Foetal Mouse Neural Stem Cells Give Rise to Ependymal Cells *in Vitro*

(neural stem cells / neurospheres / ependymal cells / ependymogenesis / *in vitro* / differentiation potential / mouse)

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Abstract. NSCs are responsible for the generation of CNS cell types derived from the neural tube. Published data resulting from experiments studying the differentiation of NSCs *in vitro* or *in vivo* have confirmed their spontaneous tripotency, i.e. their ability to generate cells of the neuronal, astroglial and oligodendroglial lineages. The relationship between NSCs generated *in vitro* and ependymal cells has not yet been studied. To confirm that ependymal cells can also be produced by NSCs, we utilized the neurosphere assay, which permits isolation and cultivation of NSCs. Cells from the forebrain of E14-15 Balb/c foetuses were grown in DMEM/F12-N2 medium supplemented with EGF and FGF-2 to form multicellular neurospheres. After 3 to 8 passages, neurospheres were plated on surfaces coated with poly-L-lysine, polyornithine and/or laminin in dishes containing the same medium where cytokines were replaced with serum. Under these conditions, neurosphere cells spread over the surface forming a cellular layer consisting of β-III tubulin$^+$ neuronal, GFAP$^+$ astroglial and O4$^+$ oligodendroglial cells. When these cells were cultivated for prolonged periods, they formed islands of epitheloid cells. Following 2 to 3 weeks *in vitro*, ependymal cells with beating cilia appeared among these cells. Ciliated ependymal cells were observed in small clusters or as single cells scattered in certain areas. Confocal microscopy confirmed the presence of α-tubulin-immunoreactive cilia arranged in tufts located on the apical surface of epitheloid cells. Our data indicate that ependymal cells are spontaneously derived from NSCs.

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Abbreviation: GFAP – glial fibrillary acidic protein, NSCs – neural stem cells.

During development of the central nervous system (CNS), all neuronal and major glial cells, i.e. astroglial, oligodendroglial and ependymal cells arise from multipotent neural stem cells (NSCs) (Carlson, 1994). NSCs can be isolated from the foetal and adult CNS parenchyma with the use of stimulatory cytokines (Anchan et al., 1991; Reynolds et al., 1992; Gage et al., 1995; Mokrý et al., 1995; Palmer et al., 1995; Gritti et al., 1996; Vescovi et al., 1999; Zhou et al., 2000). The most commonly used assay for cultivation of NSCs relies on proliferative response of NSCs exposed to growth factors, which results in formation of floating multicellular neurospheres (Reynolds et al., 1992). The assay is correspondingly referred to as the neurosphere assay. NSCs harvested from neurospheres reveal all biological properties attributed to stem cells. They lack markers specific for differentiated cells, they are capable of self-renewal and can be maintained in culture for several years (Vescovi et al., 1999; Zhou et al., 2000). They have a high proliferation potential and the ability to give rise to differentiated progeny. NSCs are transplantable and following neural grafting they differentiate into neuronal and glial cells (Gage et al., 1995; Vescovi et al., 1999; Mokrý et al., 2005). Neuronal, astroglial and oligodendroglial cells have been described upon *in vitro* differentiation of cells forming the neurospheres; the identity of generated cell types has been confirmed immunocytochemically and electrophysiologically.

Based on these published data, NSCs isolated from foetal and adult mammalian brains are generally considered as tripotent tissue-specific stem cells. Differentiation into ependymal cells has never been observed, which may reflect inability of NSCs to generate ependymal cells or necessity to develop new conditions that would facilitate ependymal differentiation.

To address this issue we modified conditions used in the differentiation assay that allowed us to identify ependymal cells in the progeny generated by NSCs isolated from the foetal mouse forebrain. In this study, we describe the conditions permitting maturation of ependymal cells *in vitro* as well as morphological criteria for their identification.

