Development/Plasticity/Repair

Human Cortical Neurons Originate from Radial Glia and Neuron-Restricted Progenitors

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Understanding the molecular and physiological determinants of cortical neuronal progenitor cells is essential for understanding the development of the human brain in health and in disease. We used surface marker fucose *N*-acetyl lactosamine (LeX) (also known as CD15) to isolate progenitor cells from the cortical ventricular/subventricular zone of human fetal brain at the second trimester of gestation and to study their progeny *in vitro*. LeX$^+$ cells had typical bipolar morphology, radial orientation, and antigen profiles, characterizing them as a subtype of radial glia (RG) cells. Four complementary experimental techniques (clonal analysis, immunofluorescence, transfection experiments, and patch-clamp recordings) indicated that this subtype of RG generates mainly astrocytes but also a small number of cortical neurons. The neurogenic capabilities of RGs were both region and stage dependent. Present results provide the first direct evidence that RGs in the human cerebral cortex serve as neuronal progenitors. Simultaneously, another progenitor subtype was identified as proliferating cells labeled with neuronal (β-III-tubulin and doublecortin) but not RG markers [GFAP, vimentin, and BLBP (brain lipid-binding protein)]. Proliferative and antigenic characteristics of these cells suggested their neuron-restricted progenitor status. In summary, our *in vitro* study suggests that diverse populations of cortical progenitor cells, including multipotent RGs and neuron-restricted progenitors, contribute differentially to cortical neurogenesis at the second trimester of gestation in human cerebral cortex.

Key words: **cerebral cortex development; primates; human fetal cell cultures; LeX immunocytochemistry; transfection; patch-clamp recording**

Introduction

Establishing the molecular and physiological characteristics of human neural stem and progenitor cells is important for understanding both the origins of cell diversity in the CNS and the potential use of these cells in replacement therapies.

In the mouse forebrain, radial glia (RGs), present in the proliferative ventricular zone (VZ), are the main cortical progenitor cells to give rise to pyramidal neurons (Malatesta et al., 2000; Miyata et al., 2001, 2004; Noctor et al., 2001). RG cells in the mouse can also generate a subpopulation of forebrain oligodendrocytes (Malatesta et al., 2003; Casper and McCarthy, 2006) and adult neural stem cells (Merkle et al., 2004).

RG cells were described for the first time in human fetal brain more than a century ago, when it was suggested that they may guide newly formed neurons to their proper positions (Magini, 1888; Rakic, 1972, 2003) (for review, see Bentivoglio and Mazzarello, 1999). However, little progress has been made in characterizing the neurogenic abilities of human RG cells. This is mainly because experimental procedures, such as Cre lox fate mapping or *in vivo* retroviral labeling, typically used in animal models cannot be applied to humans. Previous studies from our laboratory showed that at the beginning of cortical neurogenesis [5 gestational weeks (gw)], human RGs are just one of several cell subtypes proliferating side by side in the cortical VZ (Zecevic, 2004; Howard et al., 2006). This observation is consistent with the greater diversity of progenitor cells in the human cortical VZ than in lower vertebrates. The question, however, remains whether and to what extent RG cells serve as neuronal progenitors in the human cerebral cortex. One feasible approach to study this issue is to isolate progenitor cells from the human fetal cortical VZ and subventricular (SVZ) zones and analyze their progeny *in vitro*. To isolate cortical RGs by immunopanning procedure, we applied the surface marker fucose *N*-acetyl lactosamine (LeX), an extracellular matrix-associated carbohydrate, also known as SSEA1 or CD15, expressed in mouse embryonic stem cells and used to enrich them (Kim and Morshead, 2003; Capela and Temple, 2006; Imura et al., 2006; Liour et al., 2006).

Here, we present evidence that the majority of LeX$^+$ cells in

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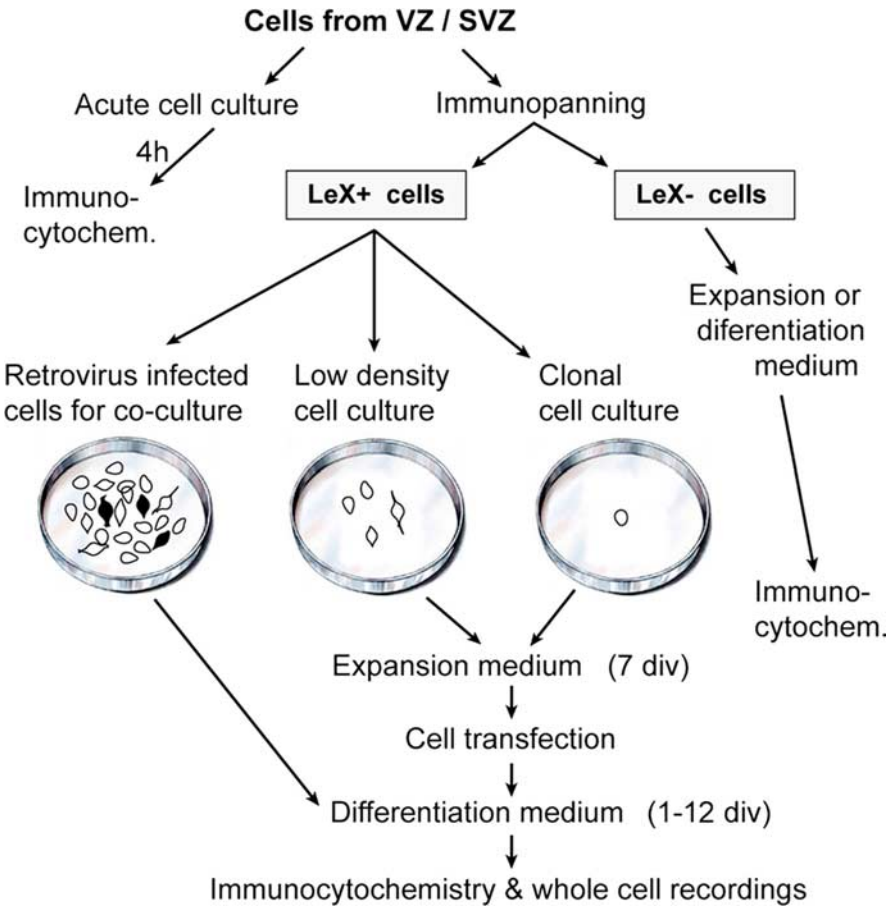

Figure 1. Paradigm of the culture methods applied in this study. Immunocytochem., Immunocytochemistry.

the human cortical VZ/SVZ are, in fact, a subset of RG cells. Our experimental results are in agreement with studies performed on animal tissue (Mai et al., 1998; Liour et al., 2006) or on fixed human fetal tissue (Mai et al., 2003). Whether cortical neurons in primates originate only from RGs or simultaneously from several progenitor subclasses has been debated for a long time. Previous conclusions were based mainly on differences in morphology and immunolabeling features of proliferating VZ cells (Levitt et al., 1981; Weissman et al., 2003; Zecevic, 2004; Howard et al., 2006). In this study, using a combination of methods, particularly the cell transfection and electrophysiological recordings, we provide the first direct experimental evidence that human cortical neurons at midgestation are generated from a subset of RG cells. In addition, we propose that neuron-restricted progenitors also contribute to the population of cortical neurons.

Materials and Methods

Human fetal brain tissue and cell culture. Human fetuses ($n = 7$), ranging in age from 14 to 23 gestational weeks, were obtained from the Tissue Repository of The Albert Einstein College of Medicine (Bronx, NY). Tissue was collected with proper parental consent and the approval of the Ethics Committees of the University of Connecticut (Farmington, CT) and The Albert Einstein College of Medicine. Ultrasonic and neuropathological examinations found no evidence of disease or abnormalities. The postmortem delay was on average 15 min. Brain tissue was collected in oxygenized HBSS containing 0.75% D-glucose and transported on ice to our lab. All procedures were performed under sterile conditions.

Dissociated cell cultures were prepared from the VZ/SVZ of the fetal forebrain, dissected from the frontally cut hemispheres as a tissue band ~2000 μm high from the VZ surface (Zecevic et al., 2005). Tissue was dissociated with 0.05% trypsin–0.02% EDTA (Invitrogen, Carlsbad, CA) and triturated through a fire-polished pipette. Cells were resuspended in DMEM/F12 (Invitrogen) containing 10% fetal bovine serum (Hyclone, Logan, UT) supplemented with B27 (Invitrogen) and seeded into poly-L-lysine (Sigma, St Louis, MO)-coated eight-well chamber slides (BD Falcon, San Jose, CA) at a concentration of 4×10^4 cells per well. Acute cell cultures were incubated at 37°C with 5% CO_2 and 95% O_2 for 4 h, fixed in 4% paraformaldehyde for 10 min, and processed for immunostaining (Fig. 1). The total number of viable cells, assessed by Trypan blue exclusion, was 95%.

Immunopanning. To isolate neural progenitor cells, we used immunopanning with a surface marker, LeX, according to a procedure described previously (Uchida et al., 2000). Petri dishes (100 mm) were prepared for panning the day before by coating them with secondary antibody [goat anti-mouse IgM; 10 μg/ml in 5 ml of Tris (50 mM), pH 9.5 (SouthernBiotech, Birmingham, AL)] and incubating overnight at 4°C (Mi and Barres, 1999). The next day, dishes were rinsed with PBS, incubated with 5 ml of anti-LeX antibody (1:100; Lab Vision, Fremont, CA) in PBS with 0.2% bovine serum albumin (BSA) at room temperature for 2 h, and rinsed with PBS. The dissociated cells (10^7) were suspended in 10 ml of DMEM/F12/B27 medium with 20 ng/ml fibroblast growth factor 2 (FGF2) and incubated on the anti-LeX-coated dishes for 20 min at room temperature with gentle agitation to ensure access of all cells to the panning surface. Thereafter, dishes were rinsed to remove the nonadherent cells [LeX-negative (LeX^-) cells], which were cultured separately (Fig. 1). LeX^+ cells were detached from the dish with trypsin–EDTA, counted, plated on poly-L-lysine-coated 12 mm coverslips (Carolina Biological Supply, Burlington, NC), and cultured in DMEM/F12/B27 supplemented with 20 ng/ml FGF2 (expansion medium) or 1 ng/ml FGF2 (differentiation medium) as indicated. Subsequent immunolabeling with LeX antibody determined that the purity of immunopanned cells was 95%.

Preparation of neurospheres. To study proliferative and self-renewing characteristics of isolated progenitor cells, we established neurosphere preparation. Neurospheres are free-floating spherical aggregates of stem or progenitor cells used to propagate stem cells *in vitro* (Reynolds and Weiss, 1992). LeX^+ cells were centrifuged at $260 \times g$ for 5 min, resuspended in DMEM/F12, transferred into uncoated culture dishes, and cultured as floating multicellular neurospheres in growth media [DMEM/F12/B27 containing 20 ng/ml human recombinant epidermal growth factor (EGF) and 20 ng/ml FGF2 (both from PeproTech, Lake Placid, NY)].

