CNS PRECURSORS

Neural stem cell systems: physiological players or *in vitro* entities?

Luciano Conti and Elena Cattaneo

Abstract | Neural stem cells (NSCs) can be experimentally derived or induced from different sources, and the NSC systems generated so far are promising tools for basic research and biomedical applications. However, no direct and thorough comparison of their biological and molecular properties or of their physiological relevance and possible relationship to endogenous NSCs has yet been carried out. Here we review the available information on different NSC systems and compare their properties. A better understanding of these systems will be crucial to control NSC fate and functional integration following transplantation and to make NSCs suitable for regenerative efforts following injury or disease.

Niche
A multicellular microenvironment supplying the factors required to maintain stem cell self-renewal and to direct their differentiation.

Antigenic
Pertaining to the expression of a specific marker or array of markers, specific parts of which are recognized by antibodies.

Neural plate
The thickened stripe of ectoderm overlying the notochord in early vertebrate embryos which contains cells that will give rise to the nervous system during embryonic development.

Department of Pharmacological Sciences and Center for Stem Cell Research, University of Milan, Via Balzaretti 9, 20133 Milano, Italy.
e-mails:
luciano.conti@unimi.it; elena.cattaneo@unimi.it
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Neural stem cells (NSCs) are self-renewing multipotent populations present in the developing and adult mammalian CNS[1,2]. They generate the neurons and glia of the developing brain and also account for the limited regenerative potential of the adult brain. *In vivo*, NSCs exist in niches that support self-renewal and regulate the balance between symmetrical self-renewal and fate-committed asymmetrical division[3–6].

For nearly 20 years growth-factor-based protocols have been developed, leading to NSC expansion in both floating and adherent conditions[7] and to a better understanding of the biological and molecular properties of NSCs. However, determining the best sources for the *in vitro* derivation of NSCs and optimizing protocols for stable, clonal proliferation are still central goals of stem cell research.

The *in vitro* synthetic milieu is thought to allow the expansion of bona fide NSCs — that is, cells that are operationally characterized by self-renewing and multipotential differentiation[8], but the physiological relevance of these NSC models for the study of neural precursors during CNS development is still a subject of debate. Several studies indicate that some antigenic and biological properties might be maintained in NSC cultures even at late passages. However, other studies have shown that exposure to growth factors can deregulate the spatial identity and differentiation potential of neural precursors.

Here we compare the functional properties of NSCs grown *in vitro* with those of NSCs present *in vivo* and review the assays developed for their isolation and expansion. Finally, we discuss recent findings indicating that NSC identity might not accurately represent that of stem cells *in vivo* owing to the alteration of the cells' genetic and epigenetic status.

NSCs *in vivo* and *in vitro*

During brain development, predetermined programmes give rise to spatiotemporally different NSC populations, making the definition of the properties of NSCs challenging[2]. Although combined transcriptomic and proteomic approaches have improved our understanding of the molecular characteristics of NSCs and progenitor cells, such findings are far from definitive.

Neurogenesis in mammals begins with the induction of the neuroectoderm, which forms the neural plate (at embryonic day 7.5 (E7.5) in mice) and then folds to give rise to the neural tube (at E8.5 in mice). These structures are made up by a layer of so-called neuroepithelial progenitors (NEPs)[9] (BOX 1), which are probably a complex and heterogeneous population. Progress in cell culture technologies has enabled researchers to induce the neuralization of mouse and human embryonic stem cells (ESCs) *in vitro*. During this neural differentiation, ESCs undergo progressive lineage restrictions similar to those observed in normal fetal development[10,11], leading to the generation of a range of distinct neural precursor populations that can be used to study the molecular and cellular events that occur during stage-specific transitions between different populations[12,13].

The identification of early stage-specific neural markers has allowed neural induction to be followed both *in vivo* and *in vitro*. SOX1 is one of the earliest known neural precursor markers in the mouse embryo[14]. SOX1-positive neural progenitors with 'primordial' properties have been described[15]. These NEPs can be rapidly (within 24 hours) induced from mouse ESCs by exposing them to conditions that minimize any contact with extrinsic factors. This situation favours the

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Neural tube
The cylindrical structure formed by the fusion of neural folds around the neural plate. The brain and spinal cord develop from the neural tube.

Rosette
Radial arrangements of columnar cells that express many of the proteins expressed in neuroepithelial cells in the neural tube. They are considered a developmental signature of neuroprogenitors in cultures of differentiating ESCs.

appearance of a colony-forming leukaemia-inhibitory factor (LIF)-dependent 'primitive' NSC population with peculiar antigenic and developmental properties (FIG. 1; TABLES 1,2). Indeed, these primitive NSCs retain vestiges of ESC identity, such as OCT4 expression and a broad differentiation potential (observed in chimeric blastocyst experiments)[15], suggesting that their neural commitment might be incomplete. Primitive NSCs are only a transient *in vitro* population as passaging switches them (the conversion being dependent on Notch signalling) to more committed neural precursors characterized by dependence on exogenous fibroblast growth factor 2 (FGF2), ceased expression of ESC markers and lost competence for chimaera formation. LIF-dependent NSCs with similar antigenic and functional properties can also be isolated from E5.5–7.5 mouse embryos[16], indicating that they are not a peculiarity of the ESC neuralization process.

A fully neuralized early human ESC (hESC)-derived NEP population has recently been described[17]. Based on the evidence of a crucial role for SMAD signalling during vertebrate neural induction, these authors developed a dual-SMAD inhibition protocol for rapid (6 days) neuralization of hESCs, generating a population of 'early' SOX1-, PAX6-, OTX2- and FOXG1-expressing NEPs (FIG. 1; TABLES 1,2). This protocol relies on strong inhibition of SMAD signalling by means of combined treatment of the hESCs with Noggin and the small molecule SB431542, the latter being a potent Lefty–Activin–Transforming growth factor-β (TGFβ) pathway inhibitor that blocks ALK receptor signalling. These early NEPs express some rosette-specific markers, although in a more primitive ES-like than polarized pattern. Interestingly, early hESC-derived NEPs are highly responsive to regionalization cues, allowing the efficient generation of neuronal subtypes relevant to that region (that is, tyrosine-hydroxylase-expressing neurons and Islet1- and HB9 (also known as MNX1)-positive motor neurons).

