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Generation of human cortical neurons from a new immortal fetal neural stem cell line

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Abstract

Isolation and expansion of neural stem cells (NSCs) of human origin are crucial for successful development of cell therapy approaches in neurodegenerative diseases. Different epigenetic and genetic immortalization strategies have been established for long-term maintenance and expansion of these cells *in vitro*. Here we report the generation of a new, clonal NSC (hc-NSC) line, derived from human fetal cortical tissue, based on v-myc immortalization. Using immunocytochemistry, we show that these cells retain the characteristics of NSCs after more than 50 passages. Under proliferation conditions, when supplemented with epidermal and basic fibroblast growth factors, the hc-NSCs expressed neural stem/progenitor cell markers like nestin, vimentin and Sox2. When growth factors were withdrawn, proliferation and expression of v-myc and telomerase were dramatically reduced, and the hc-NSCs differentiated into glia and neurons (mostly glutamatergic and GABAergic, as well as tyrosine hydroxylase-positive, presumably dopaminergic neurons). RT-PCR analysis showed that the hc-NSCs retained expression of Pax6, Emx2 and Neurogenin2, which are genes associated with regionalization and cell commitment in cortical precursors during brain development. Our data indicate that this hc-NSC line could be useful for exploring the potential of human NSCs to replace dead or damaged cortical cells in animal models of acute and chronic neurodegenerative diseases. Taking advantage of its clonality and homogeneity, this cell line will also be a valuable experimental tool to study the regulatory role of intrinsic and extrinsic factors in human NSC biology.

Key words: Neural stem cells, cerebral cortex, fetal, immortalization, v-myc, differentiation, proliferation, telomerase, growth factor.

Introduction

Recent progress in stem cell research suggests that neurons suitable for intracerebral transplantation can be generated from *in vitro* expanded stem cells isolated from different sources, and that stem cell-based therapy could be a future strategy for the treatment of neurodegenerative disorders (for review see [1-3]. Multipotent neural stem cells (NSCs) have been identified and isolated from mammalian central nervous system [4-6] including embryonic and adult human brain [7-15]. However, human NSC lines which could be useful in clinical setting are difficult to establish *in vitro* and, several different approaches have been developed to overcome this problem. One of them is based on the epigenetic propagation of NSCs, when they are grown as free-floating aggregates, so-called neurospheres, and are kept proliferating by adding growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and/or leukemia inhibitory factor (LIF) [7; 9; 10]. An alternative approach combines genetic and epigenetic immortalization strategies: cells are transduced with an immortalizing gene (the best one being v-myc) by means of replication deficient retroviral vectors, and their proliferation is also supported with growth factors. Several lines of evidence indicate that transcription factors, such as Myc family members and their target genes, could be importantly involved in the maintenance of “stemness” of cells. It has been described that Myc-dependent mechanisms are involved in the control of the pluripotency and self-renewing capability of murine embryonic stem cells [16] and neural progenitor cells [17; 18]. Several human NSC lines have been produced from whole human fetal brain or telecephalic region using the DNA sequence of the v-myc gene isolated from the avian myelocytic leukemia virus [13; 14; 19]. Recently, a new stem cell line was generated from human fetal cortical tissue by a conditional immortalizing approach [20]. The immortalizing transgene encodes a fusion protein comprising a growth promoting gene, c-myc, and a tamoxifen-regulated hormone receptor. This stem cell line gave rise to β III-

tubulin- and MAP2-immunoreactive cells *in vitro*, but the specific neuronal phenotypes acquired by these cells after *in vitro* differentiation or *in vivo* transplantation were not been investigated. The same fusion protein was not useful on human forebrain and midbrain progenitors, whilst v-myc readily immortalized human NSCs from both regions (Villa *et al.*, unpublished).

Identification of appropriate sources of human NSCs for isolation and expansion remains a crucial step in the effort to develop cell transplantation strategies for neurological disorders. Characterization of the self-renewal capacity of these cells as well as the assessment of their ability to generate different neuronal phenotypes should first be performed *in vitro*. Culture systems also allow for testing the cellular properties of the isolated lines, and for characterizing the different factors and conditions which could affect the fate of the expanded cells. In the next step, the cells should be tested *in vivo* in animal models. After intracerebral transplantation, the cells might behave differently as compared to the *in vitro* conditions depending on implantation site and local environment.

In this paper, we describe the generation of human somatic NSC lines, isolated from 8-9 weeks old fetal cortical tissue, and immortalized by a v-myc-based strategy. One of these clonal cell lines (named hc-NSC-F7b) has been maintained in culture for more than 50 passages, with an estimated population doubling time of about 24-28 h, and shown self-renewal capacity without any alteration in proliferation rate. This cell line also has the capacity to generate different types of neurons with properties characteristic of those in adult human cerebral cortex.

Material and methods

Isolation and immortalization of human fetal cortical neural stem cells

Human cells were derived from 8-9 weeks old fetal cortex. Brain tissue from aborted human fetuses was obtained from Lund University Hospital and Malmö Academic Hospital after informed consent of the women seeking abortion and in accordance with EU directives, NECTAR recommendations, and ethical guidelines set by the Lund/Malmö Ethical Committee. The cells were either immortalized by a genetic approach based on v-myc expression or expanded by treatment with bFGF and EGF. Immortalization was performed by infecting the cells with a retroviral vector coding for p110^{gag-myc} as previously described [14]. Briefly, dissected tissue was washed twice in HBSS (without Ca/Mg), re-suspended in HBSS containing 0.4 mg/ml of papain (Roche) and 0.05% w/v DNase (Sigma), and then incubated for 15 min at 37°C. Digested tissue was triturated by using fire-polished Pasteur pipettes. After 5 min of centrifugation at 1000 rpm, the cell pellet was resuspended in HBSS containing DNase, and centrifuged again. Cells were then plated at 100 000 cells/cm² in poly-L-lysine-coated plastic flasks (10 µg/ml in PBS) in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12; Gibco) containing EGF and bFGF (20 ng/ml each), 1% Albumax (Gibco), 0.6% glucose (Sigma) and N2 supplement (Gibco).

The infection with moloney murine leukemia virus (MMLV)-derived, replication defective, VSV-G pseudo-typed retroviral vector particles (multiplicity of infection of 1) was performed at a cell confluence of 30-50 %. After infection, the cells were selected by culturing them for 1 month in culture medium containing 100 µg/ml neomycin.

Non-immortalized cortical cell cultures were obtained by dissecting human fetal cortex from 8 weeks old fetuses. Pieces of tissue were mechanically triturated and dissociated cells were plated at 100 000/cm² on both 10 µg/ml polyornithine and 5 µg/ml laminin coated flasks and expanded in complete culture medium as described below.

Monolayer cell cultures

Immortalized human cortical neural stem cells (hc-NSCs) were expanded in plastic flasks (Nunc) coated with 10 µg/ml poly-ornithin and 5 mg/ml laminin (Gibco). The cells were maintained under proliferative conditions at 37°C in a 5% CO₂ atmosphere in complete culture medium (DMEM/F12 with N2 supplement (100X; Gibco), 1% Albumax (Gibco), penicillin/streptomycin (P/S;100X; Gibco)), with addition of growth factors, human recombinant EGF and bFGF (R&D; 20 ng/ml each). Cultures were passaged twice a week (splits 1:6-1:7) by trypsinization before they reached confluence.

Single-cell sorting

The polyclonal hc-NSC line was successfully expanded *in vitro* for up to 14 passages after selection with neomycin (100 µg/ml, G-418 Sulfate; Gibco). In order to generate clonal cell lines, single cell sorting was performed. First, cells were stained with 7-amino actinomycin (7-ADDH; Sigma-Aldrich) to exclude dead cells and then they were sorted according to their side and forward scatter. Individual cells were deposited by a single-cell depositor coupled to a FACSDiva cell sorter (Becton Dickinson) into wells of 96-well plates containing 100 µl of complete culture medium. After 24 h, the presence of single cells in each well was confirmed under an inverted phase contrast microscope. Two to three weeks later, colonies were generated from the single cells plated. Eleven clones were selected for further expansion and characterization.

Cell proliferation

The ability of EGF and bFGF to support cell proliferation was evaluated by growing clonal cells in culture medium with different compositions. First, cells were plated at 15 000/cm² in complete culture medium. As soon as cells had attached (after about 4 h), “complete culture

medium” was removed and replaced with either fresh “complete culture medium” or with “basal medium” (same as “complete culture medium” but without growth factors) with addition of either 20 ng/ml of bFGF or 20 ng/ml of EGF. Cell proliferation was evaluated after 48 h by quantifying the number of dividing (Ki67+) cells as percentage of total (Hoechst+) cell number.

Cell differentiation

Several protocols were used to differentiate the hc-NSC lines. For all protocols, cells were first plated with complete medium at 20 000 cells/cm² in 10 µg/ml poly-ornithin and 5 µg/ml laminin (Gibco) coated 4-well chamber slides (Nunc) or T25 flasks (Nunc). In a first set of experiments, at 24 h after plating, complete medium was removed and substituted with basal medium containing 1% fetal bovine serum (FBS) and then, every 3 days, 2/3 of the medium was replaced with fresh medium. Cells were differentiated under these conditions for 4, 5 and 7 days. In a second set of experiments, complete medium was replaced with basal medium containing either 20 ng/ml of EGF, or 20 ng/ml of bFGF, or 20 ng/ml EGF and 5 µM forskolin, or 20 ng/ml of bFGF and 5 µM forskolin. Cells were differentiated under these conditions for 7 days (old medium was replaced with 2/3 fresh medium every 3 days). In a third set of experiments, cultures which were pre-differentiated for 7 days in the presence of EGF together with forskolin, were also differentiated for an additional 2 and 3 weeks. During this period, half of the medium was basal medium and the other half was Neuro Basal Medium (Gibco) containing B27 supplement (Gibco) and 1 mM glutamine (Gibco).

