CELL TECHNOLOGY IN BIOLOGY AND MEDICINE

Spontaneous Neural Differentiation of Stem Cells in Culture of Human Olfactory Epithelium

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We studied differentiation of stem cells in dissociated cultures of olfactory epithelium. Staining with anti-nestin antibodies revealed stem cells in the primary monolayer culture of the olfactory epithelium from adult human. Proliferation of these cells during culturing in serum-containing medium in the presence of nerve growth factors FGF2 and NGF led to the formation of neurospheres freely floating in the medium or attached to the substrate. Further long-term culturing and cloning of dissociated cells from these neurospheres in media not containing nerve growth factors led to spontaneous neural differentiation of the olfactory epithelium stem cells. The cells with phenotypic signs of differentiated neurons were stained with antibodies against β-tubulin and neurospecific enolase. Differentiated neurons formed diffuse and spatially organized neuronal networks. We hypothesized that factors triggering neural differentiation of olfactory epithelium stem cells are produced by astrocytes present in these cultures.

Key Words: *olfactory epithelium; stem cells; cell cultures; neurospheres*

Death and renewal of olfactory receptor neurons in the olfactory epithelium (OE) of mammals take place continuously throughout life; these cells originate from proliferating basal stem cells located at the boundary between the olfactory epithelium and underlying connective tissue stroma [3,8,15]. Molecular mechanisms and successive stages of *in vivo* differentiation of globose and horizontal OE stem cells with the formation of progenitor cells forming, apart from olfactory receptor neurons, glial and non-neuronal cell elements in this structure are described in detail [1,4,6,10]. Multipotent properties of OE stem cells *in vitro* and *in vivo* and their capacity to differentiate into cells normally derived from the endoderm, mesoderm and ectoderm were demonstrated [12,13]. Studies on continuous cultures of human EO showed that stem, progenitor, and glial cells can form neurospheres [1,13-15].

Here we studied the possibility of neural differentiation of stem cells in OE of adult humans in long-living cultures in media not containing neurotrophic and nerve growth factors.

MATERIALS AND METHODS

Isolation and culturing of cells olfactory mucosa. Olfactory mucosa samples (*tunica mucosa nasi*) including OE and connective tissue layer (*lamina propria*) were obtained from individuals with spinal injury, patients of Neurovita Clinic (certificate of Ethical Committee of Russian State Medical University, Federal Agency for Health Care and Social Development) [2].

Mucosa fragments (10×5 mm) were dissected from the upper portion of the superior turbinate

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under local anesthesia. The tissue was transferred to the laboratory in cold Ca^{2+},Mg^{2+}-free Hanks saline (HBSS) containing antibiotics and antimycotics (1:100, Gibco) not later than 2 h after isolation. The samples were repeatedly washed with the same buffer, blood vessels were removed, and the tissue was minced and incubated with trypsin and EDTA (0.25%) dissolved in 0.01 M PBS (pH 7.4) at 36.5°C for 40 min. The enzymes were blocked by adding DMEM (Gibco) containing 3% serum; the tissue was washed 3 times with balanced Hanks' salt colution (Sigma) and dissociated by repeated pipetting in MEM (90%, Sigma), fetal bovine serum (FBS, Gibco Ivitrogen) containing 0.8% glucose, 2 mM glutamine (Gibco), B27 supplements (Sigma), 20 mM HEPES, growth factors (only for primary cultures), fibroblast growth factor (FGF2, 1 ng/ml, Sigma), and nerve growth factor (NGF 2 ng/ml, Sigma). The cell suspension was centrifuged at 1200 rpm for 3 min and resuspended in the same medium. Cell count and viability were evaluated in a Goryaev chamber after staining with 0.1% trypan blue. Only cell suspensions containing 85-95% viable cells were used for further culturing. Dissociated cells (5×10^5 cells/ml) were cultured in 12-well plates on a polylysine-laminin matrix for 14 days (36.5°C, 5% CO_2). The medium was half-replaced twice a week. Primary culture after attaining a confluent state was harvested with trypsin-EDTA, washed in HBSS, centrifuged, and resuspended in the nutrient medium. The cell suspension (10,000-12,000 cells/cm^2) was transferred to 12-well plates or 25-cm^2 flasks. This procedure was repeated 4 times. Freely floating and attached to the substrate neurospheres were collected with Pasteur pipettes and dissociated with enzymes as described previously. Isolation of neurospheres ensures their separation from ensheathing glial cells, fibroblasts, and stromal (sustentacular) cells. The suspension of cells from neurospheres after washout and centrifugation was resuspended in the nutrient medium and cultured in 12-well plates (10,000-12,000 cells/cm^2) and on

