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Non-immortalized human neural stem (NS) cells as a scalable platform for cellular assays

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ABSTRACT

The utilization of neural stem cells and their progeny in applications such as disease modelling, drug screening or safety assessment will require the development of robust methods for consistent, high quality uniform cell production. Previously, we described the generation of adherent, homogeneous, non-immortalized mouse and human neural stem cells derived from both brain tissue and pluripotent embryonic stem cells (Conti et al., 2005; Sun et al., 2008). In this study, we report the isolation or derivation of stable neurogenic human NS (hNS) lines from different regions of the 8–9 gestational week fetal human central nervous system (CNS) using new serum-free media formulations including animal component-free conditions. We generated more than 20 adherent hNS lines from whole brain, cortex, lobe, midbrain, hindbrain and spinal cord. We also compared the adherent hNS to some aspects of the human CNS-stem cells grown as neurospheres (hCNS-SCns), which were derived from prospectively isolated $CD133^{+}CD24^{-/lo}$ cells from 16 to 20 gestational week fetal brain. We found, by RT-PCR and Taqman low-density array, that some of the regionally isolated lines maintained their regional identity along the anteroposterior axis. These NS cells exhibit the signature marker profile of neurogenic radial glia and maintain neurogenic and multipotential differentiation ability after extensive long-term expansion. Similarly, hCNS-SC can be expanded either as neurospheres or in extended adherent monolayer with a morphology and marker expression profile consistent with radial glia NS cells. We demonstrate that these lines can be efficiently genetically modified with standard nucleofection protocols for both protein overexpression and siRNA knockdown of exogenously expressed and endogenous genes exemplified with GFP and Nestin. To investigate the functional maturation of neuronal progeny derived from hNS we (a) performed Agilent whole genome microarray gene expression analysis from cultures undergoing neuronal differentiation for up to 32 days and found increased expression over time for a number of drugable target genes including neurotransmitter receptors and ion channels and (b) conducted a neuropharmacology study utilizing Fura-2 Ca^{2+} imaging which revealed a clear shift from an initial glial reaction to carbachol to mature neuron-specific responses to glutamate and potassium after prolonged neuronal differentiation. Fully automated culture and scale-up of select hNS was achieved; cells supplied by the robot maintained the molecular profile of multipotent NS cells and performed faithfully in neuronal differentiation experiments. Here, we present validation and utility of a human neural lineage-restricted stem cell-based assay platform, including scale-up and automation, genetic engineering and functional characterization of differentiated progeny.

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1. Introduction

In vitro expanded human neural stem cells are a powerful tool for basic and applied neurobiology, since they provide an accessible model system to investigate human neurodevelopment and cell biology. They also offer a renewable resource for neurodegenerative disease studies and would be suitable for pharmaceutical and neurotoxicology screening. In addition, scalable production of *in vitro* human neural stem cells, or their neuronal progeny is a first step towards their use in regenerative medicine.

Fetal brain and spinal cord contain proliferating neural stem and progenitor cells, and are potential sources for deriving *in vitro* cell lines. For screening applications, it is desirable that such cell lines are clonogenic, homogeneous, can be grown adherently, and are multipotent, therefore capable of generating neurons, astrocytes and oligodendrocytes. Such properties should be achieved without the necessity of genetic immortalization. In the past, self-renewing, multipotent mammalian NSC/progenitor cells have been enriched and expanded *in vitro* as monolayers on substrate-coated tissue culture plates or as self-adherent complexes of cells, forming neurospheres (Reynolds et al., 1992; Gage et al., 1995; Ray et al., 1995; Reynolds and Weiss, 1992; Weiss et al., 1996; Svendsen et al., 1998; Vescovi et al., 1999). The inherent heterogeneity of the sphere cultures (Campos, 2004; Reynolds and Rietze, 2005) and non-adherent culture condition may not be optimal for utility in high throughput genetic and chemical screens. However, the prospective enrichment of sphere-forming neural stem cells for cell surface marker expression during the isolation process (Uchida et al., 2000; Capela and Temple, 2002) can generate homogeneous NS cell populations, which are adaptable to adherent culture conditions, lending themselves accessible to novel image-based and information-rich high-content assays. These assays are increasingly finding their way into academic and industrial pharmaceutical screens as well as basic research in the biology of stem cell self-renewal and lineage specification (Breier et al., 2008; Cromley and Fox, 2004; Chan et al., 2009). Therefore, an adherent long-term culture system of human neural stem cells is desirable.

Previously, prolonged adherent propagation of neural stem cells had required immortalization (Pollock et al., 2006). Recently, this limitation has been overcome by the establishment of protocols for the clonal derivation of non-immortalized and tripotential radial glia-like NS cells from mouse embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), fetal and adult mouse brain, and from human fetal CNS that can be propagated extensively and long-term in adherent monolayer conditions without the loss of neurogenic potential (Conti et al., 2005; Pollard et al., 2006a,b, 2008; Glaser et al., 2007; Sun et al., 2008; Onorati et al., 2010a). In the past decade, radial glia cells have been shown to be the major neurogenic neural stem cell population during fetal CNS development (Malatesta et al., 2000, 2003; Miyata et al., 2001; Noctor et al., 2001; Anthony et al., 2004), in addition to their traditional role as supporting and guiding scaffold for migration of newborn neurons (Rakic, 1971). These cells are now thought to be the cell of origin for the residing adult neural stem cell population (Alvares-Buylla et al., 2001).