After 7 d *in vitro* (div), the number of neurospheres was counted. The frequency of neurosphere formation was calculated as a percentage of neurospheres formed from the initial number of cells in the culture. The neurospheres were subsequently seeded into poly-L-lysine-coated dishes in differentiation medium, as described above.

Clonal cell culture from LeX^+ cells. To study the progeny of a single LeX^+ cell, the enriched LeX^+ cells were diluted 1 cell/20 μl and placed on coverslips in single drops removed from each other. Each drop was inspected on an inverted microscope, and those containing more than a single cell were removed. Single-cell cultures were maintained at 37°C as described above. Cells were fixed with 4% paraformaldehyde at appropriate time points (Fig. 1).

Plasmids and LeX^+ cell transfection. To selectively label RG cells, we

Table 1. Antibodies used in this study

Detection of	Name	Host	Dilution	Manufacturer
Radial glia	GFAP	Rabbit IgG	1:1000	Dako (Carpinteria, CA)
	4A4	Mouse IgG2b	1:2000	MBL (Nagoya, Japan)
	BLBP	Rabbit IgG	1:2000	Gift from Dr. Nathaniel Heintz (The Rockefeller University, New York, NY)
	Vimentin	Mouse IgG	1:1000	Sigma
Progenitor cells	LeX/CD15	Mouse IgM	1:100	Lab Vision
Young neurons	β-III-tubulin	Mouse IgG	1:500	Sigma
	MAP2a, MAP2b	Mouse IgG	1:200	Sigma
	DCX	Rabbit IgG	1:100	Santa Cruz Biotechnology (Santa Cruz, CA)
Mature neurons	NeuN	Mouse IgG	1:100	Millipore
Interneurons	Calretinin	Rabbit IgG	1:2000	Swant (Bellinzona, Switzerland)
Proliferation markers	Ki67	Rabbit IgG	1:200	Anaspec (San Jose, CA)
	BrdU	Mouse IgG	1:100	Sigma
Secondary antibodies	Alexa Fluor 488 goat anti-rabbit or anti-mouse IgG		1:400	Invitrogen
	Alexa Fluor 555 goat anti-rabbit or anti-mouse IgG		1:400	Invitrogen
	Anti-mouse FITC-IgM		1:100	Zymed Laboratories (South San Francisco, CA)

used cell transfection with enhanced green fluorescent protein (EGFP), the expression of which is under RG-specific promoters: brain lipid-binding protein (BLBP) (a gift from E. Anton, University of North Carolina, Chapel Hill, NC) (Schmid et al., 2006) or human GFAP (hGFAP) (a gift from M. Brenner, University of Alabama at Birmingham, Birmingham, AL) (de Leeuw et al., 2006). Moreover, we also used BLBP-Cre/Floxed yellow fluorescent protein (YFP) cotransfection. The pBLBP-Cre was created by inserting BLBP promoter [−1600 to +53 bp (Feng et al., 1994)] and Cre (obtained from pBS185; Invitrogen) into pGEM4 (Promega, Madison, WI). Plasmid of CAGGS-loxP-LacZ-loxP-YFP was generously provided by J. Li (University of Connecticut Health Center, Farmington, CT). LeX^+ cells were cultured onto 12 mm coverslips (Carolina Biological Supply) in expansion medium for 7 div and were transfected or cotransfected with plasmids using Lipofectamine according to the standard protocol provided by Invitrogen.

Retrovirus production and LeX^+ cell infection. To permanently label LeX^+ cells before coculturing them with region- and stage-specific cultures, we infected them with a retrovirus linked to GFP. Replication-incompetent GFP-expressing retrovirus was produced from a stably transfected packaging cell line (293gp NIT-GFP; a gift from Dr. Fred Gage, Salk Institute, La Jolla, CA). 293gp cells were transfected at ~75% confluence with pVSV-G using Lipofectamine (Invitrogen). Supernatant was harvested 48 h after transfection, filtered through 0.45 μm low-protein-binding filters (Millipore, Billerica, MA), and stored at −80°C. LeX^+ cells were suspended at a final concentration of 10^5 cells/ml in expansion medium supplemented with 1 μg/ml polybrene. Volumes of NIT-GFP virus sufficient to infect 50 cells were added to 0.5 ml of cells (50,000 cells) and then incubated for 30 min at 37°C. The cells were centrifuged, resuspended in differentiation medium, and cocultured with the cells from cortical VZ/SVZ or from the ganglionic eminence (GE) for 7 div. The medium was replaced every other day. After this procedure, cells were evaluated with immunocytochemistry.

Reverse transcription-PCR. Total RNA was isolated using Trizol reagent (Invitrogen) according to the protocol provided by the company. Identical amounts of RNA were reverse transcribed into cDNA, which was subsequently amplified by PCR with different primers: *EGF*, forward, 5′-gccaagcagtctgtgattga-3′; reverse, 5′-ccagacacgttttcccatct-3′; *FGF2*, forward, 5′-gaagagcgaccctcacatcaag-3′; reverse, 5′-ctgcccagttcgtttcagtg-3′; *Nestin*, forward, 5′-gccctgaccactccagttta-3′; reverse, 5′-ggagtcctggatttccttcc-3′; *NSE*, forward, 5′-tgaacacagacgctatgcgctcag-3′; reverse, 5′-caccttttatgtgagtggacacaga-3′; *GAPDH*, forward, 5′-ggtgaaggtcggagtcaacgga-3′; reverse, 5′-tcttccaggagcgagatccctc-3′. Thirty cycles of amplification were performed in a Peltier Thermal Cycler (PTC-100; MJ Research, Watertown, MA). Real-time quantitative reverse transcription (RT)-PCR analysis was performed starting with 500 ng of reverse-transcribed total RNA (diluted in Sybr Green buffer), with a 200 nM concentration of both forward and reverse primers in a final volume of 25 μl, using the Sybr Green PCR core reagents and the iCycler System instrument (Bio-Rad, Hercules, CA). mRNA levels for the various markers were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA. Experiments were repeated three times. The data were presented as means ± SEMs and analyzed using Student's *t* tests. The criterion for significance was set at $p \leq 0.05$.

Proliferation assay. Bromodeoxyuridine (BrdU; 20 μM; Sigma) was added to the cell cultures kept in an expansion medium for the last 6 h before immunostaining. Thymidine analog, BrdU, incorporates into DNA of dividing cells and could then be detected by immunocytochemistry. Briefly, cells were fixed in 4% paraformaldehyde in PBS for 10 min, treated with 2 N HCl for 10 min at room temperature, neutralized by rinsing in 100 mM boric acid, pH 8.5 (Sigma) for 10 min followed by incubation in a blocking solution containing 3% BSA (Sigma) in Tris-buffered saline [TBS (2.42 mg/ml Tris, 8.9 mg/ml NaCl, and 2 mM $MgCl_2$)] for 1 h. Primary antibody, monoclonal anti-BrdU (1:100; Sigma), was applied overnight at 4°C. After washing in TBS for 5 min three times, the cells were incubated with the secondary antibody.

Immunostaining. For cryosections, frozen brain blocks were serially sectioned in the coronal plane at 15 μm thickness. Sections were incubated in blocking solution [1% BSA (Sigma), 5% normal goat serum (Vector Laboratories, Burlingame, CA), and 0.5% Tween 20 in PBS] for 30 min. Primary antibodies were applied overnight at 4°C, whereas corresponding secondary antibodies were subsequently applied for 1 h (Table 1). A short incubation in bisbenzamide was used to reveal the cell nuclei.

Cell cultures were fixed with 4% paraformaldehyde for 10 min, washed with PBS at room temperature, and incubated with primary and secondary antibodies as described above. The specificity of primary antibodies was tested with corresponding isotype controls (mouse IgG1, IgG2a or IgG2b, or rabbit serum); the specificity of secondary antibodies was tested by omitting the primary antibodies from the protocol. Both tests resulted in a lack of immune reaction.

For live-cell immunolabeling, cultures were incubated with the LeX antibody in PBS with 0.2% BSA at room temperature for 20 min, rinsed, and exposed to the secondary antibody for 10 min. The immunolabeled cells were then used in electrophysiological studies.

Cell counting and statistical analysis. Cells stained with nuclear stain bisbenzimide and various cellular markers were visualized with a Zeiss (Oberkochen, Germany) Axioplan fluorescence microscope and photographed with a Spot Insight digital camera. Before quantification, 10 predesignated adjacent optical fields of view were selected in each culture and examined at a magnification of 10× (one field = surface area of 1 mm^2) or 20× (0.25 mm^2 surface area). The percentage of immunolabeled cells of total bisbenzimide-positive cells was calculated. For transfected or infected cells, the percentages of immunolabeled cells from all green cells were obtained. For the clonal cell cultures, individual clones immunostained with GFAP or microtubule-associated protein 2 (MAP2) were counted at 10× magnification. Counts of cell-type-specific clones from either 14 or 20 gw were pooled together. The data were

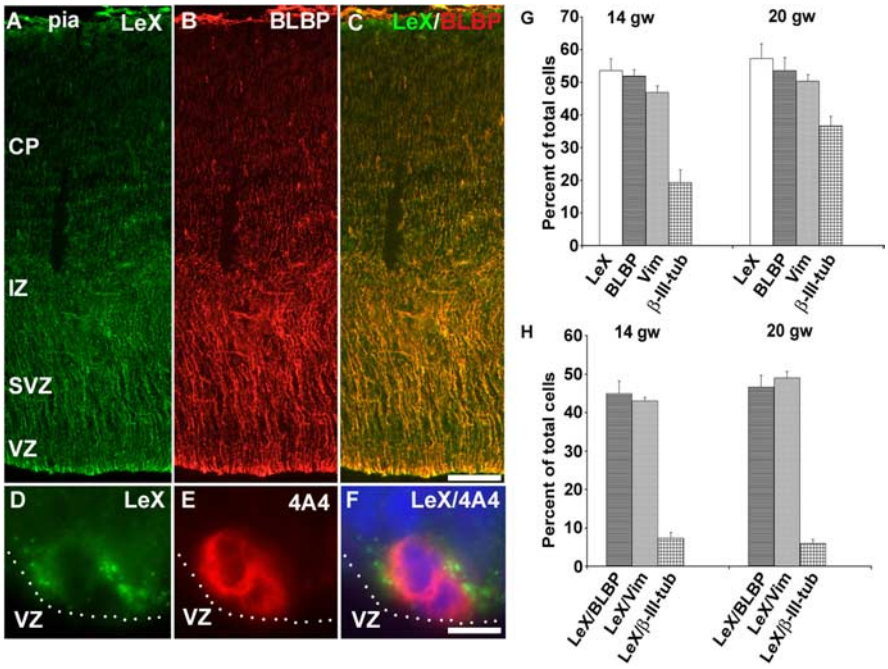

Figure 2. On cryosections of cortical VZ/SVZ at 14 gw, LeX$^+$ cells (green) are colabeled with RG markers. ***A–C***, Colabeling with LeX and BLBP (red) shows radially oriented colabeled cells in the VZ/SVZ region. ***D–F***, Two proliferating cells on the VZ surface are colabeled with the antibody 4A4 for a phosphorylated vimentin (red) and LeX (green). Cell nuclei are labeled with bisbenzimide (***F***, blue). CP, Cortical plate; IZ, intermediate zone. Scale bars: ***A–C***, 50 μm; ***D–F***, 10 μm. ***G***, Quantification of immunolabeled cells in the acute cell cultures at 14 and 20 gw shows that >50% of isolated cells are LeX$^+$, followed by a slightly smaller percentage of BLBP and vimentin$^+$ cells, whereas 10 or 35% of cultured cells belong to neuronal lineage (β-III-tubulin$^+$) at 14 or 20 gw, respectively. ***H***, Quantification of double-labeling experiments shows that ~50% of cells coexpress LeX and either BLBP or vimentin. Fewer than 10% of cells are LeX/β-III-tubulin$^+$ cells. Vim, Vimentin; β-III-tub, β-III-tubulin. Error bars represent SEM.

expressed as means ± SEMs and analyzed using unpaired one-tailed Student's *t* tests. The criterion for significance was set at $p \leq 0.05$.

