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Review

In vitro and *in vivo* characterization of neural stem cells

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Summary. Neural stem cells are defined as clonogenic cells with self-renewal capacity and the ability to generate all neural lineages (multipotentiality). Cells with these characteristics have been isolated from the embryonic and adult central nervous system. Under specific conditions, these cells can differentiate into neurons, glia, and non-neural cell types, or proliferate in long-term cultures as cell clusters termed "neurospheres". These cultures represent a useful model for neurodevelopmental studies and a potential cell source for cell replacement therapy. Because no specific markers are available to unequivocally identify neural stem cells, their functional characteristics (self-renewal and multipotentiality) provide the main features for their identification. Here, we review the experimental and ultrastructural studies aimed at identifying the morphological characteristics and the antigenic markers of neural stem cells for their in vitro and *in vivo* identification.

Key words: Neural stem cells, Progenitor cells, Neurospheres, Cell markers, Ultrastructure

Introduction

Tissue-specific stem cells have been identified in multiple embryonic and adult organs, participating in tissue growth and differentiation during development, in the normal cell turnover of adult tissues, and in the repair of damaged organs. Stem cells (which are usually slowly proliferating cells) give rise to fast-proliferating transit amplifying progenitor cells that undergo a process of progressive lineage restriction and finally generate terminally differentiated cells (Gritti et al., 2002). The adult central nervous system (CNS) has long been considered devoid of stem cells and therefore, incapable of regeneration after injury or disease. However, recent studies have shown a new form of neuroplasticity based on *de novo* differentiation of neurons and glia from the stem cells localized in specific regions of the adult vertebrate CNS (Temple and Alvarez-Buylla, 1999; Gage, 2000). Fetal and adult neural stem cells are usually defined as undifferentiated cells (lacking markers of mature cells) that display proliferative and self-renewal capacities (i.e. the capacity to maintain the number of stem cells in a given compartment at a steady level, or to increase it in particular situations) and multipotentiality (i.e. the capacity for a single self-renewing cell to generate all neural cell lineages: neurons, astrocytes and oligodendrocytes) (Gage et al., 1995; Loeffler and Potten, 1997; Gritti et al., 2002). Previous reviews have examined fundamental aspects in stem cell biology, such as the genetic and epigenetic signals that regulate their survival, proliferation and differentiation (Edlund and Jessell, 1999), the identity of the intermediate phenotypes detected in their differentiation toward the neuronal and glial fates (Compston et al., 1997; Lee et al., 2000b), the characteristics of the niches where neural stem cells reside (Doetsch, 2003), their plasticity and trans-differentiation capacity (Vescovi et al., 2001; Gritti et al., 2002), and their therapeutic potential (Gage and Bjorklund, 1998; Blakemore and Franklin, 2000; Luque and Giménez y Ribota, 2004). In this review, we focus on the characteristics that define fetal and adult neural stem cells.

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Isolation, culture and differentiation

Stem cells have been isolated from the embryonic (Reynolds et al., 1992; Davis and Temple, 1994; Uchida et al., 2000) and adult CNS (Reynolds and Weiss, 1992; Gritti et al., 1996; Weiss et al., 1996; Palmer et al., 1997, 1999). Another source of neural stem cells is embryonic stem cells, the precursors to all embryonic lineages (Tropepe et al., 2001). In liquid culture, neural stem cells proliferate in suspension as spherical cell clusters termed "neurospheres" (Fig. 1A-C) that may be dissociated and subcultured repetitively. They are usually expanded in

serum-free medium supplemented with epidermal growth factor (EGF) and/or basic fibroblast growth factor (FGF-2), which act as mitogens for these cells both in vitro (Reynolds et al., 1992; Vescovi et al., 1993; Gritti et al., 1996) and *in vivo* (Craig et al., 1996). Thus, the infusion of these mitogens into the forebrain ventricles of adult animals causes the in situ proliferation of the stem cells located in the subventricular zone (SVZ), an important germinal layer of the CNS (Craig et al., 1996). Because no specific markers are available to unequivocally identify neural stem cells, these cells are identified by their self-renewal capacity that can only be assessed by demonstrating the persistence of stem cell features in their progeny, and by their multipotentiality that can only be assessed by demonstrating that they generate neurons, astrocytes and oligodendrocytes (Loeffler and Potten, 1997). Those studies that performed clonal analyses of dissociated single sphere cells to assay for self-renewal capacity and multipotentiality, demonstrated that only a small percentage of the cells in a neurosphere are truly multipotent stem cells. The frequency estimated is in the range of 0.3%-16% depending on the different studies and culture conditions (Gritti et al., 1999; Tropepe et al., 1999). In addition, free-floating spheres contain progenitor cells, which are more restricted in their proliferative and phenotypic potentials and are derived from stem cells (Mayer-Proschel et al., 1997; Arsenijevic and Weiss, 1998; Gritti et al., 1999; Tropepe et al., 1999). Floating spheres are composed of healthy cells in the different stages of mitotic division and in interphase, and cells in the different phases of the apoptotic and necrotic processes (Bez et al., 2003; Lobo et al., 2003). Several signaling pathways have been implicated in the self-renewal capacity of neurosphere cells, such as ciliary neurotrophic factor/gp 130 (Shimazaki et al., 2001; Chojnacki et al., 2003), Pten (Groszer et al., 2001), Emx2 (Galli et al., 2002), and the Notch signaling pathway (Nakamura et al., 2000; Chojnacki et al., 2003). When floating neurospheres are plated on adhesive substrate and the mitogens are removed, stem/progenitor cells migrate out of spheres (Fig. 1D) and differentiate into neurons and glia (Fig. 1E-G) (Gage et al., 1995; Paíno et al., 2003). However, some stem cells derived from the adult CNS do not require the removal of mitogens (EGF and FGF-2) for differentiation (Reynolds and Weiss, 1992; Arsenijevic et al., 2001). In these adherent neurospheres, cell migration occurs in the absence of a neuronal or glial scaffold and therefore, is not guided by radial glia or axonal fibers (Jacques et al., 1998). Because of these features, sphere cell migration is similar to that described by Lois et al. (1996) in the adult brain as "chain migration". In addition to differentiating cells, other precursor cells remain as a proliferative pool in the subplated cultures, and a third subpopulation of cells undergoes apoptosis (Zhou et al., 2000). The survival, expansion and differentiation of stem cells appear to be regulated by environmental signals as well as the potential of the cells themselves (Kilpatrick and Bartlett, 1993; Ghosh and Greenberg, 1995; Ma et al., 2000). Different growth factors, cytokines, extracellular matrix proteins, and neurotransmitters have been implicated in the epigenetic regulation of the proliferation and differentiation processes (Burrows et al., 1997; Arsenijevic and Weiss, 1998; Edlund and Jessell, 1999; Reimers et al., 2001). Since extracellular signaling can control stem cell differentiation, several studies have determined in vitro conditions for promoting the differentiation of these cells into specific cellular phenotypes, such as dopaminergic and serotonergic neurons (Daadi and Weiss, 1999; Lee et al., 2000a). For instance, we found that EGF-expanded stem cells (Fig. 2A) isolated from the rat fetal striatum differentiate in vitro to GABAergic neurons (ß-tubulin III and GABA-immunopositive cells, Figs. 2C-F), astrocytes (glial fibrillary acidic protein [GFAP]-positive cells, Fig. 2B), and oligodendrocytes (O1-positive cells) (Bazán et al., 1996, 1998; Reimers et al., 2001). However, the addition of FGF-2 and cyclic AMP analogs (dbAMPc), or FGF-2, dbAMPc and the protein kinase C activator PMA to the culture medium induces the differentiation of some of these cells into dopaminergic neurons that express tyrosine hydroxylase (Fig. 2H) (Bazán et al., 1999). Under the same culture conditions, human neurosphere cells isolated from a 13-week old fetus (spontaneous abortion) also gave rise to dopaminergic phenotypes (Fig. 1H) (Paíno et al., 2003). Moreover, when transplanted into diverse brain locations of adult mammals, neural stem/progenitors survive (Fig. 3A), migrate from the grafting site (Fig. 3B), and differentiate into neural and glial phenotypes (Bjorklund et al., 1982; Gage and Bjorklund, 1998; Herrera et al., 1999). These studies suggest that cell replacement therapy could represent an attractive therapeutic strategy for treating several neurodegenerative disorders, such as Parkinson's disease and Huntington's disease. Another model of stem cell therapy consists of the stimulation of endogenous stem cell proliferation, migration and differentiation by the infusion of specific factors, such as transforming growth factor-α (Fallon et al., 2000), or FGF-2 and EGF (Nakatomi et al., 2002) into injured brain structures.

