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In Vitro Expansion of a Multipotent Population of Human Neural Progenitor Cells

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The isolation and expansion of human neural progenitor cells have important potential clinical applications, because these cells may be used as graft material in cell therapies to regenerate tissue and/or function in patients with central nervous system (CNS) disorders. This paper describes a continuously dividing multipotent population of progenitor cells in the human embryonic forebrain that can be propagated *in vitro.* These cells can be maintained and expanded using a serum-free defined medium containing basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), and epidermal growth factor (EGF). Using these three factors, the cell cultures expand and remain multipotent for at least 1 year *in vitro.* This period of expansion results in a 10^7-fold increase of this heterogeneous population of cells. Upon differentiation, they form neurons, astrocytes, and oligodendrocytes, the three main phenotypes in the CNS. Moreover, GABA-immunoreactive and tyrosine hydroxylase-immunoreactive neurons can be identified. These results demonstrate the feasibility of long-term *in vitro* expansion of human neural progenitor cells. The advantages of such a population of neural precursors for allogeneic transplantation include the ability to provide an expandable, well-characterized, defined cell source which can form specific neuronal or glial subtypes.

Key Words: **stem cell; development; embryonic; cell culture; progenitor; human; central nervous system.**

INTRODUCTION

Historically, the adult mammalian central nervous system has been considered incapable of regeneration. This conclusion was based, in part, on evidence that neurogenesis is complete shortly after birth (reviewed in 34). However, more recent data indicate that limited neurogenesis occurs in isolated regions of the normal adult nervous system. For instance, neuronal progenitors in the rat dentate gyrus have been found to proliferate in both late postnatal development and in the adult (1, 2, 4). A population of proliferating neural progenitor cells has also been identified in the subependyma of the adult rodent (13, 16, 20, 21). While these findings suggested that specialized populations of cells are able to proliferate and differentiate into neurons throughout an animal's lifetime, proof of a multipotent stem cell in the mammalian CNS remained elusive.

More recently, several laboratories have isolated and expanded rodent progenitor cells in the presence of EGF and/or bFGF (22, 26–28, 31). In these culture systems, removal of mitogens and the addition of serum will result in the differentiation of the progenitor cells into neurons and glia. In 1992 Reynolds and Weiss identified a population of cells in the mouse embryonic and adult CNS that could be cultured under defined conditions and could be induced to differentiate into neurons, astrocytes, and oligodendrocytes, the three main phenotypes in the CNS (26–28). These multipotent cells are maintained as nonadherent clusters, in the presence of the mitogen epidermal growth factor (EGF). These clusters can be expanded in culture for extended periods of time (7, 26, 27), and clonal analysis demonstrates their ability to self-renew and to differentiate into the major neural phenotypes (27), suggesting that these cells represent a neural stem cell population. In other work, a population of neural stem cells has been generated from the adult rat hippocampus. These cells are maintained as an adherent population in the presence of bFGF (22, 25). Although both of the cell populations expand *in vitro* and are multipotent, it is unclear whether these cells are equivalent populations. These mitogen-driven cell culture systems provide a means to exponentially expand a multipotent popula-

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tion of cells and may provide an ideal source of cells for CNS transplantation. However, while rodent neural stem/progenitor cells have been identified in these systems, attempts to expand human neural progenitor cells have not been successful. Other groups have generated human cell lines by derivation from a human teratocarcinoma (33) or by utilizing *v-myc* to drive cell proliferation (29), but these cells do not have the potential to produce the three major neural cell types.

In the experiments presented here, continuously proliferating neural progenitor cell cultures were established from first-trimester human embryonic forebrain. These cultures were maintained in the presence of human basic fibroblast growth factor (hbFGF), human leukemia inhibitory factor (hLIF), and human epidermal growth factor (hEGF). These cultures represent a heterogeneous population of cells which expand exponentially and, upon differentiation, have the potential to form the three major phenotypes in the CNS: neurons, astrocytes, and oligodendrocytes. Under certain differentiation protocols, specific neuronal subtypes can be induced, including cells immunoreactive for tyrosine hydroxylase (TH) and gamma-amino-butyric acid (GABA).

METHODS

Isolation of Tissue

Embryonic brain tissue from 5.0 to 11.0 weeks of gestation was acquired under compliance with NIH guidelines, Swedish government guidelines, and the local ethics committee and appropriate consent forms were used. Samples were collected and the forebrain was dissected and placed into sterile saline. The tissue was transferred to growth medium (see below) and was mechanically dissociated using a standard glass homogenizer. The dissociated cells were seeded into growth medium containing hEGF and hbFGF (see below) at approximately 100,000 cells/ml.

Cell Culture

Cell suspensions were grown in N2, a defined DMEM: F12-based (GIBCO) medium supplemented with 0.6% glucose, 25μg/ml human insulin, 100μg/ml human transferrin, 20 nM progesterone, 60μM putrescine dihydrochloride, 30 nM sodium selenite, 2 mM glutamine, 3 mM sodium bicarbonate, 5 mM Hepes, and 2μg/ml heparin (Sigma). This medium was supplemented with 20 ng/ml hEGF (GIBCO), 20 ng/ml hbFGF (GIBCO), and 10 ng/ml hLIF (R & D Systems). Typically, the cells grew as clusters which were passaged by mechanical dissociation approximately once every 7–30 days (depending on the mitogens used) and reseeded at approximately 75,000–100,000 cells/ml. Cell lines generated in this fashion were designated by gestational age and anatomical region, e.g., forebrain from 5 weeks of gestation was designated 5FBr.

In initial experiments, the progenitor cells were differentiated by plating on poly-ornithine-coated glass coverslips. To induce differentiation, growth factors were removed from the growth medium and the medium was supplemented with 1% FBS (GIBCO). Cells were cultured for 12–14 days before fixation for immunocytochemistry.

In some experiments, cells were differentiated in the presence of cytokines. In these experiments cells were plated onto poly-ornithine-coated coverslips at 50,000 cells/coverslip in N2 supplemented with either 400 pg/ml IL-1b (R & D Systems) or a cocktail of IL-1b (200 pg/ml), IL-11 (1 ng/ml) (R & D Systems), and GDNF (1 ng/ml) (Promega). The cells were allowed to differentiate for 20 days before fixation and immunostaining. Cultures were fed three times each week with fresh media and cytokines.

Immunocytochemistry

The cell cultures were fixed for 10–20 min at room temperature with 4% paraformaldehyde in PBS, washed three times in 0.1 M PBS, pH 7.4, permeabilized using a 2-min incubation in 100% EtOH, and washed again with 0.1 M PBS. Cultures were then incubated in 5% NGS (normal goat serum) in 0.1 M PBS with 0.1% Triton X-100 (Sigma) for at least 1 h at room temperature. Blocking was followed by incubation in primary antibodies diluted in 1% NGS + 0.1% Triton X-100 for at least 2 h at room temperature. The cultures were washed in PBS and incubated with secondary antibodies diluted in 1% NGS with 1% Triton X-100 for 30 min at room temperature in the dark.

The primary antibodies used were human specific nestin, 1:100 (generously provided by U. Lendahl, Karolinska Institute); β-tubulin, 1:1000 (Sigma); glial fibrillary acidic protein (GFAP), 1:500 (DAKO); GABA 1:200 (Sigma); BrdU, 1:1000 (Amersham); galactocerebroside (GalC), 1:25 (Boehringer); and TH, 1:200 (Chemicon). The secondary antibodies used in these experiments were goat anti-mouse FITC, 1:128 (Sigma), and goat anti-rabbit TRITC, 1:100 (Cappel).