Immunocytochemistry

Differentiated cultures were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 min. For γ -aminobutyric acid (GABA) and glutamate detection, cells were

fixed with PBS containing 4% PFA and 0.25% glutaraldehyde (Sigma). After fixation, cultures were washed with potassium-PBS (KPBS) (pH 7.4) and then incubated in KPBS containing 5% of the appropriate normal serum and 0.025% Triton X-100 (pre-incubation solution) for 1 h at room temperature. Subsequently, cultures were incubated overnight at 4°C in pre-incubation solution containing primary antibodies listed below. Slides were washed 3 times with KPBS and incubated for 2 h at room temperature in the appropriate incubating solution containing 10 µg/ml Hoechst 33342 (Molecular Probes, Eugene, OR), and the appropriate biotinylated (Vector, Burlingame, CA) and/or Cy3-conjugated (Jackson Immunoresearch) secondary antibodies at dilution 1:200.

After rinsing with KPBS, slides were incubated for 2 h with Alexa 488-conjugated streptavidin (Molecular Probes) at dilution 1:200, rinsed, and coverslipped with PVA-DABCO mounting medium (Sigma).

The following primary antibodies were used: monoclonal mouse anti-vimentin (Dako; 1:50); polyclonal rabbit anti-nestin (provided by Dr. R.G. McKay, NIH, Bethesda, MA 1:1000); monoclonal mouse anti-sox2 (R&D Systems; 1:50); monoclonal mouse anti-βIII-tubulin (Sigma; 1:330); monoclonal mouse anti-human Tau (Zymed; 1:100); polyclonal rabbit anti-neuron-specific enolase (NSE; Chemicon; 1:100); polyclonal rabbit anti-gliial fibrillary acid protein (GFAP; Dako; 1:500); monoclonal mouse anti-GFAP (Sigma; 1:200); polyclonal rabbit anti-NG2 (Chemicon; 1:150); polyclonal rabbit anti-GABA (Sigma; 1:2000); polyclonal rabbit anti-glutamate (Chemicon; 1:100); polyclonal goat anti-calretinin (Chemicon; 1:2000); polyclonal rabbit anti-Pax6 (Chemicon; 1:500); polyclonal goat anti-neurogenin 2 (Ngn2; Santa Cruz; 1:200); monoclonal mouse anti-tyrosine hydroxylase (TH; Chemicon; 1:200); polyclonal rabbit anti-vesicular monoamine transporter 2 (V-MAT; Chemicon; 1:500); monoclonal mouse anti-Ki67 (Novocastra; 1:100). Specificity of immunostaining was assessed by omission of primary antibodies during overnight incubation.

RNA preparation and reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from both proliferating and differentiated hc-NSC lines by using RNA extraction kit RNAqueous-4CPR (Ambion, Austin, TX) according to manufacturer's instructions. Possible DNA contamination was removed by two subsequent RNase-free DNase I treatments (Ambion). Total cellular RNA (1 µg) was reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen, La Jolla, CA). One-twentieth of the cDNA was amplified in 20 µl of PCR mix containing 1U RedTaqDNA Polymerase provided together with 10 X PCR buffer and containing 11 mM MgCl₂ (Sigma), 0.2 mM dNTP mixture, and 0.5 mM for each primer. To control for DNA contamination, reverse transcriptase was omitted at the cDNA synthesis step, and the sample was analyzed by PCR. PCR amplification of a fragment of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was performed as an internal control for each sample. Total RNA was also collected from non-immortalized fetal cortical cell cultures expanded for 3 passages, and amplified as described above.

All PCR programs were initiated with 4 min at 95°C. The sequence and product size of primers are presented in the table below.

Genes	Primers	Product (bp)
GAPDH [21]	F:5'- CCACAGTCCATGCCATCAC, R:5'-TCCACCACCCTGTTGCTGTA	400
Nestin [14]	F:5'-GGCAGCGTTGGAACAGAGGTTGGA R:5'-CTCTAAACTGGAGTGGTCAGGGCT	781
v-myc [14]	F:5'-CCTTTGTTGATTTGCGCCAAT R:5'-AGTTCTCCTCCTCCTCCTCG	273
hTERT [14]	F:5'-GAGGAGGAGGACACAGACCC R:5'-CAGGATCTCCTCACGCAGAC	289
Pax6	F:5'-AACACACTTGAGCCATCACC R:5'-TGTCTCGGATTTCCCAAGCA	681

Emx2	F:5'-AACTCCAGCCCCATAAATCC R:5GTTGCGAATCTGAGCCTTCT	549
Ngn2	F:5'- ATCCTTCATTCAGACGGGCT R:5-TTCTAACCTGCCCCTCTAAC	564
EGFR [22]	F:5'-CAGTCGTCAGCCTGAACATAACATCC R:5'- AGGTTGCACTTGTCCACGCATTCCC	300
FGFR [23]	F:5'-CGCTCTAGAGCAGAACTGGGATGTGGGGC R:5'-CTCGGATCCAGGGCTTCCAGAACGGTC	832/976/ 1089

Cytogenetic analysis

To explore whether the hc-NSC-7Fb line exhibited any gross chromosomal abnormalities after multiple expansions, karyotyping was performed. Briefly, cells were plated on chamber slides and arrested in metaphase using Colcemid (0.02 µg/ml for 3 h). In situ preparations were made after hypotonic shock and fixation in methanol:acetic acid (3:1) G-banding was obtained with Wright's stain. Twenty-five cells in metaphase were analyzed.

Quantification and statistical analysis

Cell counting in cultures was performed using a computerized setup for stereology driven by the Computer Assisted Stereological Toolbox (C.A.S.T. - GRID) software (Olympus, Denmark) with a 40x objective. A CCD IRIS color video camera displayed the acquired images from the epifluorescence microscope (Olympus BX-61) live on a monitor screen. Counting frame areas and stepping distances were chosen in order to sample approximately 100-200 cells per well. Each well was evaluated separately, counting both total cell number based on Hoechst staining, and cells immunoreactive for different markers. One-way ANOVA followed by Fisher's *post hoc* test were used to assess differences between groups. Data are expressed as means ± SEM and differences were considered significant at $p < 0.05$.

Results

Generation of a human cortical NSC line (hc-NSC), subclone isolation, and screening of basic neural properties. Cortical tissue from human fetal brain was mechanically and enzymatically dissociated to a single cell suspension, plated, and transduced with v-myc retrovirus. This heterogeneous cell culture was further expanded in the presence of neomycin, thus allowing for the transduced cells to generate polyclonal cell lines. In order to isolate clonal cell lines, we used preparative cell sorting and deposited single cells in each well of 96-well plates. We never observed more than one cell in each well, though many wells did not contain any cells (probably due to death of the single cells). Fifty out of 140 plated individual cells generated healthy colonies after 2-3 weeks (Fig. 1A). All colonies were passaged at least twice and based on their proliferation properties, 11 clones were selected for further analysis. When grown with bFGF and EGF on a polyornithine/laminin-coated surface these cells showed a flattened, mostly multipolar morphology, with uniform distribution on the surface of the flasks (Fig. 1B). The cells had to be passaged every 3-4 days when plated at split 1:6-1:7. Cytogenetic analysis of metaphases in 25 cells from hc-NSC-F7b clone did not reveal any numerical or structural chromosomal abnormality (Fig. 1C).

As a next step, we screened these clones for their capacity to generate neurons and astrocytes. Differentiation for 7 days in the presence of 1% FBS, and consecutive staining for the neuronal marker β III-tubulin and the astrocytic marker GFAP, revealed that these markers were expressed in different cell populations without any co-labeling. Eight clones were capable of generating both neurons and glia (Table 1). However, there were clear differences between the clones in the number of β III-tubulin+ cells, as well as in the occurrence of downregulation of v-myc expression after differentiation.

At 48 h after plating in the presence of EGF and bFGF, these clonal cell lines were positive for NSC markers, such as transcription factor Sox2 (Fig. 1D-E) and the cytoskeletal proteins nestin and vimentin (Fig.1 F-H). Virtually all cells were immunoreactive for these markers, reflecting their stem cell status. Consequently, we did not detect any β III-tubulin+ cells but observed that 18% of the cells were GFAP+.

Capacity of hc-NSC lines to downregulate v-myc and telomerase expression.

The p110^{gag-myc} protein (encoded in the genome of the avian myelocytomatosis virus) has been the most successful immortalizing protein described for NSCs. In spite of being a fusion protein involving coding exons 2-3 of chicken c-myc, it is not transforming the cells, just immortalizing them, as described in many instances [13; 14; 19]. In addition to its growth-promoting ability, v-myc enhances *tert* expression and telomerase activity [24; 25].

Despite the fact that no harmful actions have been described for v-myc on NSCs, there is a concern about the properties of the generated progeny. Here we studied both v-myc and telomerase expression after differentiation of the cells. Notably, telomerase has been reported to immortalize human NSCs [26]. Four out of 8 studied clones (Table 1) showed a clear decrease of v-myc expression when differentiated for 4 or 7 days in medium containing 1% serum (Fig. 2). Since v-myc drives telomerase expression, the mRNA for telomerase (*tert*) which was expressed in dividing cultures (in contrast to non-immortal cells) was accordingly found to be down-regulated after differentiation. Taken together, we show that v-myc and TERT expression is down-regulated in parallel with differentiation, yielding mature human neural cells (neurons and glia; see below).