The above mentioned definition of neural stem cells could be modified in a near future since several studies have shown that these cells can give rise to non-neural cell types (such as blood cells and myocytes) and that other tissue-specific stem cells (such as bone marrow stromal cells) can be induced to differentiate into neural cells. This cell fate conversion or trans-differentiation capacity of stem cells represents an emerging and promising area of study because of its therapeutic implications (reviewed in Vescovi et al., 2001; Gritti et al., 2002). The mechanisms underlying this phenomenon are largely unknown. Because cell fate conversion occurs when stem cells are transplanted into different environments, it has been proposed that stem cells are extremely plastic and have the ability to respond to

various environmental signals that regulate their change in commitment. In this sense, it has been shown that neural stem cells express some cell surface receptors that are usually present in hematopoietic and endothelial progenitors (Parati et al., 2002). If different precursors share common receptors their differentiation could be

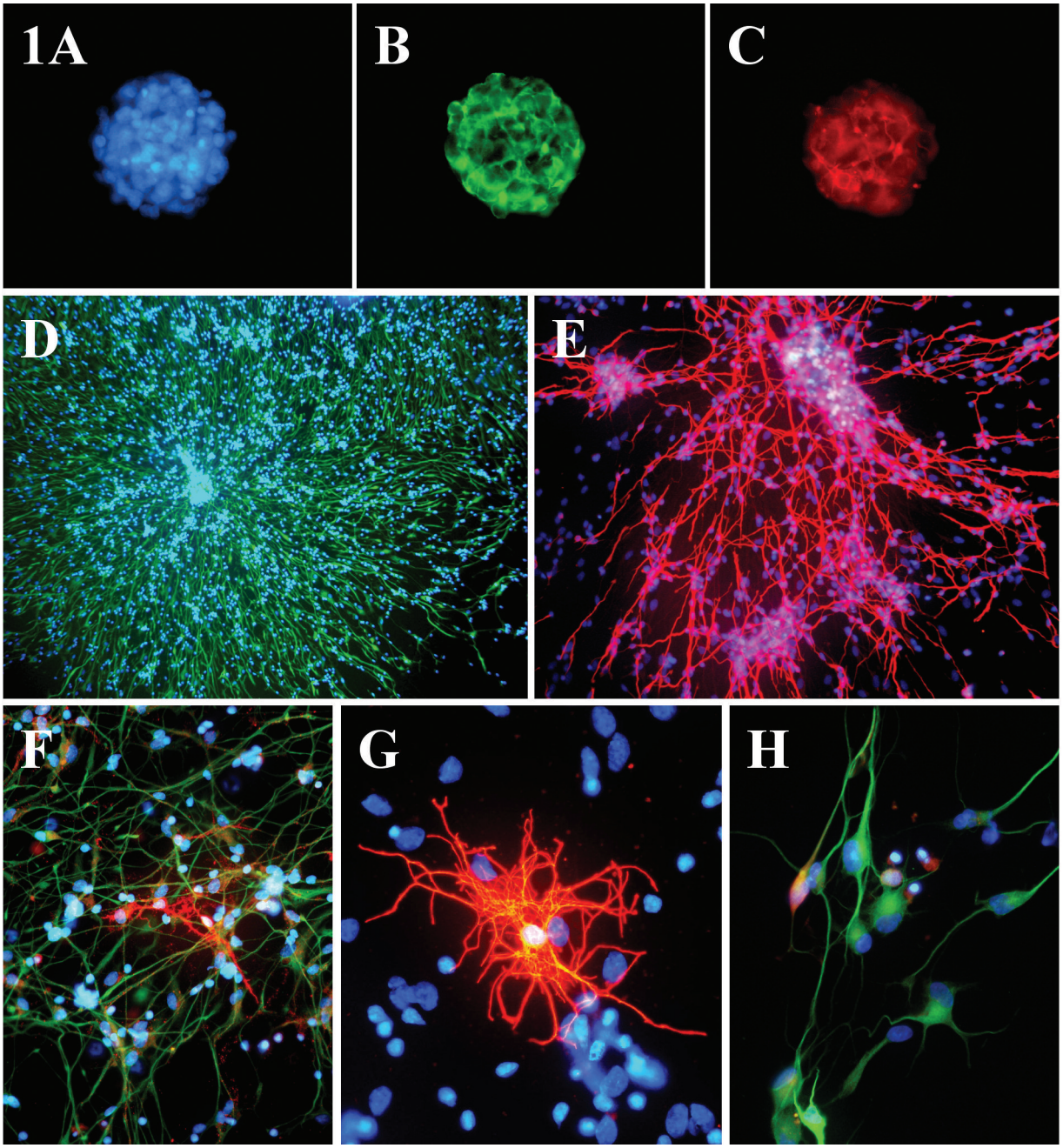

Fig. 1. Human neurospheres and their progeny. **A-C.** The forebrain of one 13-week fetus (spontaneous abortion) was mechanically dissociated and the resulting cell suspension was cultured in DMEM/F-12 (1:1) supplemented with EGF, FGF-2, and leukemia-inhibitory factor. These conditions promote cell proliferation and the formation of free-floating neurospheres. A sphere triple-stained for Hoechst 33342 (**A**, blue), the stem cell marker nestin (**B**, green), and the astroglial GFAP marker (**C**, red) is shown. **D**. Spheres were transferred onto poly-L-ornithine-coated coverslips to allow cell attachment, migration, and differentiation. Note that cells stained for nestin (green) migrate from the adherent neurosphere to the periphery. **E-G.** The progeny of neurosphere cells immunostained for the neuronal ß tubulin III (**E**, red), the early glial precursor A2B5 (**F**, red) and nestin (**F**, green), and the oligodendroglial O1 (**G**, red) markers. **H**. FGF-2+dbAMPc induces tyrosine hydroxylase expression. A representative culture double-stained for tyrosine hydroxylase (red) and ß tubulin III (green). Nuclei were counterstained with Hoechst 3342 (blue). A-C, x 190; D, x 80; E, x 110; F, x 150; G-H, x 200