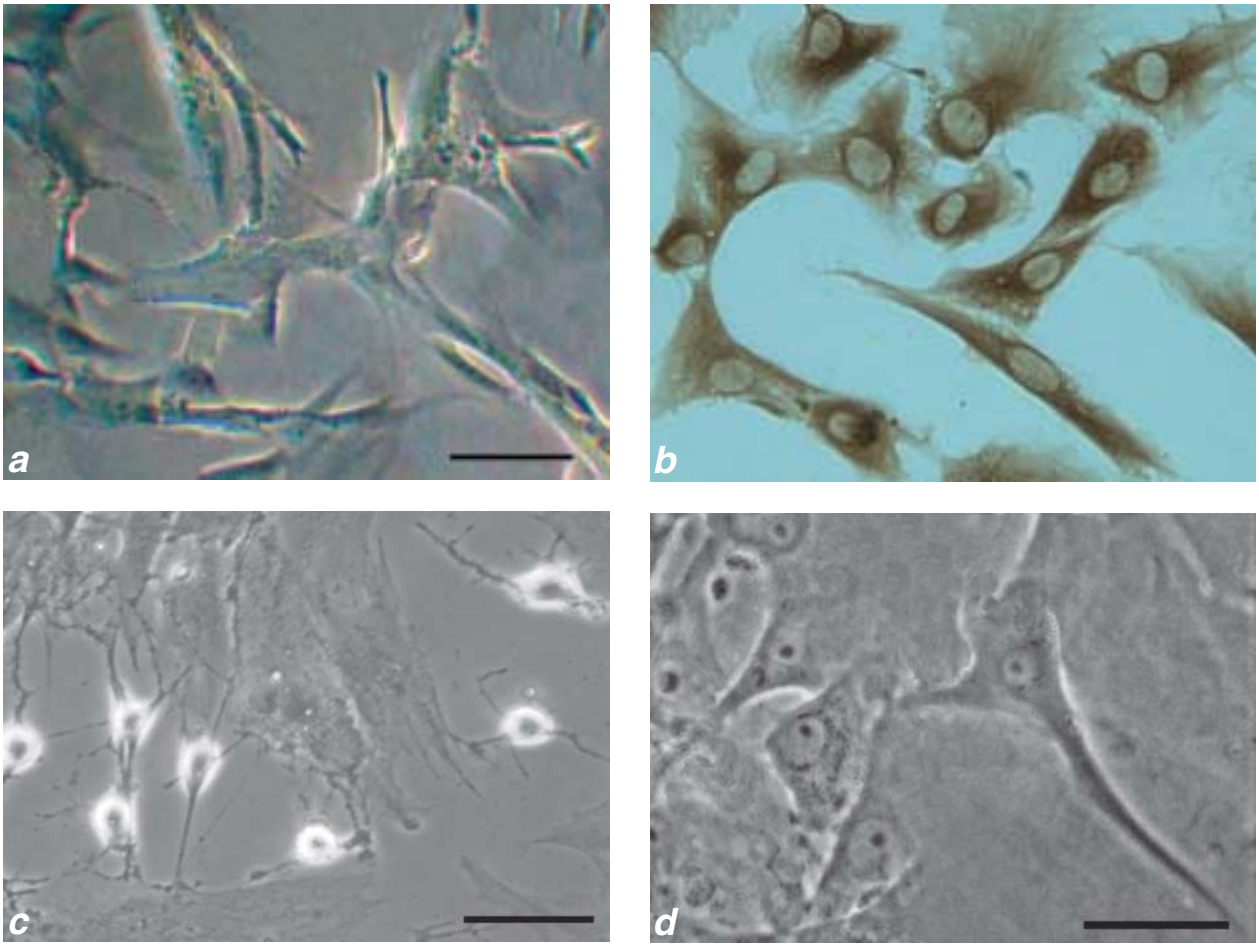

Fig. 1. Monolayer cultures of human olfactory mucosa. *a*, *b*: primary culture (6 days in culture); *c*: culture after 4 passages (12 days in culture); *d*: cells with phenotypic signs of neurons in culture after 4 passages (12 days in culture). *a, c*: phase-contrast microscopy; *b*: staining with antibodies to nestin; *d*: bright-field microscopy. Scale: *a, b, d*: 20 μ; *c*: 40 μ.

coverslips (18×18 mm) in Petri dishes until confluence. The cultures were used for cytological and immunocytochemical studies. Some cells of the last passages were frozen in a cryopreservation medium (90% serum, 10% dimethylsulfoxide) and stored in liquid nitrogen.

Immunocytochemical studies. Cell monolayer was fixed in 4% paraformaldehyde in 0.01 M phosphate buffer (pH 7.4) for 30 min. After washing with PBS (3×10 min) the cells were incubated at 4°C for 24 h with primary antibodies to β-tubulin (1:300; Chemicon), nestin (1:100; Chemicon), and neurospecific enolase (1:100; antibodies were prepared in our laboratory). The cells were washed with PBS and successively treated with biothinylated antibodies with avidin-biotin complex and (ABC, Vector Laboratories Inc.) and with diaminobenzidine dissolved in phosphate-buffered saline (DBA 0.5 mg/ml, 0.03% H_2O_2). The preparations were dehydrated and embedded into synthetic resin (Entellan, Merk) under a coverslip.

RESULTS

Vital phase-contrast microscopy showed that primary monolayer cultures of the olfactory mucosa at early terms of culturing contain flattened polygonal and spindle-shaped cells (Fig. 1, *a*) stained with antibodies to nestin specific for stem and progenitor cells (Fig. 1, *b*). Staining with these antibodies does not allow identification of neural and glial cells in these cultures. Long-term culturing and passaging led to the appearance of highly refracting cells phenotypically similar to neurons in the monolayer of flattened polygonal cells (Fig. 1, *c*). Bright field microscopy revealed cells with polygonal