Effects of growth factors on proliferation of hc-NSC lines. Previous studies have demonstrated that even after genetic immortalization, NSC lines require support of growth factors to survive and maintain mitotic activity [13; 14; 19; 27]. The hc-NSCs were expanded in the presence of bFGF and EGF. Under these conditions, at 2h h after plating, about 80% of hc-NSCs were positive for Ki67 (Fig. 3 A, B and D), a marker for mitotically active cells

[28]. The specific receptors for both growth factors were expressed by the hc-NSCs as demonstrated by RT-PCR (Fig. 3 C), and interestingly also in non-immortalized cell cultures derived from 8 weeks old human fetal cortex, expanded for only 3 passages. At 5 days after EGF and bFGF had been withdrawn and 1% FBS added to the culture medium, the number of Ki67+ cells had dropped to 20%, and at 7 days was further decreased to 10% (Fig. 3D). These data clearly show that growth factors are needed for the expansion of the hc-NSC lines. Similar results have been obtained when analyzing these parameters in another immortalized forebrain cell line called hNS1 [14].

To further evaluate the contribution of bFGF and EGF in supporting the mitotic activity of hc-NSCs, we cultured the hc-NSC-F7b clone for 2 days under either of 4 different conditions (Fig. 4): In the presence (normal expansion condition) or absence of both bFGF and EGF; or in the presence of bFGF or EGF only. The absence of both growth factors for 2 days lead to a marked reduction of the percentage of proliferating Ki67+ cells as well as of the total number of cells. When hc-NSCs were cultured only with bFGF, neither total cell number nor percentage of Ki67+ cells differed from that when both growth factors were present. In contrast, withdrawal of bFGF from the medium and culturing of cells only with EGF significantly decreased the percentage of mitotically active cells as well as the total number of cells. These data clearly indicate that bFGF promotes division of hc-NSCs much more efficiently than EGF. However, also EGF has a pro-mitotic action because when hc-NSCs were cultured with this growth factor, 43% of cells were Ki67+ as compared to 29% when cells were kept without any growth factors (Fig. 4), and the total number of cells was higher in the EGF-treated cultures.

Effect of growth factors on β III-tubulin expression in hc-NSCs. Under normal expansion conditions, in the presence of bFGF and EGF, hc-NSCs proliferate extensively and do not express the neuronal marker β III-tubulin. We tried to identify conditions when cells are

still expanding but cells in the cultures also express early neuronal markers such as β III-tubulin. When both growth factors were absent, at 7 days about 11% of the cells were β III-tubulin+ (Fig. 5A). A similar percentage of β III-tubulin+ cells was observed in cultures grown for 7 days in the presence of either EGF or bFGF (Fig. 5A). However, in line with mitotic activity of these growth factors, in their presence, in particular bFGF, cells continued to proliferate. The total number of cells cultured with bFGF was almost doubled as compared to that found in cultures with EGF (Fig. 5B). Addition of forskolin to the culture medium together with either EGF or bFGF increased the percentage of β III-tubulin+ cells to about 18% (Fig. 5A). Forskolin has previously been described to prime neuronal differentiation from neural precursor cells [19; 29].

Differentiation of hc-NSCs. Our data showed that bFGF is a more potent mitogen than EGF for hc-NSCs, and that both growth factors when combined with forskolin, give rise to approximately similar numbers of β III-tubulin+ cells. Therefore, for further investigation of the differentiation potential of the hc-NSC-7Fb line, we decided to prime cells towards neuronal lineage by expanding them in the presence of EGF and forskolin for 7 days and then switching to differentiation medium without EGF and forskolin for an additional 7 days. Under these conditions, the hc-NSC line generated about 20 % β III-tubulin+ cells. The neuronal phenotype of the differentiated β III-tubulin+ cells (Fig. 6A) was confirmed by immunostaining with antibodies against neuron-specific enolase (Fig. 6B) and human Tau (Fig. 6C). Moreover, the differentiated cells clearly down-regulated Sox2 expression, although almost half of the cells in the cultures remained positive for this stem/progenitor marker. Importantly, the β III-tubulin+ neurons did not show any co-localization with nestin (data not shown). Many cells differentiated into GFAP+ astrocytes (Fig. 6D), but the extensive network of processes made it impossible to perform accurate quantification of the number of GFAP+ cells. Only single cells were immunopositive to NG2, a marker for oligodendrocyte progenitors (Fig. 6E).

Neurochemical phenotype of differentiated neurons. The neuronal population in the adult cerebral cortex comprises glutamatergic pyramidal neurons and GABAergic interneurons [30; 31]. Interestingly, after 2 weeks of differentiation of hc-NSCs about 10% of them were glutamate+ (Fig. 7A) and about 1% were GABA+ (Fig. 7B). Virtually all GABA+ cells were also immunoreactive to β III-tubulin (data not shown). We also detected calretinin+ cells (Fig. 7C) but no parvalbumin+ or calbindin+ cells.

In order to explore whether the fetal cortex-derived cell line could also generate cells with non-cortical identity, we stained differentiated cultures with an antibody against the dopamine-synthesizing enzyme TH. Interestingly, some cells (less than 1%) were TH+ (Fig. 7D). Very few of them contained GABA (Fig. 8A-C) similar to olfactory bulb interneurons, but a significant portion (less than half) coexpressed the vesicular monoamine transporter (VMAT; Fig. 8D-E), suggesting that they could be catecholaminergic neurons.

Expression of developmental genes. We finally investigated whether the hc-NSCs maintained the gene expression profile characteristic of neuronal precursors in the developing cortex after multiple passages *in vitro*. Using RT-PCR, we assessed the expression of region-specific developmental genes that are involved in the lineage restriction of cortical progenitors and in the regionalization of the dorsal telencephalic area during embryonic development.

The expression of some of these genes was evaluated in both the v-myc-immortalized hc-NSC-F7b line and in human fetal cortex-derived cells expanded as monolayer culture without any genetic manipulation. As shown in Fig. 9 A-C, both cell types expressed Emx2, Pax6 and Ngn2. Expression of the transcription factor Pax6 was confirmed by immunostaining of proliferating as well as differentiated cells (Fig. 9 D and E). In contrast, another transcription factor, Ngn2 was not detectable immunocytochemically in dividing cultures (Fig. 9F), while being present in differentiated cells (Fig. 9G). Interestingly, Ngn2-immunoreactivity was restricted to β III-tubulin+ cells (Fig. 9H-J). The lack of Ngn2

immunoreactivity in dividing cells could reflect its low expression under this condition which is also obvious from RT-PCR. Ngn2 seemed to be markedly less expressed in the hc-NSC line as compared to cortex-derived non-immortalized cell culture. Taken together, our data indicate that the expression of some molecular determinants of cortical identity has been maintained despite immortalization and long-term expansion.

Discussion

Many studies have shown that functional recovery can be induced by cell replacement in animal models of human neurodegenerative disorders such as Parkinson's disease, Huntington's disease and stroke [32-35; 36 ; 37]. A major remaining challenge is to identify appropriate sources of cells which can be easily expanded *in vitro* and generate large numbers of the specific types of cells needed to be replaced. For example, ischemic stroke in humans most often causes extensive damage and neuronal loss in the basal ganglia and/or cerebral cortex. Thus, in order to be able to repair the brain after stroke, we need to produce cell lines which, after intracerebral transplantation, differentiate into cortical and/or striatal neurons. In the present study, we have generated a new NSC line (hc-NSC-F7b), using immortalization of cortical tissue from a 9 weeks old human fetus. Our study shows that this hc-NSC line i) expresses appropriate markers of undifferentiated NSCs; ii) shows self-renewal capacity and can be expanded for long time periods by adding growth factors, iii) is multipotential and has the capacity to generate cells of all three neural lineages, i. e., neurons, astrocytes and oligodendrocytes, iv) gives rise to neurons which exhibit some genuine characteristics of *in vivo* cortical neurons. Our findings indicate that cells in the hc-NSC-F7b line have NSC properties and could be an interesting source of cortical neurons for future cell replacement studies in neurodegeneration models.

It is believed that members of the myc family play a role during normal development. Myc-dependent mechanisms are involved in the control of pluripotency and self-renewing

capacity of murine embryonic stem cells [16], and in the expansion of neural progenitor cell populations and inhibition of differentiation [17; 18]. Several studies have demonstrated that the avian myelocytic leukemia retroviral v-myc gene, the ortholog of cellular c-myc, could be successfully used to immortalize human neural precursor cells [13; 14; 19]. Here we used retroviral v-myc transduction to generate a human fetal cortex-derived NSC polyclonal line, and then selected single cell-derived clones with defined properties: expression of neural markers, high expansion rate, decreased proliferation upon removal of growth factors and capacity to differentiate into neurons.

A major concern regarding the genetic immortalization strategy, as used in the present study, is the safety of the generated cell lines. Could v-myc act as a transforming oncogene like the mammalian c-myc counterpart? However, such an action has never been demonstrated experimentally [38]. By unknown reasons, v-myc works perfectly well for immortalization of mammalian NSCs without transforming them. In the present study we addressed the issue of transformation of the hc-NSC line in three different ways: Firstly, we performed a chromosomal analysis and found that this hc-NSC line retains a normal karyotype even after more than 40 passages in culture. No numerical or structural chromosomal abnormalities were detected. Indirectly, this finding also speaks in favor of good telomere preservation as reported previously [25].