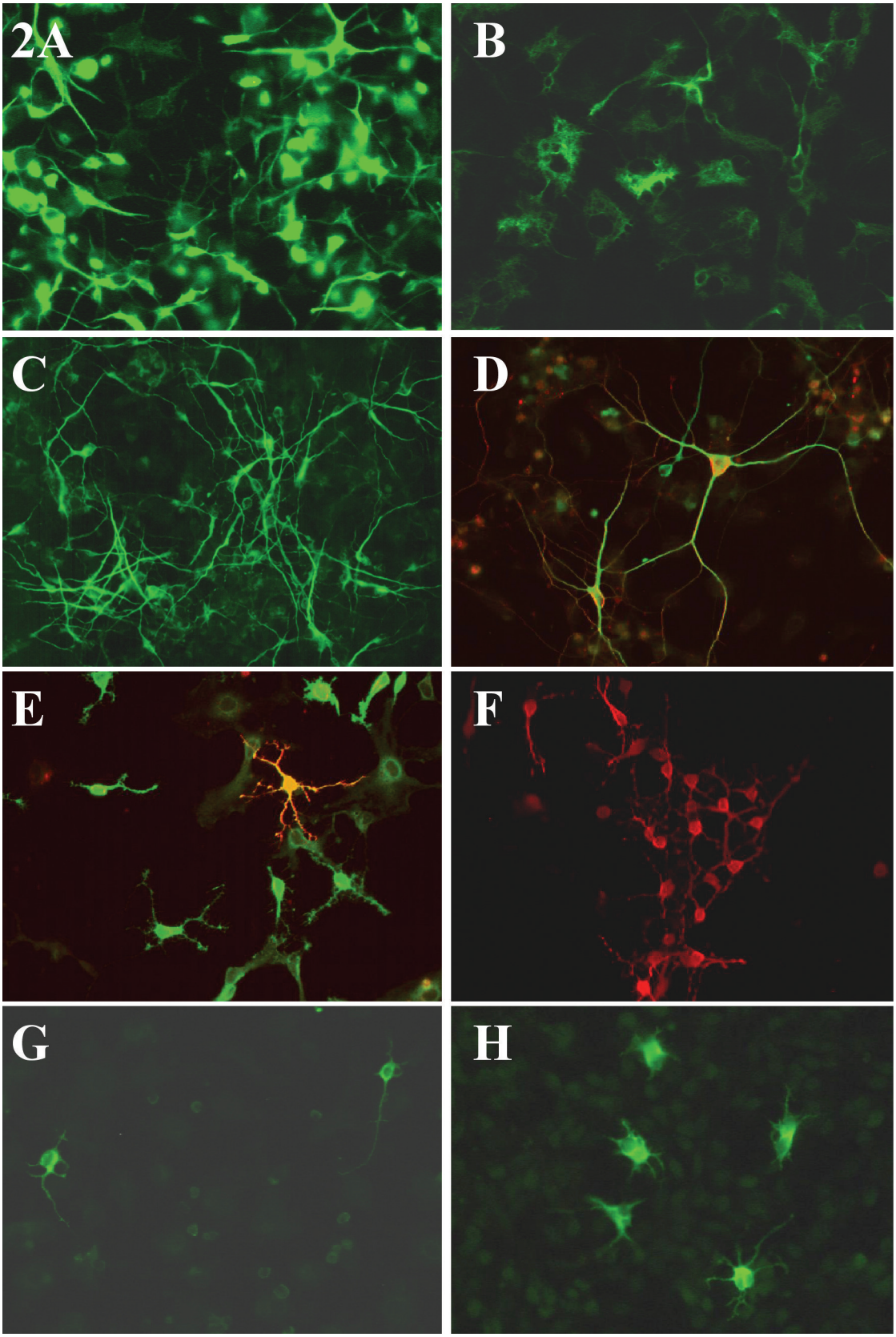

Fig. 2. The progeny of fetal stem cells. Striata from E15 rat embryos were mechanically dissociated and the resulting cell suspensions were cultured in DMEM/F-12 (1:1) with EGF. The cells grew as free-floating neurospheres. After 5 passages, spheres were plated onto poly-L-ornithine and grown in DMEM/F-12 with EGF for 3 days, and then switched to DMEM/F-12 without EGF for longer culture periods to allow cell differentiation (as described in Lobo et al., 2003). At 3 days post plating (dpp), most cells were stained for nestin **(A)**. At 10 dpp, the percentage of nestin-positive cells decreased and the cell population stained for GFAP **(B)**, a marker of astrocytes, and ß tubulin III **(C)**, a marker of neurons, increased. Of the total neuron population generated in the cultures, most of them were GABAergic **(D)** as revealed by double-staining for GABA (red) and ß tubulin III (green). Some cells **(E)** were stained for both the inhibitory (GABA, green) and excitatory (glutamate, red) amino acid neurotransmitters. Colocalization of both amino acids is visible as a yellow staining. At 10 dpp most neurons were stained for GABA **(F)** but some cells were stained for choline acetyltransferase **(G)** suggesting a cholinergic phenotype. FGF-2+dbAMPc treatment induces tyrosine hydroxylase expression **(H)**. A-D, x 250; E-H, x 200

partially regulated by the same molecules, which could be responsible for their cell fate conversion. On the other hand, using cDNA microarray techniques, Terskikh et al. (2001) found that neural stem cells express a large set of genes that are also expressed in hematopoietic stem cells, suggesting an overlap between the genetic programs of different stem cell populations.

