

# Derivation and large-scale expansion of multipotent astroglial neural progenitors from adult human brain

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The isolation and expansion of human neural cell types has become increasingly relevant in restorative neurobiology. Although embryonic and fetal tissue are frequently envisaged as providing sufficiently primordial cells for such applications, the developmental plasticity of endogenous adult neural cells remains largely unclear. To examine the developmental potential of adult human brain cells, we applied conditions favoring the growth of neural stem cells to multiple cortical regions, resulting in the identification and selection of a population of adult human neural progenitors (AHNPs). These nestin<sup>+</sup> progenitors may be derived from multiple forebrain regions, are maintainable in adherent conditions, co-express multiple glial and immature markers, and are highly expandable, allowing a single progenitor to theoretically form sufficient cells for ~4×10<sup>7</sup> adult brains. AHNPs longitudinally maintain the ability to generate both glial and neuronal cell types *in vivo* and *in vitro*, and are amenable to genetic modification and transplantation. These findings suggest an unprecedented degree of inducible plasticity is retained by cells of the adult central nervous system.

**KEY WORDS:** Astrocyte, Progenitor, Plasticity, Human, Expansion, Commitment, Transplantation

## INTRODUCTION

Significant attention has been focused on the development of primary human neural tissue sources for multiple applications in the central and peripheral nervous system (CNS, PNS). Several techniques exist for generating such cell types, including cultivation of multipotent neurogenic progenitors from developing brain (Carpenter et al., 1999; Ostenfeld et al., 2000; Vescovi et al., 1999; Zhang et al., 2001). Despite their promise in generating variegated cell types for therapeutic application, pluripotent cell sources are beset by ethical concerns surrounding their use, as well as concerns over potential tumor formation (Odorico et al., 2001) and immunorejection (Martin et al., 2005).

Primary cells derived from adult brain may provide committed cell types that are developmentally and immunologically matched for transplantation or other biological assays when expanded as proliferating precursor populations *in vitro*. However, a significant barrier to the use of normal somatic cells is an intrinsic lack of sustainable *ex vivo* mitosis in culture (Evans et al., 2003; Kiyono et al., 1998). Characterization of clonally expanded multipotent human progenitors reveals similar proliferative limits to those appreciated in somatic neural cell types (Nunes et al., 2003). Despite these observations, several recent findings suggest that barriers to expansion of postnatal progenitor populations may be more flexible than previously believed. The appreciated lack of expandability of primary human cells has been linked to the cell cycle arrest and to entry into senescence via activation of cyclin-dependent kinase inhibitor p21<sup>WAF1</sup> (and subsequent activation of the p16<sup>INK4A</sup>)

pathway, which has been reported to initially arrest growth of cultured astrocytes after ~20 population doublings (PDs) (Evans et al., 2003). Recent reports describing culture of normal rodent glia indicate that the static upper boundary for maximal cell divisions is more flexible than previously imagined (Mathon et al., 2001; Tang et al., 2001), and may be circumventable using appropriate culture conditions. A growing body of evidence indicates a disparity in replicative competency of cells *in vivo* compared with *in vitro*, particularly for stem cells of high turnover organ systems (Rubin, 2002), theoretically allowing the extensive expansion of endogenous progenitor populations upon the application of correct growth criteria. The lack of catalytic telomerase (TERT), the enzyme responsible for telomere maintenance, in non-neurogenic regions of human brain may also be a limiting factor in the long-term expansion of neural progenitor populations. Though TERT expression has been reported in the neuroepoietic regions of adult rodents (Caporaso et al., 2003), only low levels of telomerase have been reported in *ex vivo* cultured human cells (Ostenfeld et al., 2000). However, telomerase has been detected in germ cells (Kim et al., 1994) and hematopoietic cells *in vivo* (Broccoli et al., 1995; Counter et al., 1995; Hiyama et al., 1995), and can be attenuated by physiological alterations in endometrial cells (Kyo et al., 1997), suggesting it may be possible to induce and/or longitudinally maintain telomerase expression in human cell populations under appropriate conditions.

Glial cells are an acknowledged target for manipulation, as they make up the majority of CNS cell types, and are increasingly recognized for their role in development (Faissner and Steindler, 1995), injury (Silver and Miller, 2004) and as a potential tool for treatment of neurological disease [i.e. secretion of neuroprotective factors (Cunningham and Su, 2002; Kordower, 2003; Tai and Svendsen, 2004)]. Although glial cells retained in defined neuroepoietic niches have been implicated as multipotent, self-renewing neural stem cells (NSCs) in rodents (Doetsch, 2003), the capabilities of cells from non-neuroepoietic regions remains unclear. To examine the extent to which proliferative and developmental alterations may be induced in such cells, we applied growth

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conditions favoring the propagation of gliotypic rodent neural progenitor/stem cells in dissociated monolayer culture (Scheffler et al., 2005) to both neurogenic and non-neurogenic regions of postnatal human brain. Using these conditions, a single population of highly expandable neural progenitors from multiple forebrain regions was isolated and maintained as a homogenous population in vitro. These cells display multiple progenitor markers and retain morphologies consistent with type I astrocytes in culture. Cultured progenitors are highly expandable, and can be maintained for over 300 days and more than 60 PDs with minimal signs of senescence or immortalizing mutations. Interestingly, progenitors derived from neurogenic and non-neurogenic regions express telomerase, the continued expression of which appears to be linked to a synergistic mitogenic effect and is directly coupled to the continued growth of AHNPs.

To test the ability of the derived progenitors to incorporate into the CNS, expanded cells were grafted into the ventricle and cortex of early postnatal and adult rodents, and examined longitudinally for survival, integration, distribution and fate choice. Implanted cells effectively incorporate in a variety of host brain regions and adopt both neuronal and glial phenotypes. Stable and long-term genetic modification of AHNPs was achieved using both transient and long-term transfection approaches. Finally, it was possible to preferentially differentiate AHNPs to rapidly generate neuronal cell types in vitro. These findings suggest an unprecedented developmental plasticity and proliferative potential are retained in CNS glia throughout life.

## MATERIALS AND METHODS

### Isolation and derivation of neural progenitors

Primary tissue was gathered from individuals undergoing surgery related to intractable temporal lobe epilepsy ( $n=5$ , data presented from 17-year-old female, see Table S1 in the supplementary material). All procedures were performed with informed patient consent and in accordance with institutional human tissue handling and use guidelines. Primary tissue was stored overnight in ice-cold Dulbecco's modified eagle medium with F12 supplements (DMEM/F-12, Gibco, Grand Island, NY) medium containing 20  $\mu\text{g}/\text{ml}$  penicillin, 20  $\mu\text{g}/\text{ml}$  streptomycin and 25  $\text{ng}/\text{ml}$  amphotericin B (collectively abx, Invitrogen, Carlsbad, CA). Surgical samples ranging from 0.5–1.5  $\text{cm}^3$  were used from hippocampus (containing hilus), temporal cortex and subventricular zone [SVZ, including anterior horn ( $n=3$ ) and segmented lateral ventricle ( $n=1$ ), see Table S1 in the supplementary material). Tissue samples were immersed in 1 $\times$  phosphate-buffered saline (PBS, 25°C, pH 7.3) lacking  $\text{CaCl}_2$  or  $\text{MgCl}_2$ , and were manually dissociated into 1  $\text{mm}^3$  pieces under sterile conditions. Dissociates were collected by centrifugation (400  $g$ , 5 minutes) and resuspended in 0.25% trypsin (10<sup>6</sup> cells/ml, 15 minutes, 37°C, pH 7.3, Sigma). Dissociates were further triturated using restricted bore pipetting. Cells were collected by centrifugation (400  $g$ , 5 minutes), resuspended in proliferative media (10<sup>6</sup> cells/ml) and seeded onto uncoated 75  $\text{cm}^2$  culture flasks (TPP, Switzerland) overnight (12 hours, 37°C, 5% humidified  $\text{CO}_2$ ). Unattached cells were subsequently collected and plated onto uncoated 60 mm plastic dishes (Costar, Corning, NY) in 4 ml defined proliferative media at a density of  $2 \times 10^4$  cells/ $\text{cm}^2$ . Proliferative media comprised DMEM/F-12 containing N2 supplements, 35  $\mu\text{g}/\text{ml}$  bovine pituitary extract (Invitrogen), abx, 5% fetal calf serum (FCS, Hyclone, Logan, UT), and 40  $\text{ng}/\text{ml}$  of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF, R&D, Minneapolis, MN). EGF and bFGF (20  $\text{ng}$ ) were supplemented every other day. Proliferative media was changed every fourth day. Cells were frozen in aliquots of 1 million cells in DMEM/F-12 containing 10% FCS and 20% dimethyl sulfoxide (v/v, Sigma). For expansion in vitro, cells were proliferated to 90–95% visual confluency, washed with 1 $\times$  PBS, and dissociated with 0.25% trypsin (37°C, 5 minutes). Unless noted, culture media and supplements were purchased from Sigma. Cells were counted using trypan dye exclusion as primary