These early hESC-derived NEPs cannot be maintained *in vitro* as they spontaneously convert into a later-stage NEP population, which grows in rosette-like structures (named R-NSCs), with apical zonula occludens 1 (ZO1; a rosette-specific marker) expression and evidence of interkinetic nuclear migration[18]. R-NSCs can be maintained for some passages *in vitro* by sonic hedgehog (SHH) and Notch receptor agonists and show an anterior FOXG1-positive NEP identity. Studies from the same group have shown that R-NSCs can also be directly isolated, by combining the detection of the expression of forebrain-surface-embryonic (Forse-1) epitope and N-cadherin (also known as cadherin 2) cell sorting strategies, from neuralized mouse and human ESCs and from E8.25 anterior neural plate tissue exposed to SHH and Notch receptor agonists[18]. One of the most important properties of R-NSCs is their responsiveness to patterning signals, which is similar to that of early NEPs and enables them to adopt CNS or PNS fates. A similar phenomenon is observed during development, but only at the neural plate stage — not in neural precursors emerging after neural tube closure — supporting the idea that the R-NSCs represent NEPs of the neural plate stage. Owing to their intrinsic plasticity, R-NSCs would be an ideal NSC population for a range of basic and biomedical applications. Nevertheless, the effective and stable long-term maintenance of these cells in culture remains to be investigated. The overgrowth of R-NSCs that has been observed following transplantation into the adult CNS will need to be addressed before they can be used in cell therapy approaches[18].

On exposure to commonly used mitogens (that is, FGF2 and epidermal growth factor (EGF)) R-NSCs are converted into a SOX1-negative radial glia (RG)-like population (named $NSC^{FGF/EGF}$) with a more restricted differentiation potential[18]. Similar results have been shown to occur with a SOX1-positive transient NEP population obtained *in vitro* from a SOX1–enhanced green fluorescent protein (eGFP) reporter ESC line[11,19] by means of a serum-free monolayer protocol[15]. Unlike R-NSCs, this transient, not expandable SOX1-positive NEP population seems to acquire a posterior regional specification, possibly as a consequence of the caudalizing effect of retinoic acid in the culture medium, which prevents the development of rostral neuron subtypes (that is, glutamatergic telencephalic neurons)[20].

Box 1 | **How many NSC or progenitor cell types are in the brain?**

Four main types of neural stem cell (NSC) or progenitor cell can be distinguished in the brain.

- Neuroepithelial progenitors (NEPs) are radially elongated and contact both the apical and the basal surfaces of the single-layered neuroepithelium. They divide at the ventricular zone (VZ), initially symmetrically to increase the pool of progenitor cells but later asymmetrically to generate a VZ progenitor and a daughter cell that migrates radially outward. NEPs are responsible for the first wave of neurogenesis in the neural tube, after which they give rise to both radial glia and basal progenitors.
- Radial glia (RG) originate from NEPs at the beginning of neurogenesis and are the main cell type in the developing brain, where they serve both as neural progenitors and as scaffolds for migrating newborn neurons. In contrast to NEPs, RG express astroglial markers such as GLAST (also known as SLC1A3), glial fibrillary acidic protein (GFAP) (only in humans and primates) and BLBP (also known as FABP7). RG undergo symmetrical proliferative or asymmetrical neurogenic divisions. Their differentiation potential is less broad than that of NEPs. Although subsets are tripotent, being able to generate neuronal, glial and oligodendroglial lineages, most seem to be bipotent or unipotent.
- Basal progenitors (BPs) are a distinct population of neurogenic precursors predominantly present in the subventricular zone (SVZ) in the developing telencephalon. They are generated at early stages of development by NEPs and at later stages by RG. BPs do not make contact with apical or basal surfaces of the neuroepithelium. Time-lapse imaging studies have revealed that most BPs undergo a single round of symmetrical division, generating one pair of neurons, but some are subjected to a second round of mitosis, generating two pairs of neurons. Therefore, BPs may be considered neurogenic transit-amplifying progenitors that specifically increase the production of neurons during restricted time periods. BPs lack expression of key transcriptional regulators that function in RG self-renewal, including PAX6 and SOX2, but they express TBR2, CUX1, CUX2 and SVET1.
- Adult progenitors are a population of multipotent neural cells mainly present in two specialized niches of the adult mammalian brain, the SVZ of the lateral ventricle wall and the subgranular zone of the dentate gyrus. They maintain neurogenesis and gliogenesis throughout adult life. They derive directly from RG that in the postnatal brain convert into astrocytic-like NSCs. Type B NSCs in the SVZ share many characteristics with astrocytes. These are in intimate contact with all other SVZ cell types, including the rapidly dividing transit-amplifying type C cells and the lineage-committed migratory neuronal type A cells.

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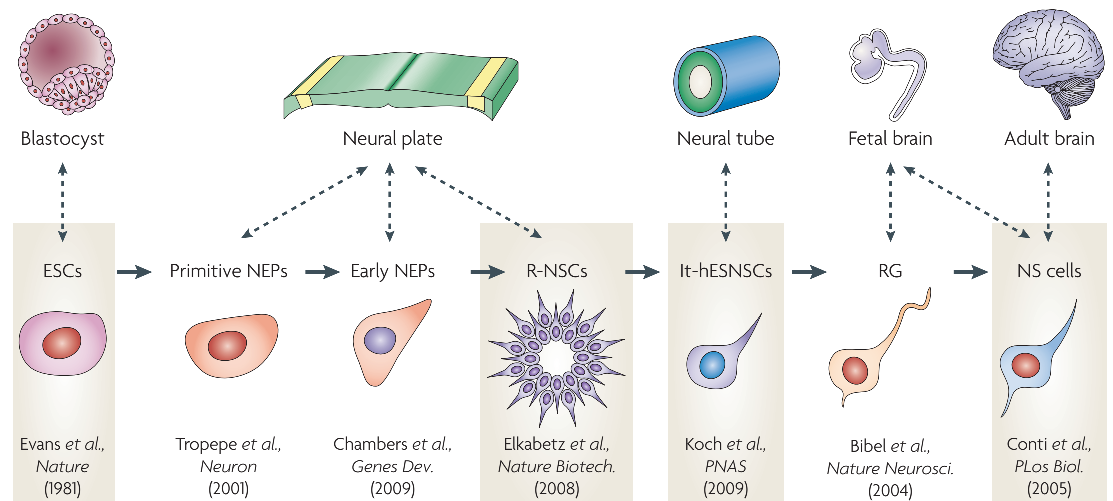

Figure 1 | **Developmental links between the different NSC populations that can be isolated or generated *in vitro*.** Shaded boxes indicate the neural stem cell (NSC) populations that can be obtained through mid-term or long-term expansion *in vitro*. The corresponding *in vivo* developmental stages and the reports that first described these populations are indicated. Induced pluripotent stem cells, generated by means of reprogramming, are thought to have the same developmental potential as embryonic stem cells (ESCs). Late NSCs have not been included as they have many similarities (in terms of growth factor requirements, antigenic profile and neuronal subtype specification) with NS cells, probably indicating that they represent an analogous population grown in different ways (monolayer and aggregation).