The neural stem cell marker nestin

The intermediate filament family consists of more than 50 cytoskeletal proteins that are usually divided into six classes, based on sequence homology and exon/intron organization in their genes (reviewed in Fuchs and Weber, 1994). Nestin (a sort of acronym for neuroepithelial stem cell protein) is a class VI intermediate filament protein (Lendahl et al., 1990). However, it could also be included in class IV (represented by neurofilaments and α-internexin) based on similarities in exon/intron structure (Dahlstrand et al., 1992b; About et al., 2000). The expression of nestin was originally studied in the CNS, where it is transiently expressed in stem and progenitor cells (Lendahl et al., 1990; Dahlstrand et al., 1995). Upon stem cell differentiation, nestin expression is down regulated and replaced by other intermediate filament proteins, such as neurofilaments in neurons and GFAP in astrocytes (Lendahl et al., 1990; Messam et al., 2000). Nestin has also been detected in endothelial cells of developing and adult brains (Dahlstrand et al., 1992a; Tohyama et al., 1992; Frisen et al., 1995; Alliot et al., 1999), in reactive astrocytes after brain injury (Clarke et al., 1994; Frisen et al., 1995; Lin et al., 1995; Holmin et al., 1997) and in a variety of human brain tumor-derived cell lines and CNS tumors (Dahlstrand et al., 1992a; Tohyama et al., 1992, 1993; Crowe et al., 2004). In addition, nestin is expressed in some normal cell types of non-neural tissues, such as the pancreas (Hunziker and Stein, 2000;

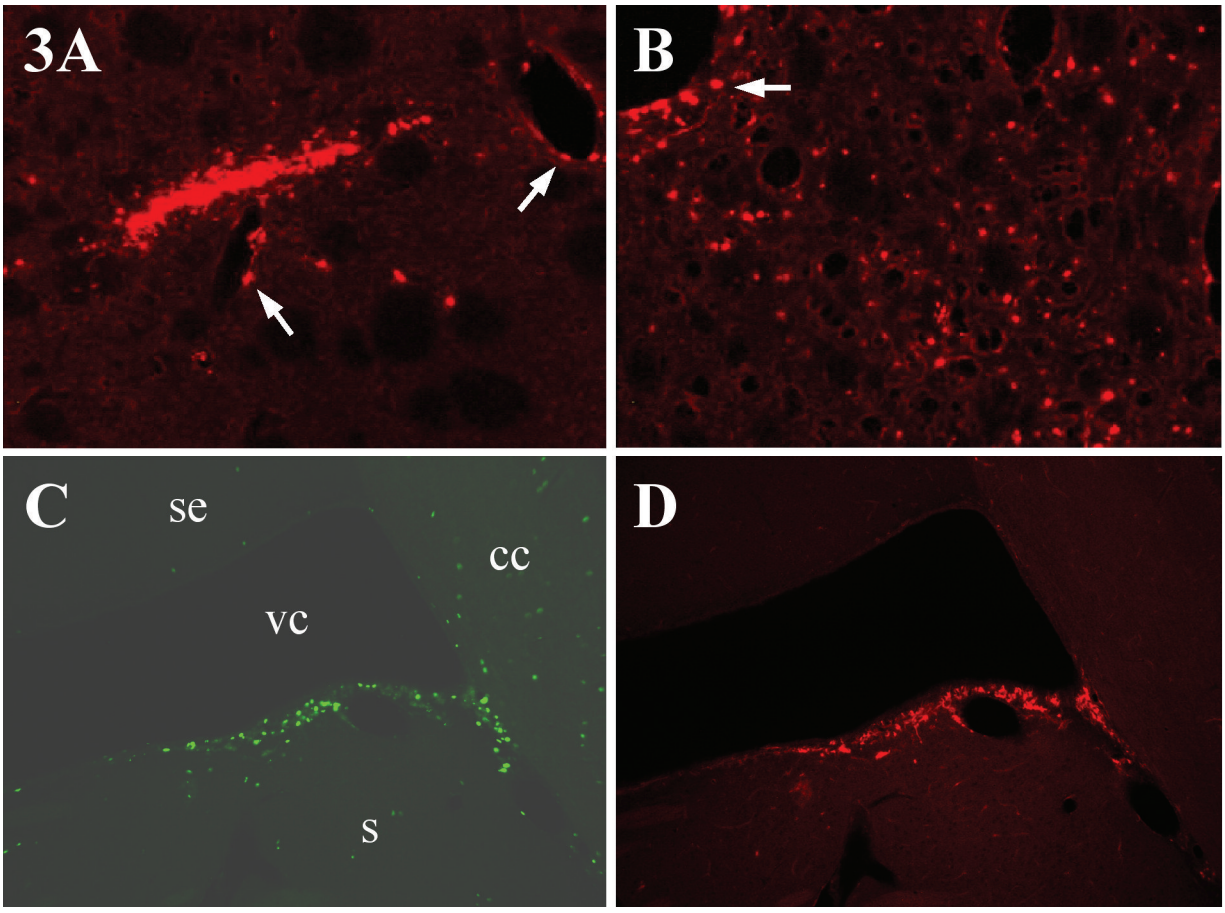

Fig. 3. A, B. The progeny of neural stem cells was grown as indicated in the legend of figure 2, labeled with red fluorescent cell linker PKH-26 for their identification after transplantation, and grafted in the adult rat striatum. Note that 3 months after grafting cells survive **(A)** and migrate from the graft site into the striatal parenchyma and in the vicinity of the blood vessels (**A-B**, arrows). **C-D**. Serial sections of the subventricular zone of adult rats stained for bromodeoxyuridine to enable the detection of proliferating cells **(C)** and nestin to enable the detection of precursor cells **(D)**. The corpus callosum (CC), striatum (S), septum (SE), and ventricular cavity (VC) are shown. A-B, x 50; C-D, x 30

Zulewski et al., 2001), muscle (Sejersen and Lendahl, 1993; Kachinsky et al., 1994), heart (Kachinski et al., 1995), tooth (Terling et al., 1995; About et al., 2000), and testis (Fröjdman et al., 1997; Lobo et al., 2004), and in some neoplastic cells of tumors, such as rhabdomyosarcomas (Kobayashi et al., 1998), gastrointestinal stromal tumors (Tsujimura et al., 2001), melanomas (Florenes et al., 1994) and teratomas (Lobo et al., 2004). In most cell types nestin colocalizes with other intermediate filament proteins, since it has been shown that nestin cannot form filaments by itself and it co-assembles with vimentin or a-internexin to form intermediate filaments (Eliasson et al., 1999; Steinert et al., 1999). Despite the evidence suggesting that the presence of nestin may not identify all CNS stem cells (Dahlstrand et al., 1995; Kukekov et al., 1997; Lobo et al., 2003) and that nestin is expressed in cells that are not progenitors, nestin is currently used as the main marker to identify neural precursors (Lendahl et al., 1990).

