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Adherent Neural Stem (NS) Cells from Fetal and Adult Forebrain

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Stable in vitro propagation of central nervous system (CNS) stem cells would offer expanded opportunities to dissect basic molecular, cellular, and developmental processes and to model neurodegenerative disease. CNS stem cells could also provide a source of material for drug discovery assays and cell replacement therapies. We have recently reported the generation of adherent, symmetrically expandable, neural stem (NS) cell lines derived both from mouse and human embryonic stem cells and from fetal forebrain (Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying QL, Cattaneo E, Smith A. 2005. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol 3(9):e283). These NS cells retain neuronal and glial differentiation potential after prolonged passaging and are transplantable. NS cells are likely to comprise the resident stem cell population within heterogeneous neurosphere cultures. Here we demonstrate that similar NS cell cultures can be established from the adult mouse brain. We also characterize the growth factor requirements for NS cell derivation and self-renewal. We discuss our current understanding of the relationship of NS cell lines to physiological progenitor cells of fetal and adult CNS.

Keywords: adult, EGF, FGF-2, mouse, neural, radial glia, stem cell

Introduction

A stem cell is an uncommitted cell that can divide repeatedly while maintaining potency to generate differentiated cell types. In contrast, cells that exhibit only a limited number of divisions before a change in potency or overt differentiation are termed progenitor cells. The functional distinction between stem cells and progenitors can only be defined by experimental determination of the capacity for self-renewal. Our current view of central nervous system (CNS) ontogeny supposes a founder population of cells capable of forming both neurons and glia in response to inductive patterning and differentiation cues conveyed by adjacent cells (Panchision and McKay 2002). This mechanism generates the appropriate diversity of neuronal and glial subtypes in the correct place and time. However, whether the cells that form the CNS in vivo are stem cells or transient progenitors is uncertain (Anderson 2001).

During brain development, neural progenitors are localized in the pseudostratified epithelium of the germinal zone surrounding the ventricles. Postmitotic neurons migrate away from this ventricular zone, guided by the elongated processes of radial glia that act as scaffolds in the maturing brain. It has recently become evident that within the developing cortex, and possibly other regions, radial glia not only fulfill this structural role but also are progenitor cells that divide to generate neuroblasts and neurons (Noctor and others 2001; Malatesta and others 2003; Anthony and others 2004). In the adult brain, ongoing neurogenesis has been convincingly documented in 2 regions, the subventricular zone (SVZ) of the lateral ventricles and the subgranular layer of the hippocampus (Gage and others 1998; Doetsch and others 1999). Cells extracted from the germinative zones of the developing and adult nervous system can be expanded in vitro (Temple 1989; Cattaneo and McKay 1990; Reynolds and others 1992; Reynolds and Weiss 1992). These findings have raised hopes that in the future it may be possible to stimulate endogenous CNS repair mechanisms or to replace dysfunctional or dead cells with cells generated in vitro. However, these remain ambitious and challenging goals (Lindvall and others 2004).

An ability to continuously expand stem cells clonally by symmetrical division offers critical opportunities for experimentation, allowing identification of factors and genes acting directly on the stem cells to regulate self-renewal and commitment (Smith 2001; Gottlieb 2002). Clonal and adherent expansion also enables efficient genome engineering and genetic screening, as established for embryonic stem (ES) cells (Bradley and others 1992). Derivation of adherent neural stem (NS) cell lines with stable neuronal differentiation potential has recently been described (Conti and others 2005). These have been termed NS cells to highlight the similar experimental attributes compared with ES cells.

Initially, NS cells were derived from ES cells differentiated into heterogeneous neuroepithelial progenitors in adherent monolayer culture (Ying and others 2003). Fibroblast growth factor (FGF-2) plus epidermal growth factor (EGF) supported expansion of a distinct subset of progenitor cells attached to tissue culture plastic in a defined basal media. These cells lost the heterogeneous morphology and marker expression of the primary neuroepithelial population (Li and others 1998; Ying and others 2003). Instead, over several passages, the cultures acquired a homogeneous morphology and were shown to uniformly express nestin and Sox2, although extinguishing the expression of Sox1. These cells are stem cells as they are clonogenic and maintain indefinitely the capacity to generate both neurons and astrocytes. NS cells display many hallmarks of radial glia by morphology and molecular markers, including brain lipid binding protein (BLBP), RC2, GLAST, and Pax6 (Gotz 2003; Rakic 2003). Thus, neural differentiation of ES cells initially entails conversion to a transient Sox1-positive panneural progenitor cell population, containing a subpopulation of cells that mature to a specific Sox1-negative state without differentiation and may then be stably maintained and expanded using EGF plus FGF-2 (Fig. 1).