More recently, the generation of a pure population of SOX1-positive self-renewing hESC-derived NEPs (named 'lt-hESNSCs') has been described[21] (FIG. 1; TABLES 1,2). These cells exhibit long-term self-renewal, clonogenicity and stable neurogenesis. Remarkably, they can be maintained for over 100 *in vitro* passages in the presence of FGF2 and EGF but still preserve some properties of the R-NSCs, such as rosette-like patterns, the expression of Forse-1, N-cadherin and ZO1 and responsiveness to instructive cues that promote the induction of distinct neuron subpopulations. Direct comparison of the gene expression profiles of R-NSCs and long-term-expanded lt-hESNSCs indicates that lt-hESNSCs partially retain rosette properties, possibly embodying an intermediate developmental stage between rosette-organized NEPs and RG. Interestingly, lt-hESNSCs show a more posterior regional identity (a ventral anterior hindbrain identity code) than R-NSCs. This specific posterior identity could be a result of the *in vitro* expansion process as freshly isolated (passage 1 and 2) lt-hESNSCs show prominent expression of anterior markers such as OTX2 and FOXG1.

Later in development (E9.5 in mice), RG arise in the neural tube (BOX 1). Like NEPs, RG are a transient population in the developing brain that exhibit definite morphological hallmarks. Purification of RG from tissue has been efficiently achieved by means of fluorescence-activated cell sorting (FACS) and transgenic mouse lines expressing the eGFP reporter under the control of RG-associated gene promoters (such as those of *GLAST* (also known as *SLC1A3*), *BLBP* (also known as *FABP7*) and human glial fibrillary acidic protein (*GFAP*))[22–24]. *In vivo*, RG are characterized by lineage heterogeneity, with spatiotemporal diversity[22,25–28]. A similar heterogeneity in RG has also been appreciated *in vitro*, as distinct differentiation potentials have been observed depending on whether the RG were isolated at early or late developmental stages[28,29]. For example, focusing on forebrain development, 60–70% of RG isolated during the period of neurogenesis (from E14.5 to E16.5 in mice) differentiated into neurons, with a negligible proportion of bipotent RG generating neurons and other glia, including RG, astrocytes or oligodendrocytes[24,30]. At the end of neurogenesis (E18 in mice) neurogenic RG disappear[31,32] and RG isolated at this stage are mostly gliogenic[33,34]. Large numbers of RG are found in primary cell populations dissociated from E10.5–18.5 CNS tissue[26,] and these are a good source of stem cells that can be expanded and maintained *in vitro*[26,35–37].

RG populations can be efficiently generated from ESCs using various differentiation protocols[35,38–42], suggesting that the transition from a pluripotent ESC or multipotent NEP state towards neurons via an intermediate RG state may be a common step during the course of neuronal *in vitro* differentiation[9,35,43]. Indeed, transient populations of nearly pure PAX6-positive RG that matured into glutamatergic neurons, mimicking the process that occurs during cortical development, have been generated[38,43] (FIG. 1; TABLES 1,2). Transplantation experiments in chicken embryos revealed that this PAX6-positive ESC-derived RG population is fate-restricted and able to generate only a limited neuronal progeny[44].

A remarkably different population of ESC-derived RG can be obtained by exposing SOX1-positive cells to EGF and FGF2, which leads to SOX1-negative, nestin-positive, BLBP-positive and PAX6-positive RG-like cells that can be expanded for over 100 passages in monolayer and at homogeneity[35] (FIG. 1; TABLES 1,2). This conversion is dependent on Notch activity and on exposure to EGF

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and FGF2 (REFS 35,41). These self-renewing ESC-derived RG-like cells (named NS cells; see next section) maintain the marker signature of RG and full capacity for tripotential differentiation[35,45]. The difference between RG and RG-like NS cells is achieved solely by the *in vitro* differentiation protocol; the protocol that produces RG does not include any amplification step and causes fast proliferation and differentiation of ESCs into glutamatergic neurons[43], whereas the protocol that produces NS cells depends on the addition of mitogens for continued proliferation as RG-like cells[35].

Along with RG, another population of neural progenitors is represented by the basal progenitors (BPs) of the subventricular zone (SVZ)[33,46,47] (BOX 1). BPs showing neuronal-restricted differentiation potential are generated both by NEPs and RG[48,49]. *In vitro* studies on BPs are very limited. BPs can be isolated from the forebrain of reporter mice based on the expression of TIS21 (also known as BTG2), a molecular marker of BPs that have switched from proliferative to neurogenic divisions[50]. A recent study has identified the production of BPs (*in vitro*) from a cortical RG subpopulation

Table 1 | **Comparative analysis of the characteristics and sources of derivation of iPSCs, ESCs and different NSC cultures**

	Source	Year of first derivation	Origin	Growth	Growth factor requirements	Long-term‡ expansion	Purity of the cultures	Long-term‡ neurogenic potential	Clonogenic assay	Cryopreservation	Tumorigenic potential	Refs
ESC	Fetal and adult tissues	1981	Human and mouse	Monolayer	LIF and BMP for mouse; ACTIVIN/NODAL and FGF2 for human	Yes	>95%	Yes	Single cell	Yes	Teratomas	12,93, 145–148
iPSC	Blastocyst	2006	Human and mouse	Monolayer	LIF and BMP for mouse; ACTIVIN/NODAL and FGF2 for human	Yes	>95%	Yes	Single cell	Yes	Teratomas	130–133
Primitive NEP	ESCs	2001	Mouse	Sphere	LIF	No	NA	NA	Single cell	No	NA	15,16
Early NEP	ESCs and iPSCs	2009	Human and mouse	Monolayer	SHH and SB43152	No	80%	NA	NA	No	NA	17
R-NSC	ESCs and fetal neural tissues	2008	Human and mouse	Monolayer (high density)	Notch and SHH	NA	90%	Yes	Single cell	NA	Overgrowth	18
lt-hESNSC	ESCs	2009	Human	Monolayer	EGF and FGF2	Yes	>95%	Yes	Single cell	Yes	ND	21
RG	ESCs	2004	Mouse	Monolayer	NA	No	85–90%	NA	NA	Yes	ND	38,43
NS cell	ESCs and fetal and adult neural tissues	2005	Human, mouse and rat	Monolayer	EGF and FGF2	Yes	>95%	Yes	Single cell	Yes	ND	35,45, 85,86,88
Late NSC	ESCs and fetal neural tissues	1992	Human, mouse and rat	Sphere	FGF2 and/or EGF	Yes	<1%	Yes/no§	Single cell and clonal density‖	Yes	ND	52,56,58, 61–65
Adult SVZ NSC	Adult SVZ	1992	Human, mouse and rat	Sphere	FGF2 and/or EGF	Yes	<1%	Yes/no§	Single cell and clonal density‖	Yes	ND	53,59,60, 67
Adult HI NSC	Adult hippocampus	1997	Human, mouse and rat	Sphere and monolayer	FGF2 and/or EGF	Yes	~85%	Yes/no§	Single cell and clonal density‖	Yes	ND	81–84