Quantitation of the different phenotypes was accomplished by counting immunolabeled cells on each coverslip. For each condition, three to six coverslips were evaluated. Four separate randomly chosen fields were counted on each coverslip using a 40× objective. The number of nuclei was counted using DAPI staining and then the number of β-tubulin-, GFAP-, or GABA-immunoreactive (ir) cells in each field was counted. These numbers were then summed to give a total representative count for each coverslip. A percentage for each phenotype was generated and these numbers were used to generate mean values for each condition.

Statistical analyses included Student's *t* test using StatView software.

RESULTS

Generation of Continuously Proliferating Human Progenitor Cell Cultures

In this paper, the term "cell line" refers to a cell line which is a continuously proliferating population of cells derived from a single embryonic source, where proliferation is induced by mitogens, rather than by oncogenes or transformation. Cell lines (cultures) were derived from nine samples of forebrain tissue from human embryos at 5, 6.5, 7, 8.5 (two separate samples), 9, 9.5 (two separate samples), and 10.5 weeks of gestation. Each cell line was designated by gestational age and anatomical region of the tissue of origin, i.e., "5FBr" is the cell line obtained from embryonic forebrain at 5 weeks of gestation. Two parallel cultures—with and without hLIF—were generated from each of these samples, for a total of 18 cell lines. One of these cell lines (5FBr without hLIF) failed at passage 2, but all other cultures continued to expand, some for as many as 35 passages, and were able to differentiate into neurons, astrocytes, and oligodendrocytes.

Cells derived from the dissociation of embryonic forebrain samples (described under Methods) were suspended in growth medium. In all cultures, many single cells attached to the flasks; however, as the cells proliferated into small clusters, they detached from the plastic and floated in suspension. The clusters continued to increase in size during a period of 7–30 days, depending on growth conditions (see below). After clusters formed, the cells were passaged by mechanical dissociation into a single cell suspension. To date, the cells have been reseeded and carried continuously for up to 17–35 passages (175–371 days), and the cell lines remain viable. Figure 1A illustrates representative clusters from cell line 9FBr at passage 6 (all cell lines appeared similar in their undifferentiated state). The spheres showed positive immunoreactivity to the neuroepithelial stem cell marker, nestin (17) (Fig. 1B), and incorporated bromodeoxyuridine (BrdU) (Fig. 1C), characteristic of actively proliferating cell populations. To date, no consistent difference in growth rate between cultures generated from tissues of different gestational age while maintained in hEGF, hbFGF, and hLIF have been observed (Fig. 1D).

Expansion: Effect of hLIF

In addition to the cultures maintained in hEGF, hbFGF, and hLIF, parallel cultures were generated by placing some of the cells into medium containing only hEGF and hbFGF. Initially, cell lines maintained in hEGF and hbFGF expanded at a rate similar to those cultured with hEGF, hbFGF, and hLIF. However, after about 50–60 days *in vitro*, the cultures without hLIF consistently showed slower expansion; conversely, the cultures that contained all three growth factors continued to expand and their growth rate appeared to increase, the population doubling every 7–10 days (Figs. 2A and 2B). To determine if hLIF would affect cultures which had been continuously maintained in only hEGF and hbFGF, hLIF was added to the $9FBr_{021997}$ cell line which had been maintained only in hEGF and hbFGF for 10 passages. The expansion of these cells was monitored for six more passages. The addition of hLIF resulted in an increase in the proliferation rate of this cell line (Fig. 3A) indicating that at least some cell lines remain responsive to LIF even when maintained in the absence of this cytokine.

LIF is a member of the IL-6 cytokine family which also includes oncostatin M, IL-11, ciliary neurotrophic factor (CNTF), and Flt3/Flk2. Other members of this cytokine family were tested to determine if this growth effect was unique to LIF. Preliminary experiments indicate that cells grown in hEGF, hbFGF, and hCNTF (10 ng/ml) show a proliferation rate similar to cells grown in hEGF, hbFGF, and hLIF. However, if hLIF is replaced with hFlt3/Flk2 (10 ng/ml) or IL-6 (10 ng/ml) the growth rate is reduced (data not shown).

Expansion: Effect of bFGF

To determine if hbFGF was essential for cell proliferation, hbFGF was removed from the $9FBr_{021997}$ and $5FBr_{072397}$ cell lines, which had been previously maintained in hEGF, hbFGF, and hLIF (as described above), and the growth rates of cells under the two culture conditions were compared. Proliferation of both cell lines declined immediately upon removal of hbFGF, although cells remained viable in the absence of this growth factor. The results for the $9FBr_{021997}$ cell line are shown in Fig. 3B. The $9FBr_{021997}$ cell line has been maintained in the absence of hbFGF for as many as 22 passages (230 days), and multipotency has been evaluated (see below). These results indicate that hbFGF, similar to hLIF, appears to promote proliferation of these cells.

Expansion: Effect of EGF

To assess whether hEGF was necessary for proliferation of the human cells, hEGF was removed from the $5FBr_{072397}$ and $8.5FBr_{032598}$ cell lines. As shown in Fig. 3C, the initial growth rates of both cell lines did not appear to be affected by the removal of hEGF. It is unlikely that this is due to the absence of the EGF receptor because RT-PCR data for these cell lines showed the presence of mRNA for the human EGF receptor (data not shown).

