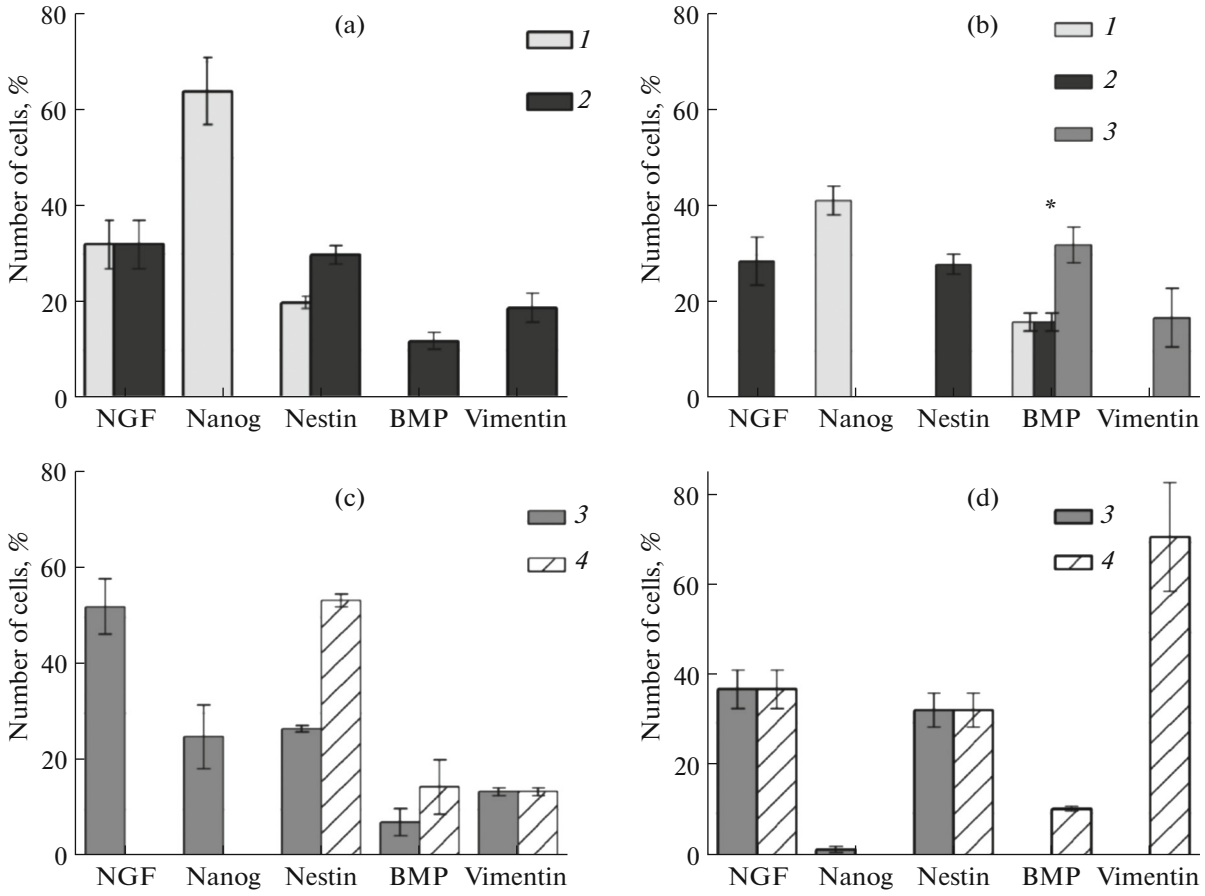


**Fig. 5.** Changes in the ratio of cells expressing NGF and differentiation markers on days (a) 5, (b) 8, (c) 14, and (d) 22 of long-term culturing of $R_1^{NGF}$ cells. Designations: *1*, colonies; *2*, monolayer; *3*, rosettes; *4*, individual cells.

numerous progenitors (glial cells that express vimentin) appeared in the populations of $R_1^{NGF}$ cells (Fig. 5d).

Thus, changes in the ratio of cells expressing NGF and neural differentiation markers during long-term culturing allow NGF to be regarded as a selective factor promoting proliferation and differentiation of ESCs in vitro towards neural progenitors. Thus, NGF-transfection with accumulation of the NGF protein, which functions as an autocrine factor of ESC differentiation in early (nestin) and late (vimentin) progenitors of neural tissue, can be used to induce neurogenesis in vitro.