Radial glia-like NS cells are readily derived from the different sources listed above, are grown in monolayer, serum-free conditions in the presence of the growth factors fibroblast growth factor (FGF2) and epidermal growth factor (EGF) and self-renew symmetrically as a homogenous culture. They show many hallmarks of radial glia *in vivo* such as expression of neurogenic neural stem cell markers including Nestin, Sox2, brain lipid binding protein (BLBP) and 3CB2, a bipolar morphology and interkinetic nuclear migration (Pollard and Conti, 2007). In contrast to the mouse, whereby adherent NS cells derived from the CNS and pluripotent cell sources possess a similar radial glia-like identity by morphology and gene expression profile (Conti et al., 2005; Pollard et al., 2008), adherent, long-term cultures of neural stem cells recently derived from both human ES cell (Koch et al., 2009) and iPS cell (Danovi et al., 2010; Falk et al., unpublished) pluripotent cell sources, appear to be distinct from radial glia-like human fetal NS cells, displaying features of more primitive neuroepithelial cells, which were previously described as transient human ES cell-derived neural rosette cultures (Elkabetz et al., 2008).

Here, we report the characterization of long-term adherently cultured human neural stem cells from either the whole fetal brain, following prospective enrichment by sorting for expression of the CD133/Prominin-1 antigen (Uchida et al., 2000; Tamaki et al., 2002; Guzman et al., 2007), or from different regions of human fetal CNS tissue. The latter retain their positional identity code along the rostrocaudal axis, and were isolated based on previously reported protocols (Conti et al., 2005; Sun et al., 2008). Some protocol modifications included the use of animal component-free media formulations. The human NS lines described herein are highly expandable, display self-renewal capacity, retain a diploid karyotype and robust differentiation potential into glia and a high proportion of excitable neurons, even after prolonged passaging. They are further amenable to genetic modification while retaining their multipotential differentiation properties. Moreover, we show the automation and scale-up of the hNS cell lines on a robotic CompacT SelecT system. These findings demonstrate that human fetal NS cell lines are self-renewing human neural stem cells *in vitro* without requirement for a specialised cellular niche or genetic immortalization, in addition to their proven therapeutic potential upon *in vivo* engraftment and characterization. As such, they may provide a new platform for a range of studies in basic and applied human neurobiology.

2. Experimental procedures

2.1. Cell lines, cell culture and protocols for in vitro differentiation

For hNS cell line derivation all studies with human tissue were performed under Ethical Approval from the Lothian Healthcare Trust (Local regional ethical committee consent number 06/S1103/2) using tissue donated with informed consent according to the Polkingthorne guidelines after elective termination of pregnancy (Polkingthorne, 1989). Human fetal brain and spinal cord tissue at embryonic 60–65 day were carefully dissected in Neurobasal medium (Invitrogen) and dissociated into single cell suspensions with Accutase (PAA Laboratories Ltd) treatment. Primary cells were then plated onto laminin (10 mg/L; Sigma) coated dishes (Iwaki) in expansion medium comprising RHB-A (StemCells, Inc., *SCS-SF-NB-01*), penicillin–streptomycin (10 ml/L final, Sigma), recombinant human FGF2 and EGF (20 ng/ml final; Peprotech). Animal component free RHB-Basal (StemCells, Inc., *SCS-SF-NB-00*) was supplemented with humanized hN2 and/or hB27 (N2-AF, *SF-NS-01-005-AF*; N27-AF, *SF-NS-02-010-AF*; StemCells, Inc.) additives or standard B27 supplement (Invitrogen) according to the experimental plan. To eliminate neurons in initial cultures, cells were transferred onto 0.1% gelatin (Sigma) coated dishes for seven days before being re-plated onto laminin (Sigma) for further expansion. Cells were split 1:2–1:3 once cultures became confluent. Cell lines were tested to be negative for CMV, HTLV1, HIV1, HepC, HepB, EBV and Mycoplasma/Ureaplasma (tests carried out by independent CPA accredited laboratory).

Human central nervous system stem cell neurosphere cultures (hCNS-SCns) were generated as described (Uchida et al., 2000; Tamaki et al., 2002). Briefly, second trimester (16–20 weeks) fetal human brain tissue was dissected and enzymatically treated with collagenase and trypsin. The resulting single cell suspension was then labelled with CD133 and CD24 antibodies (SC111; STEM24^{TM}, *AB-24-PE-050*; StemCells, Inc). The $CD133^{+}CD24^{-/lo}$ target population was aseptically sorted using a BD Vantage flow cytometer. Sorted cells were cultured in X-VIVO 15 medium (Lonza) supplemented with N2, heparin, N-acetylcysteine, FGF2, EGF (20 ng/ml) and leukemia inhibitory factor (LIF, 10 ng/ml; Millipore) at a density of 10^5/ml. When neurosphere size reached 200–250 μm, cultures were passaged by collagenase treatment and re-plated in the same medium. For adherent culture hCNS-SCns propagated for less than ten passages were dissociated into single cells and cultured either in X-VIVO 15 medium supplemented as above in tissue culture plates coated with fibronectin (Sigma), poly-ornithine (Sigma) and laminin, or in RHB-A with laminin only coating, in the presence of a combination of the growth factors LIF, FGF2 and EGF.

Table 1
Neural progenitor, radial glia and neuronal markers.

