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VERSITA

HUMAN NEUROSPHERES: FROM STAINED SECTIONS TO THREE-DIMENSIONAL ASSEMBLY

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Abstract

Human neurospheres are free-floating spherical clusters generated from a single neural stem cell and comprising cells at different stages of maturation in the neuronal and glial lineages. Although recent findings have disproved the original idea of clonally derived neurospheres according to the paradigm of one stem cell – one neurosphere, they still represent a valid model for growing neural stem cell cultures in vitro. While the immunocytochemical approach to the identification of stem cells, progenitor cells, and mature cells has been extensively used, scant data are available about the ultrastructural arrangement of different cell types within the neurosphere. This paper provides, by means of scanning electron microscopy, some new insights into the three-dimensional assembly of human neurospheres, trying to correlate some parameters such as cell density, shape and growing strategies with the immunolocalization of some antigens such as nestin, GFAP, α-internexin and βIII-tubulin. The major findings from this study are: a) regardless of the stage of in vitro maturation, the growth of the spheres is the result of mitotic divisions producing the aspect of an irregular budding mechanism in the outermost layer look like; b) analysis of the volumetric composition of the inner core has revealed the presence of two alternative shape pattern (pyramidal vs rounded cells) possibly related to both the ongoing maturation stages and GFAP and internexin expression.

Keywords

• Human neurospheres • Neural stem cells • Scanning electron microscopy • Immunocytochemistry

1. Introduction

In the last decade, a new boost to the understanding of neurodegenerative mechanisms has come from the discovery of three-dimensional floating cultures, called neurospheres that growth in vitro in a "morula"-like fashion [1,2]. Originally interpreted as neural stem cell (NSC) reservoirs, those embryoid bodies have been found by biochemical and molecular evidence to harbour a heterogeneous cell population comprising neural progenitors and precursors [3-7]. An interesting feature is that neurospheres isolated from different regions of the developing or adult brain display unique characteristics with regard to growth, differentiation, and gene expression [8-10]. Therefore, despite not being selectively committed [11], they maintain distinct regional and temporal identities.

Since then, the "neurosphere assay" has been extensively explored as a bona fide method to define and measure NSCs behavior [12] but several caveats have been raised regarding the application of clonal features to those dynamic structures [13]. On the other hand, neurosphere culture systems represent intriguing models by reason of their intrinsic multipotency, which is easily triggered by withdrawing basic mitogens (EGF and FGF-2) and ultimately leads to the maturation of all the neuronal and glial lineages [14] capable to develop in vitro, such as monolayered cells. However, their morphological and biochemical characterization at single cell level has almost exclusively relied on the use of immunocytochemical markers applied to sectioned neurospheres [15] or neurosphere-dissociated cells [16]. Indeed very few studies have investigated the closer spatial relationships between cells based on their volumetric assembly by means of ultrastructural approaches [17-19] in particular by using transmission electron microscopy. Therefore, this study set out to explore, by scanning electron microscopy (SEM), both intact human neurospheres and their fragments at different in vitro stages, to assess the effectiveness of this method in providing further information, in association with the immunolabeling of neural stem cells and their progeny. In this sense, our aim was to fill the correlation gap between the rigorous definition based on gene and/or protein expression (the latter also addressed by this paper) and the dramatic complexity of shape and geometry in contacting cells ultimately committed to building a three-dimensional model.

2. Experimental procedures

A collection of human embryos ranging from 5 to 12 gestational weeks (gw) was obtained from abortions performed at the Obstetric/Gynecology Department, "Carol Davila" University of Medicine and Pharmacy, Bucarest, Romania, according to the ethical legislation (nr. 134/25.01.2005 from the Bioethical Committee of the same university).

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The time elapsed between the abortion, dissection and cell manipulation was approximately 15 to 30 min [20]. Dissociated cells were cultured in selective medium containing DMEM-F12 (Gibco, Grand Island, NY), 1% B-27 with antioxidants (Gibco), epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) (both at 20 ng/ml, R&D Systems, Minneapolis, MN), heparin (5 µg/ml) and 1% gentamicin (Sigma-Aldrich, St. Louis, MO). Passage of the cells was performed every 14 days by selecting spheres to be sectioned when they reached at least 400 µm in diameter with a tissue chopper, in accordance with Svendsen *et al.* [21]. Those neurospheres that had not reached the critical mass for cutting after 2 weeks were allowed to grow further in culture for no longer than 1 month. Free-floating neurospheres were fed every 4 days by replacing half of the medium with fresh medium until they reached the desired size.