Other markers of neural precursors

No specific marker of neural stem cells has been found up to the present. This has been one of the main hindrances to unambiguously identify neural stem cells both in the nervous system and in neurospheres. Cell types that have been classified in different studies as progenitors, transit amplifying, neuroblasts, astroblasts or neural stem cells may, in fact, correspond to the same kind of cell or, in some cases, comprise heterogeneous groups of proliferating cells. This is a problem due to the lack of consensus between researchers, to the different behavior of cells *in vivo* and ex vivo (i.e. in culture) and to the absence of markers or procedures that identify the different developmental stages of neural cells. As a matter of fact one cannot draw a flow chart of neural development that includes all the above concepts at the same time. The identification of precursor cells is further complicated by the possible existence of de-differentiation or trans-differentiation processes.

Several molecules have been used by diverse research groups as markers of stem cells in the CNS. Besides nestin, the RNA-binding protein associated with asymmetric divisions in neural progenitor cells, Musashi1 (Sakakibara et al., 1996; Sakakibara and Okano, 1997; Kaneko et al., 2000), is expressed in both embryonic and adult proliferating pluripotent precursor cells. However, Musashi1 and nestin are not always co-expressed in CNS cells, so it is not clear if these two proteins distinguish different types of precursor cells. Additionally, Musashi1 is also expressed, at lower levels, in differentiated astrocytes and some ependymocytes (Sakakibara and Okano, 1997); to be noted, both of these cell types have been reported to have neural stem potential in SVZ (Doetsch et al., 1999a; Johansson et al., 1999). Musashi1 is not CNS-specific either, since it is also expressed by intestinal crypt basal cells (Nishimura et al., 2003), so it might be a molecule implicated in general stem-cell functions.

Transcription factors are other candidate molecules that could have specific expression in neural stem cells. The HMG-box transcription factors of the SOX family play roles in the maintenance of multipotency of stem cells from different tissues with a high degree of conservation across the species (Pevny and Lovell Badge, 1997). SOX1 is probably the earliest marker of neural commitment of embryonic stem cells, it is expressed in no other tissue but in the nervous system and lens and it marks the entire pool of proliferating precursors in the neural tube (Aubert et al., 2003). Upregulation of SOX1 expression is indeed sufficient to induce neural fate in teratocarcinoma cells (Pevny et al., 1998). Exit from mitosis and differentiation, on the other hand, is accompanied by downregulation of this transcription factor in the CNS. SOX1 is, furthermore, expressed in adult neural precursor cells and in cultured multipotent neural cells, where SOX1-positive cells constitute a subgroup of the nestin-expressing cells (Cai et al., 2004), suggesting that SOX1 identifies neural stem cells more specifically than nestin. Other members of the SOX subfamily group B1, SOX2 and SOX3, are also expressed in neural progenitor cells, although they show wider timing of up- and down-regulation and they also have expression outside the CNS.

Specific molecules that mediate cell adhesion or recognition might also identify neural stem cells. They have the additional advantage that, being antigens that show up extracellularly, they can be used for immune selection of neural progenitors. Polysialylated neural cell adhesion molecule (PSA-NCAM) has been used by several researchers for immunoselection, but, depending on the culture conditions, this selection has been reported to yield multipotent (Marmur et al., 1998) or mostly glial (Ben-Hur et al., 1998; Keirstead et al., 1999) precursor cells. In the SVZ in vivo, PSA-NCAM-expressing cells have been identified as migrating neuroblasts that become neurons in the olfactory bulb (Doetsch et al., 1997).

The fucose-containing carbohydrate "Lewis X" (LeX), also known as "stage-specific embryonic antigen-1" (ssea-1) or CD15, is a cell surface antigen present in embryonic pluripotent cells but also expressed by neurosphere-forming adult CNS cells (Capela and Temple, 2002). A low percentage of SVZ astrocytes are LeX-positive and it has been suggested that these cells are the ones that generate neurospheres in culture (Capela and Temple, 2002) and could be considered as authentic neural stem cells. However, this population corresponds to only one-fourth of the LeX-positive cells so this antigen cannot be used alone to identify neural stem cells. Another drawback for using this marker is that the above properties can be shown only in the adult mouse, while in the rat embryonic CNS its expression is not specific (Cai et al., 2004).

The transmembrane glycoprotein of unknown function AC133 (now assigned to CD133), expressed in immature hematopoietic stem and progenitor cells, has also been used as a marker for immunoselection of

human neural stem cells (Uchida et al., 2000). This marker is also expressed in stem cells from several human tissues and might identify cells that show pluripotentiality or, at least, stem cell plasticity, since CD133-positive cells show the capacity to integrate and differentiate in non-original tissues. The main drawback associated with this marker is that, up to date, it can be used solely for human cells.

Another property that appears to be characteristic of various types of stem cells is the capacity to exclude some dyes like bisbenzimide (Hoechst 33342 stain) or rhodamine 123. The stem cells thus constitute a "side population" of small size and low fluorescence level by FACS. The dye exclusion is due to the function of a multidrug resistance P-glycoprotein, the ABCG2 transporter. Both dye-exclusion and ABCG2 expression may be used to identify neural stem cells in cultures (Hulspas and Quesenberry, 2000; Engstrom et al., 2002; Cai et al., 2004).

Growth factor responsiveness of neural stem cells

Neural stem cells express EGF and FGF receptors (Vescovi et al., 1993; Burrows et al., 1997; Gritti et al., 1999; Lobo et al., 2003). They show high levels of FGF receptor-1 and -2 and very low levels of FGF receptor-3 (Reimers et al., 2001; Lobo et al., 2003), which are distributed in the nucleus and cytoplasm including the plasma membrane (Fig. 4). The stimulation of these EGF and FGF cell-surface receptors promotes the activation of specific signal transduction pathways, such as the mitogen-activated protein kinase (MAPK) and the phospholipase Cγ (PLCγ) signal pathways (Bazán et al., 1999; Learish et al., 2000). Thus, stem cells are targets of these growth factors. In this sense, it has been shown that EGF-expanded stem cells generate in vitro two subpopulations of cells responsive to FGF-2; one restricted to neuronal differentiation and the other to a bipotential cell that differentiates into neurons and astrocytes (Vescovi et al., 1993). However, other studies suggest that multipotent precursors proliferate in response to FGF-2 only, whereas cells responding to both EGF and FGF-2 are glial-restricted progenitors (Kilpatrick and Bartlett, 1995). In addition, it has been proposed that neural stem cells undergo a developmentally programmed switch in growth factor responsiveness, where FGF-2-responsive stem cells acquire EGF responsiveness and give rise to multipotent precursors responding to both factors: FGF-2 and EGF (Ciccolini and Svendsen, 1998; Ciccolini, 2001). Other studies support the existence of a single population of neural stem cells whose proliferation is controlled by