BMP, bone morphogenetic protein; EGF, epidermal growth factor; ESC, embryonic stem cell; FGF2, fibroblast growth factor 2; HI, hippocampus; iPSC, induced pluripotent stem cell; LIF, leukaemia-inhibitory factor; NA, not available; ND, not detected; NEP, neuroepithelial progenitor; NSC, neural stem cell; RG, radial glia; SHH, sonic hedgehog; SVZ, subventricular zone. *RG cells described here are highly enriched mouse ESC-derived RG populations[43]; other RG derived from mouse ESCs[40] and human ESCs[42] have not been included in the table as the quantitative description of the cultures was limited. ‡Long-term is defined as cultures that were expanded *in vitro* for at least 10 passages. §Yes/no indicates that the results of the studies considered are not consistent. ‖Clonal density refers to assays performed with cells plated at a density of 10–1,000 cells per μl.

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isolated by means of sorting from eGFP reporter mice bearing the human *GFAP* promoter[28]. This RG population is characterized by a high immunoreactivity for prominin and can produce neurons only indirectly, through the production of BPs. Transient induction of neurogenic TBR2-positive BPs has also been described during the differentiation of ESCs to glutamatergic cortical neurons[51].

In the adult mammalian brain, the presence of NSCs has been extensively investigated in two regions, the SVZ and the subgranular zone of the hippocampus, and their properties have been reviewed elsewhere[1,60–62] (BOX 1).

In vitro long-term propagation of NSCs

Isolation of NSCs from their natural niche and their purification and expansion have been problematic, as the factors and cell contacts required to maintain these cells in their physiological state are poorly understood. EGF and FGF2 have been key players in the identification of cell culture conditions that sustain prolonged cell division of cells with NSC properties[52–54]. In this section we review the strategies developed for NSC isolation and expansion, and compare their power in terms of efficiency and long-term maintenance of the cells' 'genuine' molecular and biological properties.

Table 2 | **Comparative analysis of the developmental, molecular and neuronal differentiation properties of NSC systems**

	Corresponding developmental stage	Specific markers	Rosette formation	Anteroposterior identity	Dorsoventral identity	Neurogenic efficiency	Responsiveness to instructive neuronal regionalization (short term‡)	Responsiveness to instructive neuronal regionalization (long term§)	Refs
Primitive NEPs	E5.5 (egg cylinder stage) < embryo < E7.5 (prestreak stage)	Nestin, SOX1, PAX6, SOX2 and OCT4	NA	Not restricted (OTX2 and HOXB1)	Not restricted (PAX6, neurogenin 1, NKX2.2 and MASH1)	NA	NA	NA	15,16
Early NEPs	E7.5 < neural plate < E8.25	Nestin, SOX1 and PAX6	Yes	Anterior (FOXG1 and OTX2)	NA	NA	Yes (tested: TH, GABA and motor neuron)	NA	17
R-NSCs	E8.25 neural plate	Nestin, SOX1, Forse1, N-cad and PAX6	Yes	Anterior (FOXG1, OTX2 and EMX2)	NA	NA	Yes (tested: TH, GABA and motor neuron)	Biased to GABAergic	18
lt-hESNSCs	E8.5 < neural tube < E9.5	Nestin, SOX1 and PAX6	Yes	Anterior hindbrain (EN1, GBX2, HOXA2 and HOXB2)	Ventral (NKX6.1 and OLIG2); no dorsal markers	65–70%	Yes (tested: ventral TH midbrain, hindbrain GABA interneurons and ventral spinal cord motor neurons)	Yes (tested: ventral TH midbrain, hindbrain GABA interneurons and ventral spinal cord motor neurons)	21
RG*	E10.5 < forebrain < E12.5	Nestin, RC2, SOX2, BLBP, GLAST and PAX6	No	Forebrain	Dorsal	93.4%	NA	NA	38,43
NS cells	E10.5 < CNS < E16.5; adult SVZ	Nestin, RC2, SOX2, BLBP, GLAST, PAX6 and CD44	No	Grossly maintained (depending on the area and time of derivation)	Deregulated along the dorsoventral axis	65–85%	Yes	Biased to GABAergic	18,35, 85–88, 103
Late NSCs	E9.5 < CNS < postnatal	Nestin, SOX2, RC2 and musashi	No	Grossly maintained (depending on the area and time of derivation)	Deregulated along the dorsoventral axis	10–25%	Yes	Biased to GABAergic	52,56,58, 61–65,95, 100,101, 106,107

E, embryonic day; GABA, γ-aminobutyric acid; NA, not available; N-cad, N-cadherin (also known as cadherin 2); NEP, neuroepithelial progenitor; NSC, neural stem cell; RG, radial glia; TH, tyrosine hydroxylase . *RG described here are highly enriched mouse embryonic stem cell (ESC)-derived RG populations[43]; other RG derived from mouse ESCs[40] and human ESCs[42] are not included in the table as the quantitative description of the cultures was limited. ‡Short term is defined as cultures that cannot be expanded *in vitro* for more than 3–5 passages. §Long term is defined as cultures that have been expanded *in vitro* for at least 10 passages.

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Neurosphere system. Neurospheres are free-floating aggregates of neural progenitors, each potentially derived from a single NSC[52–54]. Their generation relies on tissue microdissection (or dissociation of neuralized ESC or induced pluripotent stem cell (iPSC) cultures) followed by exposure to mitogens[55]. Commonly, mouse and rat neurospheres are harvested from neural tissue at E10.5–E18.5 or from the adult SVZ[56–65]. For their expansion, cells are plated in low-attachment tissue culture plastic dishes in serum-free media supplemented with EGF (10–20 ng per ml) and/or FGF2 (10–20 ng per ml)[66]. In these conditions, most differentiating or differentiated cells are expected to die, whereas the NSCs respond to the mitogens, divide and form floating aggregates (primary neurospheres) that can be dissociated and re-plated to generate secondary neurospheres. This procedure can be repeated several times to expand an NSC population.