2.1 Immunocytochemistry

Free-floating, undifferentiated neurospheres obtained from the complete medium were washed several times with PBS and immediately fixed in 4% buffered paraformaldehyde for 10 min at room temperature. After three rinses in the same buffering solution, several neurospheres were mounted in Tissue-Tek (Sakura Finetek Europe, Netherlands), frozen and cut on a cryostat into 10 µm-thick sections. Before immunostaining, the neurosphere sections were pre-incubated with 10% normal serum (Sigma) and 0.2% TritonX-100. Primary antibodies were mouse anti-nestin (1:200, Millipore, cat no. AB5922), rabbit anti-GFAP (1:400 Dako), rabbit anti-α-internexin (1:100, Chemicon, cat. no. AB5354) mouse anti-βIII-tubulin (1:100, Sigma). Detection with secondary antibodies (Molecular Probes, Eugene, OR) was carried out using Alexa Fluor 488 F(ab')2 fragment of goat anti-mouse IgG (1:1000) and Alexa Fluor 546 F(ab')2 fragment of goat anti-rabbit IgG (1:1000). Observations were made using an Olympus IX 71 inverted microscope (Olympus, Tokyo, Japan), using 20x and 40x water immersion objectives. Twelve-bit images were taken with a cooled CCD camera (Sensicam PCO, Kelheim, Germany) with a 1280X1024 pixel chip. Image analysis was performed by means of the ImagePro Plus package (Media Cybernetics, Silver Springs, MD).

2.2 Scanning electron microscopy preparation

2.2.1 Osmic maceration

After extensive washing with PBS, "naïve" neurospheres were immediately immersed in 0.25% glutaraldehyde and 0.25% paraformaldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for 20 min at room temperature. After an initial reduction in size of 1 mm^3, the specimens were washed in phosphate-buffered saline (PBS, pH 7.2) and then postfixed in a solution of 1% osmium tetroxide and 1.25% potassium ferrocyanide for 3 h. Specimens were further cut into 0.2 mm-thick slices, followed by a second post-fixation in 1% osmium tetroxide and 1.25% potassium ferrocyanide for 2 h. The slices were washed in PBS and immersed in 0.1% osmium tetroxide in PBS for 48 h. They were then dehydrated in graded ethanol and subjected to critical point drying with CO_2. The slices mounted on aluminum stubs were coated with 10 nm of pure gold in a Emitech K250 sputter-coater.

All the specimens were observed with a Philips SEM-FEG XL-30 scanning electron microscope operated at 10kV.

3. Results

Human neurospheres observed with an inverted microscope showed after some mitotic divisions a unique morphological feature due to appearance of cilia-like protrusions emerging from the outer cells, which tended to be reabsorbed with senescence (Figure 1A). Such physical extensions of the plasma membrane were responsible for neurosphere motility since they immediately disappeared in case of sudden adhesion to the bottom of the plastic surface (data not shown). In addition, when they underwent spontaneous fusion (Figure 1B) these tiny irregular formations retracted, and the neurospheres resumed to their original elliptical shape.

Experiments on sections taken at the equatorial plane showed intense fluorescent immunoreaction when samples were incubated with specific anti-nestin antibodies (Figure 2A). Virtually all cells were nestin-positive regardless of tissue localization. The most represented of the other markers was α-internexin (Figure 2B), while a consistent number of cells also expressed GFAP (Figure 2C). Interestingly, very few cells were found to express the neuronal specific marker βIII-tubulin which, however, was better visualized within the neuropil extending from the cell bodies (data not shown).