Electrophysiology. Electrophysiological measurements were made on human brain cells cultured at 20, 21, and 23 gw. Whole-cell patch recordings were performed between 1 and 17 div. Before recordings, cells were transferred to an Olympus (Tokyo, Japan) BX51WI upright microscope (equipped with infrared video microscopy) and perfused in aerated (95% O_2/5% CO_2) artificial CSF (ACSF) at room temperature (21–25°C). The ACSF contained 125 mM NaCl, 2.3 mM KCl, 23 mM $NaHCO_3$, 1 mM $MgSO_4$, 1.26 mM KH_2PO_4, 1 M $CaCl_2$, and 3.6 g glucose, pH 7.3. Individual cells were selected for recordings based on a small round or ovoid cell body (diameters, 5–15 μm) and typically two or more extended processes. In contrast, astrocytes were characterized by large cell body diameters (>15 μm), irregular shape of the cell body, and numerous stellate processes. Fluorescently labeled cells were identified by epifluorescence. Patch pipettes (10 MΩ) were filled with an intracellular solution containing (in mM) 135 K-glutamate, 10 HEPES, 2 $MgCl_2$, 3 ATP-Na_2, 0.3 GTP-Na_2, and 10 P-creatine Na_2, pH 7.3. In one set of experiments (see Fig. 4), rhodamine-dextran 3000 molecular weight (80 μM) was added to the intracellular solution. Recordings were performed using Multiclamp 700B and Clampex 9.2 (Molecular Devices, Union City, CA). After the rupture of the cell membrane, leak conductance subtraction and series resistance compensation (70–80%) were performed and monitored periodically. Voltage control (clamp) in our experiments was not ideal (see Fig. 7*C*) for the following reasons: (1) the small diameter of neurons and fragile membrane forced us to use high-resistance recording pipettes (10 MΩ); (2) we deliberately avoided voltage-gated channel agonists (such as tetraethylammonium, 4-AP, cesium, cadmium, etc.) to maintain the longevity of rare experimental preparation and to increase the duration of recording sessions. Also, the analysis of both inward and outward components (in the same cell) provided an important control of cell health in our experiments involving transfections (see Fig. 7*F*). Finally, (3) the majority of cells showed two or more processes longer than 100 μm. It has been shown before that cells with dendrites are difficult to clamp (Spruston et al., 1993). The accent of our study was not on quantitative characterization of transmembrane currents but rather on positive detection of the inward component. In voltage-clamp configuration, cells were given a series of voltage steps (duration, 50 ms) from −90 to +30 mV from a holding potential of −70 mV. In current-clamp configuration, we applied a series of current steps from −20 to +120 pA. Electrical traces were analyzed using Clampfit 9.2 (Molecular Devices). Data are expressed as means ± SEMs. Statistical analyses were performed in Excel (Microsoft, Seattle, WA) using the two-tailed unpaired Student's *t* test (significance of $p \leq 0.05$).

Results

The expression of LeX antigen in the human fetal forebrain

Radially oriented LeX$^+$ cells were distributed along the cortical VZ/SVZ in frozen sections of fetal forebrains at 14 and 20 gw. These cells had the typical bipolar morphology of RG cells, with two processes, one attached to the ventricular surface and the other directed toward the pia. In addition to morphological similarities to RGs, LeX$^+$ cells were regularly double labeled with different markers of RGs, such as BLBP (Fig. 2*A–C*), GFAP, and vimentin (data not shown). Moreover, dividing cells on the ventricular surface often coexpressed LeX and phosphorylated vimentin [4A4 antibody (Kamei et al., 1998)], specific for proliferating RGs (Fig. 2*D–F*).

To quantify cells that expressed LeX, we developed the acute mixed cell culture from cortical VZ/SVZ at 14 and 20 gw. LeX$^+$ cells represented 55% of the acutely dissociated mixed cells (4 h in culture). Similarly, ~50% of cells expressed BLBP and vimentin (Fig. 2*G*). Most LeX$^+$ cells coexpressed BLBP and vimentin, showing a clear overlap in these cell populations (Fig. 2*H*). The neuronal marker β-III-tubulin labeled 19 and 35% of all acutely dissociated cells in the VZ/SVZ at 14 and 20 gw, respectively. At both age points, ~6–7% of cells were double labeled with LeX and β-III-tubulin.

These immunostaining experiments demonstrated that at the second trimester of gestation, the LeX$^+$ cell population is heterogeneous, and that the majority of these cells represent RG cells.

To further analyze this cell population, LeX$^+$ cells were enriched and studied in more detail *in vitro*.

LeX-expressing cells are progenitors with the capacity for self-renewal

LeX$^+$ cells, enriched by immunopanning from the fetal telencephalon at 14 and 20 gw, were plated and analyzed 36 h later. At 14 gw, almost 95% of all plated cells (338 of 356) were labeled with LeX antibody (Fig. 3*A*). Similar to results with the acute mixed cell cultures, the enriched LeX$^+$ cells also colabeled with BLBP (92% of $n = 404$ analyzed cells) (Fig. 3*B–D*), vimentin (94%; $n = 312$) or GFAP (96%; $n = 340$) (data not shown). Only 8% ($n = 345$) of LeX$^+$ cells were colabeled with the neuronal marker β-III-tubulin (Fig. 3*E*). Similar results were obtained when LeX$^+$ cells were enriched from VZ/SVZ tissue at 20 gw, except that fewer LeX/β-III-tubulin-positive cells (4%; $n = 360$) were noted.

To determine whether enriched LeX$^+$ cells maintained their antigenic characteristics *in vitro*, we cultured them for 7 d in expansion medium and demonstrated that 94% ($n = 640$) of analyzed cells still expressed LeX antigen (Fig. 3*F*). Most of the immunopositive cells had bipolar morphology typical for RGs, and >90% of these cells were colabeled with BLBP, vimentin, or GFAP (Table 2). The average number of LeX$^+$ cells per field of view (surface area, 1 mm^2 at 10× magnification) increased more than six times, from the initial quantification at 36 h (four cells) to 26 cells at 7 div in expansion medium (Fig. 3, compare *A*, *F*). The increase in the cell number indicated multiple cell divisions and self-renewing characteristics of LeX$^+$ cells. Self-renewing characteristics were further confirmed by finding a high frequency (40%) of neurosphere formation from LeX$^+$ cells kept for 7 div in the uncoated dishes with the expansion medium (Fig. 3*G*). When cultured in differentiation medium, these neurospheres mainly generated astrocytes (84% from $n = 590$ cells) and a small number of neurons (13%; $n = 590$) (Fig. 3*H*). Similar results with neurospheres were obtained with LeX$^+$ cells isolated either at 14 or 20 gw, suggesting differentiation of LeX$^+$ cells into both astroglia and neurons.

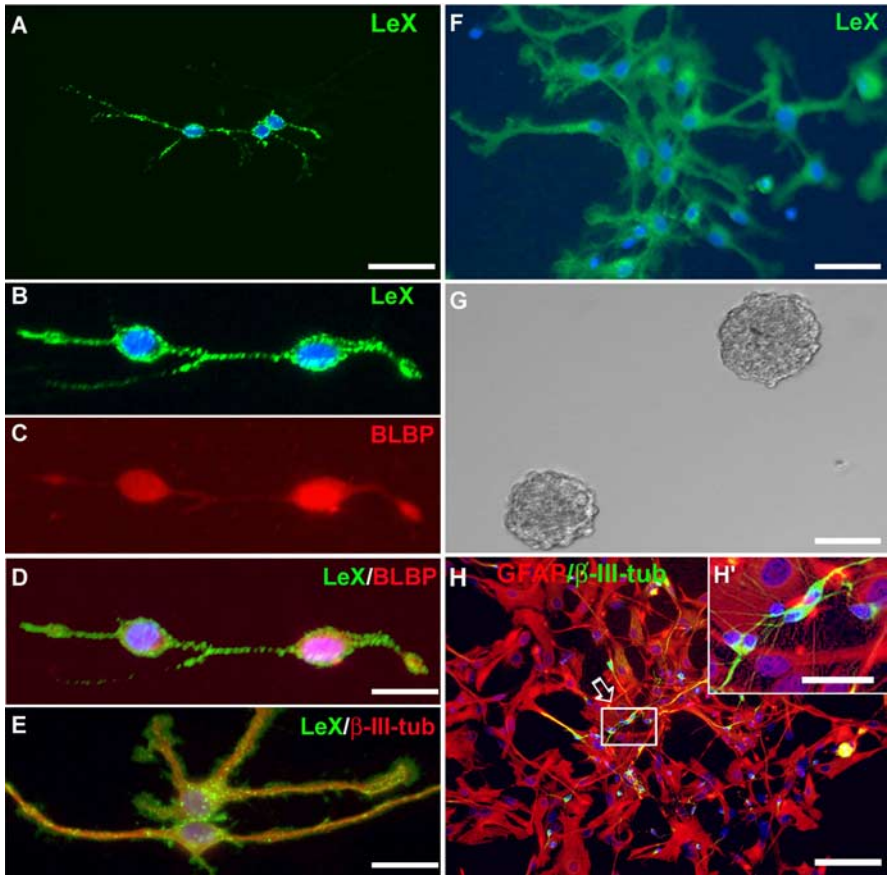

Figure 3. Expansion of LeX$^+$ cells. ***A–E***, Enriched LeX$^+$ cells from 14 gw forebrain kept in the expansion medium for 36 h. ***A***, LeX$^+$ cells (green) with nuclei stained with bisbenzimide (blue). ***B–D***, Double labeling with anti-LeX (***B***) and anti-BLBP (***C***) antibodies reveals colabeled cells (***D***). ***E***, Cells colabeled with LeX and β-III-tubulin. Note that the morphology of the cells changes in relation to antigen expression. ***F***, After 7 div in the expansion medium, the number of LeX$^+$ cells increases. ***G***, Floating neurospheres derived from cells isolated at 14 gw forebrain and kept in the expansion medium for 7 div. ***H***, In differentiation medium, neurospheres attach to the surface and differentiate mainly into GFAP$^+$ astrocytes (red) and occasionally into neurons labeled with β-III-tubulin (green). ***H′***, Higher magnification of the area labeled with an arrow in ***H***. Scale bars: ***A***, ***F***, 25 μm; ***B–E***, ***H′***, 10 μm; ***G***, ***H***, 50 μm. β-III-tub, β-III-Tubulin.