Antibody	Specificity	Company	Dilution	Secondary	Dilution
Nestin	Neural progenitor	Chemicon	1/500	GaM IgG1 546	1/1000
CD133	Neural progenitor	StemCells	1/300	DaM IgG 594	1/500
Sox2	Neural progenitor	R&D Syst	1/300	DaGT IgG 488	1/500
GFAP	Radial glia	Chemicon	1/750	GaM IgG1 546	1/1000
GFAP	Astrocytes	DAKO	1/400	DaR IgG 488	1/500
3CB2	Radial glia	DSHB	1/20	GaM IgM 488	1/200
BLBP	Radial glia	Abcam	1/250	DaR IgG 488	1/500
Pax 6	Neural progenitor	Covance	1/50	GaR IgG 488	1/1000
Tuj1	Neurons	Covance	1/1000	GaR IgG_{2a} 488	1/1000
MAP2	Neurons	Sigma	1/200	GaM IgG1 546	1/1000
DCX	Neurons	Chemicon	1/2000	DaGP IgG 594	1/500
Ser	Serotonergic neurons	Sigma	1/500	GaR IgG 488	1/1000
TH	Dopaminergic neurons	Pel-Freeze	1/500	GaR IgG 488	1/1000
GABA	GABAergic neurons	Sigma	1/1000	GaR IgG 488	1/1000
V-GLUT	Glutamatergic neurons	Chemicon	1/100	GaGP IgG 546	1/200
ChAT	Cholinergic neurons	Abcam	1/100	GaR IgG 488	1/1000
HB-9	Motor neurons	Abcam	1/100	GaR IgG 488	1/1000

G, goat; D, donkey; aM, anti-mouse; aR, anti-rabbit; aGP, anti-guinea pig; aGT, anti-goat.

For neuronal differentiation $2.5 \times 10^4/cm^2$ human NS cells were plated into poly-ornithine (Sigma) and laminin coated cell culture plates (Nunc) in expansion medium without adding EGF. After 10 days, medium was switched to the mix RHB-A/Neurobasal medium (1:1; StemCells, Inc.; Invitrogen), supplemented with NDiff™ N2 (StemCells, Inc., *SF-NS-01-005*), B27, and FGF2 (10 ng/ml). 4 days later, FGF2 was withdrawn from the medium, and after another 4 days, medium was switched to Neurobasal medium supplemented with B27 (Invitrogen) or hB27 and brain derived neurotrophic factor (BDNF, 20 ng/ml; Peprotech). For astrocyte generation, human NS cells were treated with 5% serum or 10 ng/ml BMP4 (R&D Systems) without mitogen for 2–3 weeks.

2.2. Immunocytochemistry

Cultured cells were fixed with 4% (w/v) formaldehyde solution and immunostained for the cytochemical expression of neural and glial markers using corresponding antibodies (Table 1) and suitable secondary antibodies labelled with fluorochromes (Invitrogen), following standard immunocytochemistry techniques. Visualisation of the cell culture was accomplished by fluorescence microscopy.

2.3. Imaging of calcium-response assays

Differentiated hNS cells were loaded with Fura-2 (Invitrogen), a UV light-excitable ratiometric Ca^{2+} indicator. Fura-2 exhibits an absorption shift that can be observed by scanning the excitation spectrum between ~300 and ~400 nm, while monitoring the emission at >450 nm. The buffer used contained 121 mM NaCl, 1.3 mM KCl, 0.8 mM $MgCl_2$, 6 mM $NaHCO_3$, 5.5 mM glucose, 25 mM HEPES; pH 7.5. Cultures were loaded with Fura-2 by incubation with the AM ester form of the indicator (1 μM) for 30 min, followed by a 30-min de-esterification period. Single-cell Fura-2 imaging was performed using a system configured around a Nikon TE200 inverted epifluorescent microscope equipped with a 40×, 1.3 numerical aperture oil objective, a Sutter Industries excitation filter wheel and changer and a Hamamatsu ORCA ER CCD camera. Epifluorescent illumination was provided by a 100 W Nikon mercury arc lamp. The camera and filter wheel as well as image capture and storage were controlled by PerkinElmer Ultraview software. Fura-2 was excited by alternate excitation at 340 nm (340HT15) and 380 nm (380HT15) for 200 ms periods. Between exposures, the cells were maintained in darkness. Emitted light was collected through a dichroic block containing a 400 nm dichroic mirror with a 460 nm long pass filter. The Fura2 emission caused by 340 nm and 380 nm excitation was sampled repetitively every 2 s. In a longitudinal experiment, solutions containing K^+ (54 mM), glutamate (100 μM) and carbachol (100 μM) were added sequentially (with a washout between additions). Fura2 image sequences were analysed using Ultraview software (PerkinElmer). Individual cells were selected and their corresponding raw fluorescence grey value intensities were exported into Microsoft Excel. An area devoid of cells was also selected and used to measure the 'background' signal. Background corrected ratio values (R) were determined using the equation R = (340 nm/380 nm of each cell). For each experiment, 40 different regions were distributed over 40 randomly chosen cell bodies, and the ratio of the intensities were calculated for each region and plotted vs. time.

2.4. Microarray and TLDA gene expression analysis

A simple microarray gene expression experiment was performed to determine the gene expression profile of human NS cells following differentiation, to validate existing immunocytochemistry results. RNA was extracted from cultures at days 0, 14, 24 and 32, using QIAGEN's RNAeasy kit (plus incubation with DNAse I). Samples were sent to Oxford Gene Technology (www.ogt.co.uk) for QC, labelling (test RNA: Cy3 labelled; and reference RNA: Cy5 labelled), and hybridisation to a 4 × 44K Agilent whole genome array. A Taqman low-density (real-time PCR) array (TLDA) with a set of 190 genes (in duplicates) typically expressed in neural stem cells and the neural stem cell niche, with GAPDH and 18S rRNA as endogenous controls, was designed and custom manufactured. From 1 to 3 million fetal hNS of different anterior and posterior brain regions, of passages between 18 and 21, RNA was extracted using an RNAeasy kit (QIAGEN) with on-column DNAse treatment following the manufacturer's instructions. 1 μg of RNA was used to transcribe cDNA with Superscript III (Invitrogen). The quality of the cDNA was tested by quantitative PCR of a few neural stem cells markers (e.g. Sox2 and BLBP/FABP7) before the cDNA was loaded onto the TLDA cards and run in an ABI 7900HT real-time PCR machine (Applied Biosystems). Data was analysed using the Statminer software (Integromics) by the ΔΔCt method using an average of the two anterior hNS cell lines as reference compared with the average of the two posterior hNS cell lines and was used to generate a graph of significantly up or down-regulated positional identity genes between the two populations.