Material and Methods

Neural stem cells (NSCs) were isolated from the forebrain of Balb/c mice on embryonic day 14–15. Following gentle trypsinization, mechanical trituration of the foetal brain tissue and washing in DMEM, the resulting suspension was plated in uncoated dishes in a chemically-defined serum-free medium consisting of a 1 : 1 mixture of DMEM (Gibco BRL, Gaithersburg, MD) and Ham's F12 nutrient containing N2 supplement (Gibco BRL) with 10 ng/ml FGF-2 and 20 ng/ml EGF (both from PeproTech, London, UK), 2 mM L-glutamine, 1000 U/ml penicillin and 1000 μg/ml streptomycin (Gibco BRL). The cultures were maintained in 5% CO_2 humidified atmosphere at 37°C. Under these conditions, all differentiated elements rapidly died whereas NSCs proliferated and formed multicellular neurospheres. Approximately each week (the precise time depended on the number and size of neurospheres), the neurospheres were re-dissociated and resulting single-cell suspension was plated in new serum-free media supplemented with growth factors. Such repetitive passaging (at 1 : 2 to 1 : 3 ratio) permitted us to keep NSCs in undifferentiated state. Neurospheres yielded from the third to eight passages were transferred to the differentiation assay to exploit their spontaneous differentiation potential.

To facilitate differentiation of progeny derived from NSCs, neurospheres were plated in dishes coated with a suitable substrate that allowed attachment of cells. For coating the culture dishes we used the following solutions of attachment factors: 1) 5 μg/ ml poly-L-lysine (Sigma, St. Louis, MO) (dishes incubated 30 min at 37°C, rinsed twice with distilled water and alowed to air dry), 2) 20 μg/ml polyornithine (Sigma) (30 min at 37°C, rinsed twice with distilled water and alowed to air dry), 3) 0.1% gelatin (15 min at room temperature, drained, alowed to air dry), and 4) 20 μg/ml polyornithine (see above) plus 5 μg/ml laminin (Sigma) (24 h at 37°C, rinsed with culture medium, not allowed to air dry). The cells were incubated in DMEM (devoid of growth factors) supplemented with 10% foetal cell serum (Sigma), 2 mM L-glutamine, and 1000 U/ml penicillin/1000 μg/ml streptomycin. Under these conditions, neurospheres attached to the substrate and spread in a continuous layer. The cultures were regularly examined in phase contrast with the use of a Nikon Eclipse TE 300 inverted microscope and the media were changed every 2–3 days. Following 1–3 weeks *in vitro*, the cells were fixed with 4% paraformaldehyde for 10 min and processed for immunocytochemistry.

For immunocytochemical examination, fixed cells were washed three times in PBS containing 5% Triton X-100 (Sigma). They were then incubated with primary antibody at 4°C overnight. The following antibodies were used to identify cell phenotypes: anti-β-III tubulin antibody (TU-20, Exbio, Prague, CR; diluted 1 : 200), anti-GFAP (GA-5, Sigma; 1 : 400), anti-O4 (81, Boehringer Mannheim, Mannheim, Germany; 1 : 5) and anti-α-tubulin (polyclonal, DAKO, Glostrup, Denmark; 1 : 300). Following washing with PBS, the cells were incubated with goat anti-mouse or goat anti-rabbit secondary antibodies conjugated with Cy 3 (Jackson Immunoresearch Laboratories, West Grove, PA), Alexa 488 (Molecular Probes, Eugene, OR) or horseradish peroxidase (Sigma). Nuclei were counterstained with propidium iodide or 4′,6-diamidino-2-phenylindole (DAPI) or methyl green. For immunofluorescence, the cells were mounted in polyvinylalcohol/glycerol with DABCO (1,4-diazabicyclo[2.2.2.]octane); for light microscopy the cells were mounted in DPX mountant.