Secondly, we explored whether the immortalized hc-NSCs down-regulate v-myc expression after switching from proliferative to differentiation culture conditions by removal of growth factors. V-myc stimulates the expression of growth factor-regulated genes and strongly promotes cellular proliferation. Moreover, its constitutive expression interferes with the terminal differentiation of the majority of studied cell types [16; 17; 39]. Our PCR analysis showed that the hc-NSC-F7b line down-regulates v-myc expression after growth factor deprivation. Cross-talk between myc and telomerase has been demonstrated [24; 25], and retroviral over-expression of human TERT has been tried for immortalization of neuronal

progenitor cells derived from the human fetal spinal cord [26]. Similar to previous observations in another cell line [25], our data indicate that when hc-NSCs differentiate, there is a parallel downregulation of v-myc and TERT expression.

Thirdly, we studied the growth factor-dependence of hc-NSCs for their *in vitro* proliferation. It has been shown that even if genetically immortalized, human fetal cell lines need bFGF or bFGF and EGF for their expansion [13; 14; 19; 27]. Our findings indicate that in medium containing 1% serum but without growth factors, cells stop dividing and differentiate into neurons and glia. The percentage of cells with mitotic activity (10%) agrees with previous *in vitro* findings and more recent *in vivo* data, showing that 11% of c-myc immortalized human cortical cells are Ki67+ at 5 weeks after transplantation [20].

Taken together our findings provide further evidence (i) that v-myc and telomerase are important for the expansion of hc-NSCs; (ii) that v-myc controls expression of telomerase; and (iii) that after growth factor removal, these genes are down-regulated in a coordinated way. These data are in accordance with the concept of “conditional immortalization”, i. e., that the cells are immortal as long as mitogens are present in the culture medium. In the absence mitogenes, the cells resume their standard behavior, differentiating into neurons and glia.

Our data show that bFGF has a more potent mitogenic activity than EGF on the hc-NSC line. The PCR analysis indicates that, in the presence of their respective ligands, both bFGF and EGF receptors are expressed in the hc-NSC cultures. Importantly, expression of both receptors was also detected in non-immortalized human fetal cortical cultures which had been passaged only three times. These findings argue against the hypothesis that expression of FGF and EGF receptors is induced by the v-myc immortalization of the hc-NSC line. Previous reports have demonstrated that *in vitro*, FGF can induce responsiveness to EGF in primary cells from fetal rodent striatum [40] and mesencephalon [41]. Moreover, recent data indicate the existence of a single type of stem cell responsive to both FGF and

EGF in expanded neurosphere cultures of human fetal cortex from the first trimester [42]. It is not clear from our study whether the same hc-NSCs express FGF and EGF receptors, and if FGF increases responsiveness to EGF in these cells. However, the observation that the hc-NSC line could be expanded in the presence of EGF only indicates that this factor has sufficient, though low mitogenic activity.

Experimental evidence suggests that c-myc protein has pro-apoptotic activity which can be triggered by a range of insults including growth factor deprivation [43]. To our knowledge, such an action has not been reported for v-myc. Forskolin has previously been used in combination with growth factors to prime neuronal differentiation of human fetal mesencephalic neural progenitor cells [29]. Here we found that cultures differentiated for 7 days in the presence of FGF and forskolin or EGF and forskolin showed no significant differences in terms of percentage of β III-tubulin+ cells out of total cell number. It is well known that differentiation requires cells to stop division and exit from the cell cycle. Because FGF supported cell division stronger than EGF, we decided to use a protocol based on 7 days of priming in the presence of EGF and forskolin for the further characterization of the phenotype of cells differentiated from the hc-NSC line.

In rodents, cortical projection neurons are mainly glutamatergic and derived from the dorsal telencephalon. GABAergic interneurons that are born in the ventral telencephalon reach the cortex by tangential migration [30; 44]. Interestingly, recent data have suggested that in primates, the majority of the cortical interneurons originate in the cortical ventricular and subventricular zones, and that there is a portion contributed by the ganglionic eminence of the ventral telencephalon [45-47]. We show here that differentiated hc-NSCs give rise to glutamatergic (10%) and a smaller population of GABAergic cells (about 1%). Interestingly, cells immunoreactive for the calcium-binding protein calretinin, a marker for cortical GABAergic interneurons, were also detected but we did not observe any cells containing parvalbumin or calbindin (other interneuron markers). In accordance, Zecevic and co-

workers [48] have reported that at mid-gestation development of the human brain, in contrast to rodents, the majority of cortical interneurons are calretinin immunoreactive. In relation to our data, one interpretation is that the neurons obtained after *in vitro* differentiation of hc-NSCs, had not matured to the point of expressing the whole set of phenotypic markers of their *in vivo* counterparts.

Our findings demonstrate that, at least for the parameters investigated here, hc-NSCs retain their regional identity even after multiple *in vitro* expansions. Using RT-PCR, we found that the undifferentiated hc-NSCs express the genes encoding the basic helix-loop-helix (bHLH) transcription factor Ngn2 and the homeodomain transcription factors Emx2 and Pax6. These genes have been proposed to be involved in the regionalization of the developing neocortex, and in the control of different properties of cortical progenitors such as their neuronal commitment, phenotype specification, and migration [49-55]. Cells immunoreactive to Pax6 but not to Ngn2 were detected in dividing cultures, while both transcription factors were observed in differentiated cells which also expressed the neuronal marker β III-tubulin. Although Ngn2 was found with PCR in proliferating cells, the lack Ngn2 protein immunocytochemistry most likely reflects less importance of this transcription factor during proliferation.

The PCR amplification as performed here was not quantitative, but it was obvious that Ngn2 mRNA was expressed at higher levels in non-immortalized cortical cultures as compared to the v-myc immortalized hc-NSC line. The results of the short-term culture expansion procedure applied to non-immortal cells (3 passages) provides evidence for the existence of a heterogenous cell population comprising NSCs and committed progenitors. In contrast, the monoclonal hc-NSC line is more likely to consist of a relatively homogenous population of NSCs. Nieto and co-workers [51] have proposed that the expression of *ngn-2* is restricted to committed neuronal progenitors. If this is the case, the differences in Ngn2 expression observed here could be related to different cellular composition of immortalized

and non-immortalized cell cultures. This interpretation is also supported by the finding that Ngn2 was clearly expressed in β III-tubulin+ neurons after 2 weeks of differentiation.

The gene expression profile and the ability to generate glutamatergic and GABAergic neurons demonstrate that the hc-NSCs maintain their regional identity as cortical progenitors. However, our data suggest that this cell line is also capable of generating neurons which are not normally present in the adult cerebral cortex *in vivo*. Induction of TH gene expression in neurons derived from human fetal cerebral cortex, as shown here, has been reported previously [56; 57]. In our experiments, not all V-MAT+ cells were also TH+. TH is involved in the synthesis of catecholamines (dopamine, adrenaline and noradrenaline) and V-MAT is the transporter of monoamines (dopamine, noradrenaline, histamine and serotonin) in the synaptic vesicles. Our findings provide some evidence that both catecholaminergic (TH+/V-MAT+) and serotonergic (TH-/V-MAT+) cell types can be generated after differentiation of hc-NSC (for review see [58]). Interestingly a small fraction of the TH+ cells co-expressed GABA. Such cells have previously been described as a population of interneurons in the adult human brain [59; 60].

In conclusion, our demonstration that the new human cell line generated here can produce cells *in vitro* expressing markers characteristic of some cortical neuron types represents a promising first step towards a cell replacement strategy following cortical injury such as stroke. It is now important to determine how the differentiation of these hc-NSCs after intracerebral transplantation is influenced by the external cues present in the intact and pathological brain. For maximum generation of several types of cortical neurons, it may be necessary to predifferentiate the cell line *in vitro*. However, the ability of the cortical tissue environment to drive the NSCs towards the specific cortical neuronal phenotypes should also be explored. We also have to address if the grafted new neurons can survive in large numbers and become morphologically and functionally integrated into existing neural circuitries. So far, there is very little evidence for reconstruction of cortical circuitries and, in

particular, the formation of efferent connections from the cortical graft to the host after cell transplantation in the injured brain. Moreover, whether NSCs can give rise to cells with *in vivo* electrophysiological properties specific for cortical neurons is unknown. Although we obtained no evidence for transformation of the hc-NSC line in culture, we must analyze in detail the risks for tumor formation *in vivo*. Finally, it will be of major importance to determine if intracortical implantation of hc-NSC-derived cells can induce significant functional recovery in models of neurodegenerative disorders where cortical damage plays a major pathophysiological role.

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Figure legends

Fig. 1. Phenotypic and genetic properties of immortalized human cortical neural stem cells.

A. Example of a clonal cell culture originating from a single cell of the polyclonal hc-NSC line. B. hc-NSCs grown as adherent culture for 14 passages in the presence of EGF and bFGF. C. Karyotype analysis showing a normal chromosomal complement in the hc-NSC-F7b line (n=25 cells in metaphase; after more than 40 passages). Neither numerical nor structural chromosomal abnormalities were detected. D-E. Sox2 staining (D) and Hoechst counterstaining (E) of dividing hc-NSC-F7b line. F-H. Photomicrographs of cultured hc-NSCs showing nestin (F) and vimentin (G) immunoreactivity separately or as merged image (H). Note that virtually all cells co-express both neural stem/progenitor markers. Scale bar = 50 μm .

Fig. 2. Coordinated expression of v-myc and telomerase (TERT) genes in immortalized hc-NSC-F7b line. Total RNA was purified from hc-NSCs under different conditions, and v-myc (top panel) and TERT (middle panel) mRNAs were analyzed using RT-PCR. Lane 1: molecular weight markers (100bp DNA Ladder #323-1S New England Biolabs). Lane 2: dividing hc-NSCs. Lanes 3 and 4: hc-NSCs differentiated for 4 and 7 days, respectively. The GAPDH housekeeping gene was used as internal control for each sample (bottom panel).