Similar NS cell lines were also isolated from fetal brain tissue. Either primary cell cultures or long-term expanded neurospheres allowed to settle onto gelatin-coated flasks and left

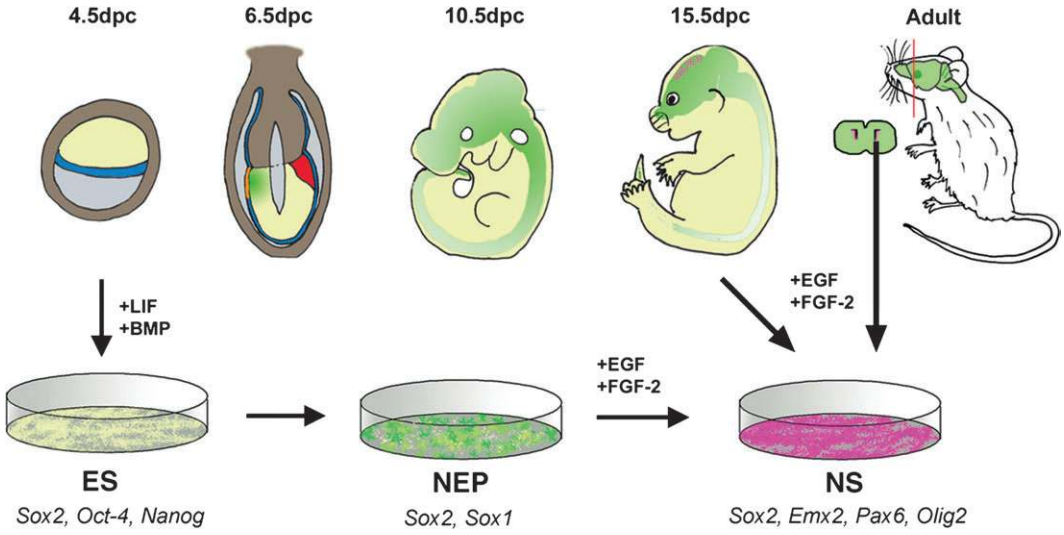

Figure 1. ES cells, neuroepithelial progenitors (NEPs), NS cells, and their in vivo sources. ES cells can be derived from and have similarities to the epiblast of late blastocyst stage embryos (left). ES cells can be readily converted to transient Sox1+ neuroepithelial cells (green) analogous to E8.5–10.5 neural tube. Some Sox1+ progenitors progress to Sox1–, Sox2+ progenitor cells that can be expanded as stem cell lines (NS cells) by the action of EGF and FGF-2 (middle/right). NS cells have similarities to forebrain radial glia and can be derived from fetal forebrain. In this report, we show that NS cells can also be derived from the SVZ of the lateral ventricle of adult mouse forebrain

undisturbed in EGF plus FGF-2 will reproducibly outgrow NS cells that are indistinguishable in all essential features from those derived from ES cells (Conti and others 2005). Our observations suggest that such cells, rather than being differentiation products of the neurosphere (Gregg and Weiss 2003), are in fact the resident stem cells. Indeed, NS cells will readily form neurospheres if detached from a culture substrate. The finding that the complexity of neurosphere cultures, which contain a mixture of stem cells, committed progenitors, and differentiated cells (Suslov and others 2002; Bez and others 2003), can be eliminated by adherent culture parallels observations with ES cells. In adherent culture, ES cells self-renew with minimal differentiation (Smith 2001), whereas suspension culture induces aggregation and differentiation into embryoid bodies (Martin and Evans 1975; Doetschman and others 1985).

In this report, we investigate whether cells capable of giving rise to NS cell cultures are restricted to developmental stages or may also be present in the adult brain. Further, we examine the requirement for EGF and FGF-2 in the derivation and maintenance of NS cells. We discuss how these data, together with other recently published findings, are shedding light on the nature of NS cells and their relationship to endogenous cell types.

Materials and Methods

NS Cell Derivation and Culture

The following protocol was used to derive the adult NS cell line ANS-1. A 2-month-old wild-type (CD1 strain) mouse was sacrificed, and an area encompassing the SVZ surrounding the lateral wall of the forebrain ventricle was dissected. Tissue was dissociated with accutase (Sigma, St. Louis, MO) for 5 min at 37 °C and mechanically dissociated using a fire-polished glass Pasteur pipette. Cells were washed once in phosphate-buffered saline (PBS) and then plated onto a 6-well untreated plastic plate (Iwaki, Iwaki, Japan) in NS-A media (Euroclone, Milan, Italy) plus N2 supplement and 10 ng/mL of both FGF-2 and EGF (Peprotech, Rocky Hill, NJ). This medium is termed expansion medium. These cultures were maintained for 3 weeks, with a half volume of medium exchanged every week. Small spheres formed within the period and were collected by centrifugation at 700 rpm for 30 s. The pellet was resuspended in accutase and cells dissociated by repeated pipetting. Cells were cultured in expansion media for a further 10 days. Upon replating of the newly formed spheres into a fresh T25 flask, attachment and outgrowth occurred within 4–5 days. These cells showed typical morphology of NS cells. They were subsequently expanded in these adherent conditions. Cells underwent rapid expansion and at 70% confluence were passaged 1:3 to 1:5 every 3–4 days for more than 35 passages to date. A procedure identical in all essential features was followed to derive a second adult NS cell line from a female mouse on a mixed 129 × MF1 background.

At higher densities and at earlier passages, NS cells can tend to aggregate and spontaneously form neurospheres. This may be reduced substantially through plating onto a tissue culture plastic precoated with gelatin (15 min treatment of 0.1% solution) or effectively eliminated by culture on poly-ornithine/laminin (30- to 60-min treatment with poly-ornithine solution 0.01%; wash twice in PBS and then add a 10-μg/mL laminin solution for at least 2 h) (Sigma). Detailed protocols for routine handling of NS cell lines are available elsewhere (Conti and others 2005).