These conclusions were based on the rationale that expression of neuronal markers is a distinctive and irreversible step in the process of neuronal differentiation. However, could β-III-tubulin expression be just a transient event in the life of an astrocyte unrelated to neuronal differentiation? To address this question, we designed experiments based on a combination of immunopanning, electrical recordings, and postrecord immunolabeling. Immunopanned LeX$^+$ cultures were kept in differentiation medium for 12 d and then cells, which appeared to have neuronal morphology under infrared differential interference contrast (see Materials and Methods), were patched with rhodamine-filled pipettes. After the detection of sodium current in voltage-clamp mode (Fig. 4*A*) or fast spikelet in current-clamp configuration (Fig. 4*B*), the recording electrode was carefully pulled out (outside-out patch), leaving rhodamine-filled cells in the culture dish. Next, human cell cultures were stained with anti-β-III-tubulin antibody. All of the rhodamine-filled "sodium-positive" cells (four of four; Na^+ current in the range 50–600 pA) expressed β-III-tubulin antigen (Fig. 4*C*). These results show that LeX$^+$ and β-III-tubulin$^+$ cells are capable of firing a regenerative, short-duration spikelet (Fig. 4*B*) in response to direct current injection (+60 pA). These results are in accord with our immunolabeling experiments (Fig. 3) and suggest that LeX$^+$ cells could serve as neuronal progenitors.

Progeny of LeX$^+$ cells

To further study the progeny of LeX$^+$ progenitor cells, we next analyzed low-density cultures (2000 cells/cm^2) after 7 div in expansion medium and an additional 7 div in differentiation medium (see Materials and Methods, Fig. 1).

Characteristic clusters of lineage-restricted cells formed regularly in low-density cultures (Fig. 5*A*,*B*). We use the term "cluster" to describe groups of cells that express the same antigen (either MAP2 or GFAP). The majority of clusters in cultures from either 14 or 20 gw contained only GFAP$^+$ cells, whereas cell clusters with pure MAP2$^+$ cells (Fig. 5*B*) or mixed GFAP/MAP2$^+$ cells (Fig. 5*C*) were several times less frequent. The morphology of cells changed during 7 div in differentiation medium. For the most part, cells became large and flat and could be labeled with GFAP, consistent with their differentiation into astroglia (Fig. 5*B*). In contrast, cells not labeled with GFAP had the typical morphology of immature neurons, with a small, round cell body and fewer processes. Such cells were colabeled with a neuronal marker, MAP2, consistent with their neuronal phenotype (Fig. 5*B*). Occasionally, in mixed clusters, the same cell expressed both neuronal and glial antigens (Fig. 5*C*,*C′*).

Double labeling with a marker of dividing RGs (4A4) and doublecortin (DCX), a marker of young, migrating neurons (Francis et al., 1999), revealed that some cells coexpressed both antigens (Fig. 5*D*,*D′*). Occasionally a pair of cells, one labeled with 4A4 and the other with DCX, was still attached, as if just

Table 2. Antigenic characterization of enriched human LeX$^+$ cells after 7 div in the expansion medium

	14 gw (%)	20 gw (%)
LeX	95.1 ± 1.2	93.2 ± 1.6
BLBP	92.0 ± 1.4	94.2 ± 1.2
Vimentin	94.0 ± 1.5	91.4 ± 1.6
GFAP	95.0 ± 1.9	91.6 ± 0.9
β-III-tubulin	8.8 ± 1.2	6.3 ± 1.1
LeX/BLBP	91.2 ± 1.0	92.6 ± 1.1
LeX/vimentin	93.3 ± 1.4	91.6 ± 1.2
LeX/GFAP	90.5 ± 1.2	88.8 ± 2.1
LeX/β-III-tubulin	6.6 ± 1.0	3.8 ± 1.0*

*$p < 0.05$ (one-tailed unpaired Student's t test); for a fetus of 14 gw, mean represents three coverslips; for 20 gw fetuses, mean represents six coverslips from three specimens.

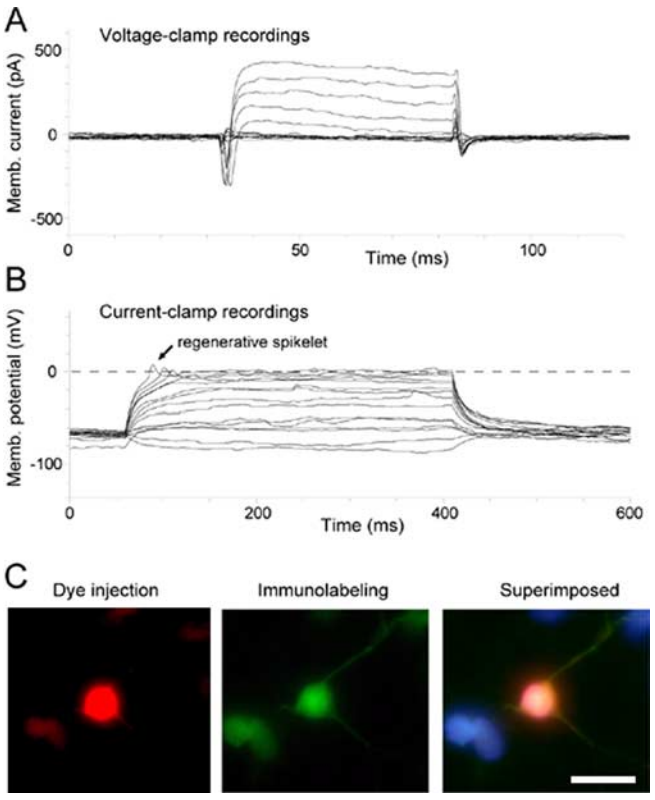

Figure 4. Generation of regenerative spikelets. ***A***, The progeny of LeX$^+$ cells exhibits transient inward currents. ***B***, The same cell as in ***A*** produces a regenerative short-duration spikelet in response to +60 pA current injection. ***C***, The same cell was injected with rhodamine (left) and immunolabeled against β-III tubulin (middle). Right, Superposition of rhodamine (red) and β-III-tubulin (green) image. Cell nuclei were stained in blue by bisbenzimide. Scale bar, 10 μm. Memb., Membrane.

completing an asymmetric cell division resulting in one neuron and one RG progenitor cell (Fig. 5*D′*). *In vivo*, double-labeled 4A4/DCX cells were also observed in the cortical VZ on cryosections of the fetal brain of a similar age (data not shown). The presence of these double-labeled cells is consistent with the notion that human RGs could generate cortical neurons.

To further confirm that some progenitors in the cultures differentiate into neurons, we used real-time RT-PCR, which allowed us to quantify mRNA levels of nestin, a well accepted progenitor marker, and neuron-specific enolase (NSE), characteristic for mature neurons. The specificity of the real-time RT-PCR products was demonstrated with gel electrophoreses, which showed a single product with the expected length (data not shown). After 7 div differentiation, the level of nestin mRNA expression was six times lower than levels obtained before differentiation (Fig. 5*E*) ($p < 0.01$). In contrast, the expression of NSE mRNA increased five times during that period (Fig. 5*E*) ($p < 0.01$), indicating that LeX$^+$ cells switched their gene expression in a process of differentiation from progenitors to neurons.

In summary, these results suggested that human fetal LeX$^+$ cells could differentiate into either glia or neurons; however, they did not address the lineage fate of an individual LeX$^+$ cell, which we next studied in a single-cell culture.

Clonal cell cultures

To study the progeny of single LeX$^+$ progenitor cells, we analyzed clonal cultures of individual LeX$^+$ cells isolated from cortical VZ/SVZ at either 14 or 20 gw (see Materials and Methods). A total of 248 cell clones were analyzed in this series of experiments. The efficiency of single cells to proliferate and establish clones was ~34%. After 7 div in expansion medium, single cells divided several times. This resulted in clones that consisted of 2–9 cells. The majority of cells in the cell clones could still be labeled with LeX antibody.

After an additional 7 div in differentiation medium, four types of clones were identified and quantified: pure GFAP$^+$ clones accounted for 67.2% (Fig. 5*F*), pure MAP2$^+$ clones made 12.8% (Fig. 5*G*), 9.5% of clones were mixed clones with a majority of GFAP$^+$ cells (Fig. 5*H*), and 6.1% were mixed clones with MAP2$^+$ cells as the major cell type (Fig. 5*I*). At 20 gw, pure GFAP$^+$ clones were more numerous, whereas the number of MAP2$^+$ clones was significantly reduced compared with 14 gw ($p < 0.05$) (Table 3). The number of cells per clone varied in respect to their antigen expression: only two to four cells in pure MAP2$^+$ clones and up to 42 cells in pure GFAP$^+$ clones were present, consistent with the known differences in proliferation capacity of glia and neurons. More glia and fewer neurons were generated at a later stage (20 gw) than at earlier stages (14 gw), suggesting that stage differences play a role in fate determination of cortical progenitor cells (Table 3).

This set of experiments further confirmed that, in the second trimester of gestation, human fetal LeX$^+$ cells in cortical VZ/SVZ represent a heterogeneous cell population that can renew itself and has a potential to generate both glia and neurons.

Cell transfection with pBLBP-EGFP and pGFAP-EGFP plasmid

As previous results showed, ~95% of enriched LeX$^+$ progenitor cells were colabeled with RG markers, but ~5% of cells were not LeX$^+$ and probably came from contamination with other cell types (Table 2). Hence, we needed to confirm more directly that cells that differentiated into neurons in our cultures are LeX$^+$ RG cells. To this aim, cell cultures were transfected with EGFP driven by RG-specific BLBP (Schmid et al., 2006) or hGFAP gene promoter (de Leeuw et al., 2006). Transfection experiments were done in mixed cell cultures and enriched LeX$^+$ cell cultures ($n = 5$). The results of these two culture systems were similar, and thus we present only the results for LeX$^+$ cell cultures. Enriched LeX$^+$ progenitor cells were cultured in expansion medium for 7 div and then transfected with the pBLBP-EGFP or pGFAP-EGFP plasmid (Fig. 1). Immunofluorescence studies showed that 12 h after transfection, 90% of transfected cells were labeled with GFAP (Fig. 6*A–C*) but not with β-III-tubulin (data not shown), suggesting that initially all transfected cells were RGs. As an additional control experiment, we also transfected enriched neuronal cultures ($n = 2$) either with the plasmids described above or CMV-GFP plasmid. In cultures transfected with pBLBP-EGFP or

pGFAP-EGFP, transfected cells were not colabeled with β-III-tubulin, whereas when cultures were transfected with CMV-GFP, transfected cells could be colabeled with both glia and neuronal markers. These results are consistent with a specificity of the hGFAP or BLBP promoter for human RGs and not for neurons.