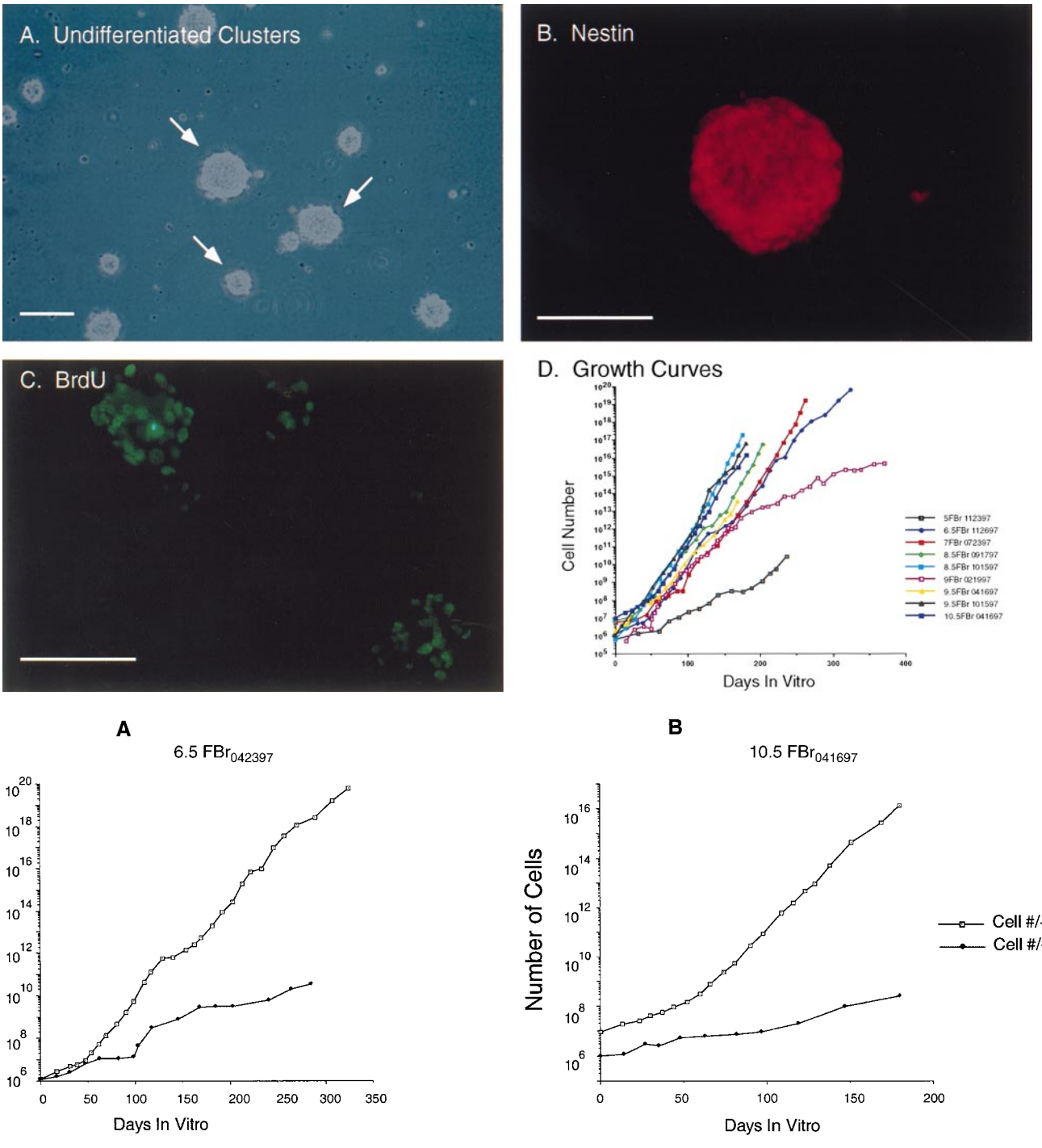
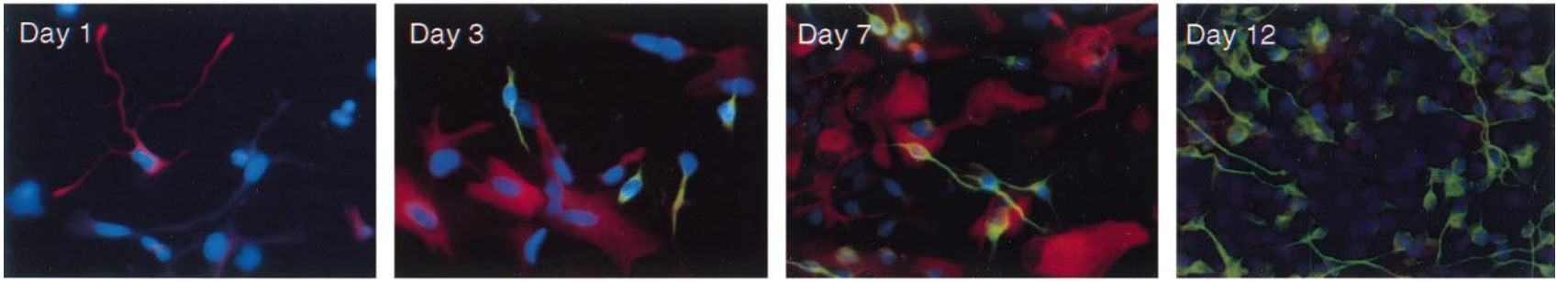


FIG. 1. Human neural progenitor cells. (A) Phase-contrast photograph of undifferentiated human neural progenitor cells ($9FBr_{021997}$) after six passages. Arrows indicate clusters of cells in suspension. (B) Nestin immunoreactivity in undifferentiated neural progenitor cell clusters. Because these cells are very tightly packed, the nestin immunoreactivity does not appear filamentous. In all experiments control cultures were included which received only the secondary antibody. These controls did not show any positive staining. (C) BrdU incorporation in undifferentiated neural progenitor cell clusters. Scale bars in all images represent 100μm. (D) Growth rates for nine "cell lines" maintained in hEGF, hbFGF, and hLIF.

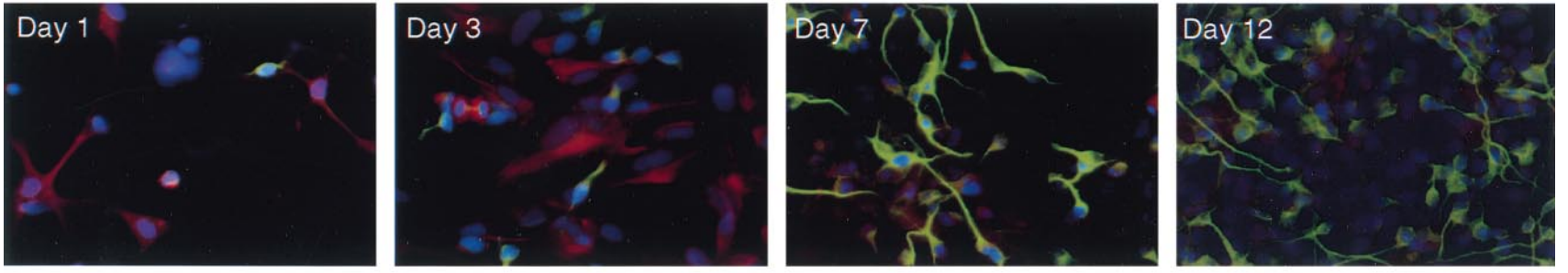
FIG. 2. Growth rates of human neural progenitor cells. Parallel cultures were maintained in hEGF and hbFGF or in hEGF, hbFGF, and hLIF. Cultures were generated from forebrain at gestational weeks 6.5 and 10.5. Cell lines maintained in the absence of hLIF showed slower proliferation rates than cell lines maintained in hLIF.

FIG. 4. Characterization of human progenitor cells during differentiation *in vitro*. Cells were plated on day 0 and were fixed and stained on days 1, 3, 5, 7, and 12. (A) β-Tubulin and GFAP expression increased with time, until about 1 week *in vitro*. In these images, fluorescein represents β-tubulin-ir and Texas red represents nestin-ir. (B) Nestin expression decreased with time *in vitro*. In these images, fluorescein represents β-tubulin-ir and Texas red represents nestin-ir. (C) Quantification of the expression of β-tubulin, GFAP, and nestin indicates that while nestin expression decreases over time, both β-tubulin and GFAP expression increase.