## REFERENCES

Antonov, S.A., Manuilova, E.S., Dolotov, O.V., Kobylyansky, A.G., Safina, D.R., and Grivennikov, I.A., Effect of nerve growth factor on neural differentiation of mouse embryonic stem cells, *Bull. Exp. Biol. Med.*, 2016, vol. 162, no. 5, pp. 679–682.

Cimadamore, F., Fishwick, K., Giusto, E., Gnedeva, K., Cattarossi, G., Miller, A., Pluchino, S., Brill, L.M., Bronner-Fraser, M., and Terskikh, A.V., Human ESC-derived neural crest model reveals a key role for SOX2 in sensory neurogenesis, *Cell Stem Cell*, 2011, vol. 8, no. 5, pp. 538–551.

Dhara, S.K. and Stice, S.L., Neural differentiation of human embryonic stem cells, *J. Cell Biochem.*, 2008, vol. 105, no. 3, pp. 633–640.

Germain, N., Banda, E., and Grabel, L., Embryonic stem cell neurogenesis and neural specification, *J. Cell Biochem.*, 2010, vol. 111, no. 3, pp. 535–542.

Inanç, B., Elçin, A.E., and Elçin, Y.M., Human embryonic stem cell differentiation on tissue engineering scaffolds: effects of NGF and retinoic acid induction, *Tiss. Eng. A*, 2008, vol. 14, no. 6, pp. 955–964.

Kim, J.M., Moon, S.H., Lee, S.G., Cho, Y.J., Hong, K.S., Lee, J.H., Lee, H.J., and Chung, H.M., Assessment of differentiation aspects by the morphological classification of embryoid bodies derived from human embryonic stem cells, *Stem Cells Dev.*, 2011, vol. 20, no. 11, pp. 1925–1935.

Li, T., Jiang, L., Zhang, X., and Chen, H., In-vitro effects of brain-derived neurotrophic factor on neural progenitor stem cells from rat hippocampus, *Neuroreport*, 2009, vol. 20, no. 3, pp. 295–300.

Lin, S., Wang, Y., Zhang, C., and Xu, J., Modification of the neurotrophin-3 gene promotes cholinergic neuronal differentiation and survival of neural stem cells derived from rat embryonic spinal cord in vitro and in vivo, *J. Int. Med. Res.*, 2012, vol. 40, no. 4, pp. 1449–1458.

Pyle, A.D., Lock, L.F., and Donovan, P.J., Neurotrophins mediate human embryonic stem cell survival, *Nat. Biotechnol.*, 2006, vol. 24, no. 3, pp. 344–350.

Schuldiner, M., Eiges, R., Eden, A., Yanuka, O., Itskovitz-Eldor, J., Goldstein, R.S., and Benvenisty, N., Induced neuronal differentiation of human embryonic stem cells, *Brain Res.*, 2001, vol. 913, no. 2, pp. 201–205.

Sofroniew, M.V., Howe, C.L., and Mobley, W.C., Nerve growth factor signaling, neuroprotection, and neural repair, *Annu. Rev. Neurosci.*, 2001, vol. 24, pp. 1217–1281.

Wyatt, T.J., Rossi, S.L., Siegenthaler, M.M., Frame, J., Robles, R., Nistor, G., and Keirstead, H.S., Human motor neuron progenitor transplantation leads to endogenous neuronal sparing in 3 models of motor neuron loss, *Stem Cells Int.*, 2011, vol. 2011, pp. 207–230.

Yamanaka, S., Li, J., Kania, G., Elliott, S., Wersto, R.P., Van Eyk, J., Wobus, A.M., and Boheler, K.R., Pluripotency of embryonic stem cells, *Cell Tiss. Res.*, 2008, vol. 33, pp. 5–22.

Zhou, J., Zhang, Y., Lin, Q., Liu, Z., Wang, H., Duan, C., Wang, Y., Hao, T., Wu, K., and Wang, C., Embryoid bodies formation and differentiation from mouse embryonic stem cells in collagen/Matrigel scaffolds, *J. Genet. Genom.*, 2010, vol. 37, no. 7, pp. 451–460.

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