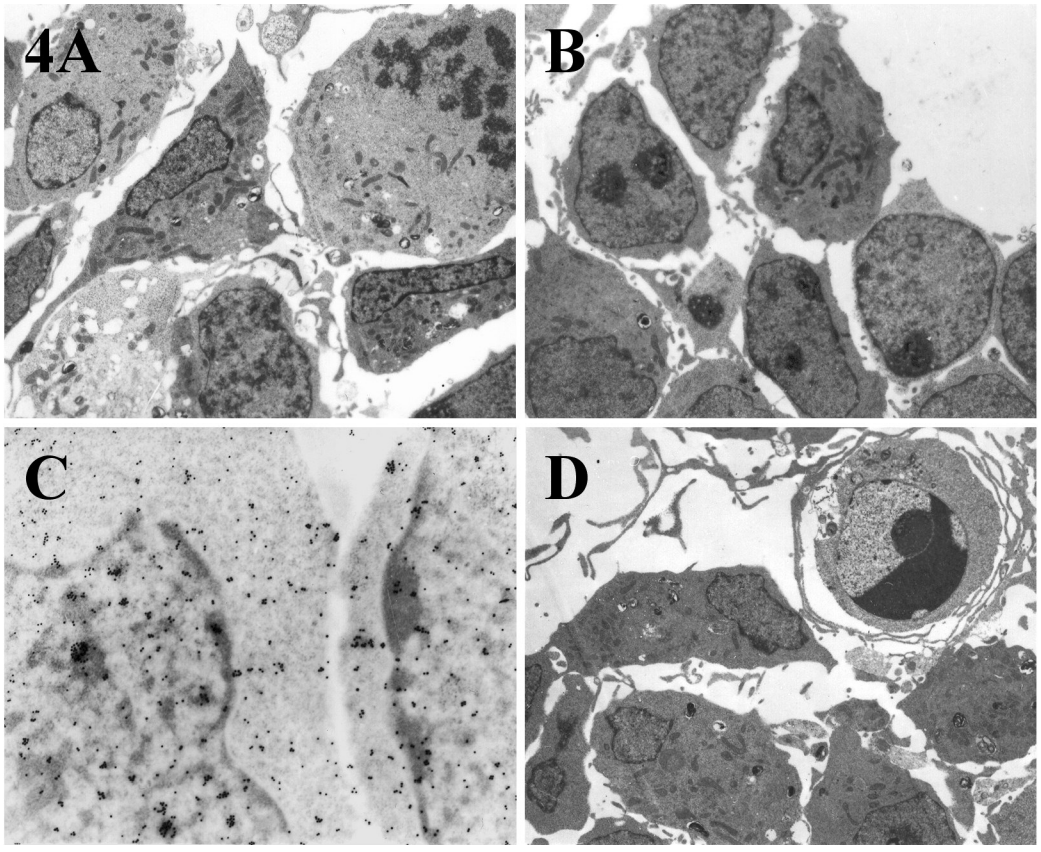

Fig. 4. A-B. Healthy cells of free-floating neurospheres showing abundant ribosomes and mitochondria. They have large nuclei and one or two nucleoli. **C.** Sphere cells immunolabeled for FGF receptor-2, which is localized in the nucleus and cytoplasm. **D.** Sphere cell at an early apoptotic phase surrounded by cell processes of healthy cells. A-B, x 3400; C, x 1800

both EGF and FGF-2 (Gritti et al., 1999), or two different cell populations: EGF- and FGF-responsive stem cells (Tropepe et al., 1999; Martens et al., 2000). This controversy may be explained by considering that neural stem cells and their immediate progeny (progenitor cells) probably represent heterogeneous cell populations. Supporting this notion, it has been shown that although the neural stem cells isolated from different species, or from different CNS areas at several embryonic and postnatal stages share common features (self-renewal and multipotentiality), they also show significant differences in relation to their culture requirements and growth factor responsiveness, or to their plasticity and differentiation repertoire. On the other hand, the above-mentioned studies examined the responsiveness of cultured stem cells after administration of exogenous growth factors, but recent studies propose that stem cells could synthesize and secrete some growth factors with autocrine/paracrine roles on these cells. Thus, it has been demonstrated that neuroepithelial stem cells synthesize FGF-1, FGF-2, and EGF, and express FGF receptors but not the EGF receptor (Kalyani et al., 1999). Consistent with these data, it has been suggested that endogenous FGF-2 is secreted by neural crest cells (Murphy et al., 1994), R1 embryonic stem cells (Tropepe et al., 2001), and free-floating neurosphere cells derived from the rat fetal striatum (Lobo et al., 2003). Other studies proposed that neural stem cells synthesize and secrete a glycosylated form of cystatin C, which acts as an autocrine/paracrine co-factor required for the mitogenic activity of FGF-2 on these cells (Taupin et al., 2000). In addition, it has been shown that expression of a threshold number of EGF receptors is required for mitotic responsiveness to EGF and that stem cells change in EGF receptor expression during embryonic development in a process that appears to be controlled by cell-cell signaling (Burrows et al., 1997; Lillien and Raphael, 2000). In conclusion, stem cells can modify their growth factor responsiveness by changing the expression of growth factor receptors and secreting some growth factors or related molecules.

Stem cell localization and identification in the CNS

In their respective organs, tissue-specific stem cells are often localized in specific compartments (termed stem cell niches) where they are provided with the environmental requirements for their proliferation and differentiation (Gritti et al., 2002). The neural stem cell niches comprise specific anatomical areas of the CNS, the characteristic cytoarchitecture of these regions, and several cellular and molecular elements, such as cell-cell interactions and somatic cell signaling, proximity to the cerebrospinal fluid and blood vessels, and a specialized basal lamina and extracellular matrix (Doetsch, 2003). Fetal neural stem cells reside in several zones of the embryonic CNS, including the olfactory bulb, ventricular zone, SVZ, hippocampus, cerebellum, cerebral cortex, and spinal cord. The ventricular zone is a transient embryonic structure that is replaced at the end of neurogenesis by the ependyma, an epithelial monolayer that separates the ventricular cavity from the SVZ. In contrast, the SVZ (postnatally termed subependymal zone) persists in neonate and adult mammals (Figs. 3C, D) as a mitotically active layer (Smart, 1961; Luskin, 1993). Fetal neural stem cells may be a specific and immature cell phenotype in the developing CNS. However, it has been suggested that radial glial cells (that have been previously considered as committed progenitors of astrocytes) can give rise to neurons and may represent a stem cell population (Gaiano et al., 2000; Malatesta et al., 2000; Noctor et al., 2001). In addition, cultured astrocytes isolated from the embryonic and early postnatal CNS show stem cell functional features, whereas those astrocytes isolated from the late postnatal and adult CNS do not show self-renewal and multipotency except those from the adult SVZ (Laywell et al., 2000). These studies should be interpreted with caution because as multipotent stem cells are extremely plastic and can generate non-neural cell types, committed progenitors (such as radial glia and embryonic astrocytes) may show a broader plasticity than previously thought. Moreover, in certain culture conditions some precursors may show a change in commitment, such as oligodendrocyte precursor cells (Kondo and Raff, 2000) and transit-amplifying precursors (Doetsch et al., 2002) that can be reprogrammed to become multipotent stem cells by the action of cytokines and EGF respectively. Therefore, although in vitro, some cells show stem cell properties, this does not imply that they are the in vivo stem cells.