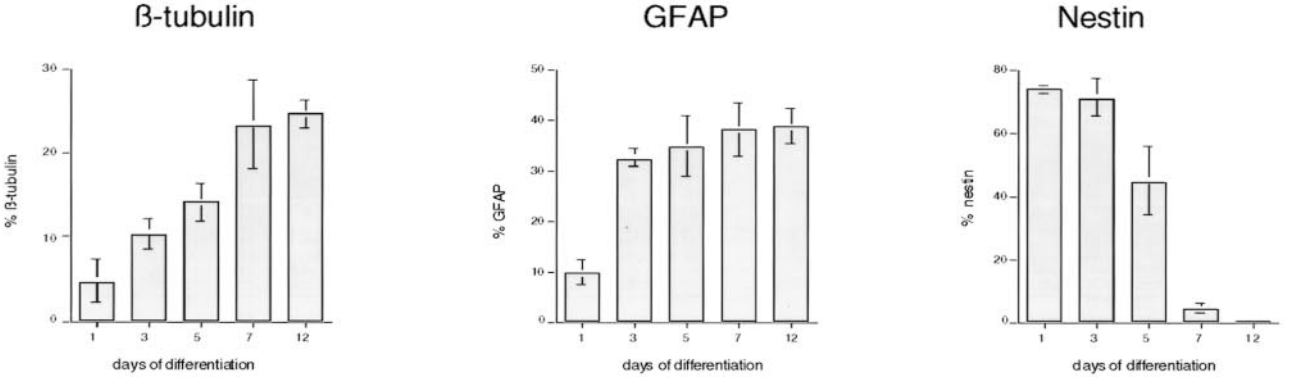
A. Expression of ß-tubulin and GFAP



B. Expression of ß-tubulin and nestin



C. Expression of Phenotypic Markers During Differentiation



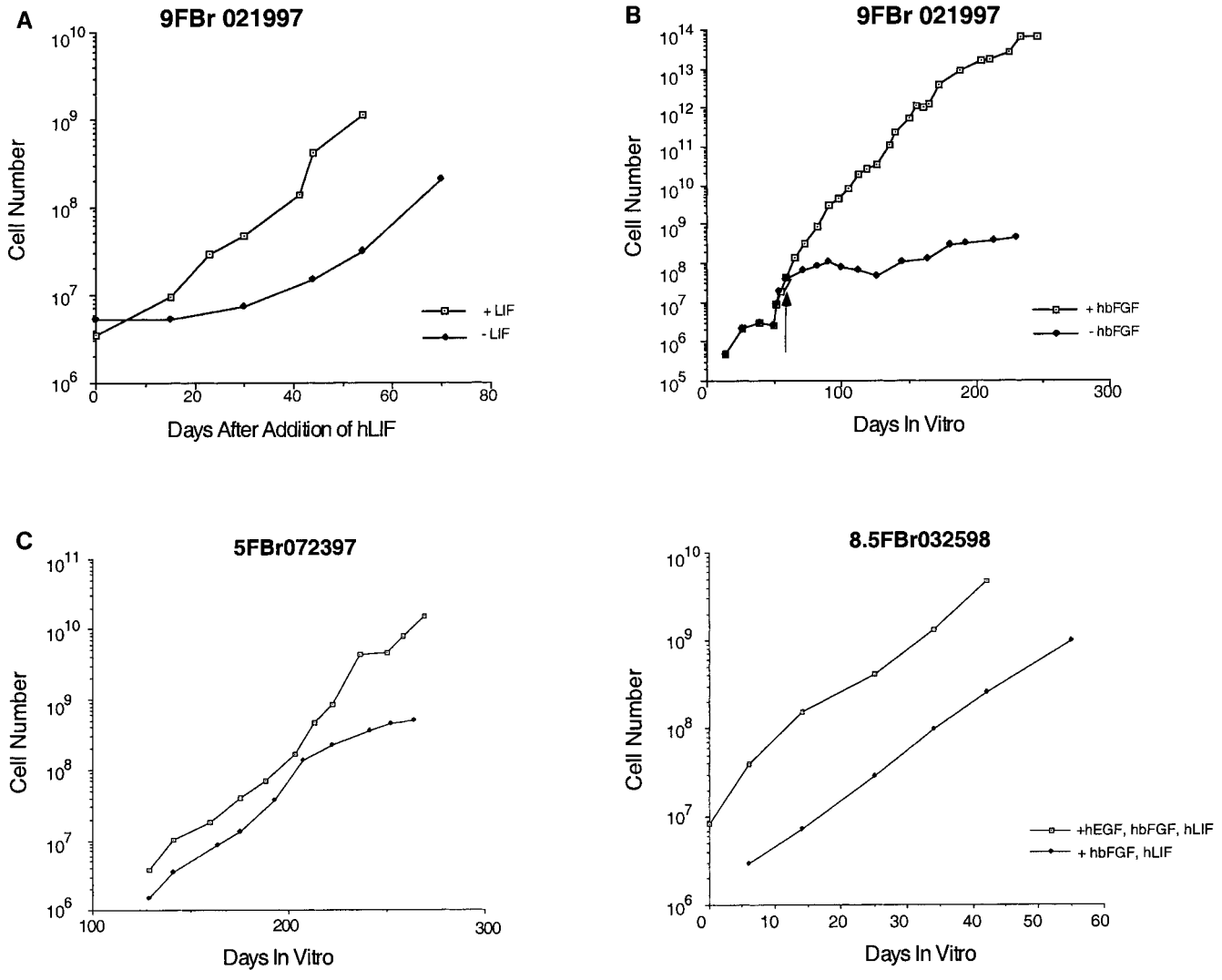


FIG. 3. The effects of hLIF, hbFGF, and hEGF on growth rates. (A) The $9FBr_{021997}$ cell line was maintained in hEGF and hbFGF for 10 passages before hLIF was added. In this graph, day 0 represents the initial addition of hLIF at P10. The addition of hLIF resulted in an immediate increase in growth rate. (B) The removal of hbFGF from the 9FBr cell line resulted in an immediate decrease in growth rate. The removal of hbFGF is indicated by the arrow. (C) Two cell lines ($5FBr_{072397}$ and $8.5FBr_{032598}$) were maintained in the absence of hEGF for 5–20 passages. No difference was seen in the growth rate of the cultures in which EGF was removed.

To date, human progenitor cell lines have been carried for as long as 35 passages in hLIF ($9FBr_{021997}$ for 371 days) and for 22 passages without hLIF ($9FBr_{021997}$ for 211 days). All cell lines continue to expand and form spheres.

Differentiation: General Observations

Unless otherwise specified, the cells were differentiated by plating onto poly-ornithine-coated glass coverslips in N2 medium supplemented with 1% FBS. Under these conditions, the cells immediately adhered to the substrate and began to differentiate, and after several days *in vitro,* different morphologies were apparent, including large flat cells and small bipolar and multipolar cells. Within 7 to 10 days the cells had proliferated to create a monolayer. Three cell lines were evaluated on days 1, 3, 5, 7, and 12 for expression of β-tubulin, GFAP, and nestin. All three displayed a similar capacity to differentiate and representative data from one of these cell lines are presented in Fig. 4. On day 1 of differentiation only a few cells were β-tubulin-immunoreactive (β-tubulin-ir) or GFAP-immunoreactive (GFAP-ir) (Figs. 4A and 4C). The percentage of β-tubulin-ir and GFAP-ir cells increased over time and seemed to reach a plateau at about 7 days (Fig. 4C). In contrast, the expression of nestin decreased over time after induction of differentiation (Figs. 4B and 4C). On day 1, about 75% of the cells were nestin-ir which declined to less than 5% within 1 week.

Cell lines generated from different gestational ages

were allowed to differentiate for 12–14 days and were evaluated by immunocytochemistry for expression of specific phenotypic markers. As seen in Figs. 5A and 5B the cells expressed GFAP, GalC and β-tubulin III, indicating the presence of glia and neurons. Each cell line showed different percentages of neurons and astrocytes. The percentage of GFAP-ir cells ranged between 15 and 53% in different cell lines. Morphologically, some GFAP-positive cells were large and flat, resembling type 1 astrocytes (Fig. 5A); others exhibited a more stellate morphology, similar to that of type 2 astrocytes (data not shown). Neither the variation in numbers nor the morphology appeared to correlate with the gestational age of the tissue used to generate the cell line. Evaluation of GFAP-ir cells was difficult in some cases where the cells became so confluent that cell boundaries were often obscured, creating an obstacle to accurate cell counting. These cases were therefore excluded from cell counts and data analysis.