Transfected cells were cultured for an additional 10–12 div in the differentiation medium and then used for either immunocytochemistry or electrophysiology. We determined that the vast majority of transfected (green) cells were still labeled with GFAP and thus represented astroglial lineage (Fig. 6*D–F*). Very rarely (~0.5%), green cells were colabeled with neuronal marker β-III-tubulin and had the morphology of a young neuron (Fig. 6*G–I*). One concern was that the promoter activity may become nonfunctional when RGs begin differentiating into neurons. However, the EGFP protein remained in transfected cells for several days after differentiation. Next, to provide the electrophysiological characterization of transfected cells, we used whole-cell patch-clamp recordings.

Sodium current in cultured human cells

Electrical membrane properties were analyzed in a total of 97 cultured cells harvested from four human fetuses at gestational weeks 20 ($n = 1$), 21 ($n = 2$), and 23 ($n = 1$). We first determined the properties of isolated LeX$^+$ cells *in vitro*. Enriched LeX$^+$ cells were grown in expansion medium for 7 div and then immunolabeled live with LeX antibody (before recordings). Live immunolabeling indicated that >90% of the cells in the cultures were LeX$^+$. Whole-cell patch recordings performed on these live-immunolabeled LeX$^+$ cells ($n = 7$) revealed typical RG characteristics (Noctor et al., 2002) with outward potassium currents and no presence of inward sodium currents (Fig. 7*B*).

In a separate series of experiments, immunopanned LeX$^+$ cells were allowed to differentiate for an additional 7 div in differentiation medium. A smaller number of cells were immunolabeled with LeX antibody after 7 div of differentiation (50%; compare with 90%), probably because LeX antigen is downregulated in a process of differentiation to lineage-specific cells. Immunolabeled LeX$^+$ cells displayed only outward potassium currents with no detectable sodium currents ($n = 5$). It is worth noting that a considerable fraction of nonimmunolabeled cells (three of five) in the same culture dish had traces of inward sodium current (peak sodium current = 39 ± 5.48 pA).

Because LeX appears to be downregulated after differentiation, a second set of experiments was performed on pBLBP-EGFP- or pGFAP-EGFP-transfected and -nontransfected human cells cultured for up to 17 d in differentiation medium. Nontransfected cells were selected for recordings based on morphology consistent with young neurons, including small diameter (5–15 μm), oval or ovoid-shaped cell body with two or more extended process, and smooth appearance of the somatic membrane. In

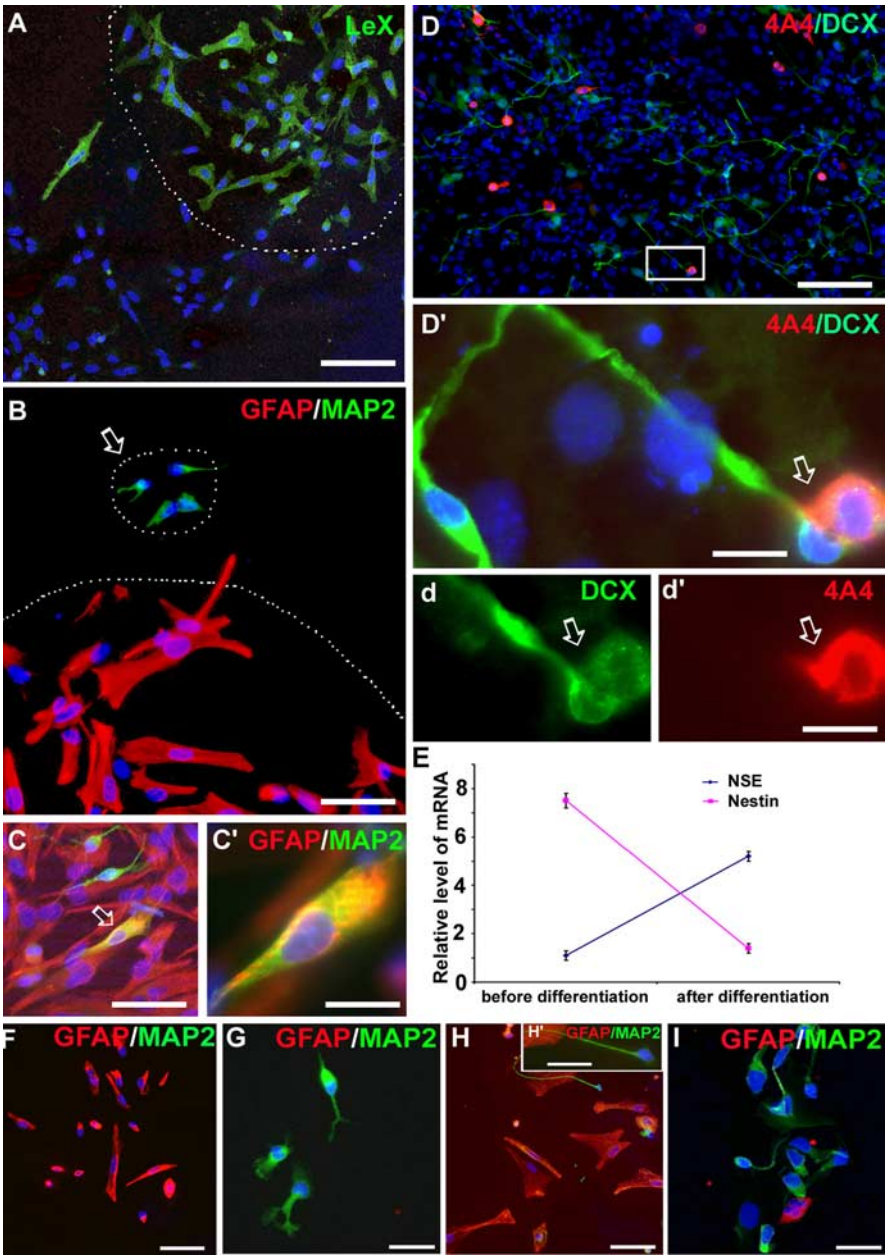

Figure 5. Differentiation of LeX$^+$ cells. ***A***, A cluster of LeX$^+$ cells (encircled) forms in low-density cultures after 14 div. ***B***, Two clusters, one containing large and flat cells labeled with GFAP (red) and the other containing only four MAP2$^+$ neurons (encircled, arrow). ***C***, An example of a mixed cell cluster; occasionally, cells express both GFAP and neuronal marker MAP2 (arrow). ***C′***, Higher magnification of the double-labeled cell. ***D***, Immunostaining with 4A4 antibody (red), a marker of dividing RG cells, and neuronal marker DCX (green), in an LeX$^+$ cell culture after 7 div in expansion and 1 div in differentiation medium. ***D′***, Higher magnification of the boxed area in ***D***. A dividing 4A4$^+$ RG cell and the attached DCX$^+$ neuron with a long process (arrow) are shown. ***d***, ***d′***, Single channels from the same field. ***E***, Real-time RT-PCR shows that nestin mRNA expression is six times lower ($p < 0.01$) after differentiation, whereas the expression of NSE mRNA increased five times ($p < 0.01$). ***F–I***, Clonal analysis reveals that a single LeX$^+$ cell generates clones which consist of only GFAP$^+$ cells (***F***), only MAP2$^+$ cells (***G***), mixed clones with a majority of GFAP$^+$ cells and a small number of MAP2$^+$ cells (***H***; inset, enlarged MAP2$^+$ cell), or mixed clones with a majority of MAP2$^+$ cells (***I***). In all images, cell nuclei are stained with bisbenzimide (blue). Scale bars: ***A–C***, ***G–I***, 25 μm; ***D***, ***F***, 50 μm; ***C′***, ***D′***, ***d***, ***d′***, ***H′***, 10 μm. Error bars represent SEM.

Table 3. Percentage of clones from immunopanned LeX$^+$ cells at two gestational ages

	14 gw (%)	20 gw (%)
GFAP	67.2 ± 5.7	82.3 ± 7.7
MAP2	12.8 ± 1.5	6.6 ± 0.9*
GFAP major/MAP2	9.5 ± 1.3	4.5 ± 1.8
MAP2 major/GFAP	6.1 ± 0.8	4.2 ± 1.4

*$p < 0.05$ (one-tailed unpaired Student's t test). Values for 14 gw represent three experiments from one case; for 20 gw, values represent seven experiments from three cases (mean ± SEM).

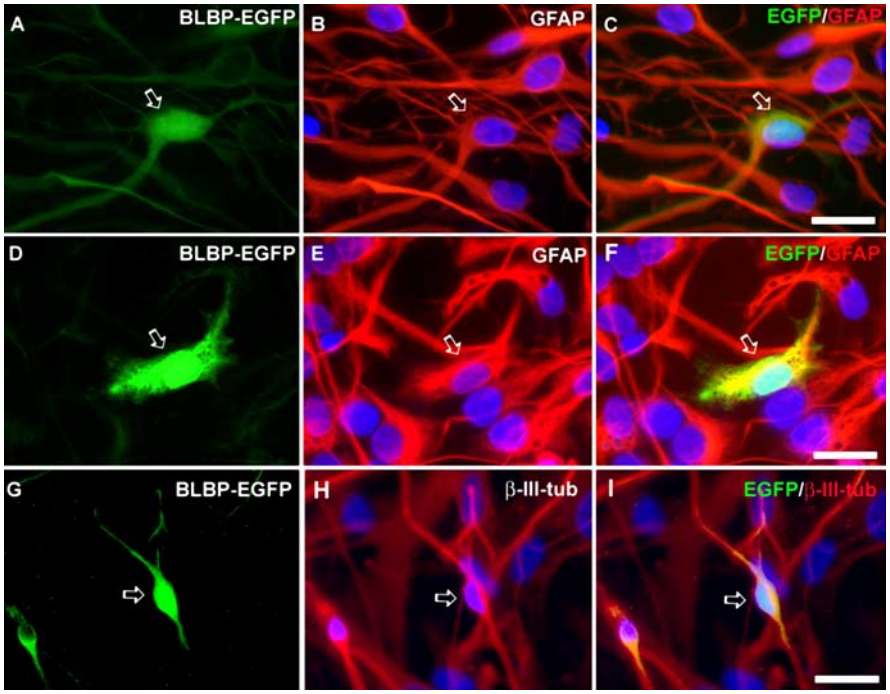

Figure 6. Transfection of RG cells with BLBP-EGFP plasmid. ***A–C***, In the expansion medium, a transfected cell with bipolar morphology (green) is colabeled with GFAP (red, arrow). ***D–I***, In the differentiation medium, the morphology of the green cells changes, as they become either typical astrocytes labeled with GFAP (***D–F***) or occasionally neurons labeled with β-III-tubulin (***G–I***). Scale bars, 10 μm. β-III-tub, β-III-tubulin.

addition to these criteria, the transfected cells were bright green under epifluorescent light (Fig. 7*A*, right). Approximately 40% of nontransfected cells (n = 19/49) and 13% of transfected cells (n = 4/31) showed traces of inward transient current (Fig. 7*C*,*D*). We determined that TTX completely abolished the fast transient current without having any effect on the outward component (n = 3; data not shown). Peak amplitudes of sodium currents in transfected cells were considerably smaller than those obtained in nontransfected cells (Fig. 7*C*,*D*, arrows). To determine whether the transfection procedure affected the overall health of the cells, we evaluated the inward sodium currents in parallel with outward transmembrane currents (Fig. 7*E*). The average outward currents obtained in 31 transfected and 49 nontransfected cells (254 ± 54.9 and 326 ± 59.5 pA, respectively) were not significantly different (p = 0.3798). However, there was a statistically significant difference in the size of the peak sodium current between transfected and nontransfected cells (30 ± 9.98 and 206 ± 63.8 pA, respectively; p = 0.0126).