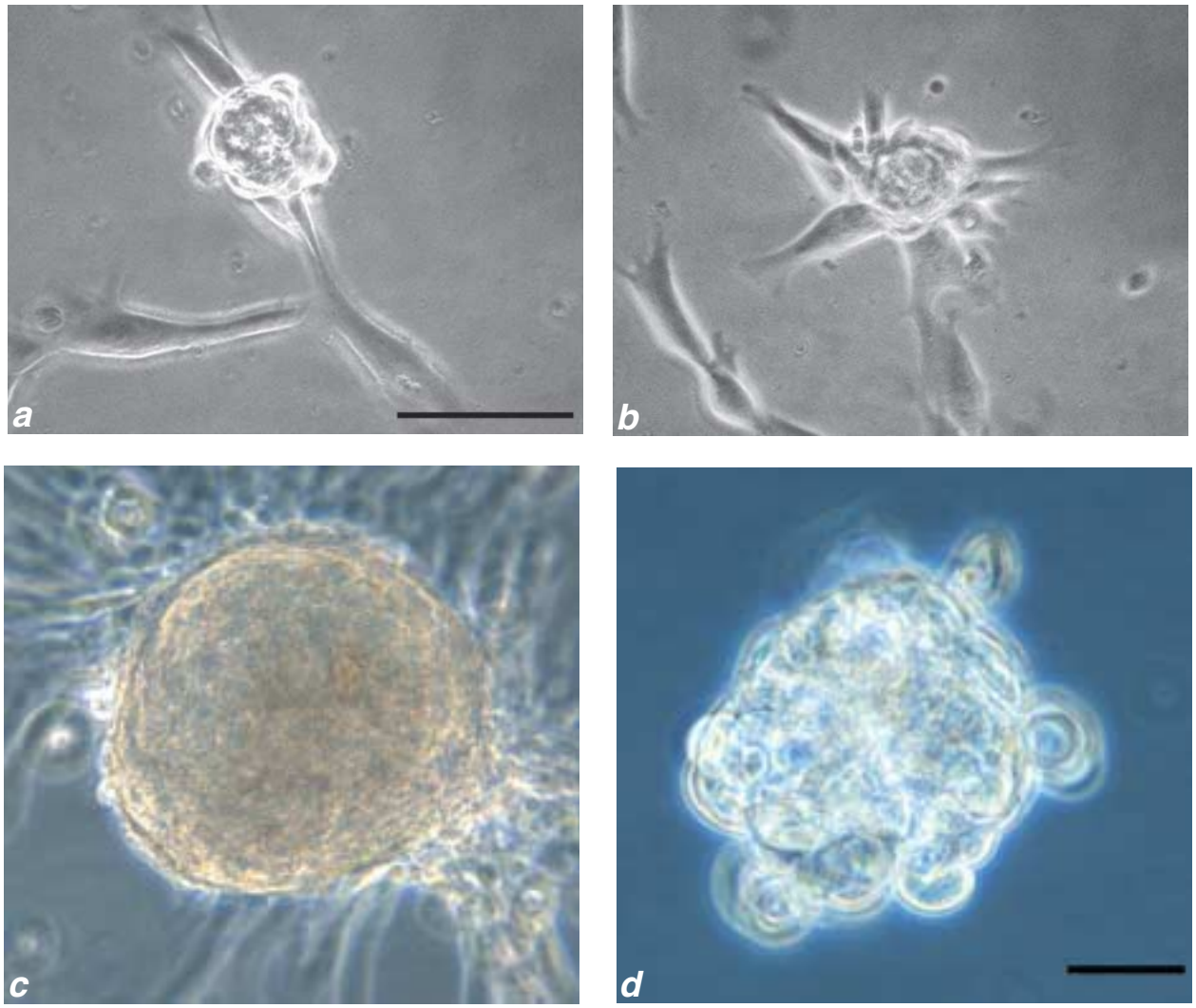

Fig. 2. Neurospheres in cultures of human olfactory mucosa: phase-contrast microscopy (scale: *a-d*: 100 μ). *a, b*: formation of primary neurospheres of glial cell feeder; *c*: neurosphere attached to the substrate; *d*: floating neurosphere.