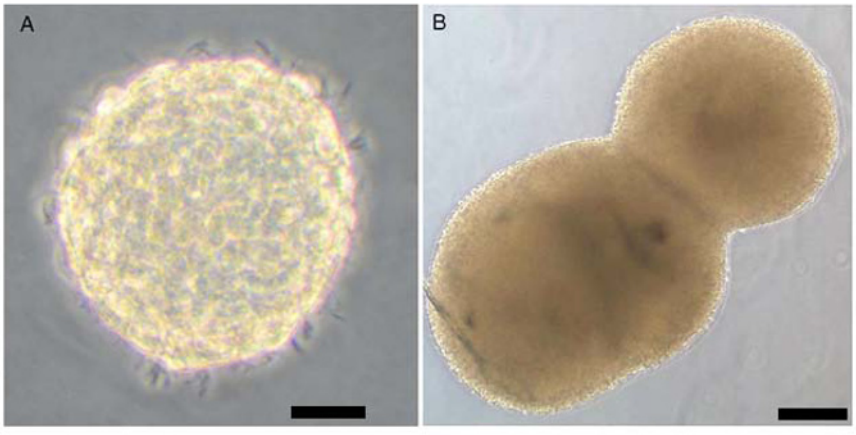

Figure 1. Morphology at transmitted light microscopy.
A. Typical morphology of human neurosphere at an early maturation stage with several cilia-like protrusions irregularly distributed on the outermost cell layer; B. Appearance of two neurospheres in the act of fusion. Notably the cilia-like formations have retracted. Scale Bars: A = 25 µm, B = 100 µm.

Experiments carried out by SEM on whole preparation of "naïve" neurospheres taken at different maturation stages confirmed that the margin of the neurospheres was characterized by an irregular surface made of various projections but also of budding elements possibly identifiable as newborn cells (Figure 3A). Another striking evidence was that sometimes the centripetal growth of the neurosphere seemed to take place progressing from one pole to the opposite as documented by the different thickness of the external profile. At higher magnifications, ultrastructural analysis revealed scattered and multidirectional crests similar to plasmalemmal specializations (cilia-like) (Figure 3B) and already described by transmission light microscopy (unpublished observation). Many adjacent areas not decorated in this way were occupied by rounded-like elements placed at regular intervals (Figure 3C) and emerging from a smoother outline.

However, as the quality of our observations was limited and conditioned by the high degree of tissue overlapping, we opted for an alternative procedure, namely osmic maceration of cut neurospheres, to dissect the complex network of intermingled structures better. In particular, by means of this technique, we were able to achieve both random and mild cellular digestion, as documented by a small hole in the centre of the neurosphere centre and by the detachment of irregular pieces (Figure 4A). Despite partial degradation of the complex architecture of the tissue, the dense cellular packing predominant on the extracellular matrix component was clearly evident. A magnification of the inset corresponding to a piece of the removed area (Figure 4B) suggested that volumetric assembly takes place according to a multilayered arrangement. In particular, as shown in the figure, the lower plane was filled with vesicle-like structures of similar size, with budding action, in contrast with a single cell with a bulging base in continuity with a slender process in contact with its outer surface. The complexity of shape and contacts between cells is documented in Figure 5A, where many cell bodies are pyramidal in shape, often with long processes asymmetrically expanding in