In the adult mammalian brain, gliogenesis continues throughout life and neurogenesis has been detected in two main areas: the dentate gyrus of the hippocampus (Altman and Das, 1965; Cameron et al., 1993; Seri et al., 2001), and the SVZ of the forebrain lateral ventricles (Altman and Das, 1966; Altman, 1969; Lois and Alvarez-Buylla, 1993; Doetsch et al., 1999a,b). Hippocampal neurogenesis has been related with learning and memory (Kempermann et al., 1997; Gould et al., 1999a; Shors et al., 2001), and consists in the development of new neurons in the subgranular layer of the dentate gyrus which differentiate into granule cells that project axons to the CA3 area of the hippocampus (Stanfield and Trice, 1988; Markakis and Gage, 1999). In the other neurogenic region, the neuroblasts generated throughout the SVZ migrate along a network of chains that course parallel to the walls of the lateral ventricles and converge on the rostral migratory stream to reach the olfactory bulb where they differentiate into interneurons to replace dead or dying granule and periglomerular neurons (Lois and Alvarez-Buylla, 1993, 1994; Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996). Cells that met the criteria for their identification in vitro as neural stem cells have been isolated and cultured from these two germinal zones of the adult brain (Reynolds and Weiss, 1992; Morshead et al., 1994; Palmer et al., 1997; Roy et al., 2000; Rietze et al., 2001). However, the in

vivo identification of adult neural stem cells is a matter of controversy. Ependymal cells have been considered terminally differentiated cells (Bruni et al., 1985), but it has been recently proposed that they are mitotically active cells and the in vivo adult neural stem cells (Johansson et al., 1999). In contrast, other studies suggest that ependymal cells are capable of forming neurospheres in vitro, although they are unipotent and give rise only to cells within the glial lineage (Chiasson et al., 1999; Laywell et al., 2000). Finally, it has been reported that ependymal cells neither divide nor give rise to neurospheres (Doetsch et al., 1999a; Capela and Temple, 2002) and instead, a population of astrocytes from both neurogenic regions (the SVZ and the dentate gyrus) are neural stem cells in the adult brain or generate neural stem cells (Doetsch et al., 1999a, 2002; Laywell et al., 2000; Seri et al., 2001; Capela and Temple, 2002). In vivo studies suggest a neurogenic model by which astrocytes (type B cells) give rise to immature precursors (type C cells) that finally generate neuroblasts (type A cells) in the SVZ, and in vitro studies demonstrated that SVZ astrocytes give rise to EGF-responsive stem cells (Doetsch et al., 1999a). In the subgranular zone of the dentate gyrus, astrocytes generate neuroblasts via an intermediate phenotype, termed type D cells (Seri et al., 2001). It should be considered that astrocytes seem to be a heterogeneous cell population. Thus, Miller et al. (1994) reported the existence of as many as five subpopulations of astrocytes based on morphological and antigenic characteristics.

In addition to the stem cells residing in the dentate gyrus and SVZ, cells that in vitro behave as stem cells have also been isolated from the spinal cord and the third and fourth ventricles of adult mice (Weiss et al., 1996), and from the amygdala and temporal and frontal cortex of adult humans, who underwent brain resections due to epilepsy, dysplasia, trauma or edema (Arsenijevic et al., 2001). Moreover, the existence of a slow physiological turnover of dopaminergic neurons in the substantia nigra of adult mice has been suggested, and that dopaminergic neurogenesis is increased after a partial lesion (Zhao et al., 2003). These studies indicate that neurogenesis in the adult CNS is more widespread than previously thought. Supporting this notion, it has been proposed that neurogenesis continues throughout adulthood in the primate neocortex and therefore new neurons are added to the prefrontal, parietal, and temporal lobes (Gould et al., 1999b, 2001). This is an extraordinary finding because the neocortex is the site of the highest cognitive functions and because no previous studies have reported cortical neurogenesis in primates and non-primate species, although it has been shown that gliogenesis persists at a low rate in the adult CNS. Moreover, those studies that tried to confirm the results of Gould et al. (1999b, 2001) using the same methods failed to detect cortical neurogenesis and instead they found dividing satellite glia (Komack and Rakic, 2001).

Although some stem cells are identified by their *in vivo l*ocalization, such as embryonic stem cells that are defined as those cells derived from the inner cell mass of the blastocyst, further in vivo studies are required about the cellular identity and localization of fetal and adult neural stem cells before we can use these criteria for their precise identification.

Cellular phenotype of putative stem cells in vivo

The early morphological studies of the SVZ in the developing and adult brains revealed the presence of several cell types in this area, including light and dark cells that were considered cells committed to the glial lineage (Tennyson and Papas, 1962; Blakemore, 1969; Stensaas and Gilson, 1972). This interpretation was performed in the context of the concept generally accepted at that time, considering the adult CNS as a non-renewable organ that lack stem cells. Recent studies have re-examined the cytoarchitecture of the adult SVZ and characterized different cell types: proliferating migratory neuroblasts (type A cells), slowly dividing astrocytes (type B cells), rapidly dividing progenitors (type C cells), tanycytes (type D cells), which are different from the type D cells described in the dentate gyrus by Seri et al. (2001), and ependymal cells (type E cells). These cells are identified on the basis of cell-cycle length, ultrastructural, and antigenic criteria (Lois and Alvarez Buylla, 1994; Doetsch et al., 1997; Peretto et al., 1999). As mentioned above and according to the different studies, types B and E cells are putative stem cells and types A, C, and E cells are possible progenitors or committed precursor cells.

The migrating neuroblasts (type A cells) described by Doetsch et al. (1997) in the SVZ probably correspond to the dark cells detected in previous studies. These type A cells show a spindle-shaped cell body with one or two cell processes. Their elongated nucleus shows a dispersed chromatin pattern with small aggregates of condensed chromatin masses, two to four small nucleoli, and occasionally exhibit nuclear indentations. Their scant and electron-dense cytoplasm shows many ribosomes, a small Golgi apparatus, few cisternae of rough endoplasmic reticulum, and many microtubules distributed along the long cell axis. No dense bodies, lipid droplets, cilia or microvilli have been found in these cells. They are immunopositive for nestin, PSA-NCAM, and TuJ1 (a peptide of ß-tubulin isotype III, which is specific for neurons), and immunonegative for GFAP and vimentin (Doetsch et al., 1997). These neural precursors migrate from the SVZ to the olfactory bulb grouped into chains of closely apposed cells connected to each other by adherens junctions and ensheathed by type B cells, which form a tubular trabecula that separates type A cells from the surrounding cells. This form of migration is not guided by radial glia or axonal fibers and allows the movement of many type A cells through a defined pathway at a high speed (30 µm/hour) in a process that has been termed chain migration (Lois et al., 1996; Doetsch et al., 1997).