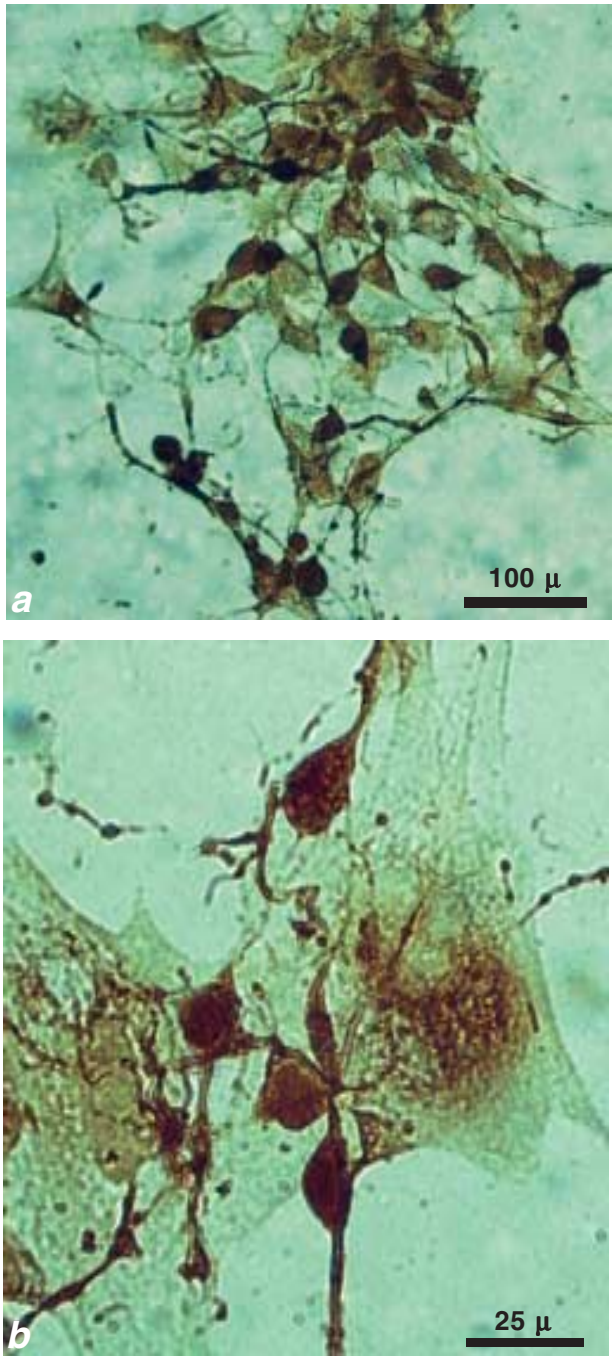

Fig. 3. Monolayer cultures of neural stem cells and progenitor cells of human OE obtained after dissociation of the fourth passage neurospheres. *a, b*: staining with antibodies to β-tubulin III; *b*: some β-tubulin-positive cells adjacent to astrocytes (nonspecific staining); *c*: staining with antibodies to neurospecific enolase. Insert in fragment *c*: differentiated neuron (arrows show dendrites, triangle shows axon).

bodies and solitary processes; light nucleus with single nucleolus was located centrally and surrounded by a wide cytoplasmic rim (Fig. 1, *d*).

In primary monolayer cultures grown in media containing nerve growth factors (FGF2 and NGF), actively proliferating stem and progenitor cells form fungiform cell conglomerations, attached primary neurospheres (Fig. 2, *a*, *b*). These neurospheres increase in size due to cell proliferation; they can be still attached to the substrate (Fig. 2, *c*) or can separate and form freely floating neurospheres (Fig. 2, *d*). Freely floating neurospheres also form in the suspension of dissociated OE cells. The formation of attached and freely floating neurospheres constantly takes place in repeatedly reinoculated cultures of the olfactory mucosa grown in the absence of nerve growth factors. Secondary and subsequent monolayer cultures of stem and progenitor OE cells can be obtained as a result of isolation and dissociation of neurospheres.

Immunocytochemical analysis of cells isolated from neurospheres of the final passage (2-week culturing) after enzyme dissociation and subsequent culturing in a medium containing no growth factors

showed that these cultures contain cells interacting with antibodies against β-tubulin (Fig. 3, *a*, *b*) and neurospecific enolase (Fig. 3, *c, d*). This attests to neural differentiation of stem and progenitor OE cells and formation of mature neurons, which can be identified by immunocytochemical methods.

Cell composition of primary cultures of human OE and after their subculturing and formation of neurospheres are described in detail [1,11,13,14, 17,18]. These studies showed that primary OE cultures contain a heterogeneous cell population including pluripotent stem cells, receptor olfactory neurons, ensheathing glial cells, epithelial sustentacular cells, and fibroblasts. In relatively poor medium (DMEM/F12 1:1 with 10% FBS) not containing neurotrophic factors, receptor olfactory neurons and ensheathing glial cells die via apoptosis within 3 weeks in culture. In 5-10% remaining cultures, neurosphere-forming cells (NSFC) survived [14] and the neurospheres include subpopulations of neural and glial cells. Dissociated cells from neurospheres actively proliferate and their number doubles every 2-3 days, which is accompanied by the appearance of multiple secondary neurospheres also including neural stem and glial cells. NSFC were cloned for 8 months and passed 70 passages [14]. NSFC were resistant to apoptosis, which can be explained by high telomerase activity and low caspase content in these cells [11].