In the past few years, the identity of the neurosphere-forming cell has been partially elucidated in the adult rodent SVZ. In the adult brain, both GFAP-positive type B cells and NG2 (also known as chondroitin sulfate proteoglycan 4)- and DLX2-positive type C cells located in the SVZ form neurospheres, but their long-term expansion has been thoroughly studied only for neurospheres derived from GFAP-positive cells[67]. The identity of the neurosphere-forming cell in the embryo is less well characterized. Most embryonic neurosphere cultures described in the literature often report only a superficial characterization in terms of NSC marker expression, hardly ever going beyond the expression of nestin. In these neurospheres, a variable proportion of cells also expresses other NSC markers, such as SOX2 and prominin, and markers of RG, such as RC2, GLAST and BLBP)[26]. Interestingly, EGF receptor signalling is sufficient to regulate both the generation and the differentiation of morphologically, antigenically and functionally defined RG from mouse neurospheres upon adhesion[68]. This effect was also seen in human neurospheres exposed to a high EGF concentration (100 ng per ml)[36] and might indicate that the NSC components of the neurosphere have an RG identity.

Neurospheres have been used *in vitro* for defining, by extrapolation, the persistence and properties of NSCs *in vivo*[69,70]. The validity of this process may be questionable because of theoretical and technical intrinsic limitations of the assay, which is often erroneously performed[66]. In the original protocol for adult SVZ-derived spheres, 1,000 viable cells were plated per 35-mm-diameter dish and the number of neurospheres was measured after 6–8 days[53]. However, we now know that unless a rigorous single-cell analysis is performed[71], the number of spheres generated at this early stage cannot be regarded as a formal index of the number of NSCs in the tissue. Indeed, cell aggregation occurring in the extremely heterogeneous populations of cells that are present in the initial cultures might undermine the evaluation of single-cell multipotentiality[66,72,73].

The cellular milieu of the neurosphere has been suggested to provide an *in vitro* counterpart to the *in vivo* neurogenic compartment, a microenvironment that is relevant for NSC maintenance, proliferation and differentiation. Although this concept of a neurosphere as an *in vitro* recapitulation of a niche-like structure has become extremely popular in the NSC field, it should be emphasized that a niche *in vivo* represents primarily a precise cell-specific microdomain with a spatial organization that helps regulate how stem cells participate in tissue generation, maintenance and repair[74,75]. The regulation of stem cell features in the niche requires both interactions between stem cells and interactions between stem cells and neighbouring differentiated cells, mediated by soluble and adhesion molecules and extracellular matrix components. In the rodent brain, the best characterized stem cell niche is the adult SVZ, where the different cell types are specifically organized with SVZ astrocytes (type B cells) located next to the ependymal layer and ensheathing chains of migrating young neurons[1]. In this respect, neurospheres do not show any cellular organization in terms of cell types and distribution that may recapitulate the SVZ structure. Indeed, electron microscopy has shown that the neurosphere is composed of different cell types, mirroring the heterogeneity of the *in vivo* niche but with no spatial organization and no specific cell–cell interactions, which are typical of tissue stem cell niches[76,77].

The heterogeneity of the neurospheres can be inherent in their three-dimensional structure (FIG. 2) as the different cells in the sphere can be exposed to suboptimal conditions; this is demonstrated by the tendency of neurospheres to generate differentiated cells in their core[74]. Consequently, the interaction between differentiating cells and precursor cells may expose the NSCs to paracrine factors that promote differentiation.

Neurospheres show multipotency, although the maintenance of the neurogenic versus gliogenic potential gradually declines with *in vitro* passages (BOX 2). Different neuronal differentiation protocols based on mitogen removal and exposure to fetal bovine serum and/or to specific substrates and cytokines have been developed[4,55,64,78,79], but none of them generates cells that are positive for the early neuronal marker β3-tubulin at a proportion greater than 20%. On the whole, this suggests that the neurosphere system is not particularly efficient in terms of neurogenic competence, but it can be useful for generating large numbers of neurons *in vitro* by means of cell sorting or genetic manipulation.

Monolayer systems. Early attempts to culture NSCs in monolayer conditions relied on plating them on polyornithine-, laminin- or fibronectin-coated dishes in serum-free media[80], but only a few examples have successfully demonstrated the possibility of expanding NSC cultures in the long term.

One such example is represented by the progenitor cells from the adult rat hippocampus grown in monolayer in the presence of FGF2 (REF. 81). These cells show a variable degree of homogeneity for nestin and SOX2 expression, and asymmetrical cell division continuously replenishes the supply of multipotent progenitors. Similar cells have also been derived from the adult

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mouse[82] and human hippocampus[83] and have been shown to undergo some degree of neuronal differentiation *in vitro*[81,84].

More recently, other strategies for the derivation and stable long-term propagation of NSC lines from different sources of rodent[35,85] and human[86] origin have been described. According to these procedures, neural precursors can be competently expanded as adherent, clonal, uniform NS cell lines by exposure to EGF and FGF2 (REFS 18,21,35). Under these conditions cells divide symmetrically, retaining their tripotential differentiation capacity, indicating that monolayer culture systems can maintain almost pure NSC populations[35], with a negligible differentiated component. The key aspect of the NS cell culture system lies in the combination of EGF and FGF2 used and the focus on cells that grow adherently. The continuous provision of EGF together with FGF2 seems to be essential for the derivation and propagation of these monolayer-growing NS cells, whether sourced from ESCs or fetal or adult brain[81]. This NS cell population shows a remarkable antigenic similarity to forebrain neurogenic RG[35,85]. These properties are also manifested by the NSC$^{EGF/FGF}$ population directly derived from R-NSCs exposed to EGF and FGF2 (REF. 18). The fact that NS cells can also be established from long-term expanded neurospheres indicates that RG-like cells might be the NSC fraction in neurospheres and that monolayer growth conditions may allow their enrichment and subsequent expansion[35].

hESC-derived lt-hESNSCs can also be maintained for a long time in monolayer conditions. Unlike NS cells and NSC$^{EGF/FGF}$ cells, lt-hESNSCs show sustained SOX1 expression and partially retain the capacity to respond to patterning cues. Interestingly, lt-hESNSCs exhibit rosette-like patterns even after extensive passaging *in vitro*. Direct gene expression comparison between rosette-type R-NSCs and lt-hESNSCs showed that many rosette-specific genes (for example, *DACH1* and *PLZF* (also known as *ZBTB16*)) are common to the two cell types, whereas other genes described in the rosette stage are undetectable in lt-hESNSCs. This indicates that lt-hESNSCs might be expandable with FGF2 and EGF, retaining intermediate properties between the R-NSCs and the RG-like NS and NSC$^{EGF/FGF}$ populations. It can be speculated that some rosette-stage properties can be maintained in lt-hESNSCs thanks to the strong expression of the Notch-downstream genes *HES5* and *HEY1*, suggesting that this pathway, which is essential for R-NSC maintenance, might be constitutively active during the proliferation of lt-hESNSCs. Successful maintenance of lt-hESNSCs also depends on the specific composition of the cell culture medium. In particular, a low concentration of B27 supplement seems to be essential, as media without or with high concentrations of B27 promote spontaneous differentiation and senescence as well as a reduced responsiveness to instructive factors. Notably, media used for both NS cells and NSC$^{EGF/FGF}$ cell growth do not contain B27.