Characterization of NS Cells

NS cells were induced to differentiate after replating 0.5–1 × 10^5 cells onto a laminin-coated treated 4-well plate. For astrocyte induction, cells were exposed to NS-A media supplemented with N2 (in-house preparation) and 1% fetal calf serum (Sigma) or 10 ng/mL of bone morphogenetic protein (BMP)-4 (R and D Systems, Minneapolis, MN) for 7–12 days. A minor fraction of adult-derived NS cells differentiated into neurons in these conditions. For more efficient neuronal induction, cells were treated for 5–7 days with NS-A media supplemented with B27 (Invitrogen, Carlsbad, CA) plus 10 ng/mL FGF-2 (Peprotech) and then switched to NS-A:Neurobasal media (1:1) supplemented with B27, 0.5× N2, and no growth factors. For all differentiations, half the volume of medium was exchanged for fresh medium every 2–3 days.

To test the effect of culture in EGF only, NS cells lines were passaged at least 5 times prior to analysis in expansion media without FGF-2. Colony formation assays were performed by plating 10 000 cells into a 9-cm culture dish and scoring colonies after 7–10 days. The fibroblast growth factor receptor (FGFR) inhibitor SU5402 (Mohammadi and others 1997) was used at 5 μM (Calbiochem). For bromo-deoxyuridine (BrdU) assays, NS cells were plated on gelatin-coated 12-well plates with expansion media, incubated at 37 °C overnight for recovery and then cultured with 10 μM BrdU (Sigma) for 2 h. Cells were then fixed in 4% paraformaldehyde for 15 min and washed 3 times with PBS (Sigma). A total of 500 μL of 2 M HCl was added to each well at room temperature for 1 h followed by a further 3 PBS washes. Cells were then immunostained with anti-BrdU antibody (Sigma). Growth curves for EGF-independent lines were determined by plating 1000 NS cells in each well of gelatin-coated 6-well plates in expansion media with or without

FGF-2. Cells in duplicate plates were cultured for 7 days with medium changes every 2 days. Cells were counted by hemocytometer every 24 h after dissociation with accutase.

For reverse transcription–polymerase chain reaction (RT–PCR), total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA) and cDNA generated using Superscript III (Invitrogen). cDNA was normalized via real-time polymerase chain reaction (PCR) determination of β-actin level (Lightcycler, Roche, Branchburg, NJ). PCR was performed for 30 cycles for all markers except β-actin (25 cycles), using primers as described in Conti and others (2005). Antibodies and protocols for immunocytochemistry are described elsewhere (Conti and others 2005).

New Results and Discussion

Derivation and Characterization of Adult NS Cell Lines

NS cell lines derived from ES cells or from fetal forebrain share a range of features with radial glia (Conti and others 2005). Although radial glia have a finite existence, differentiating shortly after birth, they may be the source of long-lived adult CNS stem cells. Recent fate-mapping experiments have indicated that the type B stem cells in the adult SVZ derive from a subpopulation of fetal radial glia (Merkle and others 2004). Therefore, radial glia and adult SVZ astrocytes appear to comprise a continuous lineage with stem cell potential (Alvarez-Buylla and others 2001; Merkle and others 2004).

We were therefore interested in determining whether NS cell lines could be established from adult mouse SVZ. We derived primary neurospheres through dissection of the lateral forebrain ventricle and culture for 3 weeks in basal media supplemented with N2 and EGF plus FGF-2. These spheres readily attached when finely dissociated and replated in a minimal volume of media and left undisturbed for several days. Upon attachment, cells outgrew rapidly and after 7 days in culture were dissociated with accutase and replated onto fresh flasks. We find that in these conditions, cell cultures could be maintained subsequently as adherent cell lines with characteristic NS cell properties. We established cell lines from a male CD1 mouse and a female 129 × MF1 hybrid animal that we have termed ANS-1 and O4ANS, respectively.

Growth characteristics and morphology of both ANS-1 and O4ANS are similar to characterized ES- and fetal-derived NS cell lines (Conti and others 2005). Cells divide approximately every 30 h, and the population forms lattice/net structures at higher densities (Fig. 1*A*). ANS-1 has been expanded for over 30 passages without crisis. The adult NS lines do have a tendency, particularly during early passages and at higher densities, for cells to aggregate and detach from the plate, spontaneously reforming neurospheres. As described previously for fetal- and ES-derived NS cells, we find that NS cells can also be expanded on a laminin substrate whereupon aggregation and detachment are reduced and cells can be expanded to confluence, facilitating routine propagation.

Interestingly, these adult-derived NS cells appear to have reverted to a radial glia–like state. Immunocytochemical staining indicates uniform expression of the markers of radial glia and NS/neural progenitor status: RC2, nestin, and BLBP (Fig. 2*B–D*). Further, RT–PCR analysis and antibody staining identify expression of the transcription factors Sox2, Olig2, Mash-1 and at lower levels both Emx2 and Pax6 (Fig. 2*G*). The transit amplifying cells within the adult SVZ express Dlx2, which is downregulated upon exposure to EGF in vitro (Doetsch and others 2002). We do not detect transcripts for Dlx2 in ANS-1 or O4ANS by RT–PCR. Further, by both RT–PCR and immunocytochemistry, we find little or no GFAP expression in NS cells cultured on laminin (not shown). These data indicate that NS cells do not correspond exactly to either transit amplifying cells or endogenous GFAP-positive stem cells. It is possible that they derive from the former, however, as has been described for neurosphere formation (Doetsch and others 2002).