Results

Cell suspension obtained from trituration of trypsinized foetal brain tissue was seeded in chemically defined culture medium supplemented with growth factors in uncoated dishes. Under these conditions, the differentiating cells were unable to attach and mature. As a result such cells soon died, whereas the undifferentiated NSCs survived. Growth factors stimulated NSCs to undergo multiple divisions, which resulted in formation of three-dimensional structures, called neurospheres. Each multicellular neurosphere contained only one or few NSCs, whereas its major progeny tended to differentiate (Mokrý et al., 1996). To interrupt the differentiation process, neurospheres were dissociated to single-cell suspension that was again replated in a new neurosphere assay. Such passaging permitted removal of all contaminating differentiated cells and preservation of undifferentiated NSCs. For experiments described in the present study, the neurospheres were passaged 3–8 times prior to plating in the differentiation assay.

To induce cell differentiation, the neurospheres yielded from the neurosphere assay were plated in dishes coated with suitable substrates and grown in serum-supplemented medium. Following neurosphere attachment, the superficial cells extended their processes radially and began to emigrate from the neurosphere core covering the surface of the culture dish in a continuous layer. Following several days *in vitro*, the cells differentiated and assumed more or less characteristic phenotypes.

Neuronal cells could be distinguished on the basis of their typical morphology. They possessed two or more processes; some occasionally with a growth cone. Their straight axons reached varied lengths and sometimes sent collaterals. Fully differentiated cells exhibited rich dendritic arborization. The identity of the cells was conclusively confirmed by immunocytochemical detection of neuron-specific marker β-III tubulin (Fig. 1A).

The majority of cells produced by differentiating neurospheroidal cells adopted morphology of astroglial