Interestingly, cells in these EGF- and FGF2-dependent monolayers retain multipotentiality and neurogenic efficiency after prolonged *in vitro* expansion and show a high competence to efficiently originate antigenically and electrophysiologically mature neurons on exposure to optimized differentiating conditions[21,35,87,88] (FIG. 2). This capacity can probably be interpreted as a consequence of the homogeneity of the starting population.

The fact that nearly homogeneous cell populations with NSC features have been grown for sustained periods under adherent conditions indicates that the NSC niche is dispensable for *in vitro* NSC propagation. The artificial two-dimensional structure ensured by monolayer-adherent cultures seems to prevent lineage restriction and minimize spontaneous differentiation, favouring niche-independent, symmetrical self-renewal and expansion of rather homogeneous cell populations.

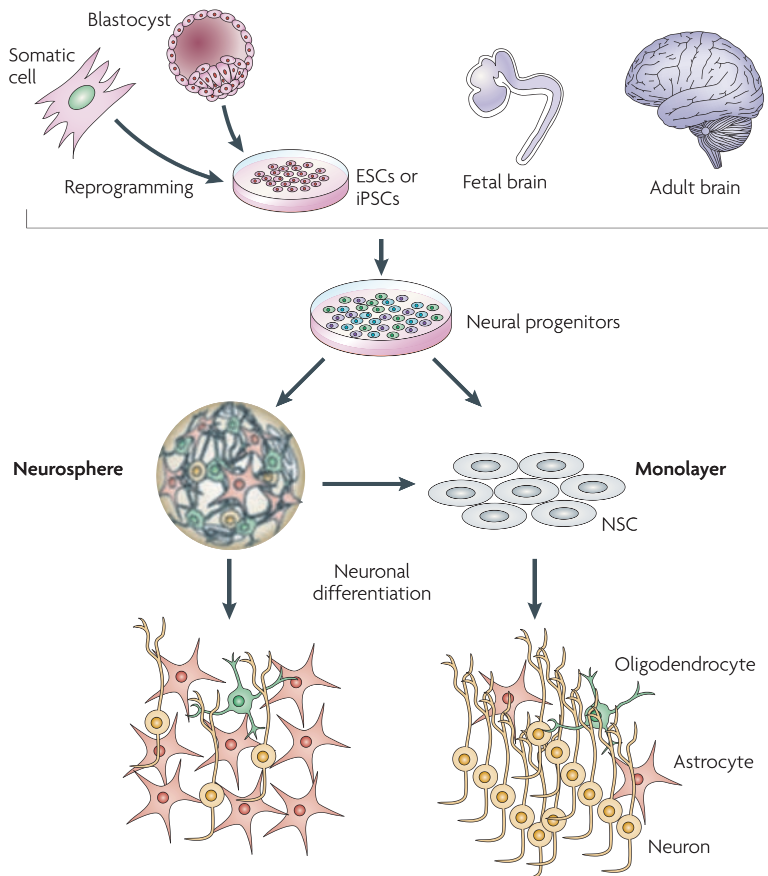

Figure 2 | **Sources of neurospheres and monolayer NSCs and results of differentiation.** Neurospheres and monolayer neural stem cell (NSC) lines can be generated from embryonic stem cells (ESCs) (derived from the inner cell mass of blastocysts), from induced pluripotent stem cells (iPSCs) (derived from reprogrammed somatic cells) and from the germinative areas of the fetal and adult brain. Neurospheres and cells grown in monolayers can be considered tripotent as they can give rise to neurons, astrocytes and oligodendrocytes. The different cellular compositions of the neurosphere (mixed, with only a fraction of the cells exhibiting NSC properties) and NSC monolayer culture (homogeneous composition) systems results in low and high neurogenic potential, respectively.

Mechanisms for the establishment of NSC systems

In vitro assays based on neurospheres or adherent colony formation are regularly regarded as a means to infer *in vivo* NSC frequency and properties[64,89–92].

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> Box 2 | **Criteria defining the value of long-term self-renewing NSC systems**
>
> **Efficiency of propagation**
> Efficiency of propagation is directly correlated to the composition of the system[137]. In neurospheres, the neural stem cell (NSC) content is variable and depends on the stage of the culture — it is high soon after the dissociation and replating but declines progressively with subsequent subculturing[64,138]. Conversely, NSCs grown in monolayers show a higher degree of homogeneity, suggesting that these conditions favour symmetrical cell division over long time periods[35,85,86].
>
> **Clonality**
> Clonality is fundamental for the retrospective identification of the NSC identity of a cell *in vitro*. In the strict sense of the term, a cell clone should derive from a single isolated cell in a separate well. This has been described for both monolayer cultures[64,89,90] and neurospheres. However, this is often achieved by plating at the so-called clonal density — that is, 10–1,000 cells per μl. Thus,what should be a clonal density is often represented by a bulk culture, hampering the initial scope of the clonal assay. Also, aggregation between separate neurospheres has been reported, resulting in the lack of clonality in any one sphere[66,72,73].
>
> **Karyotypic stability following extensive proliferation**
> The long-term stability of mouse NSCs expanded *in vitro* has been poorly documented[139–142]. Some studies support the observation that after long-term culture *in vitro* NSCs are transformed into malignant cells[142]. However, one study reported a normal karyotype in neurosphere cultures at passage 70 (REF. 140). Nevertheless, to circumvent this limitation, it is common to avoid using neurospheres beyond the tenth passage. Abnormalities in mouse cells that have been cultured for a long time are common and have also been reported in monolayers of NS cells[88] and hippocampal cell lines[81]. NSC cultures of human origin grown in monolayers seem to retain a normal diploid karyotype after long-term expansion[21,86].
>
> **Retention of neuropotency**
> Several studies indicate that gliogenesis is more prevalent in neurospheres that have undergone long-term expansion. It is thought that this switch from neurogenic to gliogenic potential is reminiscent of events that occur during development, suggesting that intrinsic cellular programmes are preserved *in vitro*. The gradual loss of neurogenic potential has often been reported for neurospheres. Conversely, NS cells, NSC$^{EGF/FGF}$ and lt-hESNSC monolayer systems retain the capability to produce a large proportion of neurons after prolonged expansion (TABLE 2), suggesting that maintenance in a symmetrical division state can preserve the cells' original neurogenic potential[88]. Alternatively, they can correspond to populations of neurogenic cells present at specific developmental stages[143,144]. It is of note that maintenance of a stable neurogenic potential does not impede prompt responses to gliogenic signals.