2.5. RT-PCR and quantitative PCR

RNA extraction and cDNA synthesis were performed as described for the TLDA analysis. The following PCR conditions were used: 94 °C for 2 min, followed by cycles of 94 °C (1 min), 58 °C (1 min), and 72 °C (1 min), with the reaction terminated by a final 2 min incubation at 72 °C. Control experiments were done without reverse transcriptase or without template cDNA to ensure that the results were not due to amplification of genomic or contaminating DNA. Each reaction was visualised using ethidium bromide after 1.5% agarose gel electrophoresis for 60 min.

The qRT-PCR was done with an ABI 7500HT real-time PCR machine, using Power SYBR Master Mix (Applied Biosystems). Thermal cycling consisted of an initial step of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The delta Ct for each sample was calculated as the difference between the critical threshold of the gene of interest and the combined critical threshold of the three housekeeping genes glyceraldehyde-3-phosphatase dehydrogenase (GAPDH), β-actin and ribosomal protein RPL13A. The ddCt was calculated as the difference in the delta Ct for two cDNA samples.

2.6. Transfection and siRNA experiments

hNS can be efficiently transfected using nucleofection technology (Amaxa Nucleofector II, Lonza) using program G013 (chosen from various programs tested, namely A-033, C-013, G-013, O-003 and O-005; being G-013 the one that gave the brightest cells with normal morphology and higher transfection efficiency, 78% at 24 h post-nucleofection) with cells resuspended in "Basic Primary Neurons" solution (Lonza) and following the manufacturer's instructions. 5×10^5–2.5×10^6 cells were transfected per reaction with Lonza's pmaxGFP or linearized pCAGGFPIP reporter plasmid and pCAGβgeo-hCD2 fusion construct, or with anti-Nestin and anti-GFP siRNA.

2.7. Automated culture of fetal hNS

Hind05 hNS cells were initially established using manual procedures before introduction onto the CompacT SelecT (The Automation Partnership, Royston, UK).

Cells were then passaged with PBS-based cell dissociation buffer (Invitrogen) instead of Accutase and expanded in standard culture conditions on the automated platform (RHB-A, StemCells, Inc.) with the addition of EGF and FGF-2 (20 ng/ml final concentration). At each passage cell viability and number was determined and after every two passages the cells were stained for markers of undifferentiated NS cells. At recurring intervals cells were also tested for their neural differentiation capacity.

2.8. Transplantation of hCNS-SCns into neonatal NOD-scid mice

Single-cell suspensions of hCNS-SCns were prepared and cultured in fresh neurosphere media for 1–2 days prior to transplantation. On the day of transplantation, cells were harvested, counted and resuspended at a density of 0.5×10^5 cells/μl. Neonatal mice (P0–P1, days post-natal) were cryo-anesthetized, aligned on a stereotaxic device (Stoelting), and cells transplanted bilaterally in 2 μl medium into each lateral ventricle. The pups were revived and returned to their mothers for nursing until they were weaned. Mice were analysed for human cell engraftment >16 weeks post-transplantation by immunostaining with the cytoplasm-localized human specific antibody SC-121 (STEM121™, *AB-121-U-050*; StemCells, Inc.).

3. Results

3.1. Isolation of neural stem cells from the human CNS

Firstly, we wanted to derive human neural stem cells (hNS) directly in adherent conditions, without an initial or transient neurosphere expansion stage, from different regions of the fetal CNS and characterize their common and unique properties. To this end, human fetal cortex, forebrain, midbrain, temporal lobe, hindbrain and spinal cord were carefully dissected according to the atlas of Bayer and Altman (2006) and dissociated into single cells by incubation with Accutase. Primary cells were then seeded onto laminin pre-coated dishes in growth medium containing both EGF and FGF2. Cells readily attached and produced a morphologically heterogeneous population containing both neural precursors (Nestin$^+$) and neurons (β-tubulinIII/Tuj1$^+$). To enrich for undifferentiated neural precursor cells, on day 7 after plating, we temporarily transferred primary human cells onto gelatin coated dishes, as under these conditions, neurons and committed neuronal progenitors fail to survive (Sun et al., 2008). 7–10 days later, viable precursor cells were re-plated back onto laminin substrate where the proliferating neural precursor cells became the dominant cell population. Three weeks after initial plating, immunostaining indicated that the primary human culture was homogeneously Nestin$^+$ and Tuj1 or MAP2 negative (Fig. 1B). At this stage, cultures were considered to be passage one human NS cells. Once established, these human NS cell lines expanded continuously in monolayer culture. On laminin substrate, the doubling time of human NS cells is approximately four days, slower than mouse NS cells grown in the same condition (Conti et al., 2005). Human NS cells were routinely split 1:2 when they reached confluence. Proliferating human NS cell cultures contain both small bipolar cells and more flattened apolar cells on the laminin substrate (Fig. 1A). It has been previously shown by time-lapse video microscopy that there is a dynamic interconversion between these morphologies in human NS cultures (Conti et al., 2005; Sun et al., 2008). However, upon three-dimensional low-density culture in PuraMatrix hydrogel (BD Biosciences) the hNS cells assume an *in situ*-like radial glia morphology with a long basal and a short apical process (Fig. 1A-d).