viability criteria, and were replated at a density of  $2 \times 10^4$  cells/ $\text{cm}^2$  onto uncoated 60 mm plastic dishes (Costar). For culture composition studies, 10  $\mu\text{M}$  5'-bromodeoxyuridine (BrdU, Sigma) was added to defined proliferative or minimal (no EGF, bFGF, and/or serum) media. For growth inhibition experiments, 1  $\mu\text{g}/\text{ml}$  aphidicolin or 20  $\mu\text{M}$  EGCG (Sigma) were added to culture media 1 hour after plating. Irradiated cells were treated with a single 3 Gy dose of X-irradiation. Following period of application, cells were washed in 1 $\times$ PBS and were resuspended in proliferative media or fixed. To assess viability of cells in various growth conditions, equal numbers of culture-matched cells were seeded into triplicate wells of various described culture conditions, and counted 7, 14 and 21 days later. For analysis of mitogenic control over expansion, EGF and/or bFGF were removed from nonconfluent proliferating (30 PD) cultures for seven days, and surviving cells were returned to proliferative media. Significance ( $P < 0.05$ ) was calculated using a student's *t*-test. In vitro images of cultured cells were captured using a Nikon Eclipse TS-100 bright field microscope and a Spot 3.1 digital camera (Diagnostic Instruments, Sterling Heights, MI).

### Immunocytochemistry

Cells were plated on coverslips coated with poly-L-lysine or polyornithine and laminin (LPO) and grown to confluency in proliferative media. Cells were fixed with 4% paraformaldehyde (15 minutes, 25°C, Sigma). After washing with PBS, cells were blocked for 20 minutes (attached cells) or 2 hours (tissue sections) in PBS containing 10% FCS, 5% normal goat serum (Sigma) and 0.1% Triton X-100 (Sigma). Primary antibodies were applied for either 1 hour at 25°C or overnight at 4°C in PBS containing 10% FCS and 0.1% Triton X-100. Primary antibodies were: anti-A2B5 (recombinant A2B5-105, 1:500, Chemicon), anti- $\beta$ -III-tubulin (mouse monoclonal, 1:300, Promega, Madison, WI; rabbit polyclonal, 1:500, Covance, Denver, PA), anti-BrdU (mouse monoclonal, 1:50, BD Biosciences, San Jose, CA), anti-CNPase (mouse monoclonal, 1:250, Chemicon, Temecula, CA), anti-doublecortin (goat polyclonal, 1:200, Chemicon), anti-GFAP (rabbit polyclonal, 1:600, DAKO, Carpinteria, CA), anti-glutamine synthetase (rabbit polyclonal, 1:100, Abcam, Cambridge, MA), anti-GFP (rabbit polyclonal, 1:300, Chemicon), anti-human ribonuclear protein (HNA, mouse monoclonal, 1:300, Acris, Hiddenhausen, Germany), anti-Ki-67 (mouse monoclonal, 1:300, BD Biosciences), anti-map2a-c (chicken polyclonal, 1:30,000, gift from Dr Gerry Shaw), anti-*nestin* (mouse monoclonal, 1:50, Chemicon), anti-*NeuN* (mouse monoclonal, 1:500, Chemicon), anti-*NG2* (rabbit polyclonal, 1:1,000, Chemicon), anti-neurofilament M (mouse monoclonal, 1:500, gift from Dr Gerry Shaw), anti-O4 (mouse monoclonal IgM, 1:150, Chemicon), anti-S100- $\beta$  (rabbit polyclonal, 1:100, Swant, Bellinzona, Switzerland) and anti-TERT (rabbit polyclonal, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were applied for 1 hour at 25°C in PBS containing 10% FCS and 0.1% Triton X-100. Secondary antibodies were: Alexa-555 goat  $\alpha$  chicken (1:300, Molecular Probes, Carlsbad, CA), Cy3 goat  $\alpha$  mouse IgG (1:300, Jackson Labs, West Grove, PA), Cy3 goat  $\alpha$  mouse IgM (1:600, Jackson Labs) and Oregon Green goat  $\alpha$  rabbit (1:600, Molecular Probes). For BrdU imaging, cells were incubated in sodium chloride/sodium citrate (SSC)-formamide (1:1, 37°C, 2 hour), washed three times for 10 minutes in SSC, incubated in 2 N HCl (37°C, 30 minutes) and washed with 0.1 M borate buffer (25°C, 10 minutes). Tissue sections were pretreated with 1%  $\text{H}_2\text{O}_2$  in 70% methanol (15 minutes, 25°C) and visualized using an ABC Elite detection kit (Vector Labs, Burlingame, CA). SA- $\beta$ Gal expression was assessed 7 days after addition of growth arrestors as described (Dimri et al., 1995). Briefly, cells were fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde (25°C, 15 minutes). Following a wash in PBS, cells were incubated (37°C, 12 hours) with fresh SA- $\beta$ -Gal solution (Dimri et al., 1995). Nuclei were stained by application of either DAPI (1  $\mu\text{g}/\text{ml}$ , 25°C, 10 minutes, Sigma) or propidium iodide (50  $\mu\text{g}/\text{ml}$ , 25°C, 10 minutes, Sigma) prior to mounting. Fluorescence microscopy and unbiased cell counting were performed on a Leica DMLB upright microscope (Bannockburn, IL) and images were captured with a Spot RT color CCD camera (Diagnostic Instruments). Confocal microscopy was performed on an Olympus IX-70 microscope (Melville, NY) using Confocal 1024 ES software (BioRad, Hercules, CA). Unbiased cell counting data was

generated from three independent experiments, with each trial comprising a minimum of 12 visual fields at 40× magnification. All values were expressed mean±s.e.m.

### Electrophysiology

Cell culture media was removed and cells were perfused with continuously oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) artificial cerebrospinal fluid containing (in mM): 125 NaCl, 26 NaHCO<sub>3</sub>, 20 glucose, 3 KCl, 2 CaCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub> and 1 MgCl<sub>2</sub> (Sigma). Cells were visualized using Axioskop-FS DIC microscope (Zeiss, Thornwood, NY). Patch electrodes were pulled from borosilicate capillary glass using a Flaming-Brown P-87 microelectrode puller (Sutter Instruments, Novato, CA) and had a resistance of 4–6 MΩ when filled with internal solution comprising (in mM): 130 K-gluconate, 10 HEPES, 0.2 EGTA, 2 ATP and 0.3 GTP (pH 7.2, osmolarity 290). Whole-cell recordings were performed with an Axopatch-1D (Molecular Devices, Sunnyvale, CA) at room temperature, and data was acquired and assembled using Clampex 8.2 software (Molecular Devices). The values of capacitance and input resistance were determined by passive membrane recording. Series resistances were 10–20 MΩ and recordings were discarded if a change of series resistances was more than 10%. Cells were held at –65 mV. Na<sup>+</sup> and K<sup>+</sup> currents were elicited by applying voltage steps to cells (–80 to +60 mV, increment: 15 mV, duration: 300 mseconds). All values were expressed as mean±s.e.m.

### Western blot analysis

Cells were lysed in a modified RIPA buffer containing (in mM): 150 NaCl, 50 EDTA (pH 7.5), 50 sodium β-glycerophosphate, 50 NaF, 5 sodium pyrophosphate, 2 EDTA, 2 EGTA, 1 DTT, 1 phenylmethylsulfonyl fluoride, 1 sodium orthovanadate with 1% Triton X-100, 10 μg/ml leupeptin and 10 μg/ml aprotinin (Sigma). Equal amounts of lysates were resolved on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked in TBST [20 mM Tris-HCl (pH 7.5), 500 mM sodium chloride and 0.05% Tween-20] containing 5% nonfat dry milk for 2 hours and then incubated with primary antibodies in TBST containing 1% BSA at room temperature for 2 hours. Primary antibodies used were: cyclin A (rabbit α human, 1:200, Santa Cruz), cyclin D1 (mouse α human, 1:2000, Santa Cruz), cyclin E (rabbit α human, 1:200, Santa Cruz), TERT (1:200, rabbit α human, Santa Cruz), p53 (mouse α human, 1:500, Santa Cruz), p21 (rabbit α human, 1:200, Santa Cruz) and p16 (rabbit α human, 1:200, Santa Cruz). Horseradish peroxidase-labeled secondary antibodies were applied in TBST containing 5% nonfat dry milk for 2 hours. Secondary antibodies: donkey α rabbit (1:10,000, Amersham, Piscataway, NJ), donkey α mouse (1:5000, Amersham). Protein was visualized by using an enhanced chemiluminescence (ECL) detection system (Amersham).