However, the actual correlation between *in vitro* stem cell lines and *in vivo* progenitors is uncertain, and there is increasing concern about the physiological relevance of studying stem cells *in vitro*. For example, it is unclear whether these cells represent a subpopulation of authentic homologues found *in vivo* or whether they are the result of 'forced' reprogramming *in vitro*. In the developing mammalian brain, both transplantation and *in vivo* fate mapping experiments have so far failed to provide definitive proof of the presence of self-renewing NSCs rather than progenitor cells[2]. In most tissues and organs, the founder cells that are present during embryogenesis either do not endure or switch to a relatively quiescent state following the conclusion of development. This suggests that the NSC state might represent an *in vitro* condition possibly induced by the experimental set-up. ESCs, for example, have a close molecular relationship to inner cell mass founder cells of the blastocyst[12,93] and express genes that are not typically detectable in the inner cell mass, which may be crucial for establishing and maintaining ESCs *in vitro*.

Clonal density
The density of cells (number of cells per μl) that should allow formation of single-cell clones. Rigorously, clonality is assured solely by plating a single cell per well, thus allowing the investigation of properties of single cells. This step is essential for formal demonstration of self-renewal and potency.

EGF and FGF2, the growth factors that are most frequently used for preserving NSCs *in vitro*, might alter the transcriptional and cellular phenotype. Many genes can be directly induced in neural progenitors by *in vitro* exposure to FGF2 or EGF[94], suggesting that these growth factors might exert a crucial role in the creation of NSC lines *in vitro*. In fact, EGF has been reported to downregulate expression of DLX2 in homogeneous NS cell lines and NSC$^{EGF/FGF}$ cultures[85] and in transit-amplifying cells of the SVZ[67], promoting their conversion into RG-like NSCs *in vitro*. Also, fetal progenitors, when exposed to FGF2 *in vitro*, rapidly activate the expression of EGF receptor (also known as ERBB1)[57,58] and OLIG2 (REF. 95), a basic helix–loop–helix transcription factor associated with the oligodendrocyte lineage[96] and ventral CNS identity. Under expansion conditions with high levels of EGF and FGF2, induction of OLIG2 is required for the proliferation and self-renewal of neurosphere cells[97].

The importance of FGF2-mediated OLIG2 induction for the self-renewal and proliferation of NSCs has been demonstrated in neurosphere systems, although with the caveat indicated above[66]. Indeed, similarly to the effect of deletion of BMI1 (a polycomb gene required for the self-renewal of stem cells from diverse tissues)[98], interference with OLIG2 severely reduces the number and size of neurospheres[97]. Besides the FGF2-mediated induction of OLIG2 (REFS 99–102), short-term exposure to FGF2 induces the expression of a broad set of genes in primary neural precursors. Among these are *CD44*, *ADAM12*, *CX3CL1*, *CDH20*, *KITLG*, *FZD9*, *GLAST*, *OLIG1* and *VAV3* (REF. 103). Many of them are likely to have substantial roles in determining the phenotype of the cells. For example, upregulation of proto-oncogenes, such as the RhoGEF family member *VAV3*, could alter cell adhesion[104] and division[105]. Interestingly, induction of some of these genes (for example *CD44* and *VAV3*) occurs rapidly (within 5 hours of FGF2 exposure)[103]. Thus, the speed and nature of the gene expression changes suggest that FGF2 action does not mediate a standard developmental progression but rather an acute transcriptional resetting.

With respect to regional identity, there are data showing profound differences between gene expression patterns *in vitro* and in primary precursors *in vivo*, which could lead to the emergence of a mixed regional identity and limited neuronal differentiation[95,101,106,107]. For example, neurospheres from the spinal cord have been shown to undergo upregulation of OLIG2 and downregulation of the dorsal spinal cord transcription factors PAX3 and PAX7 (REF. 100). OLIG2 and MASH1 (also known as ASCL1) are also induced in E14 cortex precursors grown in the short or long term as neurospheres[101]. With some exceptions[86], a similar deregulation of the regional patterning is evident in the adherent NS cell cultures[35,85,86]. RG *in vivo* are heterogeneous in terms of their transcription factor expression profile, a feature that is expected to confer positional signals[108]. However, homogeneous co-expression of PAX6, OLIG2 and EMX2 is present in virtually all NS cell cultures derived from ESCs, fetal forebrain or adult SVZ, but these cultures are negative for some ventral markers such as LHX6 (REFS 35,85).

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Box 3 | **Comparison of the main characteristics of NSCs *in vivo* and *in vitro***

- *In vivo* the presence of a niche stringently controls stem cell activity. *In vitro* neural stem cells (NSCs) can divide and differentiate in the absence of a niche.
- Asymmetrical cell division predominates *in vivo*; asymmetrical and symmetrical cell division are both observed *in vitro*.
- NSC identity *in vivo* evolves through developmental stages. *In vitro* this temporal evolution is partially recapitulated during embryonic stem cell neuralization processes. NSCs that have undergone long-term expansion mainly have a single radial glia-like identity.
- *In vivo* a clear separation between NSCs and transient amplifying progenitors can be drawn; transient amplifying progenitors can acquire an NSC identity *in vitro*.
- Precise region-specific NSC populations are present during brain development. Specific NSC positional identities can be imposed in *in vitro* neuralized ESCs. *In vitro* NSCs that have undergone long-term expansion tend to lose the codes of transcription factors that determine positional identity.
- NSCs *in vivo* generate various differentiated neuronal subtypes. NSCs that have undergone long-term *in vitro* expansion can give rise to limited assortments of specialized neuronal progeny.
- Authentic neuronal functional phenotypes are acquired *in vivo*. Only partial functional maturation can be achieved *in vitro*.

Even though it is generally evocative of a telencephalic character, this set of markers does not show an exact correlation with a specific regional identity. PAX6 and EMX2 in cortical RG are suggested to function in the maintenance of symmetrical self-renewal and neurogenic potential, respectively[109]. Hence, the co-expression of PAX6, OLIG2 and MASH1 fails to accurately define a dorsal forebrain identity.

Importantly, this relaxation in the positional code is associated with a restriction in the competence to produce diverse neuronal subtypes. Indeed, NSCs rapidly lose their original competence to generate site-specific neuronal subtypes when cultured in the presence of mitogens *in vitro*, both in monolayer and in aggregation, becoming mainly constrained to adopt a GABA (γ-aminobutyric acid)-ergic fate[35,106,107]. Notable exceptions are lt-hESNSCs[21]; nevertheless, also in this case long-term passage is accompanied by a substantial modification of their original regional identity.