Similar data were obtained about the death of dissociated stem OE cells from adult humans in serum-free medium [13]. Cell viability increases in media containing serum, fibroblast growth factor (FGF2), or transforming growth factor (TGF-α). It was also found that stromal cells from the same structure used as a feeder also maintained survival of isolated stem OE cells [12].

Neural and glial differentiations of dissociated cells from neurospheres of human OE were described elsewhere [13]. It was demonstrated that cell culture incubated for 5 days in a serum-free medium contains primarily GFAP-positive cells and only 4.50±0.29% cells express β-tubulin. In a serum-supplemented medium, 18.33±0.88% cells express β-tubulin. This value increased to 25.33±1.45% in the presence of NGF.

Neural differentiation of dissociated cells from human OE neurospheres was observed in long-term cultures grown in serum-supplemented medium in the absence nerve growth factors. Under these culturing conditions, there was no death of cells in primary and passaged cultures containing primarily stem and progenitor nestin-positive cells and astrocytes. Immunocytochemical analysis of cells from the fourth passage neurospheres after their dissociation and culturing for 14 days revealed multiple cell population with phenotypic signs of differentiated neurons stained with antibodies to β-tubulin and neurospecific enolase (Fig. 3, 4). In monolayer cultures of dissociated cells from neurospheres, some differentiated neuronal cells lay on a layer of flattened astrocytes. Multiple axons formed dense plexuses on the substrate surface (Fig. 3, *c*) and formed bundles including spindle-shaped neurons

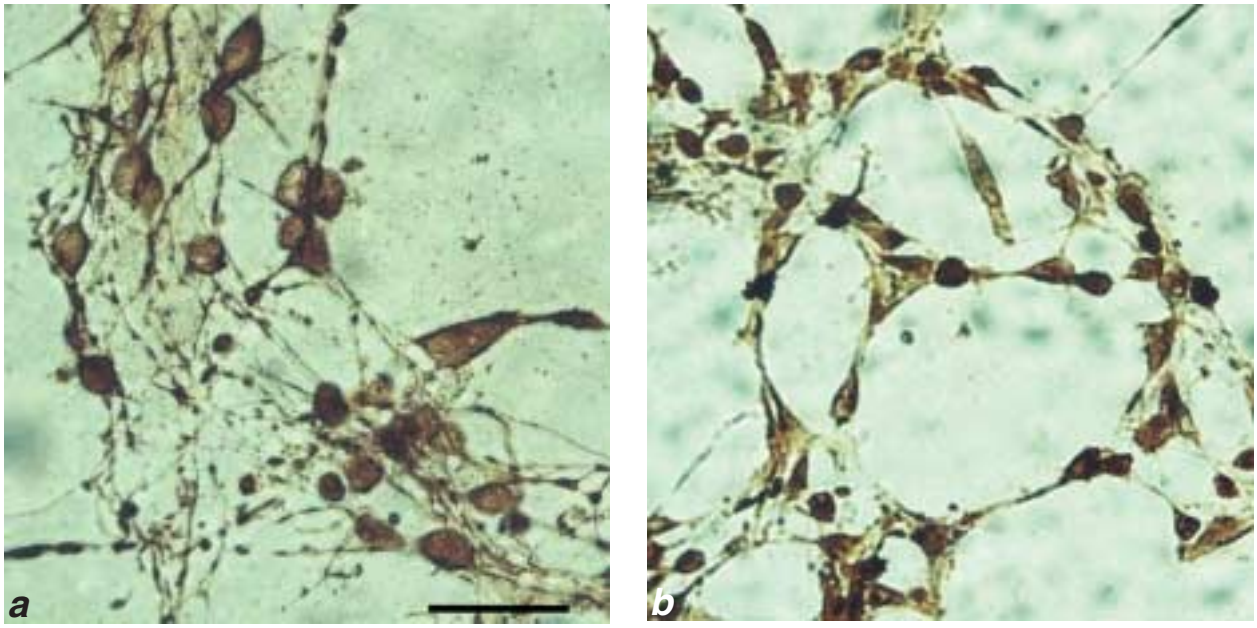

Fig. 4. Networks formed by neurons derived from stem OE cells. *a*) fragment of diffuse network: neurons in a bundle of parallel axons; *b*) circular neuronal network. Staining with antibodies to β-tubulin. Scale: 50 μ.