Because neurons have been shown to undergo developmental changes *in vitro* (Luskin et al., 1997, Carpenter et al., 1999), we systematically analyzed LeX$^+$ cells, transfected with pBLBP-EGFP, obtained from one human fetus (20 gw) over the course of 17 d. As the cells matured *in vitro*, there was a distinct difference between transfected and nontransfected cells. Whereas nontransfected cells began expressing sodium current starting at 10 div, transfected cells started showing sodium current at 16 div, and that current was smaller by an order of magnitude (e.g., 50 pA vs 450 pA). Despite the smaller size in peak sodium current, these results indicate that a subset (~13%) of transfected LeX$^+$ (RG) cells has membrane properties characteristic of young neurons (Feldman et al., 1996; Bittman et al., 1997; Noctor et al., 2001).

BLBP-Cre/Floxed-YFP transfection

As the BLBP-EGFP- or hGFAP-EGFP-transfected cells differentiated into neurons, the RG-specific promoters were downregulated. Only through the remaining GFP in the differentiated cells did we observe some immature neurons derived from RGs. To investigate whether mature neurons could be traced to RG origin, we cotransfected the LeX$^+$ cells with BLBP-Cre and CAGGS-loxP-LacZ-loxP-YFP (β-actin based promoter) plasmids, which allowed us to follow differentiation of RG progeny. Twelve hours after transfection, YFP signal was displayed only in GFAP-labeled cells (Fig. 8*A–C*) and not in GFAP-negative cells (data not shown).

The transfected RG cells gradually differentiated from GFAP$^+$ to β-III-tubulin$^+$ young neurons (Fig. 8*D–F*) and finally NeuN$^+$ mature neurons (Fig. 8*G–I*), which is consistent with the idea that RGs could differentiate into mature cortical neurons. As a control, the cells transfected with CAGGS-loxP-LacZ-loxP-YFP can only be visualized by detecting LacZ activity but not by the expression of YFP.

Effects of microenvironment on the differentiation of LeX$^+$ cells

Differentiation of progenitor cells is affected by intrinsic and extrinsic factors, and it is thus important to determine how the neurogenic properties of cortical LeX$^+$ cells are affected by their microenvironment. To this aim, LeX$^+$ cells enriched from either 14 or 20 gw fetal forebrains were genetically labeled with the retrovirus NIT-GFP, resulting in infected cells that fluoresced green. These cells were plated over cell cultures from the cortical VZ/SVZ (corticocortical cultures) or from the ganglionic eminence (cortico-GE cultures) of the same fetal brain.

After 7 div in the differentiation medium, infected LeX$^+$ cells could be double labeled with GFAP (Fig. 9*A–C*) β-III-tubulin (Fig. 9*D–F*), or calretinin (CalR), a marker of interneurons (Fig. 9*G–I*). The number of neurons generated from infected LeX$^+$ cells was correlated to the stage of development and the CNS region. At 14 gw in the VZ/SVZ cultures, only 6% of green cells were β-III-tubulin$^+$ neurons, whereas almost three times more neurons (16%) were demonstrated in the GE cultures (Fig. 9*J*). This suggests that at 14 gw, the GE microenvironment was more efficient in promoting neurogenic properties of RGs than the cortical VZ/SVZ. At 20 gw, the percentage of green cells colabeled with β-III-tubulin decreased in GE cultures and increased in the VZ/SVZ cultures (Fig. 9*K*) compared with 14 gw. Interneurons,

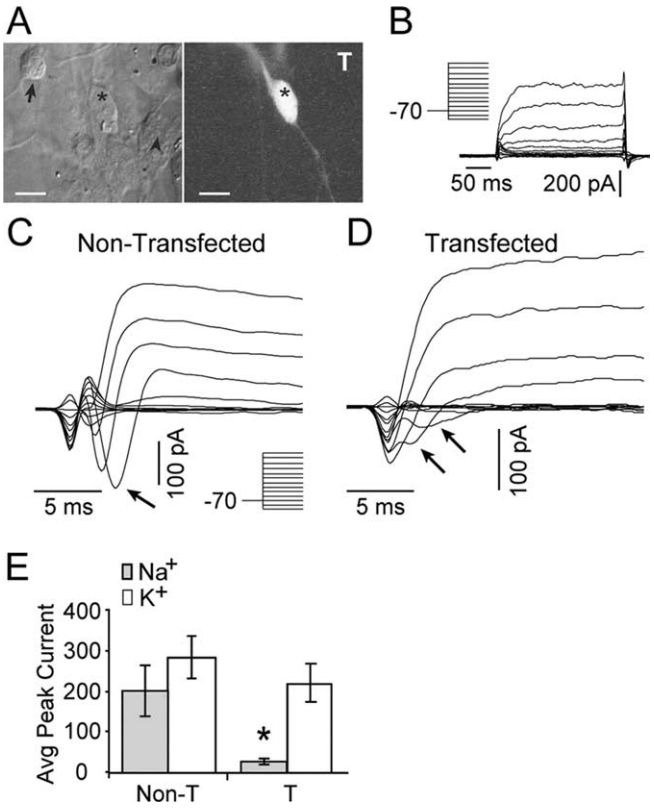

Figure 7. Transfected cells express sodium current after differentiation. ***A***, Microphotograph of a transfected cell (asterisk) captured under infrared light (left) and epifluorescent light (right). A nontransfected cell is marked by an arrow, and an astrocyte is marked by an arrowhead. Scale bars, 10 μm. ***B***, The majority of cells in our dataset exhibited zero inward current in response to a series of voltage steps (-90 to $+30$ mV from a holding potential of -70 mV). A small fraction of nontransfected (***C***) and transfected (***D***) cells do, however, demonstrate traces of transient inward TTX-sensitive sodium current (arrows). ***E***, Average (Avg) peak sodium (gray) and potassium (white) currents measured in nontransfected (Non-T) and transfected (T) cells. There is a statistically significant difference in the size of sodium current (*$p = 0.0126$), but not in potassium current ($p = 0.3798$). Error bars represent SEM.

labeled with CalR, were generated from green cells cultured in either coculture settings (Fig. 9*G–I*), but the percentage of generated interneurons also depended on the region and developmental age. In GE cultures, up to 50% of neurons generated from green cells were CalR$^+$ interneurons, whereas in the cortical VZ/SVZ cultures, the percentage of CalR$^+$ cells was <20% at either age (Fig. 9*K*). The remaining β-III-tubulin$^+$/CalR$^-$ neurons probably represent future pyramidal neurons and/or possibly some other type of interneurons (e.g., parvalbumin or cholecystokinin-positive interneurons). The highest number of interneurons was generated from cocultured green cells in the GE at 14 gw. Thus, the determination of LeX$^+$ cell fate demonstrates a dependence on both spatial and temporal factors.

Our preliminary data suggested that the growth factors EGF and FGF2 may play vital roles in the neurogenesis of LeX$^+$ cells. We studied the expression of EGF and FGF2 mRNA in the cell cultures using RT-PCR. At 14 gw, both the levels of EGF and FGF2 mRNA were higher at GE than VZ/SVZ cell cultures. In contrast, at 20 gw, the level of EGF mRNA was higher in the cortical VZ/SVZ than in GE cultures, whereas the level of FGF2 mRNA was the same in the two regions (Fig. 9*L*). Hence, EGF and FGF2 were elevated in cultures that had a higher level of neurogenic RGs, suggesting that these growth factors may have a role in neurogenesis from RG cells in a region- and stage-specific manner.

LeX$^-$ population of cells

To determine whether other progenitor subclasses are present in a population of cells that remained after immunopanning with LeX antibody, named here the LeX$^-$ population, we cultured these cells under the same culture conditions as the LeX$^+$ cells. Immediately after isolation, one-third (31%) of LeX$^-$ cells were labeled with the neuronal marker β-III-tubulin, whereas BLBP$^+$ and vimentin$^+$ cells accounted for 34 and 21% of cells, respectively, and <10% of cells were LeX$^+$. Thus, according to antigen expression, the LeX$^-$ cultures contained three times less RG cells and five times more cells labeled with the neuronal marker than the LeX$^+$ cell cultures. Surprisingly, in mixed, LeX$^+$, or LeX$^-$ cell cultures, a number of β-III-tubulin$^+$ cells were dividing, as demonstrated with double labeling with Ki67, a cell proliferation marker (Schluter et al., 1993). Importantly, the number of dividing β-III-tubulin cells was four times larger in the LeX$^-$ than in the LeX$^+$ cell cultures, 21% (51/245) and 5% (11/220), respectively (Fig. 10, compare *A*, *B*). Proliferating β-III-tubulin$^+$ cells support the notion that they are still progenitor cells, and by antigen expression, they appear to be neuron-restricted progenitors. This result was corroborated by double immunolabeling with 4A4 antibody and another neuronal marker, DCX. As expected, in LeX$^-$ cultures far fewer cells expressed the dividing RG marker 4A4, and none of the numerous DCX$^+$ cells coexpressed 4A4, in contrast to LeX$^+$ cultures (Fig. 5*D′*). The difference in the number of proliferating DCX$^+$ cells between LeX$^-$ and LeX$^+$ cultures was confirmed with BrdU labeling. Almost one-third (28%) of the DCX$^+$ cells in the LeX$^-$ cultures incorporated BrdU (Fig. 10*B*), in contrast to the LeX$^+$ cultures, which not only contained fewer DCX$^+$ cells, but only 3% of these DCX$^+$ cells incorporated BrdU (Fig. 10*D*). A far larger percentage of dividing DCX$^+$ cells combined with the low number of RG cells in LeX$^-$ cultures suggests that these proliferating DCX$^+$ cells in LeX$^-$ cultures represent a subtype of neuron-restricted progenitors. Consistent with these findings, the frequency of neurosphere formation was lower in LeX$^-$ cell cultures (18%) than in LeX$^+$ cell cultures (40%), suggesting again that in RG-rich LeX$^+$ cultures, the majority of cells were multipotent progenitors, which were more likely to form neurospheres (Capela and Temple, 2006). In contrast, in LeX$^-$ cultures, the majority of cells were lineage-restricted progenitors that rarely form neurospheres.