### Karyotyping

All cytogenetic analysis was done by the University of Florida core facility for cytogenetics. Briefly, confluent cell layers were incubated with 300 μl Karyomax (Gibco), dissociated with 0.25% trypsin and resuspended in 75 mM KCl for 6 minutes. Cells were collected and resuspended in 3:1 (vol/vol) ethanol:acetic acid. Cells were visualized on a coverslip using light microscopy. Seven metaphasic cells were observed and described.

### Transplantation

Cultured progenitors (10<sup>5</sup> cells, from 30 PDs) were trypsinized and resuspended at a density of 10<sup>5</sup> cells in 2 μl PBS. Cells were injected into the lateral ventricle or cortex of anesthetized postnatal day 3 C57/B6 mice (*n*=6) or immunocompromised adult NOD-SCID mice (*n*=3, Taconic, Hudson, NY) at the following stereotaxic coordinates: bregma, –1.06 mm; interaural +2.74 mm; 1 mm left of midline to a depth of 1 (cortical, NOD-SCID) or 2 (ventricular, C57/B6) mm. Animals were sacrificed and perfused with 4% paraformaldehyde 7 (C57/B6) or 30 (NOD-SCID) days later. Brains were removed and placed in 2% paraformaldehyde containing 30% sucrose (v/v) overnight, and were sectioned into 20 μm sagittal and coronal sections on a freezing microtome and stored in cryoprotectant. Transplanted cells were identified by size (>20 μm cell body diameter) and immunoreactivity for HNA. For studies of migration, serial sections were analyzed for anteroposterior migration. Mediolateral migration was

measured on sections containing injection site. Immunosuppressed animals (*n*=3) were injected with 10 mg/kg cyclosporin A (Sigma) immediately prior to transplantation and every other day thereafter.

### Genetic modification of AHNPs

Cultured progenitors (30 PDs) were plated on LPO coverslips at a density of 500 cells/cm<sup>2</sup> in defined proliferative media. Two hours after plating, cells were transfected with a plasmid containing Pax6-IRES-eGFP under the control of the simian virus 40 (SV40) promoter (Haubst et al., 2004) using a standard protocol for Superfect transfection reagent (Qiagen, Valencia, CA) with 1 μg DNA or Effectene transfection reagent (Qiagen) containing 4 μg DNA for 8 hours. Cells were evaluated for endogenous eGFP expression or probed with eGFP antibody 24, 36 or 72 hours later. Lentiviral vectors containing the human eGFP gene were generated as described previously (Iwakuma et al., 1999). Optimal lentiviral infection was gauged by gated FACs analysis of eGFP expression from multiplicities of infection (moi) ranging from 5–20 (presented in Fig. S1 in the supplementary material). Cells were evaluated 7 and 30 days post infection.

### Neurosphere formation and generation of neuronal cell types

PD 3 progenitors (cultured under adherent conditions 3 days) from SVZ and temporal cortex were placed in neurosphere conditions as described (Kukekov et al., 1999). Briefly, cells were seeded at a density of 5×10<sup>4</sup> cells/ml in proliferative media containing 1% methylcellulose in anti-adhesive conditions. EGF and bFGF were supplemented bidaily, and neurosphere formation was visually tracked using light microscopy at 7, 14 and 21 days. Matched cultures of clonal seedings were prepared every fifth passage. To differentiate adherent progenitors, serum, EGF and bFGF were removed from the culture media and supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.5 mM 1-dibutyl cAMP, and 25 ng/ml NGF (Ronnett et al., 1990). Media supplements were replaced every third day. FGF8 (100 ng/ml), sonic hedgehog peptide (500 ng/ml), pleiotrophin (100 ng/ml) and retinoic acid (0.5 μM) were purchased from Sigma. Matched differentiating cells were co-cultured with 10 μM BrdU for 2 days following factor addition. Cells were immunocytochemically evaluated 2, 3, 5 and 7 days later, and were electrophysiologically evaluated 7 days after differentiation.

### Electron microscopy

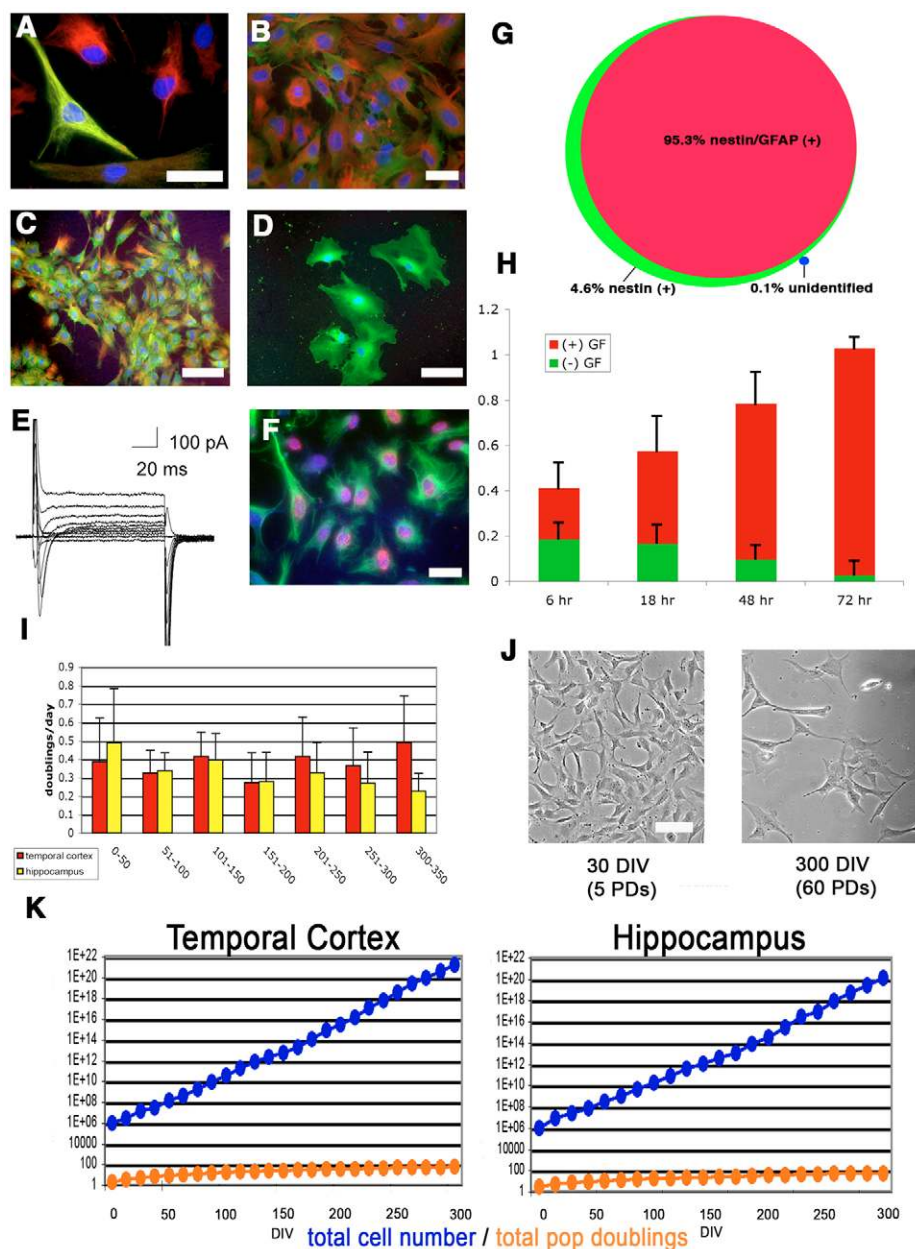
PD 5 SVZ cells were grown in defined proliferative media on LPO-coated aclar coverslips. Fixation and processing of thin sections was standard. Samples were visualized on a Leica EM10A transmission electron microscope at magnifications between 1 and 16,000×. Images were captured using a CCD digital camera (Finger Lakes Instrumentation, Lima, NY).