To investigate whether these human NS cell culture conditions can be used reproducibly, we repeated the derivation and

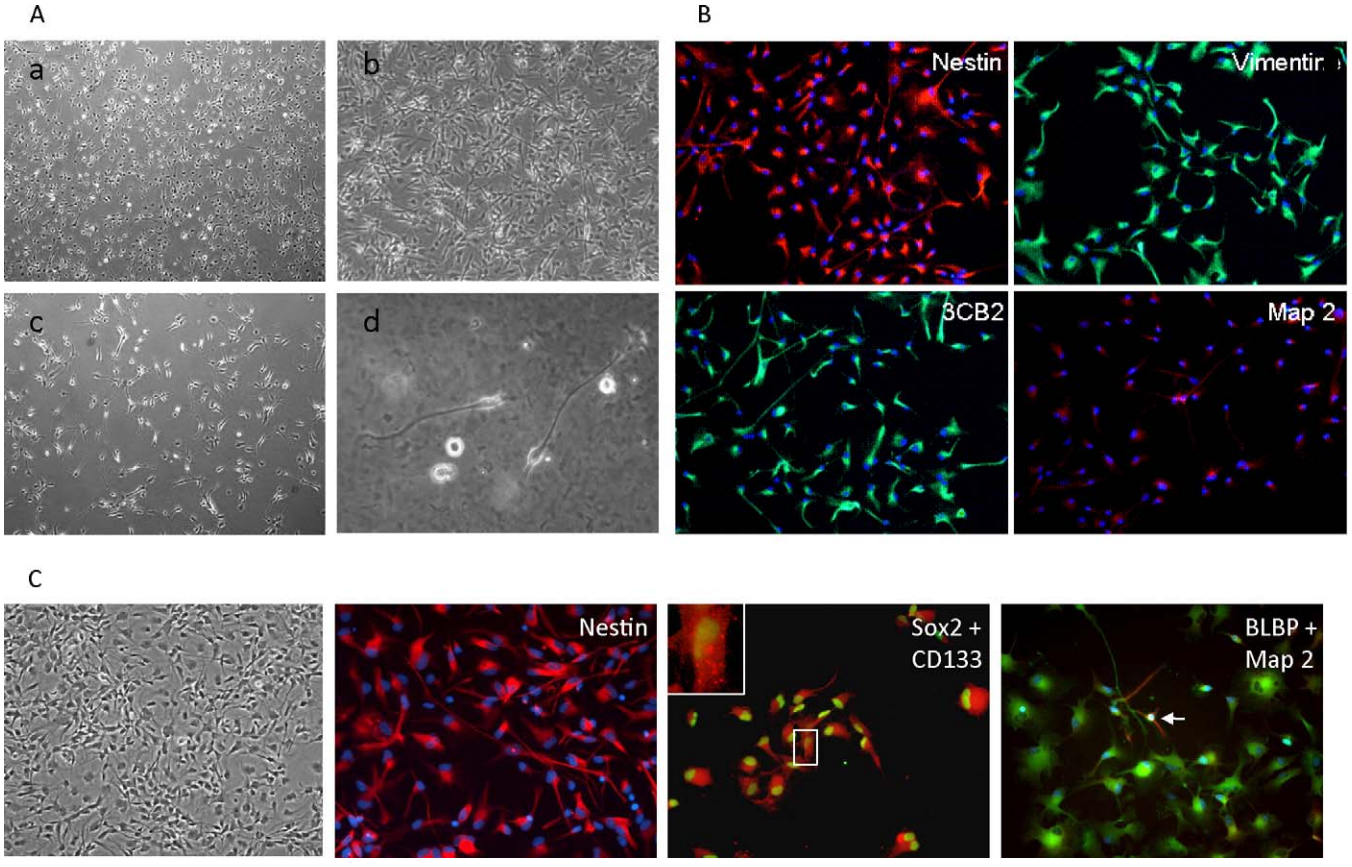

Fig. 1. Morphology and immunocytochemical characterization of undifferentiated hNS and hCNS-SC. (A) Cell morphology of the hNS lines (a) Cortex04, (b) Hind05 and (c) Spinal04 on laminin and of (d) Hind05 embedded in PuraMatrix. (B) Immunocytochemical markers (Nestin, Vimentin, 3CB2, MAP2) applied to characterise undifferentiated Cortex04 cultures. (C) Live cell morphology of passage 7 neurosphere hCNS-SC propagated in adherent culture for ten further passages in RHB-A and immunocytochemical profiling (Nestin, Sox2, CD133, BLBP, and MAP2) of undifferentiated cultures. Inset is magnification from same image showing extracellular cell surface receptor staining of the CD133 antigen and arrow points to single MAP2 positive neuron in field of view.

expansion experiments using a total of 7 independent specimens from human tissues at embryonic day 60–65. Human NS cell lines could be generated from all specimens if the neural tissue was well preserved. Only one sample was discarded due to a failure during the derivation process that was attributed to the low quality of the starting material, suggesting that the continuous adherent procedure is efficient, as described previously (Conti et al., 2005).