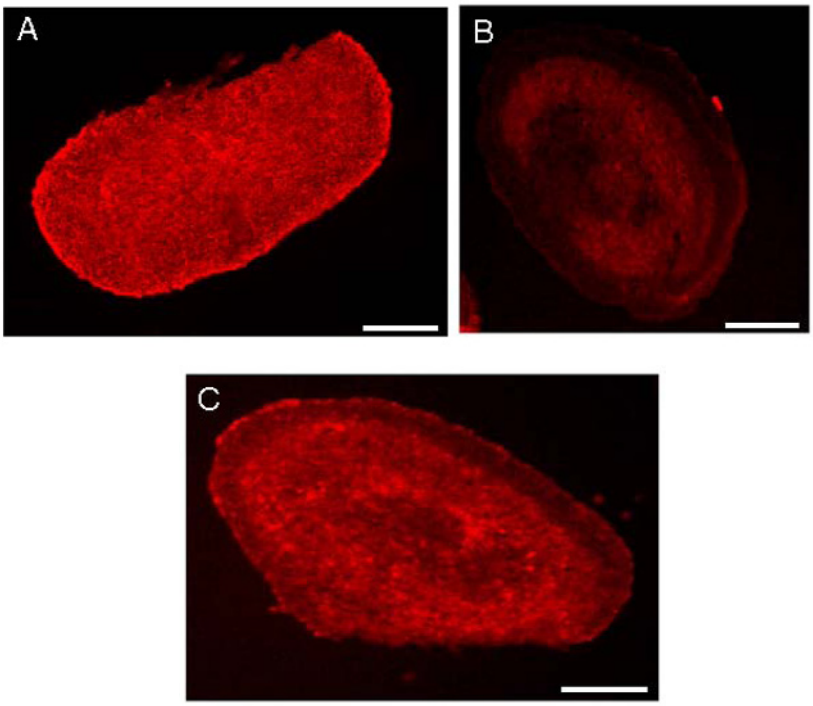

Figure 2. Immunocytochemical staining of cryostat sections.
Photomicrographs of cryostat sections of neurospheres after immunostaining with antibodies against nestin (A), α-internexin (B) and GFAP (C). Scale bar = 100 µm.

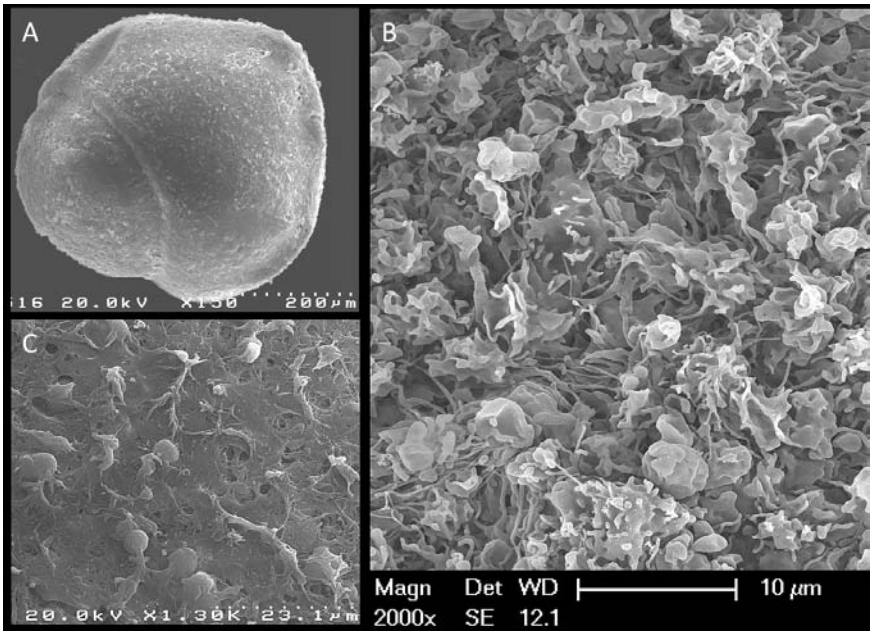

Figure 3. Scanning electron microscopy of a whole undifferentiated neurosphere.
A. Scanning electron microscopy of a whole undifferentiated neurosphere taken at 2 weeks in vitro characterized by an irregular surface comprising both various projections and budding elements. At higher magnification photomicrographs show scattered and multidirectional crests sprouting from the outer profile (B) alternating to a network of rounded-like cells emerging from a smoother outline.

various directions. Smaller cells had a rounded conformation with lesser structured, very thin branches, which were difficult to track. In some other samples (Figure 5B), the architecture of the inner core revealed the prevalent presence of spherical cells so tightly packed that intercellular space was dramatically reduced. By contrast, pyramidal cells were virtually absent together with their contribution in terms of fibers and peripheral arborizations.

4. Discussion

Since the advent of stem cell biology in the field of neuroscience, a major challenge for the scientists has been to identify cytological and molecular markers for tracking and defining the expanding population of cells derived from single neural stem cells by coupling immunological techniques with highly sophisticated technology such as confocal microscopy, fluorescence-activated cell sorting (FACS) and other ad hoc equipment. The ultimate goal of this morphofunctional analysis is to establish a "hierarchical tree" similarly to the already established haematopoiesis pathway. Furthermore, the "neurosphere system" has attracted considerable attention because it can be considered to be a self-sustaining tissue which may be studied ex vivo from the earliest mitotic divisions, in a classical cell culture setting. In practice however, the high rate growth rate of human neural stem/progenitor cells [22] together with their natural tendency to aggregate and merge into multiple neurospheres [23] gives rise to an extremely compact cluster of cells. This makes it necessary to cut neurospheres into sections in order to maintain mutual cell organization and ensure that the different types of cells can be identified on the basis of their positive correlation to immunological markers. On the other side, a tridimensional reconstruction by section stacking remains an unattainable goal. In fact, before we performed processing for ultrastructural analysis, our immunocytochemical results confirmed that nestin-positive cells, generally regarded as stem cells [24], are ubiquitous cells in the neurosphere. Moreover, the substantial amount