## RESULTS

### Characterization and expansion of primary cells as AHNPs

Human brain tissue (anterolateral temporal lobe) was derived from individuals undergoing resection associated with medically intractable epilepsy. Tissue was microdissected into regions containing hippocampus, SVZ or temporal cortex gray matter (see Fig. S1 in the supplementary material). Isolated dissociates were maintained as a monolayer on uncoated plastic dishes throughout culture in defined proliferative media, modified from a standard protocol for the culture of neural stem cells (Scheffler et al., 2005).

To identify cultured cell types, primary cells were examined for expression of phenotypic markers. Following dissociation, immunocytochemistry on primary cells 3 days in vitro (DIV) revealed a heterogeneous population containing predominantly astrocytic (GFAP<sup>+</sup>) cells, but included rare neuronal (NeuN<sup>+</sup>, PSA-NCAM<sup>+</sup>) and oligodendrocyte (CNPase<sup>+</sup>, O4<sup>+</sup>) phenotypes. Following expansion in defined growth medium, a population of progenitors is established as the sole proliferating population by 14 DIV. These cells are defined by the conserved expression of nestin



**Fig. 1. Expansion of primary neural cells as a homogenous population of AHNPs.** (A) Highly expanded (more than 60 PDs) cells ubiquitously express nestin (red), with a large subset of GFAP<sup>+</sup> cells (green). (B) AHNPs express widespread immature neuronal and glial markers, including A2B5 (red) and NG2 (green). (C,D) AHNPs (nestin<sup>+</sup>, green) express astrotypic markers in a large subset of cells, including S100 $\beta$  (C, red) and glutamine synthetase (D, green). (E) Voltage-clamp profile of these cells reveal prominent Na<sup>+</sup> and K<sup>+</sup> channel activity. Data shown for temporal cortex-derived cells. (F) Nestin<sup>+</sup> (green) AHNPs proliferated in the presence of BrdU (red) uniformly incorporate thymidine analog. (G) Stereological evaluation of proliferating AHNPs reveals a uniform nestin<sup>+</sup> population that frequently co-expresses glial cell markers (GFAP shown). Maintaining these cells in growth medium supplemented with BrdU results in label saturation in AHNPs (BrdU<sup>+</sup>Nestin<sup>+</sup> cells) at a rate of incorporation analogous to previously characterized proliferative dynamics (H). Removal of mitogenic stimuli (GF=EGF+bFGF) results in failure of AHNPs to divide (see Fig. 2F). (I,J) Both hippocampal and temporal cortex-derived AHNPs maintain comparable stable doubling rates and uniform protoplasmic morphologies throughout culture. (K) AHNPs derived from temporal cortex and hippocampus reveals continuous logarithmic expansion throughout culture. Scale bars: 25  $\mu$ m in A,B,F,J; 50  $\mu$ m in C; 75  $\mu$ m in D. Images counterstained with DAPI.

(Fig. 1A), with the retention of the morphological and antigenic properties ascribed to type I protoplasmic astrocytes (Cammer and Tansey, 1988; Norenberg and Martinez-Hernandez, 1979; Raju et al., 1980). Cells present 14 DIV frequently co-express both immature and astrotypic markers, including A2B5 (97.2 $\pm$ 1.3%), nestin (99.8 $\pm$ 0.1), NG2 (96.7 $\pm$ 2.2%), GFAP (95.4 $\pm$ 3.2), S100 $\beta$  (89.8 $\pm$ 4.1), and glutamine synthetase (90.4 $\pm$ 4.4) (% positive $\pm$ s.e.m.) (Fig. 1A-D,G), and do not express mature neuronal (NeuN) or oligodendroglial (O4) markers. To further characterize these cells, we performed single cell patch clamp recordings for highly expanded (30 PDs) cells ( $n=4$ ). Recorded cells exhibited ubiquitous gliotypic membrane potentials (Sontheimer, 1994), with a RMP of  $-28.3\pm 4.2$  mV, a  $C_m$  of  $277.2\pm 189.7$  pF, a  $R_m$  of  $214.5\pm 156.1$  M $\Omega$ , and a  $R_a$  of  $14.9\pm 3.1$  M $\Omega$ . Recorded cells did not fire action potentials, but displayed prominent Na<sup>+</sup> channel activity and K<sup>+</sup> channel activity (Fig. 1E). To determine the composition and dynamics of proliferating populations, cells undergoing 10, 20 and

30 PDs were cultured in the presence of the thymidine analog BrdU. In our culture conditions, only nestin<sup>+</sup> cells appear to re-enter the cell cycle, as shown by their rapid increase in prevalence (55.2 $\pm$ 17.2% at 3 days in culture versus 99.7 $\pm$ 0.2% at 30 days in culture) and near-ubiquitous incorporation of BrdU throughout culture [average 98.9 $\pm$ 0.8% (nestin<sup>+</sup>/BrdU<sup>+</sup>)/BrdU<sup>+</sup> for 10, 20 and 30 PD populations following 48 hours BrdU administration]. This finding was confirmed through appreciation of extensive intermediate filaments in cells subjected to ultrastructural examination (data not shown). Furthermore, the rate at which BrdU increases in culture is contiguous with the known doubling rate of expandable populations derived from multiple forebrain regions (Fig. 1H). In the absence of defined growth factors, BrdU incorporation is rapidly attenuated in nestin<sup>+</sup> cells, which accompanies a cessation of growth (Fig. 1H). Neurons (PSA-NCAM<sup>+</sup>) and oligodendrocytes (CNPase<sup>+</sup>) were not appreciated in proliferating culture conditions after 14 DIV. Cells displaying a stellate or reactive morphology were rarely detected in

culture. Selecting for and proliferating unattached cells 12 hours after initial plating decreased microglia (CD11b<sup>+</sup>) presence in culture to nearly undetectable levels. FACs analysis of 30 PD cell cycle revealed a single proliferating population with minimal side scatter (data not shown).

Immature astrocyte-like cells have been implicated as 'immortal' neural stem-like cells maintained throughout life in the hippocampus and subventricular zone (Laywell et al., 2000; Sanai et al., 2004; Seri et al., 2001). In rodents and humans, these cells have been described as existing throughout life (Sanai et al., 2004; Tropepe et al., 1997) and cells cultured from these regions may represent unique stem cell populations that may behave differently from somatic cells (Potten and Morris, 1988). To detect potential NSCs, proliferating temporal cortex and hippocampal astrocytes were clonally seeded and assayed for neurosphere formation as described (Kukekov et al., 1999) every fifth passage ( $n=3$  wells/assay). Cultured cells fail to generate multipotent neurospheres at clonal seeding densities at any point, suggesting they were a separate population from neurosphere-forming cells. Despite a lack of proliferation in neurosphere-forming conditions, clonally seeded cells remained viable for up to 14 days, and were expandable as adherent cultures following substrate reattachment. Cells isolated in this manner were expandable as individual clones (153/182 clones examined for a minimum of five PDs). To assess whether clonally isolated cells recapitulate the progenitor populations isolated from initial derivation, low density adherent cultures (500 cells/cm<sup>2</sup> from primary and 10 PD temporal cortex) were created from both adherent cells and cells surviving 14 days under clonal anti-adhesive conditions. Cells derived from both culture conditions ubiquitously express nestin (>99.9%), with a large subset (similar to previously described for initial derivation) expressing GFAP (Fig. 1G). Similarly, addition of BrdU immediately following attachment resulted in the progressive increase in BrdU incorporation to >99% within 72 hours (Fig. 1H), suggesting virtually all cells maintain a constitutively proliferative state. In both initial and clonal derivations, BrdU incorporation was limited to nestin<sup>+</sup> cells, indicating a homogenous population of proliferating progenitors with frequent astrotypic immunophenotypes compose the proliferating culture, rather than a heterogeneous culture composed of proliferating lineage-negative proliferating progenitors that generate postmitotic, but immature, astroglial cells. Removal of mitogenic stimuli results in a progressive attenuation of BrdU incorporation and subsequent failure to thrive (Fig. 1H). Thymidine analog incorporation in GFAP<sup>+</sup> cells does not significantly differ from that of nestin<sup>+</sup> cells, suggesting no additional proliferative ability of GFAP<sup>+</sup>/nestin<sup>-</sup> cells. Nestin expression remains in over 99% of GFAP<sup>+</sup> cells, suggesting a single, mitogen-dependent progenitor population comprises expanding cultures. Based on these observations it appears we have established conditions for the isolation of a unique population of widely distributed, highly expandable forebrain cells.