Procedures for the derivation of fetal hNS were established with the serum-free RHB-A media formulation plus the two neural supplements N2 and B27, which both contain animal-derived components. However, for a potential therapeutic use, or to have better defined conditions for drug discovery applications of these or other human neural stem cell lines, it would be desirable to have a process in place that is devoid of exposure to such components. We therefore developed humanized versions of the N2 and B27 neural supplements containing recombinant human protein components and initially derived hNS cultures in RHB Basal with a humanized version of N2 only (Table 2), which had no detrimental effect on NS cell derivation and initial growth, with a consistent marker profile of Nestin, Vimentin and 3CB2 expression and absence of MAP2 in expansion cultures (data not shown). Even though it was possible to derive and propagate the cells in medium containing humanized N2 only, we previously had indications that human fetal NS appear healthier in the presence of B27 during long-term expansion (Sun et al., 2008). For this reason,

Table 2
List of hNS cell lines obtained and conditions used for their adherent derivation and of hCNS-SCns tissues examined in this study.

Sample ID	Region	Media	Substrate	Karyotype
STEM1	Whole brain	XVIVO 15	N/A	P7, 46XX♀
STEM2	Whole brain	XVIVO 15	N/A	P2, 46XX♀
SCS/09/06/01	Spinal cord	EuroMed-N	L	
	Spinal cord	RHB-A	L	
	Brain A	RHB-A	L	
	Brain B	RHB-A	G	
SCS/09/06/03	Spinal cord	RHB-A	L	P8, 46XX♀
				P22, 46XX♀
	Mid region	RHB-A	L	P12, 46XX♀
	Lobe region	RHB-A	L	P17, 46XX♀
	Spinal cord	RHB-A + LIF	L	
	Mid region	RHB-A + LIF	L	
	Lobe region	RHB-A + LIF	L	
SCS/10/06/04	Spinal cord	RHB-A	L	P5, 46XY♂
	Spinal cord	RHB-A + hN2 + B27	L	P14, 46XY♂
	Cortex	RHB-A	L	P10, 46XY♂
	Cortex	RHB-A + hN2 + B27	L	P13, 46XY♂
				P26, 46XY♂
SCS/11/06/05	Forebrain	RHB-A	L	
	Hindbrain	RHB-A	L	
	Spinal cord	RHB-A	L	
	Forebrain	RHB-Basal + hN2	L	P12, 46XY♂
				P22, 46XY♂
	Hindbrain	RHB-Basal + hN2	L	P12, 46XY♂
				P20, 46XY♂
	Spinal cord	RHB-Basal + hN2	L	P9, 46XY♂
				P21, 46XY♂
SCS/06/07/06	Lobe	RHB-Basal + hN2 + hB27	L	
	Forebrain	RHB-Basal + hN2 + hB27	L	
	Forebrain	RHB-Basal + hN2	L	P2, 46XX♀
SCS/06/07/07	Lobe	RHB-Basal + hN2 + hB27	L	P6, 46XX♀
	Forebrain	RHB-Basal + hN2 + hB27	L	P5, 46XX♀
	Spinal cord	RHB-Basal + hN2 + hB27	L	P4, 46XX♀
	Lobe	RHB-Basal + hN2	L	P4, 46XX♀
	Forebrain	RHB-Basal + hN2	L	
	Spinal cord	RHB-Basal + hN2	L	

Humanized additives (hN2 and hB27) were used in some cases. hNS samples were named according to: [SCS/month/year/sample no]; L, laminin; G, gelatin.

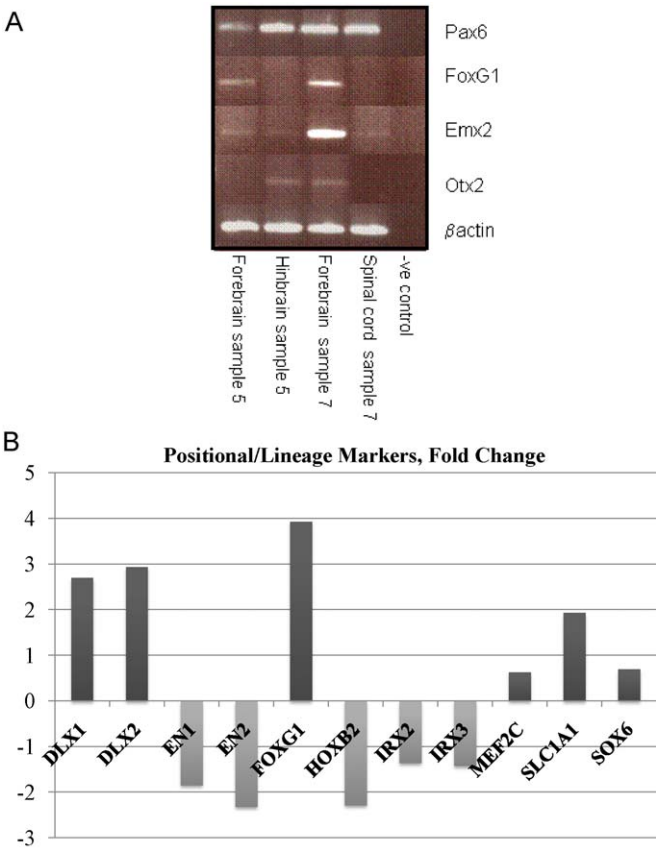

Fig. 2. hNS derived from different brain regions maintain positional gene signature. (A) Expression of regional markers (Pax6, FoxG1, Emx2, and Otx2) in human NS cell lines derived from forebrain, hindbrain and spinal cord. (B) Fold changes of significantly up and down-regulated gene expression in hNS of anterior (forebrain and lobe) related to hNS of posterior (midbrain and hindbrain) origin. The *y*-scale is logarithmic ($\log_2$).