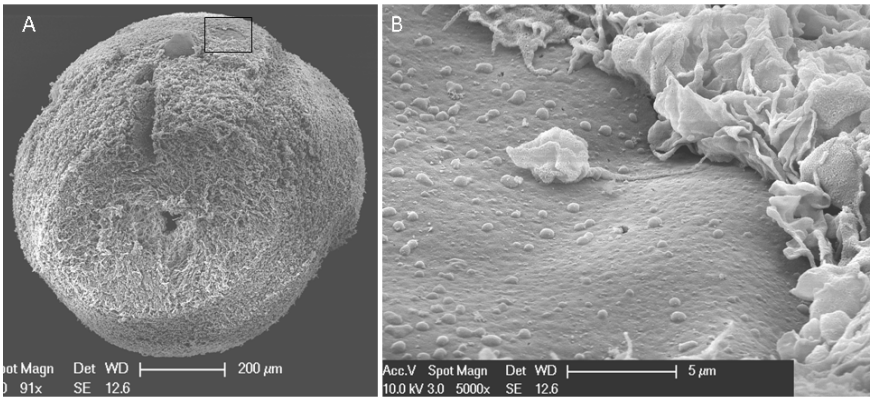

Figure 4. SEM neurosphere preparation by means of osmic maceration.
A. SEM neurosphere preparation frontally cut after osmic maceration. The high degree of cellular packing is clearly visible, while the extracellular matrix is poorly distributed. Note a small hole in the centre of neurosphere. B. At higher magnification, the removal of a small fragment of the outermost profile (see inset in panel 4A) revealed a collection of vesicle-like cells in the process of budding. Note also the presence of a single larger cell with a bulging base continuing with a tiny process.

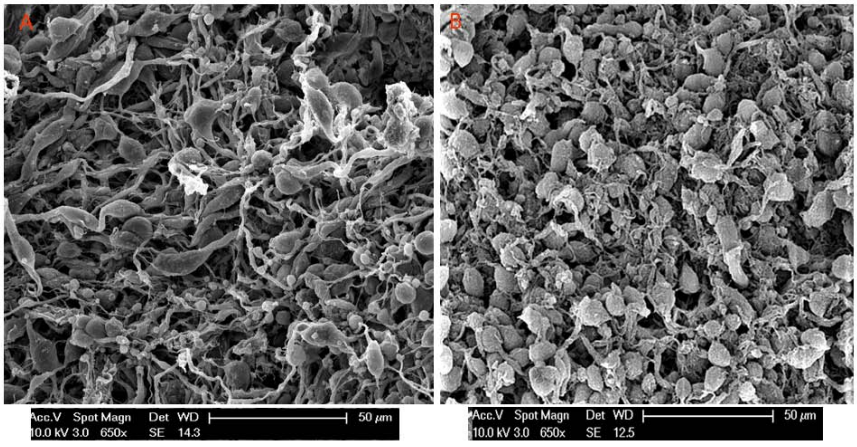

Figure 5. SEM analysis of the neurosphere core.
SEM observation of the neurosphere core. A. The complex network of cells and structure is well documented by a mixture of pyramidal cells with long and convoluted processes and smaller cells with round conformation with less structured very thin branches. B. The overall internal organization of the neurospheres shows an abundance of small, tightly packed spherical cells. By contrast pyramidal cells seem to be absent.