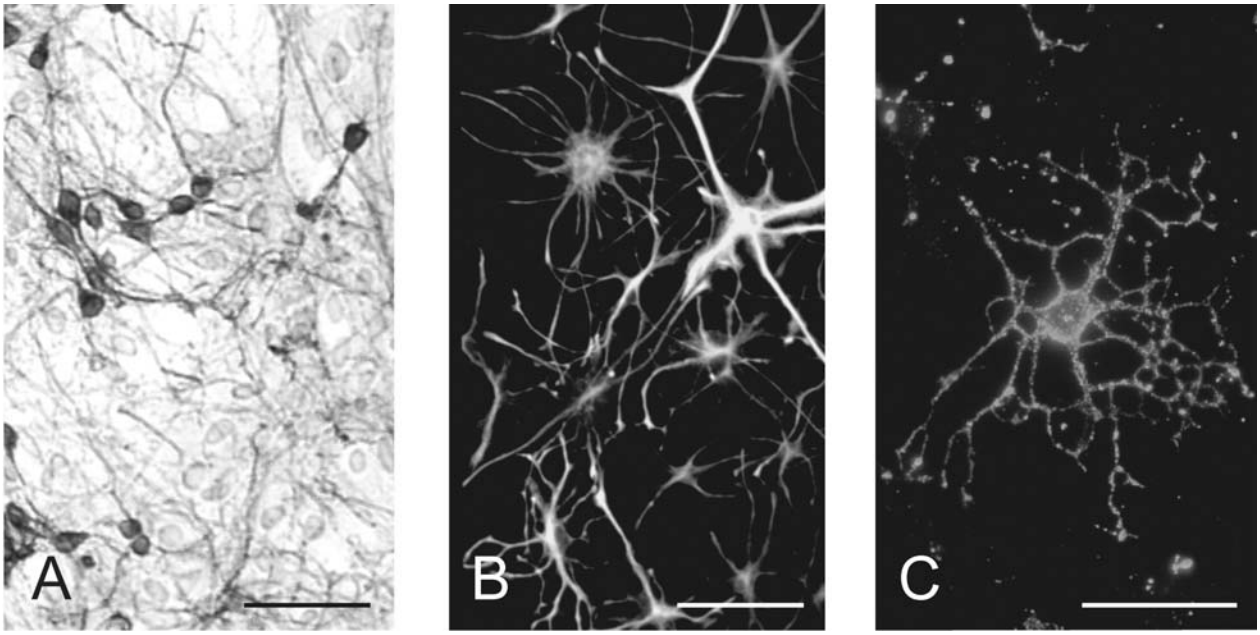

Fig. 1. Bipolar and multipolar neuronal cells expressing β-III tubulin were detected with the use of peroxidase immunocytochemistry (A). By day 8 in the differentiation assay, tertiary neurospheres gave rise to multiple cell types. Astroglial cells immunopositive for GFAP arose in the progeny derived from neurospheres (B). The specific immunoreactivity was confined to cytoplasmic intermediate filaments. The cells *in vitro* adopted morphology of protoplasmic and fibrillary astrocytes. Punctate immunofluorescence of O4 antigen (red), a marker specific for oligodendroglial cell lineage, permitted visualization of cell bodies and their ramified extensions (C). Scale bars: 50 μm.

cells. The cells were flat and exhibited numerous flat cellular processes. Their cytoplasm was immunoreactive for glial fibrillary acidic protein (GFAP) (Fig. 1B). Although the astroglia represented the most numerous cell types (especially after prolonged cultivation), counterstaining of cell nuclei revealed that they grew in admixture with other, i.e. GFAP-negative cells.

After the end of the first week in the differentiation assay, oligodendroglial cells could be distinguished. Their perikaryon contained a relatively large nucleus surrounded by a thin rim of the cytoplasm. Numerous thin and branched processes emerged from the cell body. The phenotype of oligodendroglial cell was identified immunocytochemically since the plasmalemma covering the extensions and cellular body expressed O4 antigen (Fig. 1C).

When the cells spreading over the culture surface formed almost a confluent layer, small islands of flat epitheloid cells became visible (Fig. 2A). Well-delimited intercellular boundaries of these cells gave the islands a characteristic mosaic appearance (Fig. 2A, B). Basal parts of the cells were attached directly to culture dishes and the cells grew in a monolayer, i.e. they did not require an underlying feeder layer formed by other cells. Since the epitheloid cells were mitotically active, islands enlarged their sizes gradually. The interface between the area occupied by polygonal epitheloid cells and adjacent cell types with different morphology remained sharply demarcated. Soon beating cilia extending from the apical surface of some epitheloid cells could be recognized

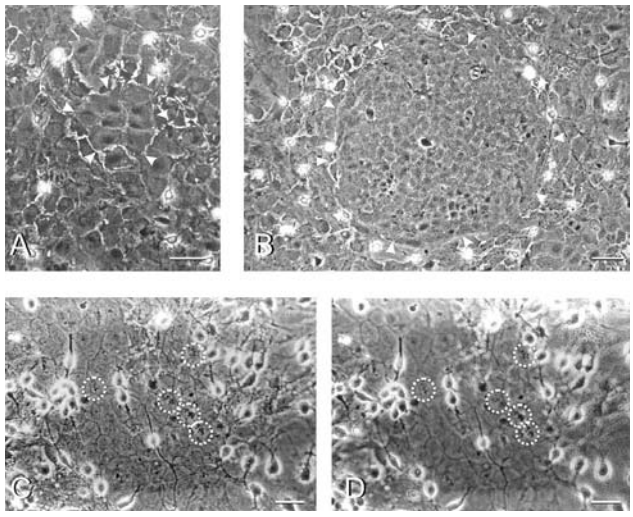

Fig. 2. Islands of epitheloid polygonal cells could be distinguished after reaching a confluent layer. Boundaries of the islands on day 17 and day 25 were demarcated by white arrowheads in Fig. A and B, respectively. Islands increased their size by mitotic divisions; cells at different phases of mitosis are clearly visible in Fig. B. Ependymal cells arranged in a single layer in phase microscopy focalized on intercellular boundaries (C) revealed a typical mosaic arrangement of polygonal cells in the island. Spindle-shaped cells occasionally grew over the monolayer of epitheloid cells. The identical field was focalized on apical poles of polygonal cells (D) and revealed that these were studded with numerous cilia. Five distinct ependymal cells were encircled but many more were visible in the island. Scale bars correspond to 50 μm in A, C and D and 100 μm in B.