in later derivations we included a humanized version of B27 as soon as it became available, together with humanized N2 and observed a faster growth rate in medium containing both humanized N2 and B27 than with humanized N2 alone. The humanized version of B27 was also tested in the neuronal differentiation protocol and gave satisfactory results (data not shown). On average, it takes about one month to derive an adherent and morphologically homogeneous human NS cell population with a total number of ~2 million cells directly from the fetal brain. To date, 19 female and male human NS cell lines from various regions of the brain have been successfully derived (Fig. 1, Suppl. Figs. 2 and 3, and Table 2). In addition, ten human NS cell lines were derived from fetal spinal cord using the same culture conditions. Spinal cord NS cells and brain NS cells are indistinguishable in their expression of general neural stem cell and radial glia markers and their ability to differentiate into both neurons and glia (Fig. 1A-c, Suppl. Fig. 4 and data not shown). Human NS cell lines can be expanded for extended periods of time (over passage ~35) without displaying cell senescence, crisis, or spontaneous differentiation (see Table 2 for karyotype results of cell lines derived from either sex at different passages).

Additionally, we wanted to confirm that hCNS-SCns, which are established long-term neurospheres derived from purified $CD133^{+}CD24^{-/lo}$ cells from gestational weeks 16–20 of fetal brain tissue (Uchida et al., 2000), could also be grown as an adherent culture, with a phenotype and characteristics equivalent to hNS derived adherently from the onset. While hCNS-SC are grown as neurospheres or adherently, they express NS markers such as

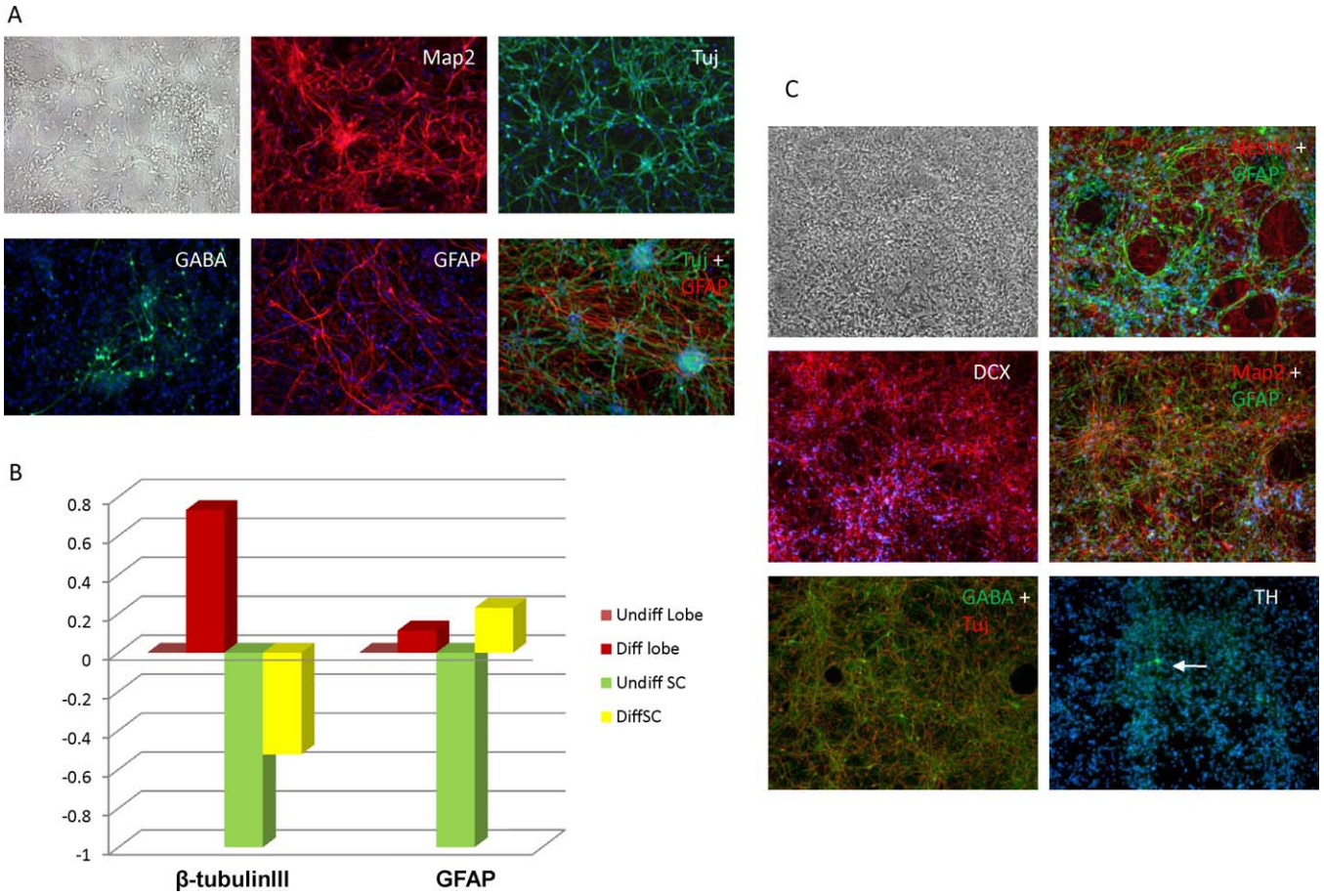

Fig. 3. Differentiation of hNS and hCNS-SC into neurons and glia. (A) Passage 19 Cortex04 cells (expanded in RHB-A) differentiated for 4 weeks and immunostained for the markers MAP2, GFAP, GABA and TuJ1. (B) Quantitative RT-PCR for β-tubulinIII and GFAP expression from samples of undifferentiated and differentiated cultures of Lobe07 and Spinal04 hNS normalized to expression level of undifferentiated Lobe07. The *y*-axis corresponds to $\log_2$ test samples/reference sample. (C) Early passage neurosphere hCNS-SC propagated as adherent culture for six passages differentiated for 4 weeks and immunostained for the markers Nestin, doublecortin (DCX), MAP2, GFAP, TuJ1, tyrosine hydroxylase (TH) and GABA.