of GFAP-expressing cells could not only be attributed to the presence of this intermediate filament protein in the astroglial lineage [25] but also be considered as a possible marker of immature precursors and neuroblasts [26]. The most interesting finding from the immune experiments was the abundant presence of internexin-labeled cells with a specific ring-like distribution in the central section, which are likely to represent a percentage of neuroblast-differentiated cells [27]. Moreover we could not find any cells with a detectable fluorescent signal for βIII-tubulin except for very scant positive fibers (data not shown). The last finding should be in agreement with the undifferentiated status of neurospheres even though, on this matter, there exist conflicting data in the literature; such discrepancies are partly explained by minor differences in medium composition, sphere passages and gestational ages of the neurosphere-derived brains [28]. We have found scanning electron microscopy to be powerful investigation method for elucidating some questions regarding morphological

parameters such as size, shape, density of cell and processes, plasmalemma specializations etc. On initial examination, the observation of intact neurospheres by SEM revealed that, in addition to tufts of cilia-like structures on the outermost layer, interpreted as motility tools [29], neurosphere boundaries were not closed by a regular juxtaposition of cells because of the rough profile of the neural body. This finding was validated at higher magnification where newly formed cells were seen to be randomly budding and increasing the total volume of the spheres. The hypothesis that budding is the process whereby neurosphere size increases is corroborated by Figure 4B where experimental maceration unmasked a limited portion of the tissue suggesting that the size increase takes place in a laminar fashion giving rise to a multilayered spatial arrangement. The appearance of vescicle-like structures on the surface should be viewed in this perspective. The other major finding concerns inner assembly, which is based on two different tissue organization processes. The first one is represented by more compact cells with small round boies, giving rise to short processes (15 days in vitro); the second one consists of larger pyramidal and long process-bearing cells intermingled with smaller spherical cells (30 days in vitro). In this regard, it has already been shown that even cells within the neurospheres can migrate from the periphery to the inner core [29,23], although there is no definitive proof of shape changes following these active movements. Therefore, we suggest that this shape transition may be the result of gradual escape from the undifferentiated state (stem cells and progenitors) to a more restricted phenotype as documented by the presence of internexin and GFAP-expressing cells where the typical spherical profile of stem cells is replaced by a more functional but irregular shape. This could be in agreement with the fact that a higher presence of intercellular spaces possibly filled with extracellular matrix fits better with more evolved cell functions.

Further experiments are in progress in our laboratory to apply immnocytochemical procedures to SEM preparations in order to ascertain whether the geometry pattern of differentiation-committed cells is linked to the expression of specific protein or cytological markers. This is not a trivial point considering that shape development often depends on functional features.

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References

[1] Reynolds B.A., Tetzlaff W., Weiss S., A multipotent EGf-responsive striatal embryonic progenitor cell produces meurons and astrocytes, J. Neurosci., 1992, 12, 4565-4574

[2] Reynolds B.A., Weiss S., Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell, Dev. Biol., 1996, 175, 1-13

[3] Hulspas R., Quesenberry P.J., Characterization of neurosphere cell phenotypes by flow cytometry, Cytometry, 2000, 40, 245-250

[4] Seaberg R.M., Smukler S.R., van der Kooy D., Intrinsic differences distinguish transiently neurogenic progenitors from neural stem cells in the early postnatal brain, Dev. Biol., 2005, 278, 71-85

[5] Barraud P., Stott S., Møllgård K., Parmar M., Björklund A., In vitro characterization of a human neural progenitor cell coexpressing SSEA4 and CD133, J. Neurosci. Res., 2007, 85, 250-259

[6] Suslov O.N., Kukekov V.G., Ignatova T.N., Steindler D.A., Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres, Proc. Natl. Acad. Sci. USA, 2002 , 99, 14506-14511

[7] Anderson L., Burnstein R.M., He X., Luce R., Furlong R., Foltynie T., et al., Gene expression changes in long term expanded human neural progenitor cells passaged by chopping lead to loss of neurogenic potential in vivo, Exp. Neurol., 2007, 204, 512-524

[8] Hitoshi S., Tropepe V., Ekker M., van der Kooy D., Neural stem cell lineages are regionally specified, but not committed, within distinct compartments of the developing brain, Development, 2002, 129, 233-244

[9] Ostenfeld T., Joly E., Tai Y.T., Peters A., Caldwell M., Jauniaux E., et al., Regional specification of rodent and human neurospheres, Dev. Brain Res., 2002, 134, 43-55

[10] Horiguchi S., Takahashi J., Kishi Y., Morizane A., Okamoto Y., Koyanagi M., et al., Neural precursor cells derived from human embryonic brain retain regional specificity, J. Neurosci. Res., 2004, 75, 817-824