under phase contrast (Fig. 2C, D). When focused on cilia, these were apparent as dots and dark diffuse areas. While dots corresponded to individual cilia and perhaps to their basal bodies, the dark areas represented cilia tufts. To provide an unambiguous evidence of multiciliated ependymal cells, we processed the specimens for anti-α-tubulin immunostaining (Fig. 3). The immunopositive signal was distributed in the cell cytoplasm and concentrated in cilia. Laser scanning confocal microscopy allowed us to examine transverse sections of cilia arranged in bunches in apical poles of ependymal cells. Tufts consisted of 20–50 cilia and covered only a central part of the cell surface. Three-dimensional shape of an ependymal cell reconstructed from serial sections revealed that the cilia were very long (up to 20 μm). Individual cells differed in the amount of cilia; some had tufts, some possessed only few cilia, whereas other polygonal cells were non-ciliated. In young cultures, only some cells appeared to be ciliated. More ciliated cells appeared during the third week when cell density in the cultures became quite high.

Sometimes some other cells were found lying on top of the ependymal cell layer; these cells were not polygonal and usually had elongated cell extensions. Although most ependymal cells were localized in distinct islands, some individual ciliated cells were scattered throughout the culture, but these cells were difficult to recognize. We observed the first ciliated cells in younger cultures (during the second week *in vitro*). Nevertheless, by that time all the other neural cell types (neurons, astroglia and oligodendroglia) had differentiated *in vitro* and therefore ependymal cells could be considered as the last cell type that was generated. The precise time of appearance and maturation of cilia-bearing cells was influenced by at least two factors: the substrate used for neurosphere attachment and the resulting cell density. Of the four substrates tested (poly-L-lysine, polyornithine, gelatin and polyornithine-laminin), the latter one was the best in promoting ependymogenesis and permitted detection of numerous ependymal cells at earlier time points. When the neurospheres were plated at densities allowing the cells to establish a confluent layer, the ependymal cells were regularly detected in all differentiation assays examined.

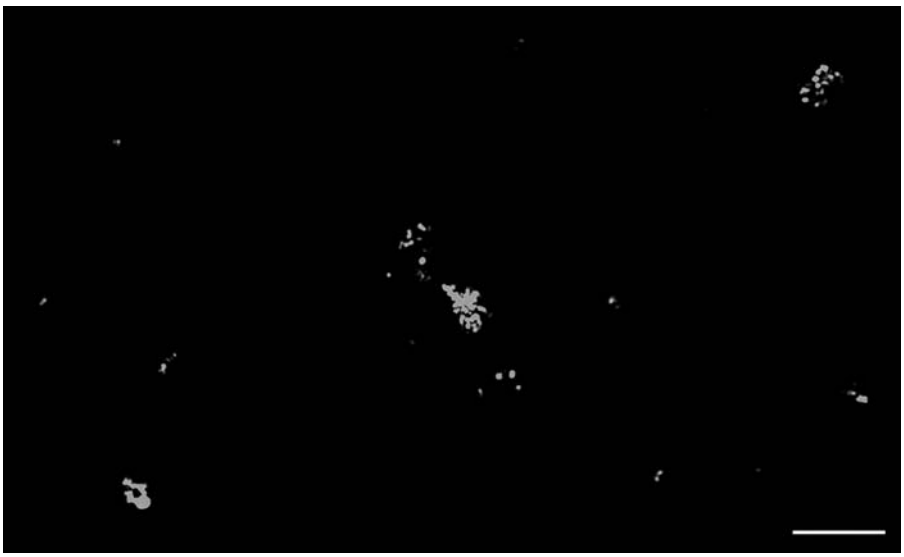

Fig. 3. α-tubulin immunofluorescent cilia provided evidence of ependymal cells in culture when visualized with laser scanning confocal microscopy. Several cells bear cilia tufts, whereas the other are studded with few cilia only. Scale bar represents 30 μm.