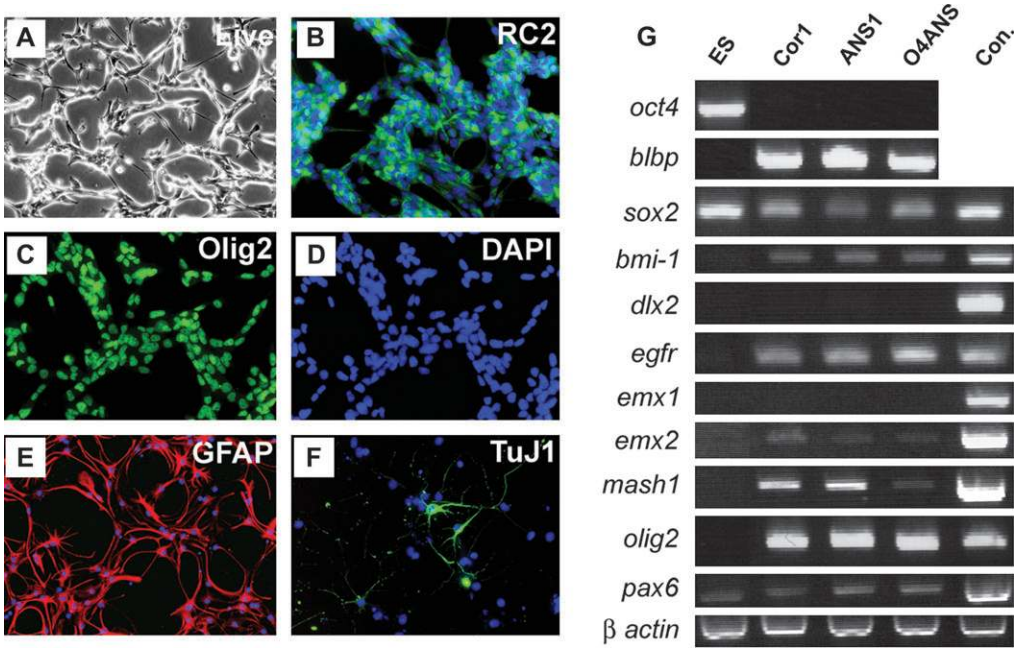

Figure 2. NS cells derived from adult SVZ are similar to fetal forebrain-derived NS cells. ANS-1 cells proliferate as an adherent monolayer and have morphological characteristics similar to ES cell–derived and fetal-derived NS cells (*A*). All cells express RC2 (*B*) and Olig2 (*C*) (corresponding DAPI, *D*) and can differentiate into astrocytes (*E*) and neurons (*F*) at passage 30. By RT–PCR, adult SVZ-derived NS cells, ANS-1, and O4ANS express mRNA for transcription factors and radial glia markers observed in the fetal line, Cor1 (*G*). Positive control is E12.5 + E16.5 embryonic head (Con).

Upon differentiation through exposure to serum on a laminin substrate, we find that the adult NS cells are capable of differentiating into neurons and astrocytes (Fig. 2*E,F*). Differentiation is also induced by treatment with BMP4, possibly reflecting its endogenous role as a paracrine factor released by ependymal cells that promote astrocyte fate (Lim and others 2000). Together, these results suggest that adult SVZ cells isolated and expanded with EGF and FGF-2 can be maintained as adherent stem cell lines in vitro with characteristic markers of NS cells. We have also analyzed a set of NS cell marker genes, identified through an Affymetrix oligonuclelotide microarray approach, and find common expression between adult-, fetal-, and ES cell–derived NS cell lines (data not shown).

A set of master transcription factors expressed by NS cells may confer capacity for self-renewal in the context of a permissive epigenome. These transcription factors may not coexist in any in vivo progenitor (see below) but reflect the in vitro environment and growth factor stimulation. Sox2 is expressed in most cell types within the adult SVZ niche and may be required to maintain neural progenitors in an undifferentiated proliferative state during development (Ellis and others 2004; Pevny and Placzek 2005). Mash-1 has recently been shown to mark the adult forebrain SVZ transit amplifying cell population (Parras and others 2004). Furthermore, Emx2 has been ascribed a role in maintaining multipotent neural progenitors (Heins and others 2001). Both Pax6 and Olig2 are expressed within the adult SVZ progenitor domain (Hack and others 2005; Kohwi and others 2005). Olig2 expression has also been associated with amplification of gliogenic progenitors derived from ES cell differentiation (Xian and Gottlieb 2004). Interestingly, several of these transcription factors are known to cross-regulate and corepress one another (Schuurmans and Guillemot 2002). The combination of FGF-2 plus EGF may create a synthetic cell state with an appropriate balance of these key transcription factors to suppress lineage commitment and allow self-maintaining divisions, analogous to the ES cell state (Smith 2001).