The number of neurons was assessed by counting the cells that demonstrated immunoreactivity to β-tubulin isotype III, a marker for neurons *in situ* and *in vitro* (3, 5, 9, 11). All of the cell lines generated neurons; however, different cell lines generated different percentages (20–37%) of β-tubulin III-positive neurons (Table 1). Many neurons showed elaborate arborizations, but markers for more mature neurons (such as neurofilament or NSE) were not evaluated in these studies. However, these cells demonstrated positive immunoreactivity to human neurofilament antibodies after transplantation into the adult rat brain, indicating their capacity to form mature neurons (12). In all cell lines assessed *in vitro,* a number of neurons showed immunoreactivity to GABA (GABA-ir) (Figs. 5C and 5D) which colocalized with β-tubulin III (Fig. 5D). The number of GABA-ergic neurons varied (9–51% of neurons) between cell lines generated from different gestational ages, but no developmental trends were seen in these data (Table 1).

Using an induction protocol recently described by Ling *et al.* (1998), immunoreactivity to TH was observed (Fig. 5F). This protocol uses a combination of IL-1b (200 pg/ml), IL-11(1 ng/ml), and GDNF (1 ng/ml) to induce the formation of TH-ir cells. In the experiments presented here, cells differentiated in this cocktail of factors or differentiated in IL-1b (400 pg/ml) alone formed TH-ir cells after 20 days *in vitro.* At this time, small patches of cells were identified which were TH-ir. In addition, these cells colocalized β-tubulin, indicating neuronal expression of TH. These data were supported by positive RT-PCR results indicating the presence of mRNA for human TH (data not shown). Because these cells were found in patches, it was difficult to accurately determine the percentage of the population which became TH-ir. This finding is currently being further characterized.

Differentiation: Effect of hLIF, hbFGF, and hEGF

As described above, cell lines maintained with and without hLIF exhibited very different growth rates. The multipotency of these parallel cultures was also investigated. Four cell lines ($6.5FBr_{042397}$, $7\ FBr_{072397}$, $8.5FBr_{091797}$, and $9FBr_{021997}$) were induced to differentiate following the standard differentiation protocol described above, and the number of neurons formed was assessed. Cells cultured in hLIF consistently produced significantly more neurons than cells cultured without hLIF ($P < 0.001$ and $P < 0.0001$) (Fig. 6A). Furthermore, qualitatively, the neurons generated in the cultures carried in hLIF before differentiation appeared to have more elaborate processes (data not shown).

Cells maintained in the presence of hbFGF produced significantly more neurons than parallel cultures maintained without hbFGF. As described previously, hbFGF was removed from the $9FBr_{021997}$ cell line, resulting in a decrease in proliferation. As shown in Fig. 6B, the number of neurons formed upon differentiation was dramatically reduced after hbFGF removal ($P < 0.05$). In addition, the neurons which were observed had short processes resembling immature neurons (Fig. 5E).

The presence or absence of hEGF did not appear to affect the differentiation of neurons or astrocytes. hEGF was removed from the $5FBr_{072397}$ and $8.5FBr_{032598}$ cell lines for five passages. In the $5FBr_{072397}$ cell line, hEGF was removed at passage 10 and the cells were evaluated for multipotency at one and five passages after this change. At both time points, the percentage of neurons remained similar ($5FBr_{072397}$ shown in Fig. 6C). The percentage of astrocytes appeared to drop after five passages without hEGF. hEGF was also removed from the $8.5FBr_{032598}$ cell line at passage 1 and multipotency was evaluated at passage 5. In this cell line, differentiation into neurons and astrocytes did not seem to be affected by the removal of hEGF.

Differentiation: Effect of Long-Term Culture

Differentiation of three of the cell lines ($6.5FBr_{042397}$ + LIF, $9FBr_{021997}$ + LIF, and $10.5FBr_{041697}$ + LIF) was characterized at various passages (Fig. 7). As mentioned previously, in some cases it was difficult to assess the number of GFAP-ir cells because accurate cell counts could not be obtained at some time points when the cells tended to grow in a monolayer. In cell line $9FBr_{021997}$ the percentage of cells expressing β-tubulin III remained stable (about 20%) until passage 21, and then it decreased by passage 30 to 14% (Fig. 7A). In the other two cell lines, the number of neurons generated decreased with passaging: in line $6.5FBr_{042397}$ they decreased from 37% at passage 5 to 13% at passage 20 (Fig. 7B); and in line $10.5FBr_{041697}$ they decreased from 29% at passage 5 to 12% at passage 20 (Fig. 7C). These