Discussion

In the course of neurogenesis, the ependymal cells are derived from neuroepithelial cells that constitute the wall of the neural tube (Das, 1979; Carlson, 1994; Fu et al., 2003). These neuroepithelial matrix cells are responsible for production of CNS neuronal and glial cell types and act *de facto* as neural stem cells (NSCs). As new cells are generated, the neural tube wall becomes thicker, NSCs accumulate in the ventricular zone that faces the ventricular system and the wall of the neural tube is spanned with specialized elongated radial glial cells. Despite their complex morphology, radial glia retain some undifferentiated potential and serve as progenitors for astroglial and neuronal cells (Malatesta et al., 2000; Tamamaki et al. 2001). Recently Spassky et al. (2005) provided evidence that in mice, some precursors of ependymal cells are also derived from radial glia. At later stages of development, NSCs pass deeper to the subventricular zone (Angevine et al., 1969). Some of the ependymal cells or their precursors proliferate for some time after birth (Spassky et al., 2005). Ciliogenesis begins around birth and ependymal cilia appear on the walls of lateral cerebral ventricles between P0 and P4. Mature ependymal cells in the forebrain are thought to be terminally differentiated and uncapable of cellular divisions (Spassky et al., 2005). Therefore, the fully differentiated ependyma is vulnerable and does not regenerate at any age (Sarnat, 1995). Mitotic activity in the ependyma is considered as evidence of neoplasia (Sarnat, 1995). However, in mammals, region-specific differences in ependymal cells exist. The spinal cord ependymal cells of the central canal were described to proliferate after injury (Liu et al., 2002; Takahashi et al., 2003).

There exists a phylogenic lineage relationship between ependymal cells, radial glia and neuroepithelial cells in lower vertebrates and invertebrates, where non-specialized ependymoglial cells maintain a radial morphology and perform a variety of functions.

Following spinal cord injury in amphibians, these cells become activated, they re-express NSC markers and regenerate the injured tissue (Echeverri and Tanaka, 2002; Walder et al., 2003). Although adult mammalian ependymal cells share features and properties of terminally differentiated cells, they can be, under certain conditions, reverted to more primitive cells. After intraventricular administration of EGF, adult mouse forebrain ependymal cells adopt morphology of radial glia (Gregg and Weiss, 2003) and undergo cellular divisions (Itokazu et al., 2006). Neurospheres generated from ventricular and choroid plexus ependymal cells can produce neuronal, astroglial and oligodendroglial cells (Johansson et al., 1999; Itokazu et al., 2006). Mouse NSCs forming neurospheres are able to generate radial glia (Gregg and Weiss, 2003), which can also act as precursors of ependymal cells (Spassky et al., 2005). All these data support the relationship that exists between mammalian ependymal cells, radial glia and neural stem cells.

In 1992, Reynolds et al. described the neurosphere assay permitting isolation of NSCs from *in vitro* that soon became a major tool of neural stem cell biologists worldwide. The assay confirmed a clonogenic potential and self-maintenance of NSCs derived from foetal and adult human and mouse brains (Vescovi et al., 1999). When the neurospheres are allowed to anchorage and are grown in serum-supplemented medium, the cells emigrate from the neurosphere and rapidly differentiate into neuronal, astroglial and oligodendroglial cells (Reynolds et al., 1992; Vescovi et al., 1999), and therefore NSCs are generally described as tripotent tissue-specific stem cells. In none of the studies concerning the NSC differentiation, the development of ependymal cells has been described. When NSCs derived either from neurospheres *in vitro* or from the developing brain were grafted into the adult brain parenchyma or intraventricularly (Gage et al., 1995; Winkler et al., 1998; Uchida et al., 2000), the signals necessary for triggering ependymogenesis were obviously missing. Even in case of intraventricular grafting, ependymogenesis was not induced. On the contrary, transplants of foetal neuroepithelia that became attached to cerebral ventricle wall eroded the underlying ependymal layer that did not regenerate (Němeček et al., 1995).