Derivation of NS Cell Lines Requires FGF Together with EGF but Does Not Depend on Neurosphere Formation

As described above, NS cells have been established from adult and fetal tissues via an initial suspension culture/neurosphere step prior to plating down and expanding as adherent cell lines. This raises the question whether suspension culture may be a necessary event for formation of NS cells. Direct isolation and culture of neural progenitors in adherent conditions have been reported over many years by several investigators using FGF-2 for survival/expansion of the cells (Johe and others 1996). However, these cultures change their properties over time (Qian and others 2000). Such primary cultures should not be considered equivalent to expanded long-term stem cell lines.

Surprisingly, few studies have reported the application of EGF and FGF-2 in combination in adherent culture. Accordingly, we plated dissociated E12.5 cortex directly onto laminin in the presence of EGF and FGF-2 to determine whether NS cell lines could be established directly without intervening neurosphere formation. Cells readily attach in these conditions and rapidly acquire a homogenous morphology. In the presence of both growth factors, cells are uniformly immunoreactive for Olig2, RC2, BLBP, and nestin (Fig. 3). These cells behave similarly to previously characterized NS cell lines cultured on laminin. We have named a line of NS cells derived in this manner Cor2. Cor2 can be serially passaged with retention of ability to differentiate

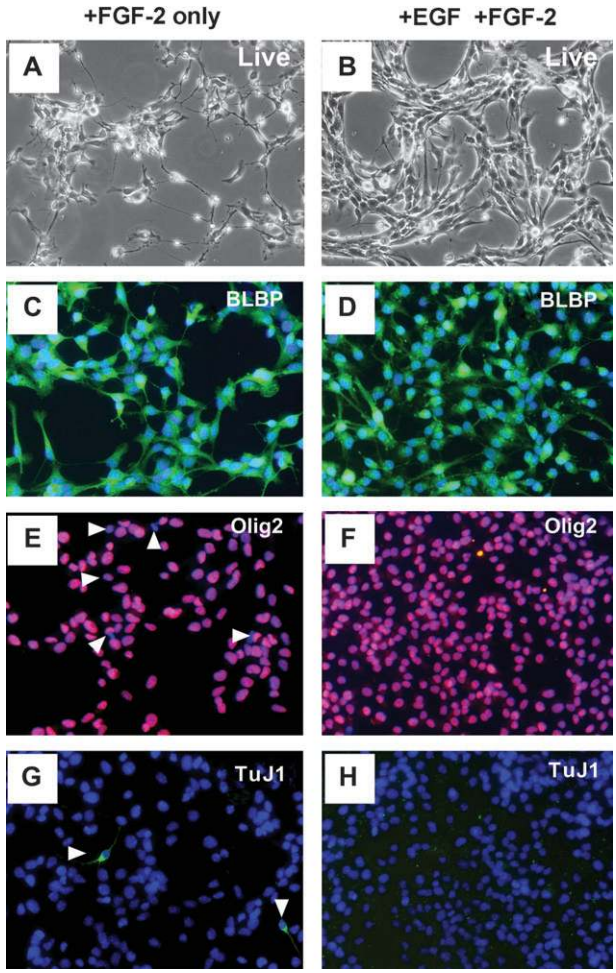

Figure 3. NS cell derivation requires both FGF and EGF but not neurosphere formation. E12.5 cortical progenitors were directly plated onto a laminin substrate without neurosphere formation. Cor2 was expanded in EGF and FGF-2 and resembles previously established NS cell lines by both morphology (*B*) and antibody staining (*D*, *F*, *H*). Cor2F was expanded in FGF-2 without EGF and has a more heterogeneous appearance (*A*). Olig2 and TuJ1 immunocytochemsitry also reveal heterogeneity and spontaneous neuronal differentiation for Cor2F (*E*, *G*, white arrowheads).

into neurons and astrocytes. We conclude that neurosphere formation is not necessary for derivation of fetal NS cells.

Previous studies have indicated that EGF alone is insufficient to generate embryonic neurospheres until late embryogenesis, whereas FGF-responsive cells are present earlier (Tropepe and others 1999). The distinction between early and late progenitor cells is thought to reside in levels of epidermal growth factor receptor (EGFR) that may relate to the developmental switch from a neurogenic to a gliogenic phase (Burrows and others 1997; Qian and others 2000; Sun and others 2005). We have been unable to isolate NS cells from ES cells using EGF or FGF-2 alone (Conti and others 2005). Here we tested whether either of these factors alone was sufficient to generate adherent NS cell lines from fetal cortex. Similar to previous studies, we found that EGF alone is not able to expand E12.5 progenitors and cultures rapidly die in suspension culture without attaching to the laminin substrate (not shown). In FGF-2 alone on a laminin substrate, we find that cells can be expanded through more than 10 passages and can be cryopreserved. We termed these cells Cor2F. Importantly, however, compared with cells expanded with EGF and FGF-2 in combination, FGF-2 cultures are more

heterogeneous by morphology; contain a proportion of immature neurons, identified by TuJ1 staining; divide more slowly; and at early passages, contain a relatively high incidence of dying cells (Fig. 3). It is possible that paracrine EGFR ligands can sustain stem cells in these conditions, but are in limiting supply.