Fig. 3. A-B. Expression of the mitotic marker Ki67 (A) in hc-NSC-F7b cells cultured in the presence of growth factors and visualized by Hoechst staining (B). Note that virtually all cells are Ki67+. C. Expression of receptors for bFGF and EGF in human fetal cortex-derived cells. RT-PCR analysis of the expression of these receptors (FGF-R and EGF-R, respectively) in dividing hc-NSC-F7b line- and human fetal cortex-derived, non-immortalized cells cultured for 48 h in the presence of bFGF and EGF. GAPDH was used as internal control for each sample. Lane 1: molecular weight markers. Lane 2: human fetal cortex-derived, non-immortalized cells. Lane 3: immortalized hc-NSC-F7b line. D. Stereological quantification of the percentage of dividing, Ki67+ cells when immortalized hc-NSCs had been cultured under proliferation conditions in the presence of bFGF and EGF, and at 5 and 7 days after withdrawal of the growth factors. Data are obtained from quadruplicate experiments and are expressed as means \pm SEM. *p<0.05, one-way ANOVA and Fisher's *post-hoc* test. Scale bar = 50 μm .

Fig. 4. Percentage of proliferating, Ki67+ cells (A) and total number of cells (B) in the immortalized hc-NSC cultures. NSCs were plated with or without bFGF and EGF, or with bFGF or EGF only. Data are obtained from quadruplicate experiments and are expressed as mean \pm SEM. * $p < 0.05$, one-way ANOVA and Fisher's *post-hoc* test.

Fig. 5. Percentage of β III-tubulin+ cells (A) and total number of cells (B) in the immortalized hc-NSC cultures under various differentiation conditions. Cells were cultured for 7 days without growth factors and with FBS; or with EGF and bFGF only; or with EGF and forskolin or bFGF and forskolin. Data were obtained from quadruplicate experiments and are expressed as means \pm SEM. * $p < 0.05$, one-way ANOVA and Fisher's *post-hoc* test.

Fig. 6. Immunocytochemical demonstration of neuronal and glial markers in differentiated hc-NSC-F7b line. Cells were plated and differentiated for 7 days with EGF and forskolin and another 7 days only with B27. Cells immunoreactive for the immature neuronal marker β III-tubulin (A) co-express another neuronal marker, neural specific enolase (NSE; B). Cells positive to β III-tubulin and NSE were visualized after double-fluorescent immunostaining by using Cy3-conjugated secondary antibody (A) and Alexa 488-conjugated streptavidin. After differentiation, the hc-NSCs also express the mature neuronal marker hTau (C) and astrocyte marker GFAP (D). Few cells in the differentiated hc-NSC cultures are positive to the oligodendrocyte marker NG2 (E). Arrows depict examples of immunoreactive cells. Scale bar = 30 μ m.

Fig 7. Immunocytochemical demonstration of specific neuronal phenotypes in differentiated hc-NSC-F7b line. Cells were plated for 7 days with EGF and forskolin, and another 7 days only with B27. Photomicrographs illustrate cells immunoreactive for glutamate (A), GABA (B), calretinin (C), and tyrosine hydroxylase (TH, D). Arrows depict examples of immunoreactive cells. Scale bar = 30 μ m.

Fig. 8. Characterization of phenotype of TH-positive cells generated from hc-NSC-F7b clone. Epifluorescence images of differentiated hc-NSCs showing TH (A), GABA (B) and merged image (C), or TH (D), V-MAT (E) and merged image (F). Arrows depict TH+/GABA+ (A-C), and TH+/V-MAT+ (D-F) double-stained neurons; arrowheads indicate TH+/GABA- (A and C) and TH+/V-MAT- (D and F) neurons. Scale bar = 30 μ m.

Fig. 9. Expression of mRNA and protein of cortical developmental transcription factors in proliferating and differentiated hc-NSCs. Total RNA was collected from dividing, hc-NSC-F7b and from human fetal cortex-derived, non-immortalized cells cultured for 48 h in the presence of EGF and bFGF. RT-PCR was used to determine the expression of Emx2 (A), Pax6 (B) and neurogenin2 (Ngn2) (C). GAPDH served as internal control for each sample. Lane 1: molecular weight markers. Lane 2: fetal cortex-derived non-immortalized cells. Lane 3: immortalized hc-NSC-F7b line. Photomicrographs of proliferating (D and F) and differentiated (E and G) hc-NSCs stained with antibodies against Pax6 (D and E) or Ngn2 (F and G). Epifluorescence photomicrographs of differentiated hc-NSCs showing Ngn2 (H) and β III-tubulin (I) immunoreactivity separately or as merged image (J). Arrows depict two double-labeled immunoreactive cells. Scale bar = 30 μ m.

Table 1. Properties of clones isolated from polyclonal hc-NSC line

Clone name	β III-tubulin + cells	GFAP+ cells	Ki67+ cells	Downregulation of v-myc
F9B	+++	++	N.D.	No
D10B	+++	++	N.D.	No
F7B	+++	++	+	Yes
E6C	++	++	+	Yes
D11C	++	++	+	Yes
G5B	+	++	+	Yes
G7C	+	++	N.D.	N.D.
C11A	+	++	N.D.	N.D.

Clones were plated for 7 days without growth factors in the presence of 1%FBS. Semi-quantitative assessment of number of β III-tubulin, GFAP, and Ki67 immunoreactive cells and occurrence of v-myc mRNA (RT-PCR) downregulation. “+” - low number; “++” - moderate number; “+++” - high number; N.D. - not determined.

Figure 1
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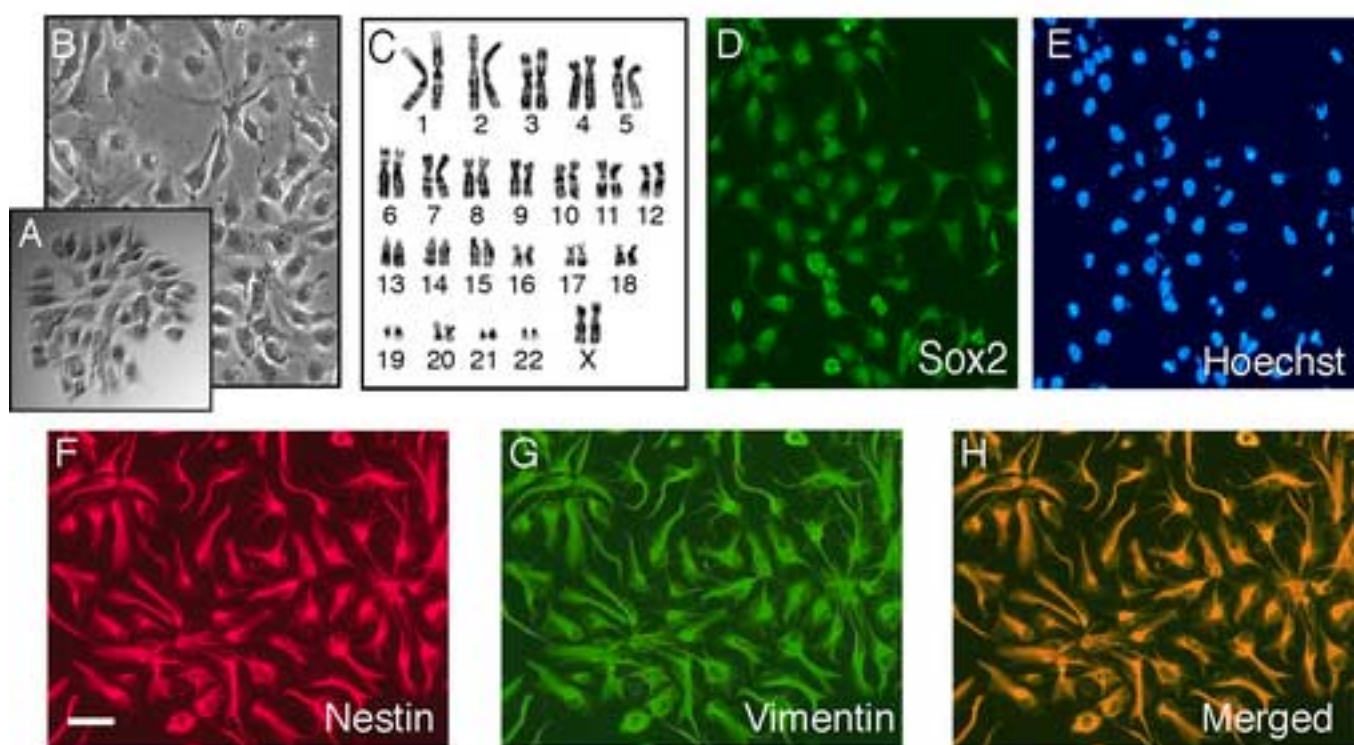