To determine the proliferative limits for purified gliotypical cells in these conditions, cells were grown *in vitro* and their expansion quantified via cell counting. To ensure a homogenous population for measurement of proliferation, neural cell dissociates were grown continuously for 30 days prior to quantification of growth (~10 PDs) to remove postmitotic cells. Following this initial culture period, 10<sup>6</sup> cells from hippocampus and temporal cortex were plated in defined proliferative media and supplemented with EGF and bFGF bi-daily. Proliferating cultures derived from multiple regions maintain a constant contact-inhibited growth rate for both hippocampal- and temporal cortex-derived cells (0.34±0.04 and 0.35±0.04 doublings/day respectively) (Fig. 1I), while retaining constant

morphology and size throughout culture (Fig. 1J). Upon reaching confluency, cultured cells were passaged 1:2 and total cell number counted. Both temporal cortex, subventricular and hippocampal astrocytes exhibited logarithmic growth expansion in defined growth medium for over 300 DIV, with a maximal expansion of >60 PDs (Fig. 1K), equivalent to one cell giving rise to >10<sup>16</sup> cells.

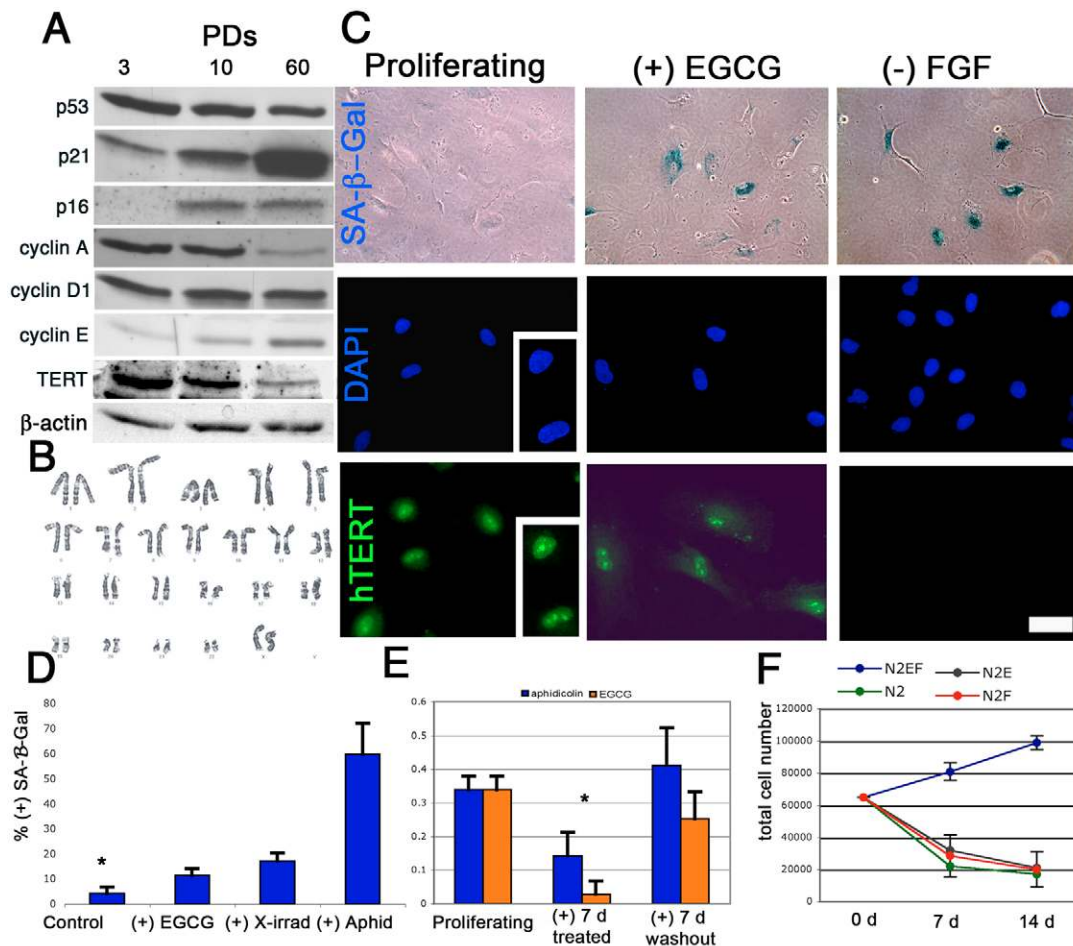
### AHNPs maintain growth sensitivity and avoid immortalization

Purified expanding cell populations may undergo growth-specific genetic modification(s) resulting in circumvention of cell cycle regulatory mechanisms and manifesting in an immortalized phenotype, allowing for extensive clonal expansion similar to that observed. Immortalized cells frequently contain accumulated neoplastic mutations in genes linked to cell cycle control, apoptosis, and survival, and may be characterized by a lack of response to physiological or chemical arrestors of the cell cycle. Furthermore, transformed cells often exhibit irregular or hyperplastic growth rates, and can be tumorigenic when transplanted. To determine whether such immortalizing mutations were present in AHNPs, we examined the molecular and cytogenetic profiles of proliferating populations.

Immortalization of human cells is frequently marked by the aberrant expression of key regulatory proteins. To determine the activation status of cell cycle proteins in expanding cells, protein expression levels for major cell cycle regulatory proteins were measured throughout the culture period (Fig. 2A). AHNPs longitudinally express major cell cycle checkpoints, including p53, a key initiator of cellular senescence. Expanding AHNPs also express p16, the deletion of which is reported to be essential for immortalization in both epithelial cells (Kiyono et al., 1998) and astrocytes (Evans et al., 2003) in humans (Fig. 2A). Though p53 remains constant throughout culture, other cyclin-dependent kinase inhibitors (i.e. p21) and cyclins (i.e. cyclin E) increased throughout the culture period. This observation agrees with noted increases in both promotive and inhibitory proteins during the extended culture of glial progenitors in rodents (Mathon et al., 2001; Tang et al., 2001). Interestingly, robust TERT expression was appreciated in cultured cells initially, matching a report of initial expression of telomerase in cultured fetal human brain tissue (Ostenfeld et al., 2000). TERT is expressed at progressively lower levels during expansion in defined proliferative conditions. None of the populations examined ( $n=6$  from 4 individuals) was capable of indefinite growth (62.1±2.31 PDs on average, range 59-65). Karyotypic analysis of metaphasic high passage cells ( $n=7$ ) revealed no gross cytogenetic abnormalities in highly expanded cells (Fig. 2B).

To determine whether AHNPs remain sensitive to chemical and physiological regulators of the cell cycle, highly expanded AHNPs were treated with the DNA synthesis inhibitor aphidicolin or X-irradiation and were assessed for the senescent cell marker SA-β-gal (Dimri et al., 1995) (Fig. 2D,E), 7 days later. Treated cells expressed significantly higher levels of SA-β-gal than did age-matched controls, suggesting cell cycle checkpoint mechanisms remain sensitive throughout culture period.