Taken together, these results suggest that although FGF-2 is specifically required for initial derivation of NS cells, addition of EGF is important for expansion of homogenous NS cell cultures with efficient suppression of differentiation and apoptosis. FGF-2 may serve to induce EGFR expression in culture and/or to configure the epigenetic state of key regulatory loci such as *Sox* genes. There are data to support each of these possibilities; EGFR can be induced in culture by FGF-2 (Ciccolini and others 2005), and EGFR overexpression in early progenitors confers characteristics of late SVZ progenitors (Burrows and others 1997). Oligodendrocyte precursor cells can be converted to multipotent neurosphere-forming stem cells, and this is associated with modulation of chromatin at the *Sox2* locus leading to its reexpression (Kondo and Raff 2004). Interestingly, the initial requirement for FGF in setting up the NS cell state is reminiscent of its transient requirement in the reprogramming of primordial germ cells into pluripotent self-renewing embryonic germ cells (Matsui and others 1992; Resnick and others 1992).

EGF Alone Can Maintain NS Cell Self-Renewal

Established NS cells expanded in EGF/FGF-2 cannot be maintained in FGF-2 alone as removal of EGF from culture media results in massive cell death within 24 h (Conti and others 2005). Death can be avoided by provision of a laminin substrate, but FGF-2 is insufficient to maintain NS cells as passagable cell lines and cultures begin to undergo differentiation. This is more extreme than the situation described above for primary isolates in FGF-2 on laminin that can be continuously propagated, albeit with accompanying differentiation. The reason for this difference is unclear at present, but one possibility could be different levels of paracrine EGFR stimulation. The requirement for EGF-mediated signaling to maintain NS cell self-renewal may reflect endogenous neuregulin or transforming growth factor (TGF)α signals (Tropepe and others 1997; Schmid and others 2003; Ever and Gaiano 2005).

We investigated whether the converse was true, namely, can established NS cell lines be maintained with EGF alone upon withdrawal of FGF-2? We cultured ES cell–derived (CGR8-NS, NS5) and fetal-derived (Cor1, Cor1-3) NS cells in EGF alone. NS5 and Cor1-3 are clonally expanded cell lines. Surprisingly, withdrawal of FGF-2 did not result in any striking change in NS cell morphology or behavior (Fig. 4). NS cells withdrawn from FGF-2 maintain their homogenous morphology and marker staining, and differentiation to GFAP-expressing astrocytes or β-tubulin–expressing neurons remains fully suppressed (Fig. 4*J,K*). EGF-only propagated cells retain the capacity to generate astrocytes and neurons at a frequency similar to NS cells expanded in parallel in EGF plus FGF-2. Furthermore, we found that NS cells plated at clonal density formed colonies with similar frequency in EGF alone as in EGF plus FGF-2 (Fig. 4*G*).

The frequency of NS cell colony formation in either condition is around 1%. This low efficiency raises the possibility that there is a hierarchical organization within NS cell cultures, belying their homogeneity in marker expression. However, all colonies examined expand continuously similar to parental cells. Thus, there is no evidence for progenitors with finite proliferative lifespans, for example, as are observed in keratinocyte cultures (Barrandon and Green 1987). It seems more likely therefore that low colony-forming frequency is the result of culture stress at low density and/or dependence on autocrine stimulation rather than arising from heterogeneity of potency within the population.

In order to evaluate a potential role for autocrine FGF signaling in NS cell self-renewal, we employed a pharmacological inhibitor of FGFR (SU5402) (Mohammadi and others 1997). Addition of this inhibitor at a concentration (5 μM) effective at blocking FGFR signaling in other assays (Ying and others 2003) did not impair frequency of colony formation, although colonies were slightly smaller. SU5402 does impact upon cell population doubling time. EGF-only cultures show a lag phase in expansion after replating compared with parallel EGF plus FGF-2 cultures. BrdU pulse labeling indicates that the proportion of cells in S phase is similar in each condition, suggesting that the increased population doubling time may be due to higher cell death. In the presence of SU5402, the slower doubling rate of EGF-only cultures does not increase with cell density (Fig. 3*E–H*). These data suggest that autocrine FGF signaling can contribute to NS cell propagation. Therefore, more detailed analyses are required to determine whether there is an absolute requirement for FGF.

Are NS Cell Lines Equivalent to Forebrain Radial Glia?

A critical issue to consider when characterizing any stem/progenitor cell source in vitro is whether these cells have an in vivo counterpart and which cell type is the cell of origin (Doetsch and others 2002; Buehr and others 2003; Joseph and Morrison 2005). NS cells isolated from all sources express markers of radial glia (RC2, GLAST, BLBP, vimentin). Radial glia emerge during mouse development from Sox1-expressing neuroepithelial cells. In vivo fate-mapping studies along with characterization of purified radial glia in vitro have revealed a function for these cells not only as scaffold/guides for migrating neuroblasts (Rakic 1971) but also as proliferative founders of neuronal and, later, astrocyte, oligodendrocyte, and ependymal cell types (Alvarez-Buylla and others 2001; Gotz 2003; Merkle and others 2004).