Fig.1

Figure 2
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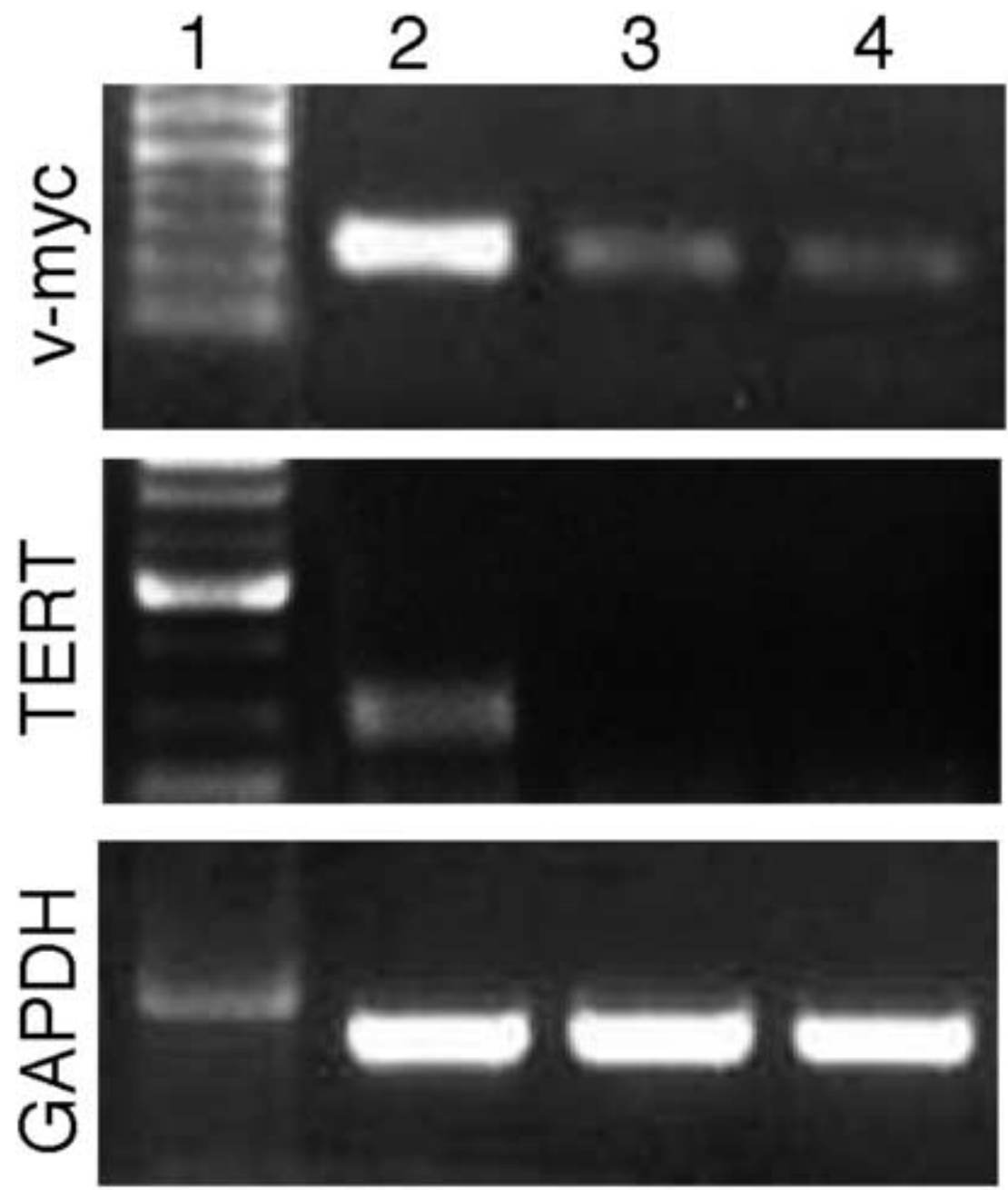


Fig.2

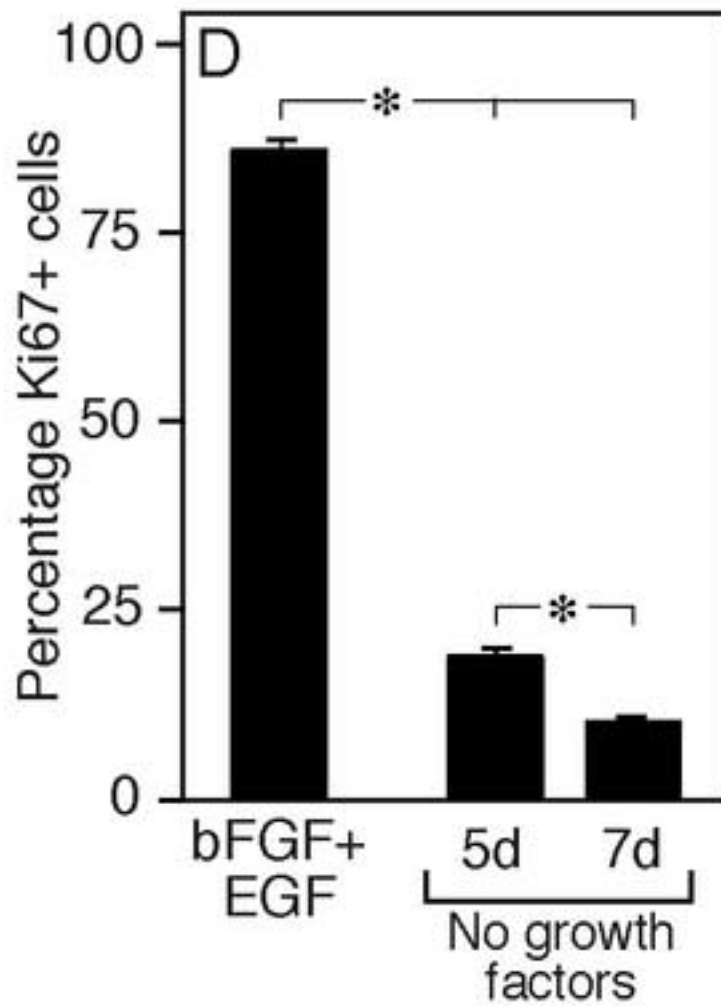
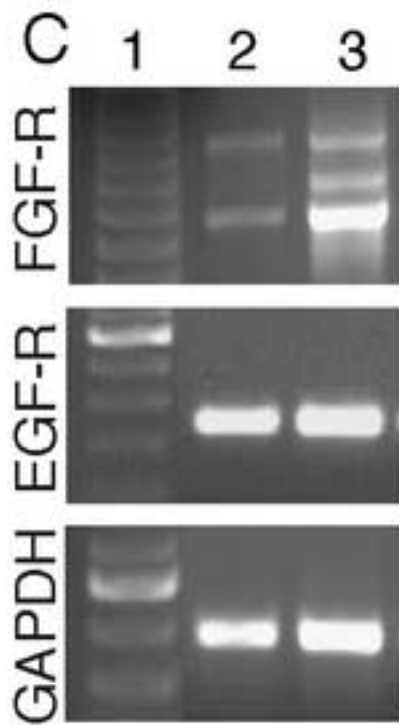
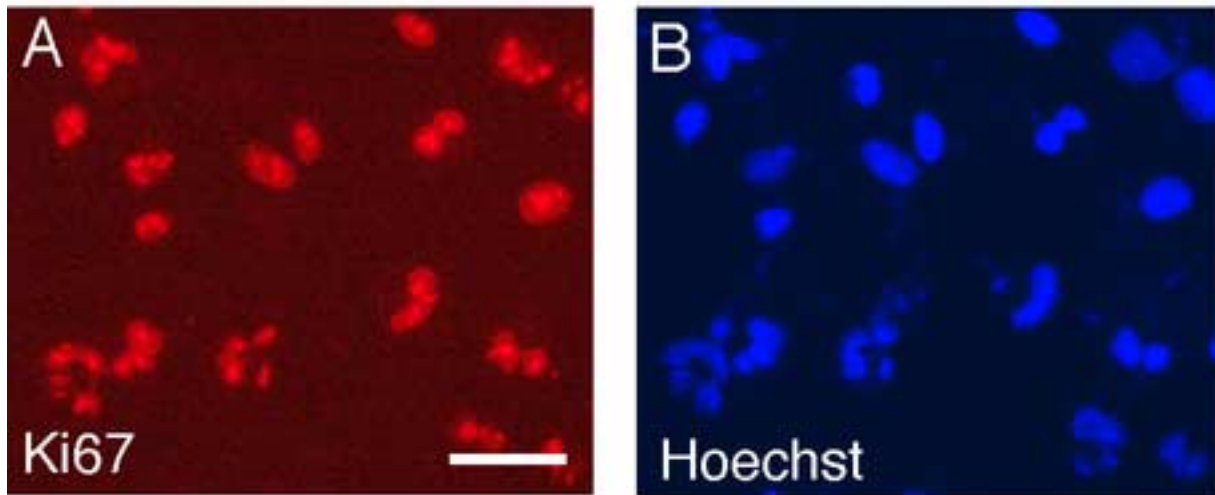