Telomerase, the holoenzyme responsible for telomeric extension, is longitudinally expressed during the observed period of growth. To investigate the relationship between telomerase expression and continued growth in culture, the telomerase inhibitor epigallocatechin-3-gallate (Naasani et al., 1998) was added to highly expanded cells, and telomerase expression and growth rate were measured 7 days later (Fig. 2C-E). Despite significant reduction in the rate of cellular proliferation, TERT



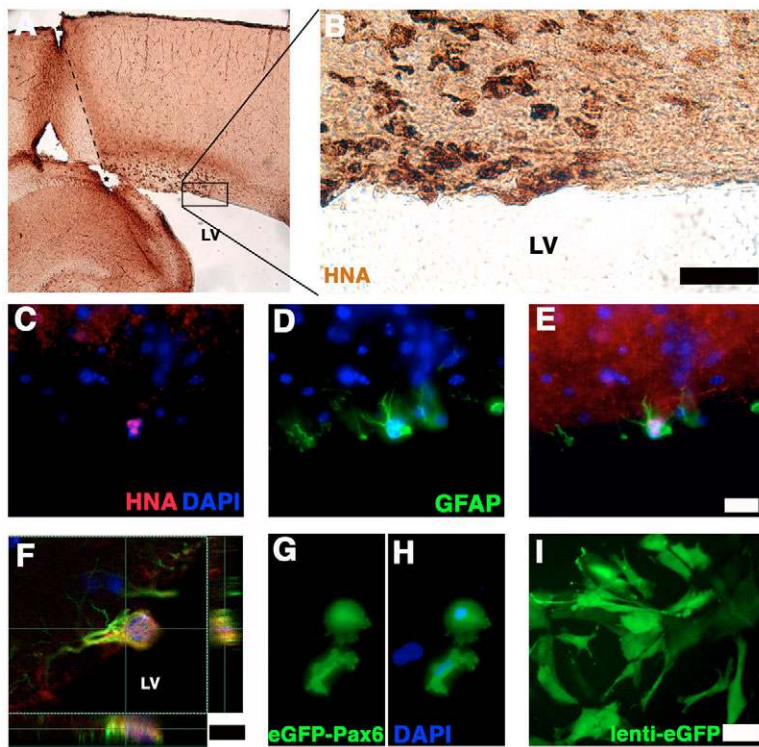
**Fig. 2. AHNPs avoid immortalizing mutations, and exhibit mitogen- and telomerase-dependent growth.** (A) Cultured AHNPs express major growth regulatory proteins longitudinally throughout culture. (B) Karyotyped AHNPs display normal ploidy and have no gross cytogenetic malformations. (C) Following growth arrest by an exogenous TERT inhibitor (EGCG) or growth factor withdrawal cultured cells express SA-β-Gal. However, only mitogen-withdrawn (-bFGF) cells lose TERT expression when evaluated 7 days later. (D) Physiological (x-irradiation) or chemical inhibitors (aphidicolin, EGCG) consistently increase the fraction of cells expressing SA-β-Gal. (E) Application of reversible growth inhibitors yields a significant reduction in growth rate. AHNPs revert to previous proliferative levels following arrestor washout. (F) Age-matched AHNPs placed in either basic media (N2) or media containing EGF or bFGF only (N2E, N2F) enter irreversible growth arrest compared to defined proliferative conditions (N2EF) and subsequently become unviable. Data shown for temporal cortex derived cells. \* $P < 0.05$ , Student's *t*-test. Scale bar: 75  $\mu\text{m}$  in C.

expression remained ubiquitous in expanding AHNPs (Fig. 2C). To further examine the potential relationship between growth conditions, telomerase expression and expandability of progenitors, EGF, bFGF and serum were selectively removed from culture medium of highly expanded cells. Removal of EGF and/or bFGF resulted in the loss of telomerase expression within 7 days accompanied by a failure to continue to proliferate (Fig. 2D,E). Interestingly, AHNPs treated with EGCG or aphidicolin returned to normal growth rates within 7 days after replating in proliferative media (Fig. 2E), while cells deprived of growth factor failed to regain previous proliferative levels and subsequently became unviable (Fig. 2F).

Less than 4% of AHNPs exhibited SA-β-gal in defined proliferative conditions, suggesting cells continue to be mitotically active and are expandable for over 60 PDs (Fig. 2D). Multinucleated and/or giant cells, characteristic of senescent cells, were rarely observed at any point throughout culture period. These findings suggest cultured progenitors are highly expandable, do not spontaneously immortalize and remain mitogen dependent.

### AHNPs demonstrate phenotypic plasticity, and are a transplantable, modifiable cell source

To assess the ability of AHNPs to survive, integrate and assume a committed phenotype *in vivo*, AHNPs were injected into the right lateral ventricle of early postnatal C57/B6 mice. Engrafted cells were assessed for patterns of incorporation and immunophenotype 7 days later using human ribonuclear protein (HNA) to identify engrafted cells. Moderate reactive gliosis was appreciated in transplanted animals, which increased with animal age. Immunosuppression of young animals with cyclosporin A substantially reduced reactive gliosis and increased survival and engrafted cell distribution. Engrafted cells were primarily detected within the ependymal wall of the injected ventricle, with increasingly frequent distribution immediately adjacent to the injection site (Fig. 3A,B; see Fig. S2 in the supplementary material). HNA<sup>+</sup> cells were also frequently detected in the choroid plexus, adjacent to the third ventricle, cerebral aqueduct and (rarely) in the cerebellum. Immunocytochemistry revealed integrating cells infrequently adopt mature neuronal morphologies. Though many



**Fig. 3. AHNPs maintain viability and assume glial phenotypes upon ventricular transplantation.**

(A) AHNPs cells injected into the right lateral ventricle of postnatal day 3 mice were detectable with HNA in periventricular tissue adjacent to injection site (\*). (B) HNA<sup>+</sup> cells (boxed in a) were primarily located within 100  $\mu$ m of the ventricular wall in the ependymal and subependymal cell layer. (C-E) HNA<sup>+</sup> cells (red) integrating into the LV wall display conserved morphology of astrotypic cells, and frequently co-express GFAP (green). (F) Confocal micrograph shows GFAP<sup>+</sup> process extension from an AHNPs into the subependymal zone. (G,H) Activated dendrimer transfection of Pax6-eGFP of 30 PDs AHNPs 3 days post-transfection. (I) Lentiviral-eGFP transfection of 30 PD AHNPs (20 moi). Scale bars: 40  $\mu$ m in B; 25  $\mu$ m in C-E; 8  $\mu$ m in F; 20  $\mu$ m in G-I. Images in C-F,H are counterstained with DAPI.

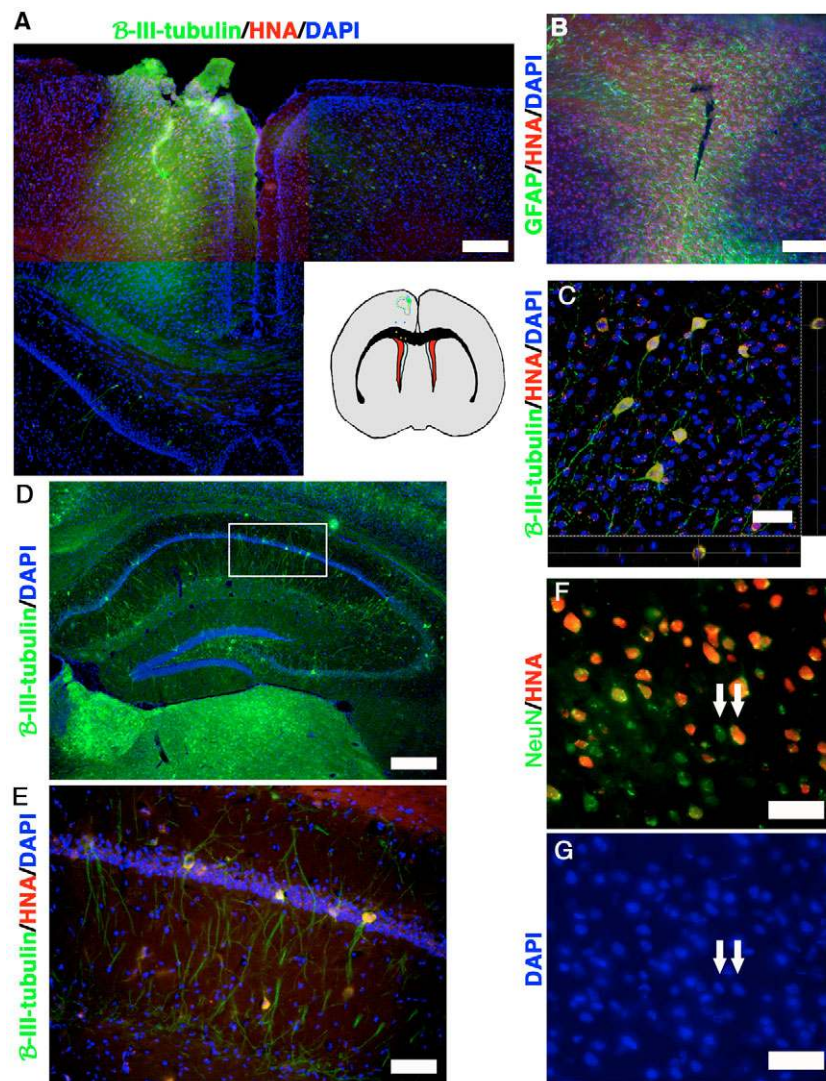
ventriculary engrafting cells did not express mature phenotype markers, engrafted cells were frequently found to co-express GFAP (Fig. 3C-F). Surviving ventriculary engrafted cells did not co-express neuronal markers and rarely expressed nestin, suggesting they mature to largely postmitotic astrotypic cell types upon integration.