Discussion

Evidence collected here by multiple methods suggests that cortical neurons in the human cerebral cortex are generated from various subpopulations of cortical progenitors, including LeX$^+$ RGs and neuron-restricted progenitors. The RG origin of human cortical neurons is consistent with results in the mouse, in which most if not all pyramidal neurons originated from RGs or their ancestors. In contrast to mouse, it seems that RGs in human do not generate all, or even the majority, of cortical neurons at the gestational age we studied. An additional progenitor subtype, restricted neuronal progenitors, is contributing to neuronal population of the human cerebral cortex.

Multipotent progenitor cells

Studies in rodents (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001, 2002, 2004; Tamamaki et al., 2001) and humans (Weissman et al., 2003) suggested that in the cortical proliferative zone, RGs are the sole proliferating cells to give rise to projection neurons. In contrast to these views, previous studies, from the classical ones of His (1887) and Cajal (1911) to more recent ones (Levitt et al., 1981; Grove et al., 1993; Tan et al., 1998;

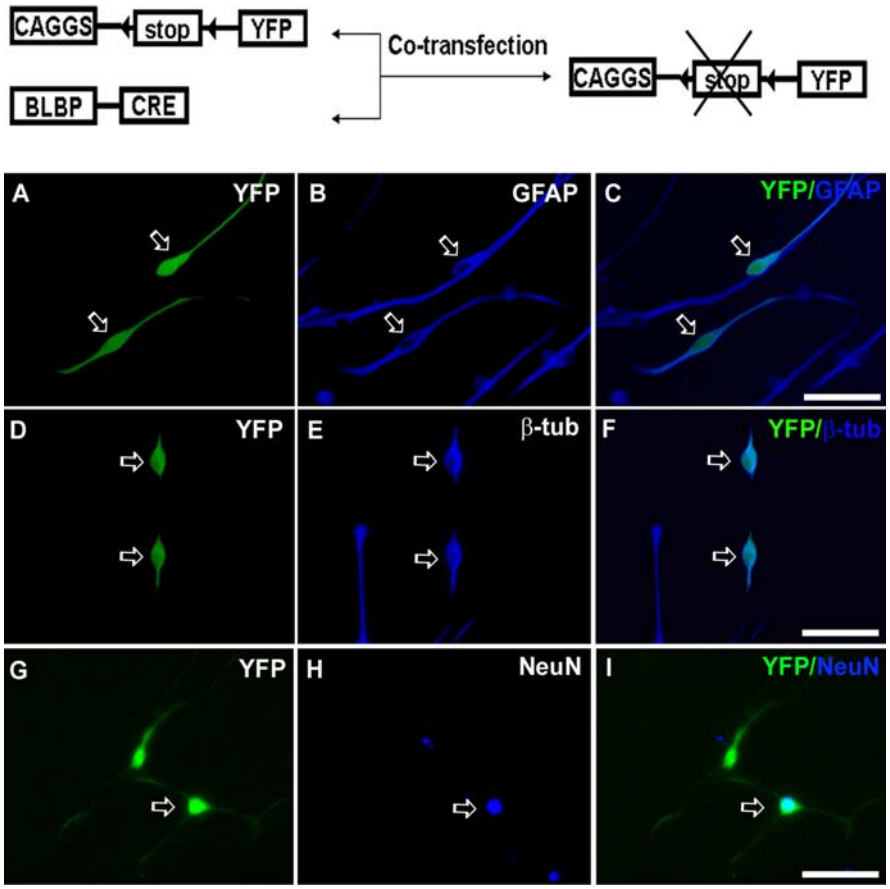

Figure 8. The schematic diagram of the pCAGGS-LoxP-LacZ-LoxP-YFP and BLBP-Cre [LoxP sites are indicated by triangles]. ***A–C***, Twelve hours after transfection, cells cultured in the expansion medium have bipolar morphology, and all transfected cells are colabeled with GFAP. ***D–F***, After 3 div in differentiation medium, some of the transfected cells express immature neuron marker β-III-tubulin. ***G–I***, After 7 div in differentiation medium, transfected cells are colabeled with mature neuronal marker NeuN. Note that YFP appears green on a green channel (wave length, 488 nm). Scale bars, 20 μm. β-tub, β-III-Tubulin.

Zecevic, 2004; Howard et al., 2006, Maric et al., 2007), argue that different classes of progenitors in the cortical VZ proliferate simultaneously during corticogenesis. Studies in primates, however, were based on differential immunolabeling, and so far the origin of human cortical neurons has not been demonstrated directly.

In this study, cortical progenitors were enriched using a surface marker LeX/CD15, from the human forebrain at the second trimester of gestation. We confirmed that the majority of isolated LeX$^+$ cells could be colabeled with known RG markers (Choi and Lapham, 1978; Dahl et al., 1981; Feng et al., 1994; Kamei et al., 1998) and had a typical elongated morphology, qualifying them as a subset of the RG cell population (Mai et al., 2003). Although almost all LeX$^+$ cells express RG markers, not all RGs expressed LeX, which is in accord with described heterogeneity of RG antigen profile in both rodent (Hartfuss et al., 2001; Malatesta et al., 2003) and human fetal brain (Howard et al., 2006).

Human LeX$^+$ cells *in vitro* were highly prolific progenitor cells that formed neurospheres and differentiated mainly into astroglia, and less often into neurons, similar to what has been reported in the mouse (Abramova et al., 2005; Capela and Temple, 2006; Liour et al., 2006). Clonal analysis confirmed heterogeneity of human fetal LeX$^+$ cell population and showed that some of them are cell-type restricted and differentiate into either neurons or glia, whereas others were multipotent and gave rise to mixed clones containing both neurons and glia.

Because we were primarily interested in whether RGs generate cortical neurons, we transfected RG cells and followed their progeny *in vitro*. Previous electrophysiological studies reported that undifferentiated RGs have low membrane resistance and no voltage-gated sodium conductance, but as they differentiate into young neurons, voltage-gated sodium currents and a higher membrane resistance appeared (LoTurco and Kriegstein, 1991; Bittman et al., 1997; Noctor et al., 2001). Studies performed here on human cells harvested at 20–23 gw demonstrated that a fraction of human LeX$^+$ RG cells (13%) transfected with BLBP-EGFP displayed a detectable transient inward current sensitive to the sodium channel blocker TTX, suggesting their differentiation into neurons. Cotransfection experiment with BLBP-Cre and CAGGS-loxP-LacZ-loxP-YFP plasmids confirmed that RGs can differentiate into NeuN$^+$ mature neurons.

Moreover, we observed a small number of cells that were double labeled with specific RGs and neuronal markers, similar to previous reports (Skogh et al., 2001; Zecevic, 2004; Howard et al., 2006; Kim et al., 2006). These cells may represent multipotent progenitors, neurogenic RG cells, or intermediate progenitors (IPs) on their way to differentiate into neurons (Miyata et al., 2004; Noctor et al., 2004; Englund et al., 2005; Martinez-Cerdeno et al., 2006). It is likely that IPs contribute to the total cell population that we observed in the SVZ cultures, because this zone is a major proliferative region in primate brains in the later stages of corticogenesis studied here (Smart et al., 2002; Zecevic et al., 2005).

Both electrophysiological and immunofluorescence findings indicate that human fetal RGs at this developmental stage generate mainly astrocytes and also a small but significant percentage of cortical neurons.

Neuron-restricted progenitors

A substantial population of dividing cells in the fetal VZ/SVZ cultures was labeled with the neuronal markers β-III-tubulin or DCX. Comparable proliferating cells labeled with neuronal markers were identified in both the human fetal (Piper et al., 2001, Howard et al., 2006) and murine cortex (Haubensak et al., 2004; Gal et al., 2006).

These dividing cells could represent IPs known to be dedicated neuronal progenitors, or they might represent a separate subclass of neuron-restricted progenitors. It is difficult to exclude the possibility that neuron-restricted progenitors were not generated in an earlier time point from RGs. However, several lines of evidence are consistent with the presence of at least two progenitor subtypes, neuron-restricted progenitors and RGs. First, adjacent proliferating cells in the embryonic ventricular zone at the onset of human corticogenesis were immunolabeled either with RGs or neuronal markers (Howard et al., 2006). Second, in this study, LeX$^-$ cultures had three times more neuronal restricted progenitors and only a small percentage of RGs and mul-

tipotent progenitors. In LeX$^-$ cultures, neurospheres were less likely to form both in mice (Capela and Temple, 2006) and humans (this study), consistent with the notion that they contained more lineage-restricted progenitors. Finally, the heterogeneity of human cortical progenitor cell population, suggested here, is well correlated with results from other laboratories on human fetal stem cells (Carpenter et al., 2001; Suslov et al., 2002; Messina et al., 2003; Kim et al., 2006) or human fetal forebrain (Piper et al., 2001).

Heterogeneity of cortical progenitors has been described also in other mammals, and thus it is not characteristic only for primates. Neural stem cells in rodents include temporally and regionally restricted subpopulations from the very beginning of corticogenesis (Grove et al., 1993; Tan et al., 1998; McCarthy et al., 2001; Temple, 2001; Capela and Temple, 2006, Maric et al., 2007). In the mouse VZ, two precursor subclasses, distinct neuronal precursors and RG cells, were discerned using *in utero* electroporation with different reporter constructs (Gal et al., 2006). In the Tis21-GFP knock-in mouse, a novel neuron-specific progenitor population at the basal border of the VZ was described (Haubensak et al., 2004).

Fate determination of RG progeny

It has been described that both intrinsic and extrinsic factors probably influence fate determination of RG progeny (Fishell, 1995; Hartfuss et al., 2001; Götz et al., 2002; Malatesta et al., 2003; Anthony et al., 2004).

Another level of complexity is that regional identities of progenitors are stage dependent. Mouse cortical progenitors produce diverse cell types according to a precise schedule, with generation of neurons early followed by generation of glia in later stages of development (Qian et al., 1998, 2000; Abramova et al., 2005). In agreement with this, our clonal study showed that individual LeX$^+$ cells generated predominantly gliogenic clones, as was expected at the developmental stage studied here when neurogenesis was slowing down (Sidman and Rakic, 1973). The number of neuronal clones was higher at 14 gw than at 20 gw, following the predicted timetable of generation of neurons first and glia later. In either age, a relatively small percentage (9–15%) were mixed clones, similar to results reported by others (Parnavelas et al., 1991; Luskin et al., 1993; McCarthy et al., 2001), indicating that multipotent progenitors still exist in a relatively late stage of human corticogenesis.