Fig.3

Figure 4
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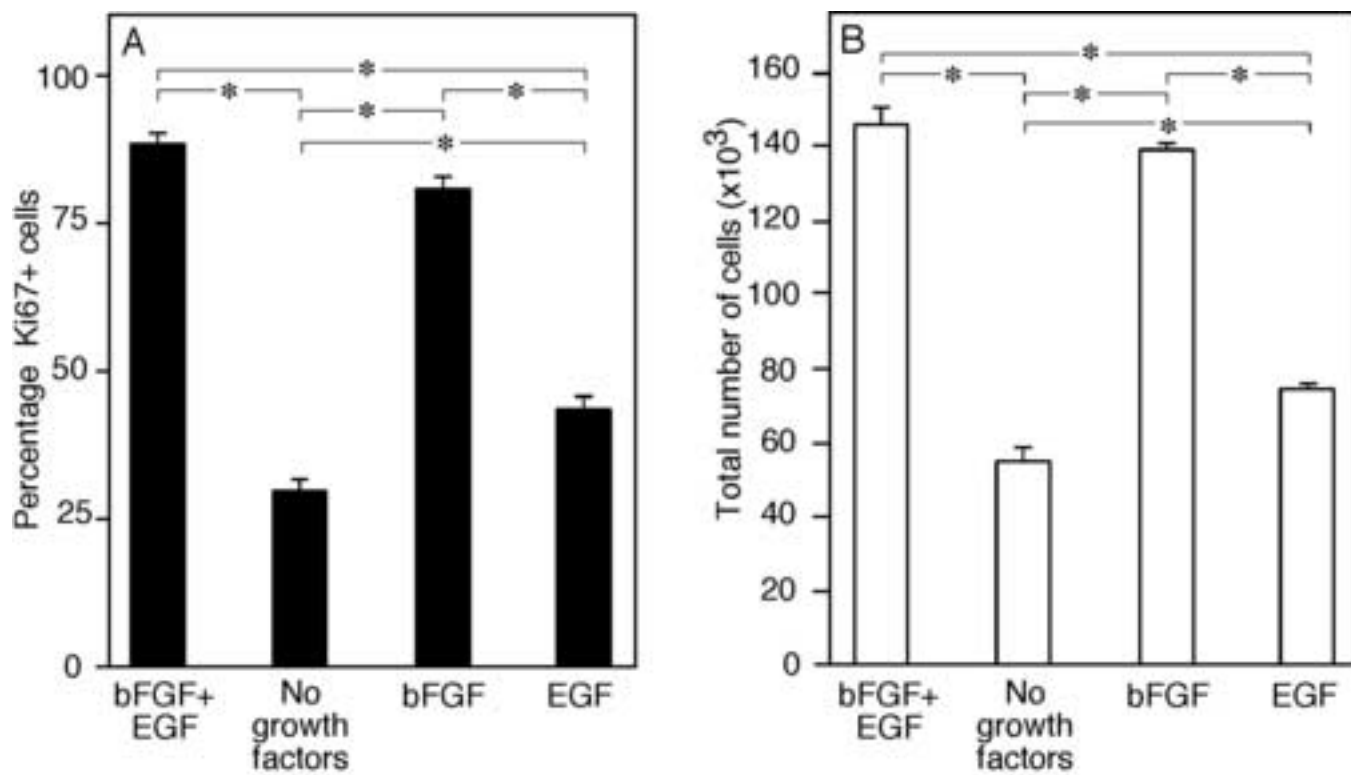


Fig.4

Figure 5
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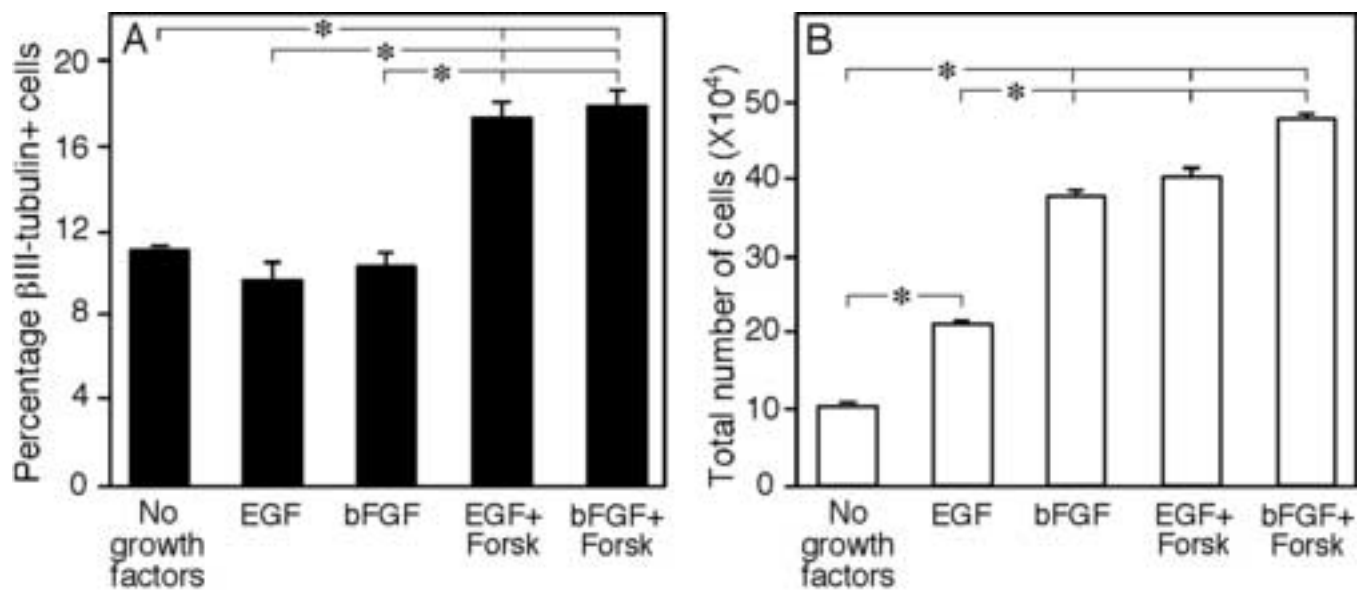