SVZ astrocytes (type B cells) are characterized by

their light cytoplasm containing few free ribosomes, dense bodies, and bundles of intermediate filaments that are immunopositive for GFAP. They show irregular contours, cell processes, and irregular-shaped nuclei that frequently show deep indentations of the nuclear envelope (Doetsch et al., 1997). Occasionally, SVZ astrocytes extend a process between ependymal cells to contact the lateral ventricle and exhibit a short single cilium (Doetsch et al., 1999b). Two types of astrocytes have been described in the adult SVZ, type B1 and B2 cells, which differ in their ultrastructural characteristics and distribution (Doetsch et al., 1997). In addition to GFAP, type B cells express vimentin and nestin but are immunonegative for PSA-NCAM and TuJ1 (Doetsch et al., 1997). Astrocytes also express the cell-surface carbohydrate LeX (Capela and Temple, 2002).

Progenitor cells (type C cells) show smooth and spherical contours. Their large nuclei show deep indentations, a dispersed chromatin pattern, and a large reticulated nucleolus. Their cytoplasm contains a large Golgi apparatus, fewer ribosomes than type A cells, and no bundles of intermediate filaments. They are larger and more electron-lucent than type A cells but more electron-dense than type B cells. In the SVZ, type C cells are found grouped into small clusters and as isolated cells. These cells are stained by anti-nestin antibodies and are immunonegative for PSA-NCAM, TuJ1, GFAP, and vimentin. These type C cells may correspond either to precursors of type A cells or to multipotent precursors that may generate neurons and glia (Doetsch et al., 1997). It has been recently shown that these cells express the EGF receptor, that EGF converts transit-amplifying C cells into multipotent stem cells, and that the majority of EGF-responsive cells both in vitro and in vivo are the C type (Doetsch et al., 2002).

Ependymal cells are characterized by the presence of multiple cilia (40-50 per cell) and microvilli in the surface exposed to the ventricular cavity. In their lateral surface, they show cell processes interdigitated with those from adjacent ependymal cells, and junctional complexes. Their nuclei are spherical and their cytoplasm is electron-lucent and exhibits many mitochondria. They are immunopositive for nestin, vimentin and GFAP, and immunonegative for PSA-NCAM and TuJ1 (Doetsch et al., 1997). They are also LeX-negative (Capela and Temple, 2002).

Cellular phenotype of putative stem cells in culture

The cellular phenotypes of cultured stem cells have also been analyzed (Kukekov et al., 1997, 1999; Bez et al., 2003; Lobo et al., 2003). Kukekov et al. (1997) examined neurosphere cells isolated from the adult mouse brain and treated with mercaptoethanol, which inhibits cell-cell contact. This treatment yields two types of neurospheres: I and II. Under phase microscopy, type I spheres are phase-dark. They consist of rings of electron-dense cells that surround a core of flocculent material. Early type I spheres are immunonegative for nestin, GFAP, ß-tubulin III, and L1 adhesion molecule. They progress through a phase-dark nestin-negative state and then through a phase-dark nestin-positive state, and finally they become phase-bright type II spheres. Type II spheres are nestin-positive, consist of less electron-dense cells than type I spheres, and give rise to neurons and glia. Thus, using mercaptoethanol, Kukekov et al. (1997) identified two types of neurosphere cells: nestin-negative and nestin-positive populations.

We have examined the ultrastructural and antigenic features of EGF-expanded free-floating sphere cells obtained from rat embryos (Lobo et al., 2003). When these spheres were dissociated and subcultured they formed new neurospheres, suggesting that they retain the ability to self-renew, and when these spheres were seeded onto adherent substrate and the mitogen (EGF) was withdrawn, they generated neurons and glia (Bazán et al., 1998; Reimers et al., 2001; Lobo et al., 2003). Therefore these cultures should contain stem cells. They also contain progenitor cells because some sphere cells were immunopositive to A2B5 (a marker of early glial precursors). Two ultrastructurally and antigenically distinct populations of cells were found in these neurospheres: dark cells that were immunopositive for actin, weakly positive for vimentin, and nestin-negative, and light cells that were positive for actin, vimentin, and nestin. Both cell types showed a variable appearance in morphology and size, irregular contours, many free ribosomes and mitochondria, and moderately developed Golgi complexes, and rough and smooth endoplasmic reticula. They had large nuclei, which exhibited a dispersed chromatin pattern, one or two large nucleoli with a reticulated structural configuration, and frequent nuclear indentations (Fig. 4). These cells were attached to each other by adherens junctions and express E- and N-cadherin, and α- and ß-catenin. These results lead us to propose that the process whereby clonally derived cells aggregate into spherical clusters (sphere cell compaction) could be mediated by adherens junctions through specific cadherin/catenin/cytoskeleton complexes. Sphere cells also show cell processes that spread through the intercellular space and have phagocytic capability. The phagocytosis process observed in these neurospheres involves the following stages: a) the early recognition of dying cells; b) the engulfment of these dying cells by cells processes from healthy cells that form a nest-like structure around each dying cell; c) the phases of apoptosis and necrosis that occurs within these nest of cell processes, and finally d) the phagocytosis of the cell fragments and cell debris by nearby cells (Lobo et al., 2003). These cultured cells and those described in other studies do not have clear homologs with the cells characterized *in vivo*. This is probably due to the differences between the in vitro and *in vivo* environments. For instance, we do not add cholesterol to the culture medium and neurosphere cells may obtain the cholesterol and other nutrients (lipids and non-lipids) required for their survival by a phagocytosis process, which may not be necessary for the *in vivo* stem

cells. Moreover, ultrastructural studies have demonstrated that cultured neurospheres lack a defined basal lamina, whereas a specialized basal lamina has been described in the SVZ, which extends from blood vessels, makes extensive contacts with all SVZ cell types, and terminates in small bulbs adjacent to ependymal cells that may correspond to concentrated foci of signaling molecules (Mercier et al., 2002). This basal lamina is an important component of the stem cell niche, playing a role in cell anchoring and ligand binding and regulation (Mercier et al., 2002; Doetsch, 2003). Despite these studies, the ultrastructural features of neural stem cells are still unknown because the only way to identify a single cell as a truly multipotent stem cell is to assay for self-renewal and multipotential capacities, but then the cells under study are lost since stem cells differentiate into their progeny.

In summary, fetal and adult neural stem cells are neurosphere-forming cells with self-renewal capacity that proliferate in response to the mitotic stimuli of EGF and/or FGF-2, that frequently express nestin, that give rise to neurons, glia and non-neural cells, that appear widely distributed in the fetal CNS or at restricted germinal zones of the adult CNS, and that show an undifferentiated, ependymal or astrocytic phenotype. However, this is an imprecise definition and therefore, further studies are required to get a better identification of these cells.

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