[11] Kim H.T., Kim I.S., Lee I.S., Lee J.P., Snyder E.Y., Park K.I., Human neurospheres derived from the fetal central nervous system are regionally and temporally specified but are not committed, Exp. Neurol., 2006, 199, 222-235

[12] Reynolds B.A., Rietze R.L., Neural stem cells and neurospheres--re-evaluating the relationship, Nat. Methods, 2005, 2, 333-336

[13] Coles-Takabe B.L., Brain I., Purpura K.A., Karpowicz P., Zandstra P.W., Morshead C.M., et al., Don't look: growing clonal versus nonclonal neural stem cell colonies, Stem Cells, 2008, 26, 2938-2944

[14] Ferrari D., Binda E., De Filippis L., Vescovi A.L., Isolation of neural stem cells from neural tissues using the neurosphere technique, Curr. Protoc. Stem Cell Biol., 2010, Chapter 2:Unit2D.6

[15] Piao J.H., Odeberg J., Samuelsson E.B., Kjaeldgaard A., Falci S., Seiger A , et al., Cellular composition of long-term human spinal cord- and forebrain-derived neurosphere cultures, J. Neurosci. Res., 2006, 84, 471-482

[16] Maric D., Barker J.L., Neural stem cells redefined: a FACS perspective, Mol. Neurobiol., 2004, 30, 49-76

[17] Kukekov V.G., Laywell E.D., Suslov O., Davies K., Scheffler B., Thomas L.B., et al., Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain, Exp. Neurol., 1999, 156, 333-344

[18] Bez A., Corsini E., Curti D., Biggiogera M., Colombo A., Nicosia R.F., et al., Neurosphere and neurosphere-forming cells: morphological and ultrastructural characterization, Brain Res., 2003, 993, 18-29

[19] Lobo M.V., Alonso F.J., Redondo C., López-Toledano M.A., Caso E., Herranz A.S., et al., Cellular characterization of epidermal growth factor-expanded free-floating neurospheres, J. Histochem. Cytochem., 2003, 51, 89-103

[20] Nat R., Cretoiu D., Popescu L.M., In vitro differentiation of human embryonic neural stem cells, J. Cell. Mol. Med., 2001, 5, 324-325

[21] Svendsen C.N., ter Borg M.G., Armstrong R.J., Rosser A.E., Chandran S., Ostenfeld T., et al., A new method for the rapid and long term growth of human neural precursor cells, J. Neurosci. Methods, 1998, 85, 141-152

[22] Mori H., Ninomiya K., Kino-oka M., Shofuda T., Islam M.O., Yamasaki M., et al., Effect of neurosphere size on the growth rate of human neural stem/progenitor cells, J. Neurosci. Res., 2006, 84, 1682-1691

[23] Wang T.Y., Sen A., Behie L.A., Kallos M.S., Dynamic behavior of cells within neurospheres in expanding populations of neural precursors, Brain Res., 2006, 1107, 82-96

[24] Hockfield S., McKay R.D., Identification of major cell classes in the developing mammalian nervous system, J. Neurosci., 1985, 5, 3310-3328

[25] Reeves S.A., Helman L.J., Allison A., Israel M.A., Molecular cloning and primary structure of human glial fibrillary acidic protein, Proc. Natl. Acad. Sci. USA, 1989, 86, 5178-5182

[26] Doetsch F., Caillé I., Lim D.A., García-Verdugo J.M., Alvarez-Buylla A., Subventricular zone astrocytes are neural stem cells in the adult mammalian brain, Cell, 1999, 97, 703-716

[27] Fliegner K.H., Kaplan M.P., Wood T.L., Pintar J.E., Liem R.K.H., Expression of the gene for the neuronal intermediate filament protein α-internexin coincideswith the onset of neuronal differentiation in the developing rat nervous system, J. Comp. Neurol., 1994, 342, 161-173

[28] Jensen J.B., Parmar M., Strengths and limitations of the neurosphere culture system, Mol. Neurobiol., 2006, 343, 153-161

[29] Singec I., Knoth R., Meyer R.P., Maciaczyk J., Volk B., Nikkhah G., et al., Nat. Methods, 2006, 3, 801-806