(Fig. 4, *a*). Differentiated neurons formed diffuse and spatially organized neuronal networks (Figs. 3, 4).

In long-term cultures grown in serum-supplemented medium in the absence of exogenous nerve growth factors, spontaneous neural differentiation of stem and progenitor cells of OE from adult human can be triggered by not only bioactive components of embryonic serum [13], but also by growth factors produced by satellite glia. This assumption is confirmed by the data on the production of nerve growth factors, in particular, astroglial neurotrophic factor (ADNF, activity-dependent neurotrophic factor) by astrocytes [5,7,9]. It was found that in mature brain these properties are primarily exhibited by astrocytes in structures characterized by postnatal neurogenesis (*e.g.* hippocampus [16]). OE also belongs to these structures, because it is characterized by constant generation of olfactory receptor neurons, which explains the ability of astrocytes in this structure to stimulate neural differentiation of stem and progenitor cells.

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REFERENCES

1. I. V. Viktorov, E. A. Savchenko, O. V. Ukhova, *et al., Kletochn. Tekhnol. Biol. Med.*, No. 4, 185-193 (2006).
2. E. A. Savchenko, N. A. Andreeva, T. B. Dmitrieva, *et al., Ibid.,* No. 2, 95-98 (2005).
3. J. Altman, *J. Comp. Neurol*., **137**, No. 4, 433-457 (1969).
4. C. L. Beites, S. Kawauchi, C. E. Crocker, and A. L. Calof, *Exp. Cell Res*., **306**, No. 2, 309-316 (2005).
5. D. E. Brenneman and I. Gozes, *J. Clin. Invest*., **97**, No. 10, 2299-2307 (1996).
6. L. A. Carter, J. L. MacDonald, and A. J. Roskams, *J. Neurosci.*, **24**, No. 25, 5670-5683 (2004).
7. G. W. Glazner, A. Boland, A. E. Dresse, *et al., J. Neuochem*., **73**, No. 6, 2341-2347 (1999).
8. P. P. Graziadei and G.A.Graziadei, *J. Neurocytol*., **8**, No. 1, 1-18 (1979).
9. E. Hansson and L. Ronnback, *FASEB J*. **17**, No. 3, 341-348 (2003).
10. J. M. Huard, S. L. Youngentob, B. J.Goldstein, *et al., J. Comp. Neurol.,* **400**, No. 4, 469-486 (1998).
11. C. T. Marshall, Z. Guo, C. Lu, *et al., Brain Res*., **1045**, Nos. 1-2, 45-56 (2005).
12. J. S. Mumm, J. Shou, and A. L. Calof, *Proc. Natl. Acad. Sci. USA*., **93**, No. 20, 11,167-11,172 (1996).
13. W. Murrell, F. Feron, A. Wetzig *et al., Dev. Dyn*., **233**, No. 2, 496-515 (2005).
14. F. J. Roisen, K. M. Klueber, C. L. Lu, *et al., Brain Res*., **890**, No. 1, 11-22 (2001).
15. J. E. Schwob, *Anat. Rec*., **269**, No. 1, 33-49 (2002).
16. H. Song, C. F. Stevens, F. H. Gage, *Nature*, **417**, 30-44 (2002).
17. X. Zhang, J. Cai, K. M. Klueber, *et al., Stem Cells*, **24**, No. 2, 434-442 (2006).
18. X. Zhang, K. M. Klueber, Z. Guo, *et al., Exp. Neurol.*, **186**, No. 2, 112-123 (2004).