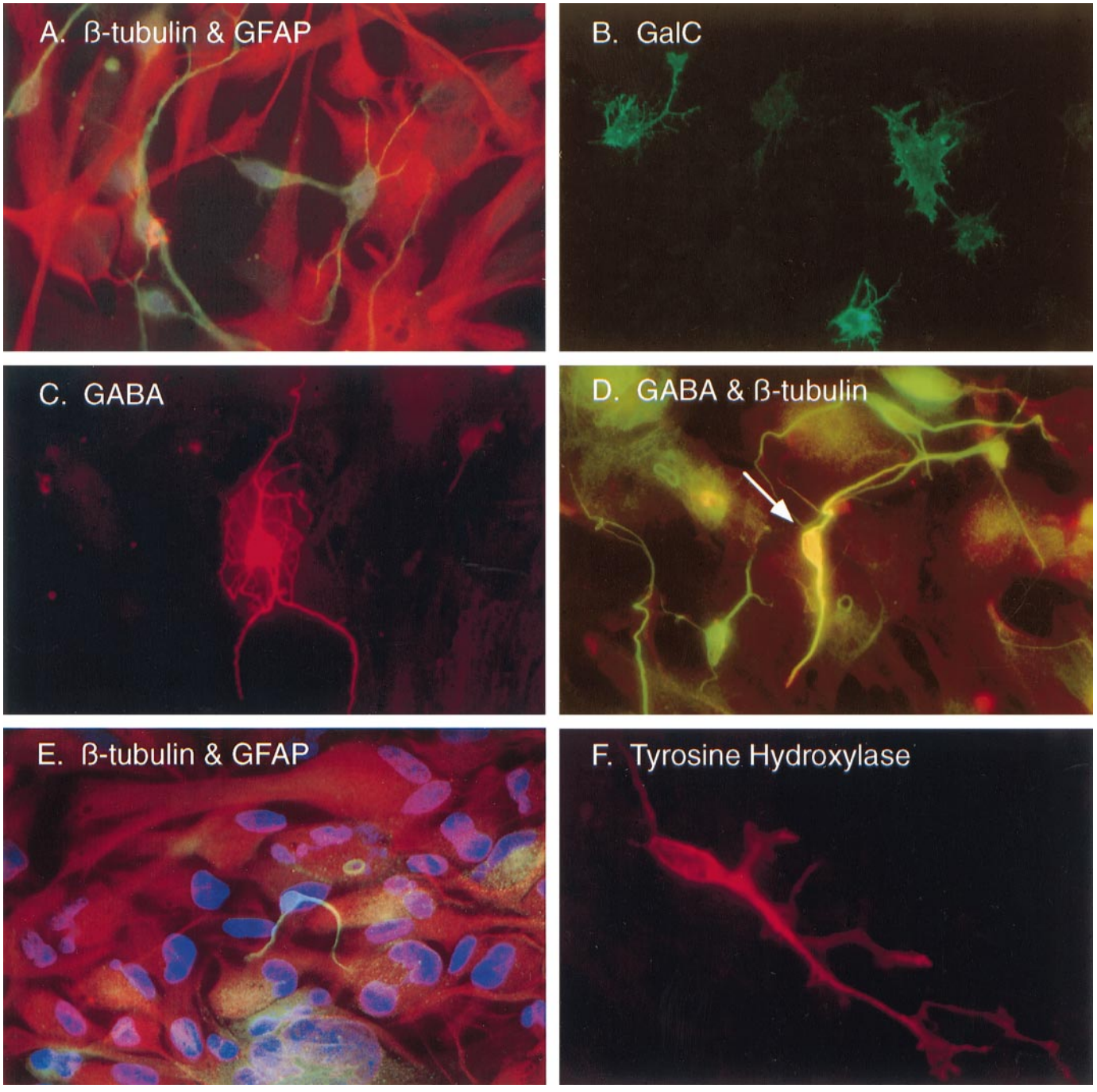


FIG. 5. Trilineage potential of human progenitor cells upon differentiation. (A) $9FBr_{021997}$ cell line maintained in hEGF, hbFGF, and hLIF for 30 passages before differentiation expresses β-tubulin (green) and GFAP (red). (B) GalC immunoreactive cells in the differentiated $9FBr_{0219997}$ cell line maintained in hEGF and hLIF (no hbFGF) for 22 passages. (C) GABA immunoreactive neuron from the $5FBr_{112397}$ cell line maintained in hEGF, hbFGF, and hLIF for 5 passages. (D) GABA (red) and β-tubulin (green) immunoreactivity in differentiated cultures from the $5FBr_{112397}$ cell line maintained in hEGF, hbFGF, and hLIF for 5 passages. One cell is double labeled with both markers (yellow, indicated with arrow). (E) GFAP (red) and β-tubulin (green) immunoreactivity in the $9FBr_{021997}$ cell line maintained in the absence of hbFGF (hEGF and hLIF only) for 22 passages. The β-tubulin cell has short processes indicative of an immature neuron. Cell nuclei are labeled with DAPI (blue). (F) Positive TH immunoreactivity found in human neural progenitor cells differentiated in cytokines for 20 days. The $8.5FBr_{032598}$ cell line was passaged five times in hEGF, hbFGF, and hLIF and then differentiated in N2 + 1% FBS supplemented with IL-1β (200 pg/ml), IL-11 (1 ng/ml), and GDNF (1 ng/ml) for 20 days.

TABLE 1

Quantitation of Multipotentiality of Human Neural Progenitor Cell Lines Generated from Forebrain at Gestational Weeks 6.5, 7, 9, and 10.5

Age of gestation (weeks)	% GFAP	% β-tubulin	% GABA (% of neurons)
6.5 (P5)	15 ± 3	37 ± 3	21 ± 4
7 (P7)	N/A	22 ± 1	9 ± 3
9 (P7)	53 ± 4	20 ± 0.4	35
10.5 (P5)	50	29 ± 2	51 ± 5

Note. Cell lines were carried for five to seven passages before quantification. The percentage of cells expressing GFAP and β-tubulin was evaluated. The percentage of neurons expressing GABA is also shown. Data are presented as mean ± SEM.

data may be indicative of shifting ratios of progenitor cell populations in these cultures.

All of the cell lines evaluated produced oligodendrocytes, as indicated by immunoreactivity to GalC. GalC-ir cells were typically found in patches and the overall number GalC-ir cells was generally quite low and varied between cell lines. Because the distribution of these cells was sparse and uneven, it was difficult to accurately quantify the percentage of cells which formed oligodendrocytes. GalC-ir cells were identified in cell lines maintained in hEGF, hbFGF, and hLIF as well as in hEGF and hLIF. Although the presence/absence of different mitogens affected the number of neurons formed, it is unclear if the mitogen cocktail affects the number of oligodendrocytes formed. Figure 5B illustrates GalC-ir in the $9FBr_{021997}$ cell line after 22 passages.

DISCUSSION

The ideal type of cells for CNS transplantation will be an expandable population of cells which can differentiate into appropriate phenotypes. The cell culture system described here meets these criteria. Other human cell lines exist that may be used for transplantation. Both the human teratocarcinoma cell line (NT2) (33) and the conditionally immortalized tetracycline-responsive v-*myc* cell line (29) can be expanded and induced to form postmitotic neurons; however, these cell lines do not form all three neural phenotypes. More recently, human neural stem cells have been derived using v-*myc* which can be expanded for long periods *in vitro* (10). It is unclear whether the proliferation rate of these cells is similar to that of the cells described in this report.

The data presented here indicate that a population of human neural progenitor cells exist in the first trimester which can be expanded *in vitro* and have the capacity to differentiate into neurons, astrocytes, and oligodendrocytes. This expansion is dependent upon mitogens and does not require genetic modification of the cells. The greatest expansion of cells is observed using hEGF, hbFGF, and hLIF as growth factors in the medium. Although EGF and bFGF have been used in a variety of progenitor and stem cell culture systems, the use of LIF in this culture system appears to be unique. These cultures contain a mixture of progenitor cell types with various differentiation potentials. It is likely that each of the mitogen conditions which were investigated affected subpopulations of progenitor cells differently, resulting in the observed variations in multipotency. This conclusion is most clearly illustrated by the differences seen in neuronal formation in cultures maintained with and without hbFGF and hLIF. Although the described cultures proliferate and remain multipotential for extended periods of time in the three mitogens, it is not clear that this is the optimal growth condition for these cells. Upon removal of EGF, two cell lines continued to proliferate and maintained their multipotency for at least five passages. The requirement of EGF and different media and mitogen cocktails is currently being evaluated to determine the appropriate growth conditions for the progenitor cultures.

It is of particular interest that cultures maintained in the presence of LIF consistently produced more neurons than parallel cultures carried in the absence of LIF. LIF is known to be an essential factor in the maintenance of embryonic stem cell (ES) cultures. While the action of LIF in ES cultures is believed to be via the inhibition of differentiation (23, 30, 32), it is not clear what the action of LIF is in the human neural progenitor cell cultures presented in this study. It is possible that LIF is acting to inhibit the differentiation of cells such that the multipotent nature of these cells is maintained for longer periods of time. However, it is also possible that LIF is selectively supporting the survival of a population(s) of neural progenitor cells. Neuronal formation was found in all nine cell lines grown in the presence of hEGF, hbFGF, and hLIF. In fact, the percentage of neurons (20–37%) observed in these cultures was considerably greater than that seen in murine cultures (5–7%). To further examine this finding, both rat and mouse neurosphere cultures were grown in this mixture of mitogens. However, these cultures failed within two passages (M. K. Carpenter, unpublished observations) indicating that the population of cells isolated from rodent and human sources may be different populations of cells.