AHNPs are highly expandable, suggesting a potential role as a substrate for a transplantable cell source for delivery of gene products (Kordower, 2003; Tai and Svendsen, 2004) or alteration of fate choice. To concurrently examine the amenability of AHNPs to ex vivo genetic modification and examine the feasibility of single-gene alteration in directing progenitor fate choice, AHNPs were transfected with a plasmid containing a 2 kb gene encoding the neural patterning gene Pax6 and enhanced green fluorescent protein (eGFP) using both activated dendrimer transfection and non-liposomal lipid transfection. Stably transfected (eGFP<sup>+</sup>) cells were detected 3 days following transfection (Fig. 3G,H), but did not result in generation of neuronal phenotypes. Introduction of a lentiviral vector expressing recombinant human eGFP under the SV40 promoter was used to identify optimal transfection conditions for expression of lentiviral vector expression in AHNPs (Fig. 3I, see Fig. S3 in the supplementary material). Stably infected (eGFP<sup>+</sup>) cells were present 30 days after infection.

To examine survival and integration of AHNPs in the adult CNS, AHNPs (30 PDs) were transplanted into the cortex of adult (P90) NOD-SCID immunocompromised mice. Transplanted AHNPs (HNA<sup>+</sup>) were detectable following a 30-day engraftment period. In contrast to ventriculary engrafted cells, cortically implanted cells were found to express  $\beta$ -III-tubulin and adopt neuronal morphologies with significant process extension (Fig. 4A,C). Astrocytic (GFAP<sup>+</sup>, Fig. 4B) or oligodendrocytic (CNPase<sup>+</sup>, data not shown) phenotypes were rarely detected in cortically integrating HNA<sup>+</sup> cells. Transplanted AHNPs were largely concentrated around the injection site, with limited migration along the dorsoventral axis. Serial analysis of adjacent sections revealed

modest anteroposterior migration and more extensive migration on the medial-lateral axis (see Fig. S2 in the supplementary material). Very infrequently, HNA<sup>+</sup>/ $\beta$ -III-tubulin<sup>+</sup> cells were present in the CA1 and CA3 regions, which adopt morphologies and extend process characteristic of endogenous pyramidal neurons (Fig. 4D,E). Engrafted cells very rarely co-express the proliferative cell cycle marker Ki-67 and were nestin<sup>-</sup> (data not shown), suggesting they were largely postmitotic following transplantation. Transplanted cells adopt mature neuronal phenotypes, as demonstrated by the expression of NeuN in cortically transplanted HNA<sup>+</sup> cells 30 days following engraftment (Fig. 4F,G).

Controlled alteration and in vitro manipulation of cellular phenotype are increasingly envisaged in tissue culture paradigms. Attempts to induce in vitro differentiation in adherent cells as previously described for attached human neurospheres (Ostenfeld and Svendsen, 2004), and adherent rodent NSCs (Scheffler et al., 2005) were unsuccessful in producing multiple differentiated cell types. To further test the potential for alteration of phenotype in AHNPs, expanded cells (more than 20 PDs) were subjected to multiple combinations of culture supplements, including FGF8, retinoic acid, sonic hedgehog, dibutyl cAMP, nerve growth factor (NGF), 1-isobutyl-3-methylxanthine (IBMX), retinoic acid and serum. Application of dibutyl cAMP, NGF and IBMX, combined with the removal of serum and growth factors, was found to induce a rapid phenotypic alteration in proliferating cells (Fig. 5A) that yielded morphologically and electrophysiologically characteristic immature neurons within 7 days. Three days after this induction of phenotypic alteration, a transient subset of cells displayed a hybridized somatodendritic morphology intermediate to astrocyte and neuron, and displayed both neuronal and astrocytic markers (Fig. 5B). These transitory cells progressively disappear, and are supplanted by cell types displaying characteristic morphologies and immunophenotypes of newborn neurons (Fig. 5C,D). Immature neurons produced 5 days after induction of differentiation ( $n=4$ ) display electrophysiological properties reminiscent of immature



**Fig. 4. Cortically implanted AHNPs adopt predominantly neuronal fates.** (A) Coronal section of engrafted left hemisphere shows  $\beta$ -III-tubulin<sup>+</sup>/HNA<sup>+</sup> donor cells adjacent to engraftment site. Schematic representation includes two-dimensional proximodistal and lateral distribution of the majority of AHNPs and ectopically migrating cells in two transplanted animals (blue and yellow). (B) Fate analysis indicates few cells adopt an astroglial identity. (C) Integrating AHNPs within the primary engraftment site adopt neuronal morphologies and immunophenotypes. (D)  $\beta$ -III-tubulin<sup>+</sup> cells present within the hippocampus of engrafted animals occasionally displayed HNA (E, from boxed area in d) in CA1 and CA3, where they adopted apparent pyramidal neuron morphologies. (F,G) Single plane confocal image of cortically implanted AHNPs. HNA<sup>+</sup> cells form mature neuronal (NeuN<sup>+</sup>) cell types, which co-exist with endogenous neurons (arrowheads). Scale bars: 200  $\mu$ m in A,B,D; 50  $\mu$ m in C,F,G; 100  $\mu$ m in E.

neuronal characteristics: RMP of  $-33 \pm 20.8$  mV, a  $C_m$  of  $32.6 \pm 1.3$  pF, a  $R_m$  of  $1.3 \pm 0.3$  G $\Omega$ , and a  $R_a$  of  $16.7 \pm 5.5$  M $\Omega$ , with prominent Na<sup>+</sup> and K<sup>+</sup> channels (Fig. 5E). Immature neurons fire single evoked action potentials (Fig. 5E). Oligodendrocytes were not detected following induction of neurogenesis. Defined cells displaying an exclusively neuronal phenotype universally incorporate BrdU (Fig. 5f) and express Ki-67 (data not shown), suggesting they are the product of subsequent divisions of cells following differentiation. New neurons continue to express immature neuronal markers (PSA-NCAM, Fig. 5G) and maturing neuronal markers (Fig. 5H). Although FCS is not essential to the propagation of AHNPs, serum prevents the generation of neuronal phenotypes, regardless of concomitant mitogen addition (data not shown).