In vivo, radial glia are heterogeneous in terms of their transcription factor expression profile, likely associated with elaboration of positional cues (Kriegstein and Gotz 2003). However, we found that NS cell lines, derived from either ES cells or fetal forebrain (cortex or striatum), express Pax6 and Emx2, although being negative for ventral markers such as Lhx6 (Schuurmans and Guillemot 2002). Pax6 and Emx2 within cortical radial glia are present in forebrain where they are suggested to function in the maintenance of neuronal potential and symmetrical self-renewal, respectively (Heins and others 2001, 2002). An unresolved issue is whether radial glia from regions other than forebrain, such as midbrain and spinal cord, can give rise to NS lines and if so whether they retain any positional specification. Reports using neurospheres and adherent neural progenitor cultures have suggested a respecification of positional markers when cells are exposed to growth factors in vitro (Gabay and others 2003; Santa-Olalla and others 2003; Hack and others 2004).

So are NS cells equivalent to fetal cortical radial glia? Expression of Pax6 and Emx2 by NS cells is consistent with a dorsal forebrain identity, but Mash-1 and Olig2 are found ventrally within the developing forebrain. There may exist within the developing CNS a rare cell population that coexpresses all

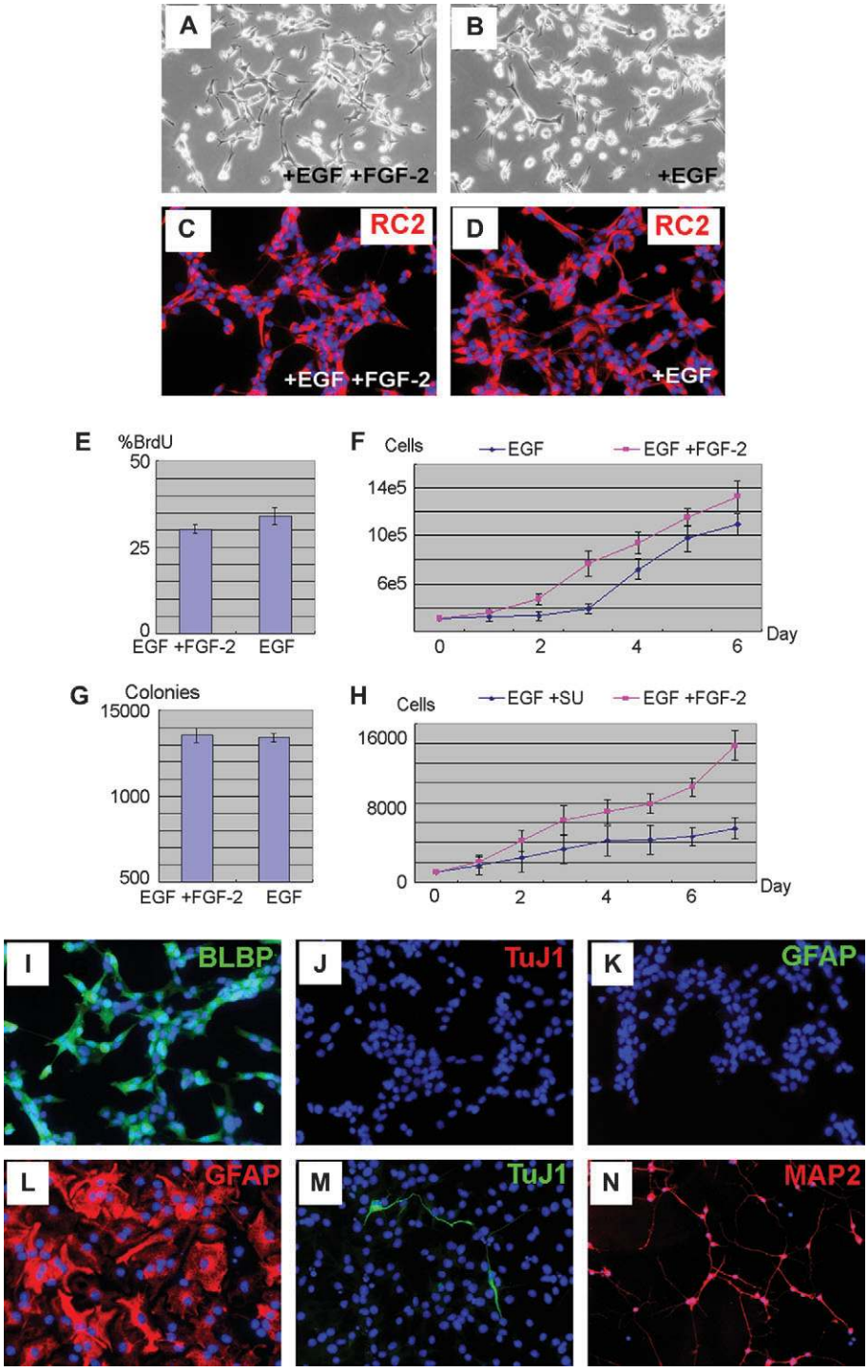

Figure 4. Addition of EGF is sufficient to maintain NS cell self-renewal. CGR8-NS cells (passage 19) were expanded for at least a further 5 passages with EGF alone. Compared with parallel cultures expanded with EGF plus FGF-2, we find no dramatic change in cell morphology (*A*, *B*) or radial glia marker RC2 (*C*, *D*). BrdU incorporation assays (*E*). Growth curves for CGR8-NS cells in EGF compared with EGF/FGF-2 (*F*) and in the presence of FGFR inhibitor (SU5402, 5 μM) (*H*). NS colony formation; 10 000 cells were plated and colonies scored after 7–10 days in culture (*G*). CGR8-NS cells expanded in EGF alone retain homogenous BLBP expression (*I*) but lack spontaneous differentiation to neurons (*J*) or astrocytes (*K*). Differentiation to astrocytes can occur to either astrocytes (upon serum treatment, *L*) or neurons (upon EGF withdrawal, *M*; or FGF-2 exposure and subsequent withdrawal, *N*).