The effect of LIF in enhancing growth rate was not evident until 50–60 days in culture. The mechanism for this delayed effect is unclear. It is possible that these first 50–60 days in culture represent the typical life span of one population of progenitor cells which is responsive to LIF, and after this initial period the cells maintained in the absence of LIF die. Alternatively, this may be due to a change in the ratio of progenitor

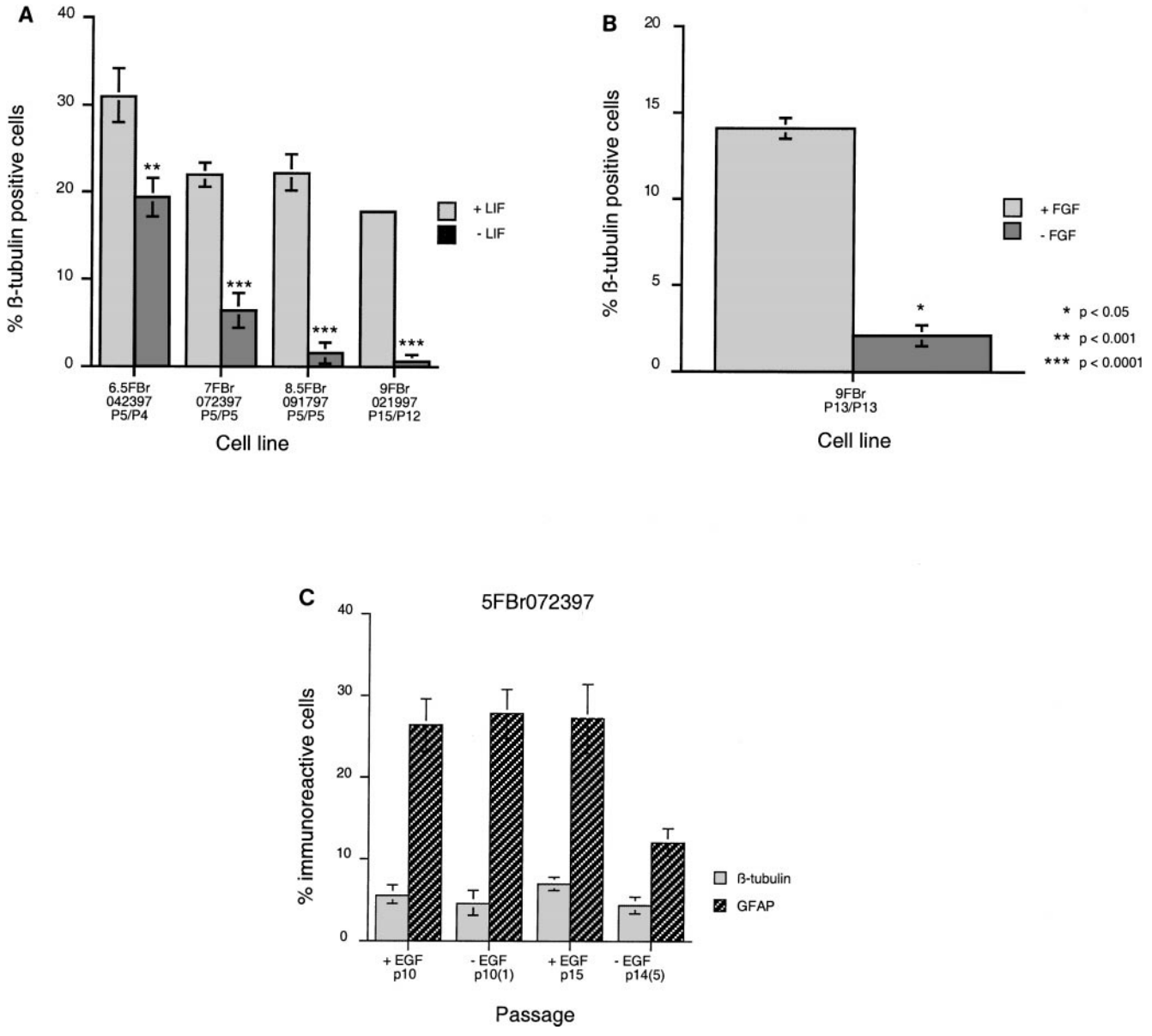


FIG. 6. Effect of mitogens on neuronal and glial differentiation. (A) Neuronal formation was significantly greater in cultures maintained in hEGF, hbFGF, and hLIF than in parallel cultures maintained in only hEGF and hbFGF. Cultures generated from forebrain at gestational weeks 6.5, 7, 8.5, and 9 were evaluated. Parallel cultures were evaluated at similar passages (the specific passage numbers for each cell line are indicated with the cell line designation), indicated by each bar. $^{**}P < 0.001$ and $^{***}P < 0.0001$. (B) Neuronal formation was significantly greater in cultures maintained in hEGF, hbFGF, and hLIF than in cultures maintained in hEGF and hLIF. The $9FBr_{021997}$ line was evaluated at P13 with and without hbFGF as indicated by each bar. $^{*}P < 0.05$. (C) Removal of hEGF did not appear to affect the formation of either neurons or astrocytes. The $5FBr_{072397}$ cell line was evaluated at one and five passages after EGF removal (at P10). For all graphs, error bars indicate SEM.

cells in the cultures or to the temporal expression of mitogen receptors on the cells.

LIF is a member of the gp130 signaling family, which signals through shared components of their multisubunit receptors. The effects of the other family members on the proliferation and differentiation of the human neural progenitor cells are currently being evaluated to determine whether additional mitogens will influence the characteristics of these cultures. CNTF is known to have many effects on very early neuronal precursors (17) and also maintains the pluripotentiality of ES cells (8). Our preliminary data indicate that CNTF supports the expansion of the population of cells. Although CNTF and LIF appear to have a similar effect on cell expansion, neither Flt3/Flk2 nor IL-6 appears to support cell expansion. These factors