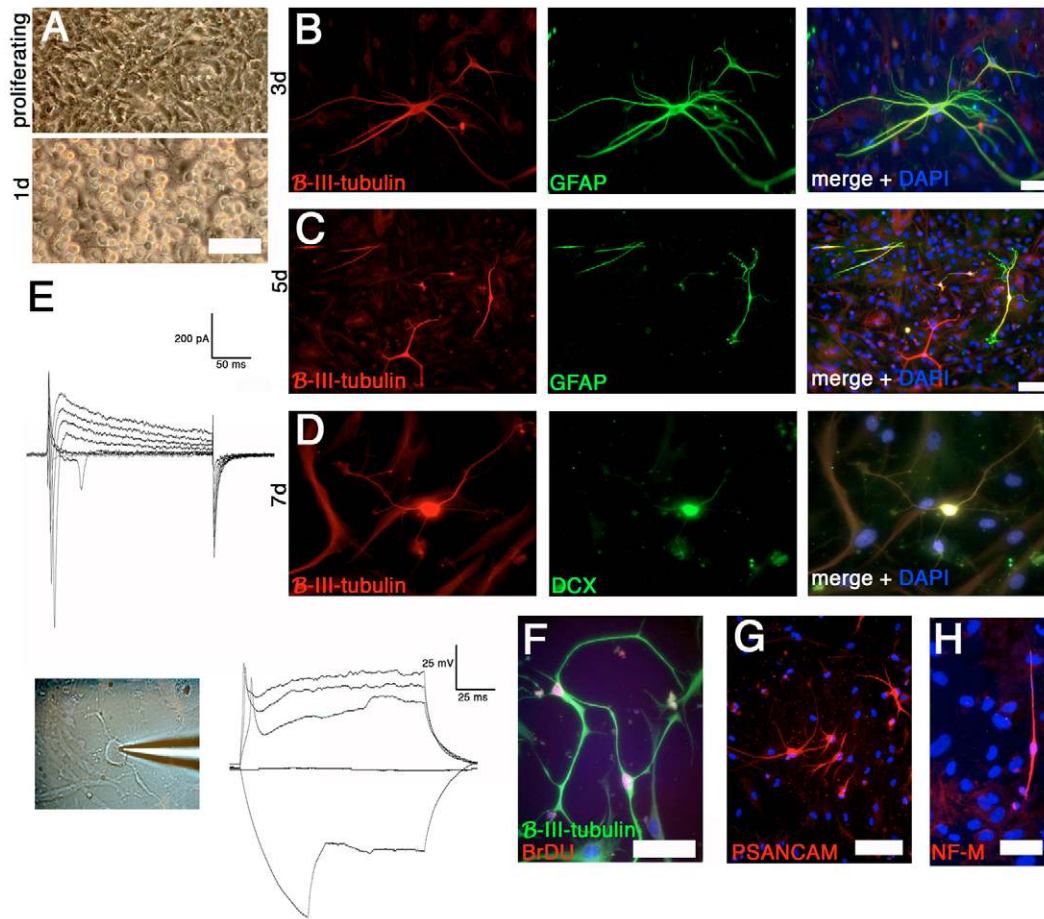
## DISCUSSION

We demonstrate a method for isolating multipotent astrocytic progenitors from primary neural tissue and describe culture conditions necessary for their extensive expansion as a homogenous population. AHNPs maintain a stable doubling rate throughout culture, and do not exhibit characteristics of transformed cells, including loss of key cell cycle checkpoint proteins, loss of sensitivity to arrestors of the cell cycle and cell contact inhibition of

growth. Our results suggest it is possible to isolate and expand astroglial cell lines from epileptic temporal lobe resections with characteristics of relatively uncommitted progenitor cells without senescence or cellular transformation.

Cultured cells display conserved primordial markers, with a large subset displaying characteristics of type I protoplasmic astrocytes. Thymidine analog incorporation reveals a uniform proliferative capacity among AHNPs, suggesting we have identified dedicated culture conditions for the isolation and expansion of a homogenous progenitor population rather than conditions which favor or maintain a subset of primordial cells that constitutively generate progressively more differentiated/postmitotic cell types. Interestingly, telomerase expression is appreciated in these cells, which appears to be linked to mitogenic dependence and continued proliferation in culture. AHNPs were unresponsive to Pax6, and did not express RC2, GLAST and minimally expressed BLBP, suggesting they are distinct from radial glial cell types. Similar to previously described neurogenic cells (Nunes et al., 2003), AHNPs maintain similar immunological expression and mitogenic dependency throughout culture. However, our cells were expandable as an adherent monolayer, were derived from multiple regions, and display a uniform potential for generating neuronal cell types.





**Fig. 5. Differentiation of AHNPs into neuronal cell types.** (A) Proliferating cells (30 PDs) assume a compacted morphology immediately after removal of mitogens and addition of dibutyl cAMP, IBMX and NGF. (B) Three days after induction of differentiation, intermediate cells displaying a developmentally intermediate phenotype are appreciated. (C) Five days after induction of differentiation, maturing cells concurrently lose GFAP and continue to strongly express  $\beta$ -III-tubulin. (D) Seven days after induction of differentiation, newly generated neurons in vitro frequently co-express immature neuron markers, and assume typical bipolar morphologies. (E) Current and voltage clamp analysis of 7-day-old neurons. New neurons exhibit prominent  $\text{Na}^+$  and  $\text{K}^+$  channels, and were able to fire elicited action potentials when polarized to  $-60$  mV. (F)  $\beta$ -III-tubulin neurons generated in the presence of thymidine analog universally incorporate BrdU. Cells generated in this manner display additional type-specific neuronal markers, including PSA-NCAM (G) and neurofilament M (NF-M, H). Scale bars:  $75 \mu\text{m}$  in A;  $25 \mu\text{m}$  in B,H;  $100 \mu\text{m}$  in C,G. Cells counterstained with DAPI.

Comparison of AHNPs derived from the hippocampus and temporal cortex reveals no difference in growth rates, cellular composition, or significant physiological factors. This is interesting, as the hippocampus is believed to contain astrocyte-like NSCs that have documented self-renewal and multipotentiality in vitro and in vivo (Eriksson et al., 1998). Thus, our findings present data suggesting the possibility of a broadly distributed population of neurogenic progenitor cells. These cells are not inherently multipotent or self-renewing in clonal conditions, indicating they do not meet the existing criteria for definition as NSCs. As a caveat, a potential role of epilepsy and its consequences in the brain may unusually affect astrocytes, endowing these cells with a tendency to act as NSCs.

Despite a lack of inherent multipotentiality, AHNPs can be induced to form neuronal cell types in vitro and in vivo. Using a paradigm adapted from the generation of neuronal cell types from cortical neuronal cell lines (Ronnott et al., 1990), it was possible to generate immature neurons in a similar manner. Similar to studies of postnatal neurogenesis in rodents and humans (Laywell et al., 2000; Sanai et al., 2004; Scheffler et al., 2005; Seri et al., 2001),

neurogenesis is accompanied by cell division, as evidenced by ubiquitous BrdU incorporation and cell cycle marker expression characteristic of dividing cells. Although PSA-NCAM, which labels both neuronal (Doetsch, 2003) and oligodendrocyte precursors (Keirstead et al., 1999), this marker was transiently expressed and did not result in oligodendrocyte production.

Neuronizing AHNPs frequently display an ‘asteron’ hybrid phenotype, similar to recent findings in cultured rodent neural cells (Laywell et al., 2005; Okano-Uchida et al., 2004). Whereas those studies report a neuron-to-glia transdifferentiation, the transition reported here represents a glia-to-neuron commitment, suggesting the isolation of conditions (Ronnott et al., 1990) that promote the phenotypic alteration of widely distributed endogenous AHNPs may be possible. Similarly, neurons derived in this manner possess electrophysiological profiles occasionally appreciated in cells derived from astrocytic tumors (Bordey and Sontheimer, 1998) and embryonic astrocytes (Gritti et al., 2000). However, when transplanted to the cerebral cortex, AHNPs frequently assume neuronal phenotypes, suggesting the appreciated electrophysiological development is at least partially

a product of in vitro culture. Transplanted cells display limited migration, which (along with phenotypic development), may be affected by the lack of functioning immune cells in immunocompromised host animals. As these cells have been implicated in neurogenesis (Ziv et al., 2006), it is not entirely clear which (if any) persisting neuronal subtypes may be generated in vivo from AHNPs.

AHNPs remain sensitive to exogenous cell cycle inhibitors (aphidicolin, EGCG), but continue to express telomerase and return to previous levels of proliferation upon removal of exogenous growth inhibitors. EGCG is able to inhibit proliferation of primary cells rapidly in this culture system, in contrast to previous work carried out in immortal cell lines (Naasani et al., 1998), but does not abrogate TERT expression. However, upon mitogenic withdrawal, telomerase expression is promptly lost and continued proliferation ceases, neither of which is restored upon reversion to defined proliferative media. This anecdotal coupling of TERT expression to cellular proliferation and mitogenic dependence provides for several interesting possibilities. First, these results suggest a system whereby environmental mitogens (provided in constant supply) provide a condition-specific synergistic growth effect, allowing for both TERT expression and continued expansion. A loss of environmental support factors may trigger a demonstrably irreversible loss of TERT expression, which may effectively mortalize cells or possibly, in the case of AHNPs, trigger their immediate and irreversible entry into a state of replicative senescence. The appreciation of rapid senescence with the loss of TERT expression in aged cells suggests that both telomere length and telomerase expression may be crucial to the maintenance of continued cell division in populations of proliferatively active cells, such as AHNPs. This agrees with a number of reported examples of poor correlation between telomere length and replicative senescence, including one example in which cells rescued from replicative senescence by viral transfection of telomerase maintained shorter telomere length than replication incompetent counterparts (Yang et al., 1999; Zhu et al., 1999).

The ability to expand progenitor cell populations massively has implications for diagnostic neurobiology, as well as for therapeutic approaches involving tissue replacement. By extensively expanding primary cells from various brain regions, it is possible to create a substrate for neural cell bioassays (i.e. primary cell drug testing) without relying on clonally derived cell lines that contain potentially masking genotoxic mutations or inaccurately reflect the homeostasis of target cells. Recent efforts for cell replacement therapies in the brain have prompted a focus on transplantation biology, including the use of cells genetically modified to express neurotrophins (Kordower, 2003; Tai and Svendsen, 2004). The condition-specific constant expandability of AHNPs, combined with the previously undocumented fate choice plasticity and amenability to genetic modification, provides an exciting substrate for further investigations addressing disorders and repair of the human CNS.

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/18/3671/DC1>

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