Fig.5

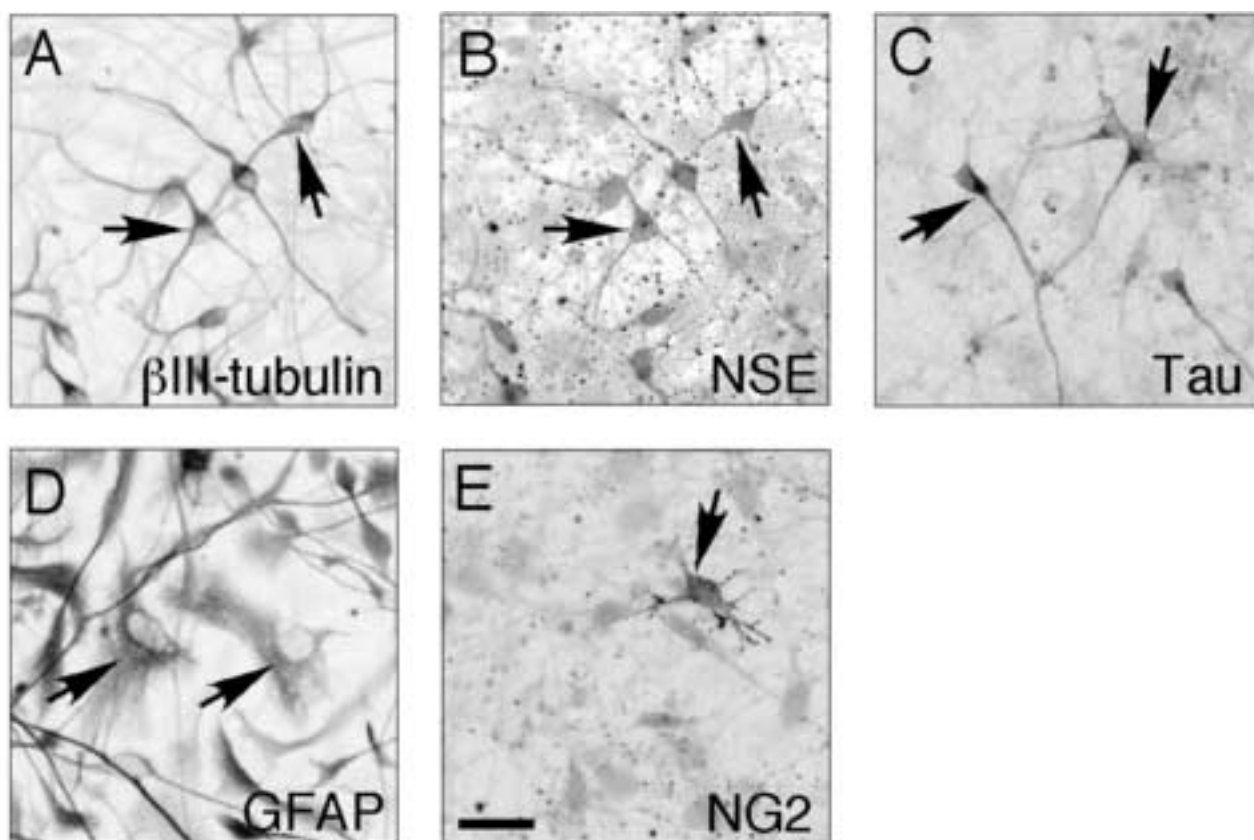


Fig.6

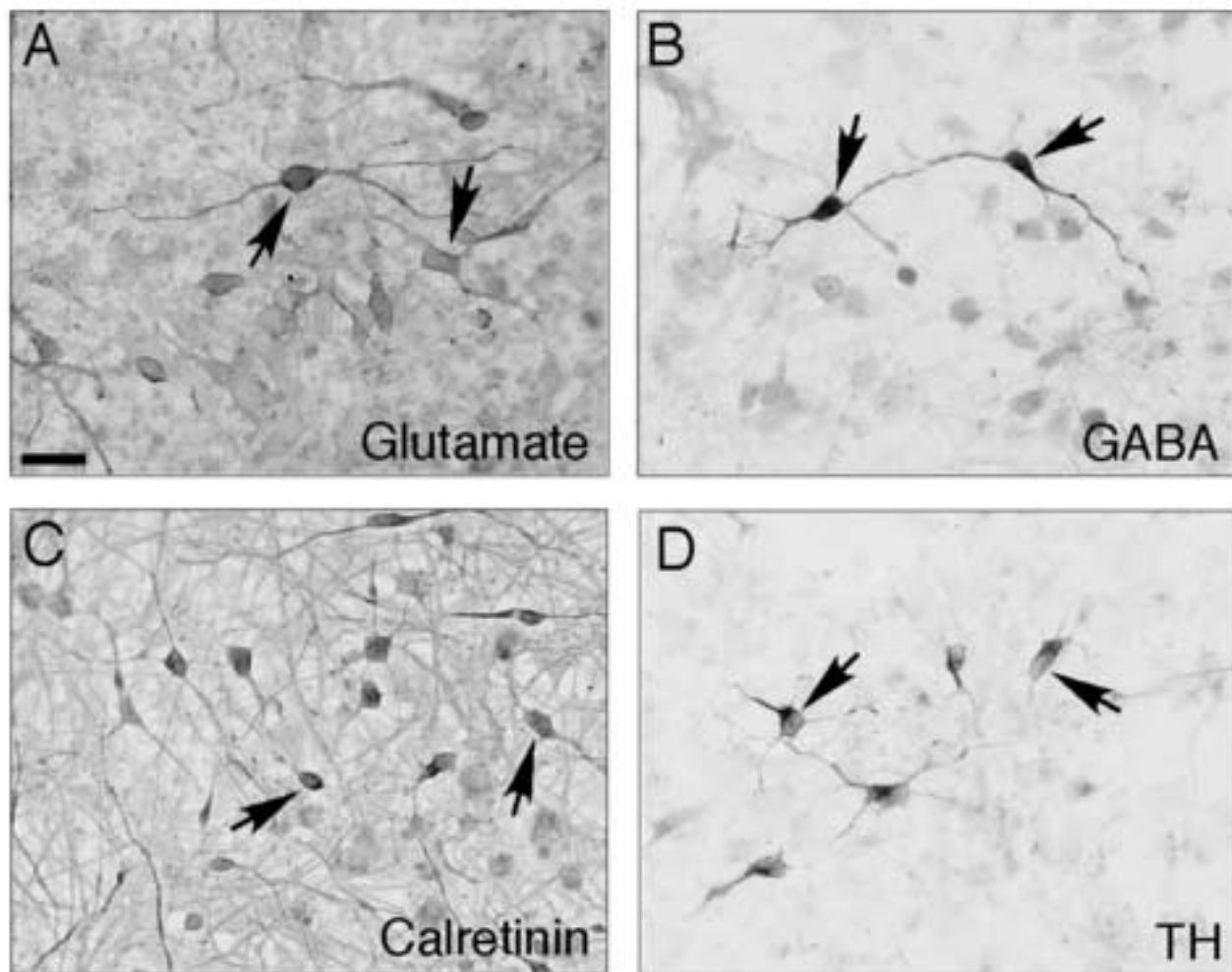


Fig.7

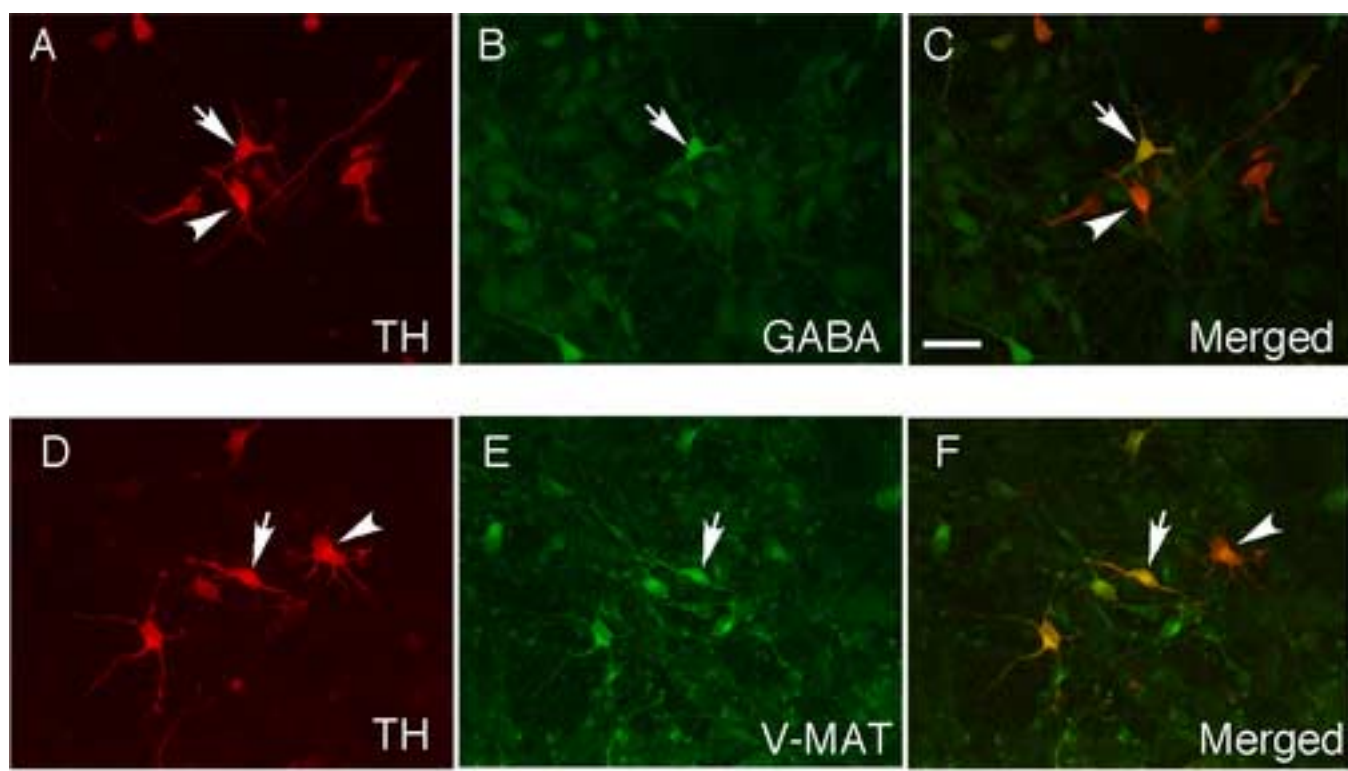


Fig.8

Figure 9
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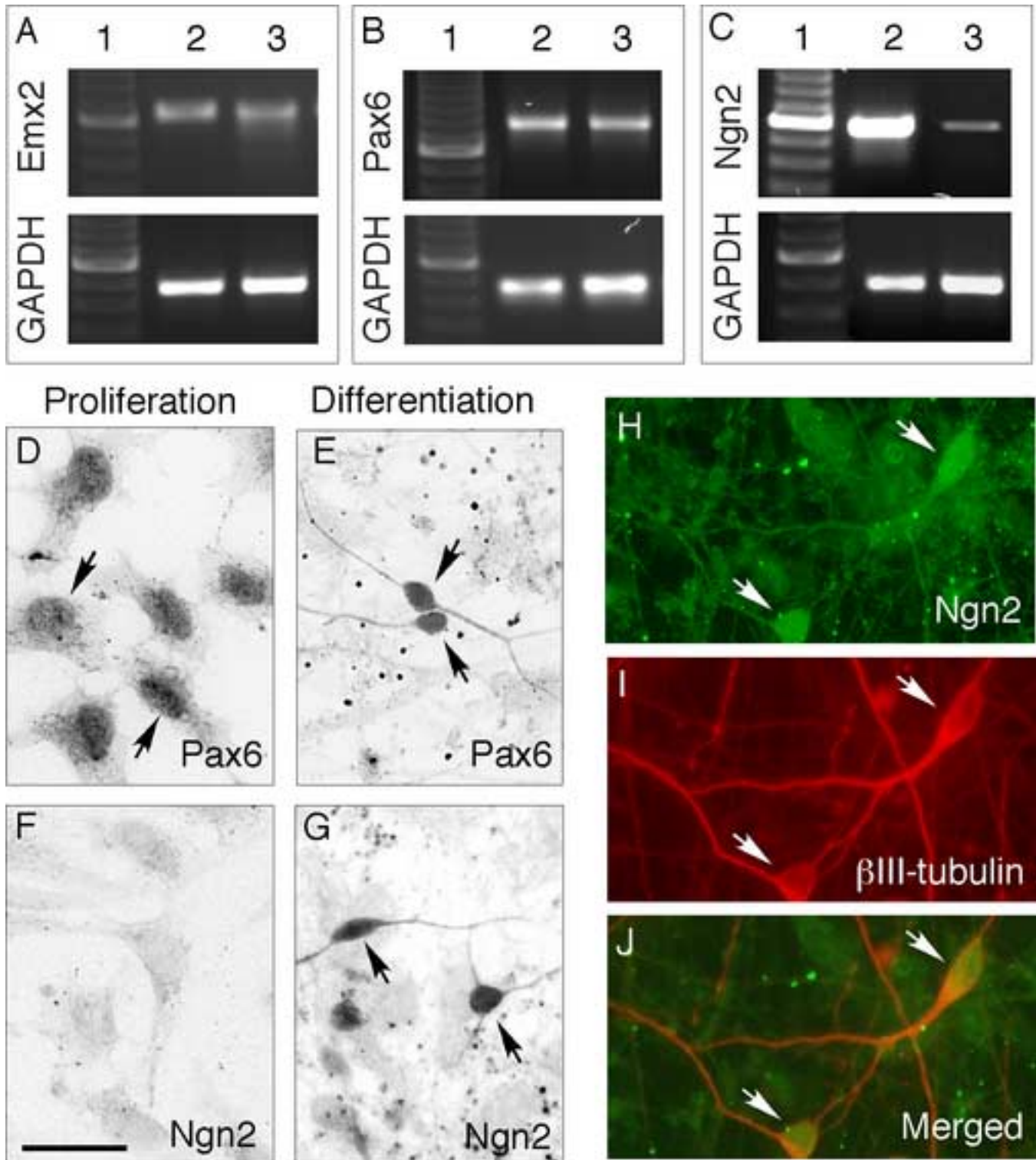


Fig.9