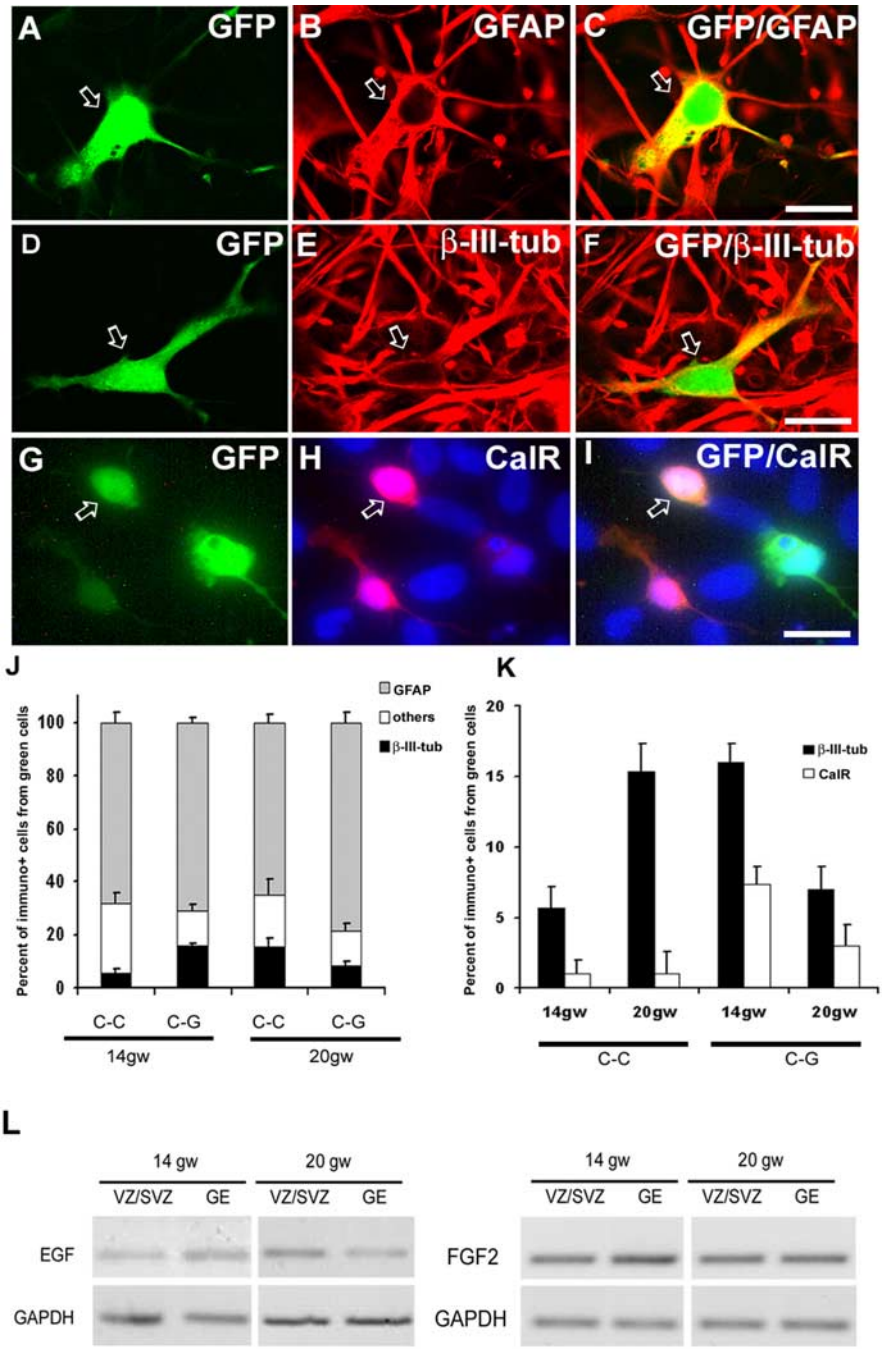

Figure 9. Coculture experiments. ***A–F***, When LeX$^+$ cells enriched from cortical VZ/SVZ at 14 gw are infected with NIT-GFP retrovirus and cocultured with cells from cortical VZ/SVZ (C-C) from the same fetal brain, they differentiate into astroglia cells that are colabeled with GFAP (***A–C***, arrows) or into neurons and are colabeled with β-III-tubulin (***D–F***, arrows). ***G–I***, Some infected LeX$^+$ cells from cortical VZ/SVZ cultured with GE are colabeled with interneuron marker calretinin (CalR; red, arrow). Scale bars, 10 μm. ***J***, Quantification of the progeny of NIT-GFP-labeled LeX$^+$ cells. Note that more neurons (black bars) are generated at 14 gw when infected LeX$^+$ cells are cocultured with GE (C-G), but at 20 gw, more neurons are produced in the coculture with cortical VZ/SVZ (C-C). ***K***, The graph depicts the percentage of CalR$^+$ (white bars) and β-III-tubulin$^+$ cells (black bars) derived from NIT-GFP-labeled LeX$^+$ cells in the coculture experiments. The highest number of CalR$^+$ cells is demonstrated at 14 gw in GE coculture. ***L***, The mRNA expression of EGF (left) and FGF2 (right) revealed by RT-PCR confirms that the level of EGF is higher in GE at 14 gw and in VZ/SVZ at 20 gw. The level of mRNA FGF2 is also higher in GE at 14 gw and equal in both regions at 20 gw. β-III-tub, β-III-Tubulin; immuno+, immunopositive. Error bars represent SEM.

Coculture experiments suggested that both the stage of development and the local environment play a role in determining RG progeny in the human brain. At the earlier age (14 gw), more neurons were generated when infected LeX$^+$ cells were cocultured with GE, but at the later age (20 gw), more neurons were produced in the coculture with cortical VZ/SVZ. This pattern is

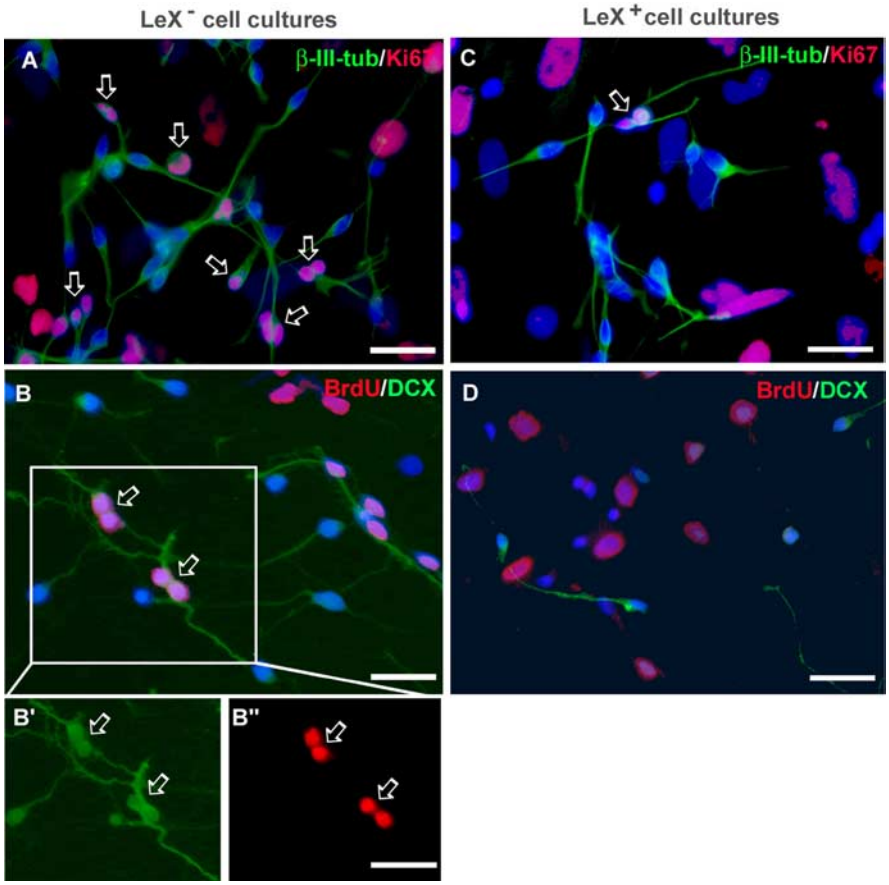

Figure 10. Dividing neuronal progenitors. ***A***, ***B***, LeX$^-$ cell cultures. ***A***, In LeX$^-$ cultures in the expansion medium, numerous β-III-tubulin-labeled cells (green) are proliferating, as indicated by their colabeling with Ki67 (red, arrows). ***B***, Under the same *in vitro* conditions, numerous DCX$^+$ cells (green) are observed, and several of them incorporate BrdU (red, arrows). ***B′***, ***B″***, Single channels of the boxed area in ***B***. Closely juxtaposed pairs of daughter cells indicate cell proliferation (arrows). ***C***, ***D***, LeX$^+$ cell cultures. ***C***, In LeX$^+$ cultures, dividing β-III-tubulin-labeled cells are very rare (arrow). ***D***, The number of DCX$^+$ neurons is small compared to LeX$^-$ cultures, and BrdU rarely incorporates in these cells (red). Scale bars, 25 μm. β-III-tub, β-III-Tubulin.

in accord with results obtained in the animal preparation (Anthony et al., 2004).

Our results showed that regional cues can influence not only the total number but also the type of neurons generated from neurogenic RGs (Skogh et al., 2001; Hall et al., 2003). Considerably more calretinin$^+$ interneurons were generated from LeX$^+$ cells cocultured with the GE (50%) than with cortical cocultures (20%). Accordingly, progenitors from cortical VZ/SVZ were observed to generate interneurons not only in GE but also in the cortical environment (Letinic et al., 2002; Rakic and Zecevic, 2003). The cell population labeled with β-III-tubulin and not with calretinin probably represents pyramidal neurons and/or various other interneuronal subtypes.

The environmental influence on the neurogenetic potential of LeX$^+$ RG cells was correlated with the level of growth factors, particularly EGF and to some extent FGF2. This is in line with the reported effects of these growth factors on neural stem and progenitor cells in animal and human studies (Vescovi et al., 1993; Qian et al., 1998; Tropepe et al., 1999; Vaccarino et al., 1999; Sun et al., 2005, Maric et al., 2007).

In conclusion, establishing an *in vitro* system of human fetal cortical progenitors allowed the use of a combination of experimental approaches previously possible only with animal models. These experiments revealed that a small subset of human fetal RG cells is able to generate cortical neurons and that these neurogenic abilities of RGs are region and stage dependent and could be modified by the environment. In addition to neurogenic RGs, a population of restricted neuronal progenitors contributed substantially to the generation of cortical neurons during midgestation. These results emphasize that a complex variety of cortical precursors with neurogenic potentials may be necessary for the proper development of the human cerebral cortex.

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