Currently, no ependyma-specific marker is known. In certain phases of their development, ependymal cells express GFAP, S 100 protein, vimentin, nestin, rat neural antigen-2 (Ran-2), cytokeratin 68 kDa, and bind Limax flavus agglutinin lectin (Raff et al., 1979; Abney et al., 1980; Bartlett et al., 1981; Weibel et al., 1986; Grondona et al., 1996). Unfortunately, none of these proteins is expressed exclusively by the ependymal cells. Therefore, *in vitro* grown ependymal cells must be recognized solely on the basis of their morphology. The most significant and distinguishing feature is the presence of beating cilia. However, not all ependymal cell types are ciliated and in cilia-bearing ependyma the surface specializations develop late in the process of their terminal differentiation. Tiny cilia are difficult to discern in culture especially when investigators focalize on cell bodies. Conditions for cultivation of ependymal cells also differ from those that are routinely used in *in vitro* differentiation assays.

To allow cultivation of foetal or newborn ependymal cells the dissociated cells must be plated at sufficient densities (approx. $5 \times 10^4/\text{cm}^2$) for establishment of a continuous layer. Cell-to-cell contacts are likely involved in triggerring maturation of ependymal cells and reflect a physiological situation occurring *in situ*, where ciliated ependymal cells cover the surfaces of cerebral ventricular system. In low-density cultures ependymal cells never arise. The substrate used for cell attachment also significantly influences the resulting amount of ependymal cells (Weibel et al., 1986; Jordan et al., 1987). Although we were able to detect small numbers of ependymal cells on the surface of all substrates tested, we obtained the highest yields from dishes coated with polyornithine-laminin. The temporal pattern of appearance of ependymal cells in culture represented another relevant feature. Ciliated ependymal cells were the latest phenotypes that matured in cultures, i.e. after the major CNS cell types including neurons, astrocytes and oligodendrocytes had been detected. In our assay used for evaluation of the differentiation potential of NSCs, many ciliated cells appeared during the third week *in vitro*. This peak correlated well with data resulting from cultures of foetal ependymal cells (Jordan et al., 1987).

In the present study, we provided the evidence that NSCs isolated from the foetal mouse forebrain via the neurosphere assay were able to generate ependymal cells and we described the conditions required for growth of ependymal cells *in vitro*. Ependymal cells in culture can be distinguished by direct visual observation in phase contrast. One of the most important morphological features is identification of epitheloid polygonal cells that are usually clustered in small islands (Fig. 2A, B). These areas predict the sites where ciliated cuboidal cells mature in subsequent developmental steps. The second distinguishing feature relies on recognition of beating cilia arranged in tufts on apical poles of ependymal cells. However, this criterion does not permit recognition of non-ciliated types of ependymal cells, which are represented *in situ* by secretory cells lining the choroid plexus or by cystic ependymal cells distributed in the aquaeductal wall (Fukuda et al., 1987; Grondona et al., 1996). Thus, the total number of ependymal cells identified in our differentiation assay may be underestimated.

Our results raise the question whether the ciliated cells detected by us in the described assay could not be derived from pre-existing ependymal cells aggregating with NSCs in cell suspension used for formation of

neurospheres. Ependymal cells themselves are able to form three-dimensional multicellular spheroids that resemble neurospheres. Because the motile cilia face the cultivation medium, their activity generates a rotation movement that allows distinguishing such spheroids at the first sight (Chiasson et al., 1999; Johansson et al., 1999). Moreover, upon their dissociation such spheroids are not capable of forming secondary spheroids (Chiasson et al., 1999), which likely reflects a low regenerative potential of foetal and adult ependyma. For the same reason it is unlikely that any ependymal cell could survive repetitive passaging used in our experiments. Therefore, our data suggest that NSCs derived from the foetal mouse brain produce ependymal cells *in vitro*, in addition to neuronal, astroglial and oligodendroglial cells.

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