It can therefore be proposed that the RG-like features that are common to NS cells, $NSC^{EGF/FGF}$ and neurosphere-forming cells might be reminiscent of those conferred by a peculiar combination of transcription factors present in a rare subset of cells in the embryonic brain. Alternatively, they might be the result of a relaxation of the developmental molecular patterns, due to exposure to the *in vitro* environment and growth-factor stimulation, as several of these transcription factors are known to cross-regulate and co-repress each other[103].

Authentic or synthetic?

The data described above call for extreme caution when extrapolating *in vitro* results to normal development or physiology without corresponding *in vivo* data and suggest that the self-renewal and multipotency demonstrated by NSCs *in vitro* might result from exposure to growth factors that create a synthetic transcriptional and biological state. Indeed, NSC culture systems may be best viewed as an environment that represses regional or cell type-specific differentiation and promotes high rates of proliferation. Recent studies support the idea that the NSC cell cycle may influence cell fate. In fact, it was shown that cell cycle lengthening caused cortical progenitors to undergo differentiative divisions, whereas cell cycle shortening resulted in more self-renewing divisions[110]. Numerous cell cycle-specific genes have been identified as important regulators of NSC proliferation *in vivo* and many of them are important for the establishment and maintenance of NSCs *in vitro*[111–114] (these include *BMI1* (REFS 98,115–117), *CDKN1A* (also known as p21, WAF1 and CIP1)[118] and nucleostemin (also known as *GNL3*)[119,120]). Interestingly, some of these genes are under the direct regulation of FGF2-induced transcription factors, such as OLIG2, which may mediate proliferation by regulating CDKN1A levels[97].

Derivation of NSC lines could hence be interpreted as a fate reprogramming assay rather than a direct measure of the number and properties of endogenous NSCs. In this respect, it has been shown that oligodendrocyte precursor cells can be converted to a tripotent state *in vitro* through bone morphogenetic protein- and FGF-induced chromatin modifications[121–123]. Similarly, in the adult brain only transit-amplifying type C cells, not GFAP-immunoreactive type B cells, respond to growth factors and can be expanded *in vitro* as an NSC population[67].

The actual criteria that define an NSC might have to be reconsidered in the light of recent findings, such as the observation that epigenetic modifications may permit the generation of cells with some of the antigenic properties of neural-like cells and neurons from non-neural lineages[124–126], although stringent biological, molecular and functional analyses indicated that these cells cannot be considered true NSCs and neurons[127]. The recent advent of iPSCs has questioned our notion of what constitutes a terminally differentiated somatic cell[128–132]. The fact that methyltransferase and histone deacetylase inhibitors greatly enhance iPSC generation points to chromatin modification being a crucial determinant of cellular reprogramming[133].

Epigenetic regulation of transcription as a means of reprogramming cell fate is attracting a lot of attention[134]. DNA methylation plays a key part in the determination of neuronal or glial fate in NSCs[135], and methylation status, which can be modulated by FGF2, has been shown to be closely linked to the multipotency of NSCs both *in vivo* and *in vitro*[136].

Together, these findings suggest that NSCs *in vitro* are distinct from neural progenitors *in vivo* (for a concise comparison of their properties, see BOX 3) and that the self-renewal and multipotency demonstrated by NSCs *in vitro* might not be an accurate representation of stem cells *in vivo*, owing to the exposure to growth factors acting at both the transcriptional and the epigenetic levels. Although this NSC state might be a synthetic stem cell state created *in vitro*, a similar phenotype may also emerge in pathophysiological conditions. In this case, studying NSCs *in vitro* may contribute to our understanding of these conditions and lead to potential biomedical applications.

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Conclusions and future directions

Our knowledge of neural progenitor identity and properties during development has been revolutionized by the ability to isolate and expand NSCs *in vitro*. In this article we have reviewed the current and most commonly used sources of NSCs and *in vitro* methodologies to isolate, expand and functionally characterize NSC populations (for a summary see TABLE 1). The real identity of and the potential lineage relationships between diverse types of stem or precursor cells isolated and cultured *in vitro* by these different methodologies are still being investigated.

Nonetheless, *in vitro* settings necessarily result in a disruption of the three-dimensional tissue structure, loss of specific cell–cell contacts and modification of the extracellular environment and intracellular signalling cascades, possibly altering the biological and molecular properties responsible for the acquisition of stem cell features.

Given that NSC biology holds tremendous potential for therapy, it will be crucial to be able to manipulate the properties of NSCs and to impose particular developmental programmes *in vitro*. Although it remains to be determined whether any particular human CNS disease will benefit from NSC transplantation, it is also becoming clear that the regenerative capacity and plasticity of the brain requires not only NSC competence but also the ability of other cells to participate in the repair process. Careful planning and extensive animal testing will be required before clinical studies with NSCs can be considered, and even then these trials should be performed in concert with other traditional therapies that aim to ameliorate degeneration and promote neuroprotection.

Emerging knowledge of the molecular biology and genetics of NSCs and their bioactive products, as well as of the injured microenvironment, will refine our judgment of when and how to use NSCs. In the meantime, several steps are required to move the field towards the ultimate goal. We should better standardize methods and protocols of isolation and culture of NSCs of human origin, improve the evaluation of the clinical efficacy of NSC transplants in adequate animal models, study the molecular mechanisms of the limitations of intrinsic brain repair, learn to promote the long-term survival of these cells by creating a more permissive environment (and niche) in the diseased brain and incorporate into future stem cell transplantation trials more detailed knowledge of the specific disease mechanisms.

Although current NSC systems are not perfect, propagation close to homogeneity has set the stage for the next round of discoveries. One can anticipate that a rigorous assessment of the functional features of NSC populations isolated and propagated by means of different cell culture systems, combined with new knowledge about cellular reprogramming, will allow us to exploit the advantages offered by these different systems to the full.

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/gene
ADAM12 | *BLBP* | *CD44* | *CDH20* | *CDKN1A* | *CX3CL1* | *DACH1* | *FZD9* | *GLAST* | *HES5* | *HEY1* | *KITLG* | nucleostemin | *OLIG1* | *PLZF* | *VAV3*

UniProtKB: http://www.uniprot.org
BMI1 | FOXG1 | HB9 | Islet1 | Noggin | OTX2 | PAX6 | SOX1 |

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Elena Cattaneo's homepage: http://www.cattaneolab.it

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ERRATUM

Neural stem cell systems: physiological players or *in vitro* entities?

Luciano Conti and Elena Cattaneo

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In Table 1 of the above article, the source of ESCs should be “Blastocyst” and the source of iPSCs should be “Fetal and adult tissues”.

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