these transcription factors. However, it is equally likely that this situation might reflect a despecification of progenitors when exposed to in vitro conditions. Recent studies have described the activation of Olig2 in culture through the action of FGF-2 and a relaxation of in vivo fate restriction (Chandran and others 2003; Gabay and others 2003; Hack and others 2004). By real-time quantitative RT–PCR, we find that Olig2 is upregulated in NS cells in the presence of FGF-2. Levels of Olig2 mRNA are around 5-fold higher in NS cells grown with EGF and FGF-2 compared with EGF alone in 3 different NS cell lines (not shown). These results highlight the artificial nature of cell culture, emphasizing the need for caution in extrapolation of in

vitro findings to normal development or physiology without parallel in vivo data.

Appreciation of the positional identity present within NS cell lines is invaluable for designing strategies to generate the desired neuronal subtypes. For cell-based pharmaceutical screening or transplantation to be viable, the critical factor is access to an unlimited stem cell resource with the potency to generate a range of cell types of the mature CNS. A range of neuronal subtypes can be generated through differentiation of ES cells, using opportunistic protocols or conditions designed to mimic developmental inductive events (Kim and others 2002; Wichterle and others 2002; Barberi and others 2003). NS cells and neurospheres generate largely γ-aminobutyric acidergic neurons, whereas ES cell–derived radial glia precursors generate glutamatergic neurons if they are not exposed to EGF/FGF-2 (Bibel and others 2004). EGF/FGF-2 may act to induce dominant ventral specification factors, such as Mash-1, that determine neuronal phenotype. For NS cells, it remains to be seen if environmental signals may promote diverse neuronal subtype differentiation. Genetic fate-mapping studies of radial glia suggest that these cells serve as founders for a range of neuronal subtypes within the nervous system, although this remains controversial (Anthony and others 2004; Gotz and Barde 2005). Therefore, a key question is whether the NS cell positional values established in vitro through the action of EGF and FGF-2 can be overridden by other extrinsic signals. Alternatively, do mitogens exist that allow capture and expansion of NS cells similar to NS cells but with distinct regional specification reflected in different repertoires of key transcriptional regulators?

Conclusions

Here and elsewhere, we have reported the ability to isolate and expand adherent NS cell lines from ES cells, fetal forebrain, and adult forebrain (Fig. 1) (Conti and others 2005). NS cells are clonogenic, apparently homogenous, symmetrically expandable, adherent cultures. They thus constitute a pure stem cell resource with experimental advantages comparable with ES cells. Derivation of these NS cell lines requires FGF-2. However, once established, NS cell self-renewal can be sustained by exogenous EGF alone (Fig. 5). NS cells may be related to brain cancer stem cells (Singh and others 2004), notably those of aggressive gliomas resulting from activation of EGFR. Identification of functional regulators of NS cell self-renewal might therefore lead to novel therapeutic strategies to target cancer stem cells. Further investigations will determine whether the characteristics of NS cells represent a unique cell state acquired in vitro or mirror features of rare endogenous CNS progenitor cells.

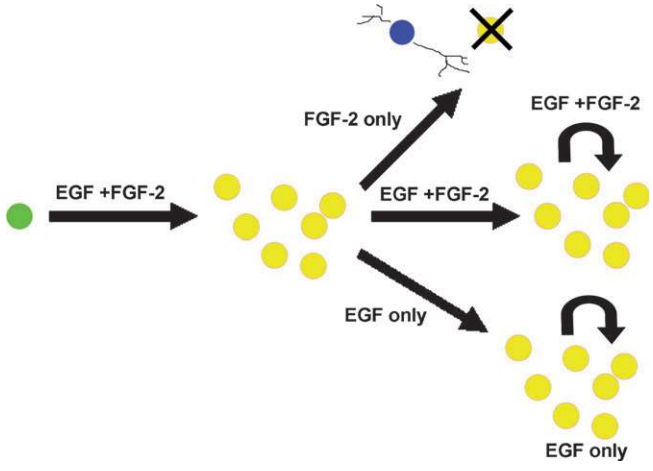

Figure 5. Roles of EGF and FGF-2 in the derivation and maintenance of NS cells. Fetal forebrain progenitors or ES cell–derived neural progenitors (left, green) can be converted into NS cell lines (yellow) using a combination of EGF with FGF-2. Once established, NS cells can be maintained in added EGF alone, whereas in FGF-2 alone, they undergo differentiation (blue) and apoptosis.

Notes

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