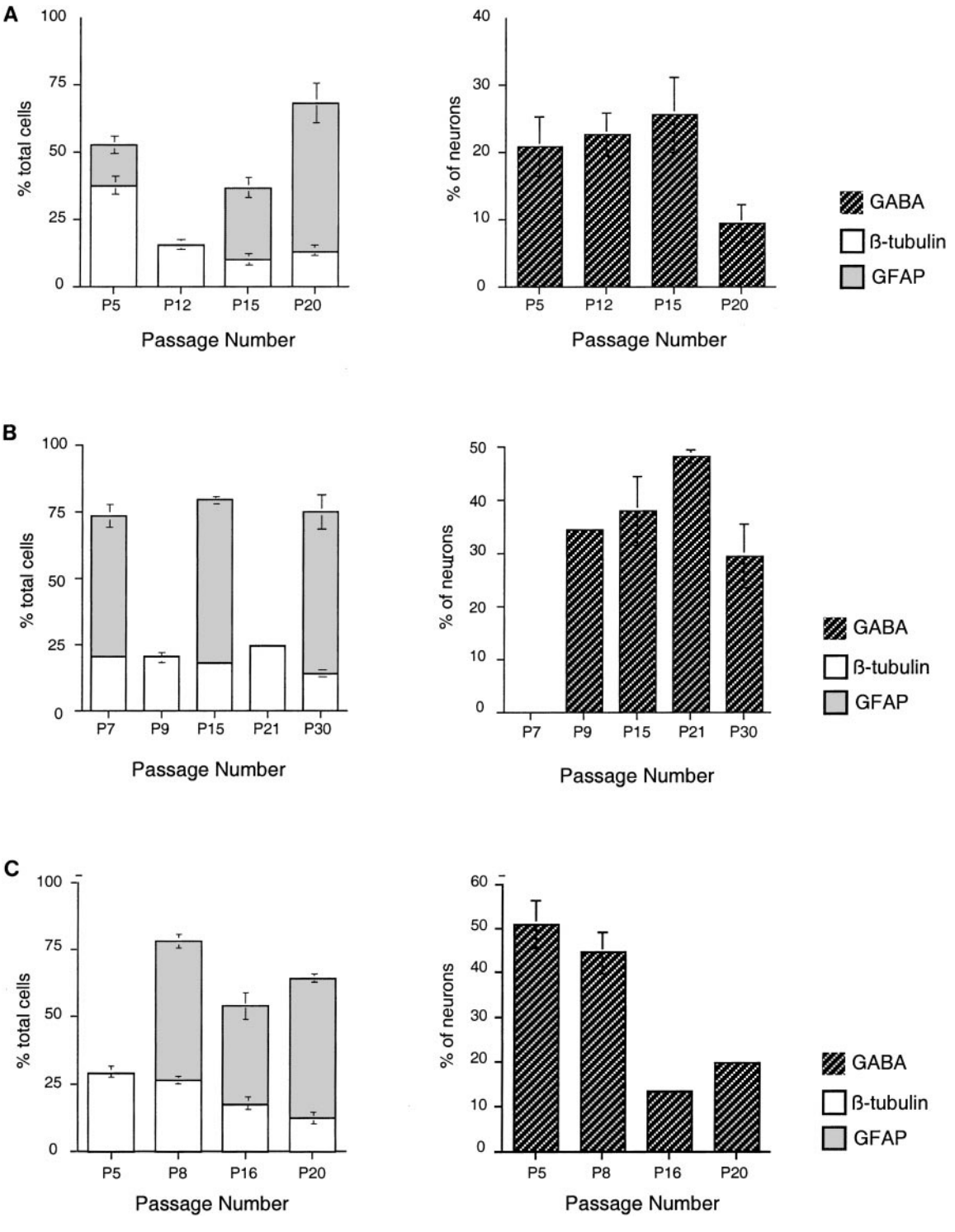


FIG. 7. The effect of long-term culture on the multipotentiality of neural progenitor cells. The percentages of neurons and astrocytes produced by the 6.5 FBr_{042397} (A), $9FBr_{021997}$ (B), and $10.5FBr_{041697}$ (C) cell lines were evaluated at different passages. Note that for cell line $9FBr_{021997}$, passages 9 and 21, and cell line 6.5 FBr_{042397}, passage 12, the number of astrocytes could not be accurately quantified and the values were not included. The number of GABA-positive neurons was assessed in each cell line at different passages. In the $6.5FBr_{042397}$ and $10.5FBr_{041697}$ cell lines, the percentage of cells appeared to decline by passage 20.

are currently being characterized for their effects on multipotency.

bFGF is known to have a mitogenic effect on many different progenitor and stem cell culture systems, including mouse and human cells (14, 15, 22). It is therefore not surprising that hbFGF increased the expansion of the human neural progenitor cell lines described here. However, survival of some portion of the population was not dependent on bFGF, since all of the cells did not die when hbFGF was removed. Furthermore, the removal of hbFGF resulted in a dramatic decrease in the formation of neurons, much like the effect of hLIF removal. bFGF has also been shown to have a mitogenic effect on a variety of forebrain progenitor cells (reviewed in 35). In murine cortical stem cells, bFGF regulates the induction of neurons and glia in a concentration-dependent fashion (24). Exposure to low concentrations of bFGF (0.1 ng/ml) results in the formation of neurons, while exposure to higher concentrations (1–10 ng/ml) results in the formation of oligodendrocytes (24). In the studies presented here, removal of hbFGF from the human progenitor cells resulted in a decrease in neuronal formation. However, various levels of hbFGF were not evaluated to determine the optimal concentration of hbFGF for neuron formation. It is unclear whether the removal of hbFGF results in the death of neural precursors or whether this results in other fate choices for these cells.

Both bFGF and LIF appeared to be necessary for the long-term expansion and for maintenance of the multipotency of cell lines. These growth factors appeared to affect the ratios of progenitor cells in the population. Therefore, it may be possible to enrich a population of cells for neuronal progenitors or oligodendrocyte progenitors by altering the growth factors to which the cells are exposed in their undifferentiated state.

It is particularly interesting that the removal of EGF did not affect the growth rate or the formation of neurons. The initial characterization of neurospheres derived from rodents was accomplished using EGF as the only mitogen (26–28). These findings indicate that, although the cell population expresses the mRNA for the EGF receptor (data not shown), EGF may not be necessary for the maintenance of the human progenitor cells. However, long-term survival experiments will be necessary to confirm this finding. Furthermore, it is unclear if the presence or absence of EGF affects the specific neuronal phenotype (GABA-ergic, dopaminergic, etc.) or the ability to form oligodendrocytes.

The data presented here indicate that the cells isolated from the human CNS respond to mitogens differently than the cells isolated from the rodent CNS. This is further supported by the finding that the rodent-derived neurospheres cannot be maintained in the presence of LIF. In addition, murine neurosphere cultures have been previously derived from LIF receptor knockout mice. These cells expanded well in the presence of EGF and generated neurons, astrocytes, and oligodendrocytes (M. K. Carpenter, unpublished observations). Taken together, these data indicate that the mitogen requirements for the expansion of the rodent and human cells may be different.

In all cell lines that underwent the *in vitro* differentiation process there was a population of cells (20–25%) which did not stain positively for any of the markers used, including nestin. Morphologically, these adherent cells appeared flat and often were polygonal. They may represent a population of nestin-negative progenitor cells, which persist under the conditions used to induce differentiation. Previous experiments using murine cultures have demonstrated that a population of tripotential progenitor cells can persist under differentiating conditions (6). It remains to be seen whether this population of cells represents a progenitor cell population, a differentiated population of cells which has not been identified, or a nonneuronal population of cells.

Upon differentiation, GABA-ergic neurons were identified in all of the cell lines examined. Using the standard differentiation protocol TH-ir cells were not identified. However, this population of human neural progenitor cells did show the capacity to differentiate into TH-expressing neurons. The addition of the hematopoietic cytokine IL-1b resulted in the expression of TH which was colocalized with β-tubulin. This is consistent with previous work using rat mesencephalic progenitor cells (19). The expression of TH in murine-derived neurosphere cultures has also been demonstrated using a combination of E-C-L attachment matrix and NGF during the differentiation process (18). These data indicate that, although TH expression is not a "default" fate choice for these progenitor cells, the cells have the capacity to form TH-expressing neurons *in vitro*. Furthermore, upon transplantation into the adult rat subventricular zone or rostral migratory pathway, these human neural progenitor cells have demonstrated the ability to migrate to the olfactory bulb and form TH-positive neurons (12). Therefore, upon exposure to appropriate environmental cues, this population of human neural progenitor cells has the ability to form different subtypes of neurons both *in vitro* and *in vivo*.

In addition to neurons and astrocytes, oligodendrocytes were identified in human neural progenitor cell cultures. Immunoreactivity to GalC was seen in all cell lines. Although proteolipid protein (PLP) immunoreactivity was occasionally identified (data not shown), immunoreactivity to myelin basic protein (MBP) was not identified. It may be necessary to differentiate the cells for longer periods of time to find robust expression of mature oligodendrocyte markers such as PLP and MBP. Alternatively, the environment used for differen-

tiation (1% FBS) may not provide the factors necessary for the survival of mature oligodendrocytes.

Although these data indicate the presence of an expandable, multipotent population of cells, they do not provide definitive proof of the presence of a human neural stem cell. Stem cells are defined as having the capacity for self-renewal. The human cells described here can be passaged and expanded over 1 year. However, clonal analysis of this culture system is necessary to determine if this population contains neural stem cells. Initial attempts at clonal analysis by limiting dilution of these cells have been hindered by their sensitivity to population density.

At the time of this writing, the ultimate capacity for expansion of these cell lines is unknown; however, the minimum expansion period appears to be at least 1 year. These cells have the capacity to form astrocytes, oligodendrocytes, and neurons. Furthermore, the neuronal phenotype of the cells can be influenced by the environment, indicating that these cells will respond to environmental cues. As potential material for replacement therapy in CNS degenerative diseases, this culture system may therefore offer many advantages, including the ability to expand, safety test, and bank these cells before transplantation.

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