CD133, Sox2 (Suppl. Fig. 1) and Nestin (data not shown), and under prolonged adherent culture conditions they possess radial glia-like NS cell morphology (Fig. 1C).

3.2. Fetal neural stem cells have a marker profile consistent with neurogenic radial glia and retain a region-specific gene expression profile in vitro

After the initial characterization of all human NS cell lines derived from fetal tissue, we further characterized two cell lines in greater detail: SCS/10/06/04 cortex (Cortex04; RHB-A) and SCS/11/06/05 hindbrain (Hind05; RHB-Basal + hN2). Both cell lines, as well as all the other human NS cells reported in this study; homogeneously express the immature neural precursor markers Nestin, Vimentin, BLBP and 3CB2 in expansion culture (Fig. 1A). Microarray gene expression analysis confirmed the presence of mRNA for radial glia-like neural stem cell markers in undifferentiated Cortex04 cells, such as astrocyte-specific glutamate transporter (GLAST), Vimentin, BLBP and CD44 (data not shown). In contrast to mouse NS cells, human NS cells are weakly immunoreactive to anti-GFAP antibodies (Sun et al., 2008 and data not shown), consistent with the known activity of the human GFAP promoter in radial glia progenitor cells (Malatesta et al., 2000). However, real-time PCR showed that the level of GFAP mRNA expression in undifferentiated human NS cell cultures is much lower than in those differentiated *in vitro* following growth factor withdrawal (Fig. 3B).

hCNS-SC in adherent culture maintain homogenous cytoplasmic and membranous (inset in Fig. 1C) expression of the prospective isolation marker CD133 (Fig. 1C and Suppl. Fig. 1) and further homogeneously express the neural precursor markers Nestin, Sox2 and BLBP, consistent with a neurogenic radial glia NS cell phenotype (Fig. 1C). Expression of neuronal markers (Tuj1 or MAP2) is rarely detected (<1%), or very faint, in these cells under expansion conditions (Fig. 1B and C).

Regional identity of the human NS cell lines was tested on some lines by RT-PCR expression analysis of markers such as orthodenticle homolog 2 (Otx2) a prosen-, mesencephalic homeobox gene, the forebrain genes forkhead Hox G1 (FoxG1) and empty spiracles homolog 2 (Emx2), and paired box gene 6 (Pax6), which is expressed in the whole developing CNS and later on in subpopulations of cells in the forebrain and hindbrain. Both forebrain from samples 5 and 7 expressed FoxG1 and Emx2, but at different levels; spinal cord from sample 7 only expressed Pax6 and none of the markers for brain; hindbrain sample expressed Pax6 and some levels of Otx2 (Fig. 2A).

To examine the preservation of a region-specific profile of transcription factors and other positional genes in greater detail, we employed a 190 gene custom neural stem cell qRT-PCR Taqman low-density array (TLDA) which included a set of 49 positional and lineage-specific genes. We compared the average gene expression of two anterior hNS lines of forebrain and temporal lobe origin with the average gene expression of two posterior hNS lines derived from midbrain and hindbrain. Consistent with the

observed common immunostaining profile of several undifferentiated regional lines, there were no significant differences in expression levels of the general neural stem cell and radial glia markers CD44, BLBP/FABP7, HES1, Pax6, Sox2 and CXCR4 (data not shown). Transcription factors and other genes defining positional identity along the anteroposterior axis were examined and two anterior CNS-derived hNS cell lines expressed significantly higher levels of the forebrain-associated genes; distal-less homeobox 1 + 2 (Dlx1 and Dlx2), FoxG1, myocyte enhancer factor 2 (Mef2c), solute carrier family 1 member1 (Slc1A1) and SRY-related HMG-box gene 6 (Sox6). Most prominent expression among these genes was FoxG1, whose expression is enriched more than 15-fold compared to posterior lines. The hindbrain/spinal cord associated genes, engrailed 1 + 2 (En1 and En2), homeobox B2 (HoxB2) and Iroquois homeobox 2 + 3 (Irx2 and Irx3) were found to be expressed in significantly higher levels in the two posterior CNS hNS cell lines. The level of enrichment found for the posterior genes ranged between 2.6- and 5-fold and is therefore relatively lower than for the most highly enriched anterior gene FoxG1 (Fig. 2B). These results suggest that hNS cells retain, after *in vitro* expansion (up to passage 20), considerable aspects of their anteroposterior transcriptional code according to their CNS region of derivation.

3.3. Human NS cells generate neurons and glia

Human neural stem cells, as a tissue-derived stem cell, should generate the three major cell types of the CNS: neurons, astrocytes, and oligodendrocytes. The differentiation potential of human NS cells was assessed by adapting protocols previously developed for mouse NS cells (Conti et al., 2005). Cells plated into poly-ornithine and laminin coated tissue culture plates were triggered for neuronal differentiation by removing EGF from the growth medium. Over 14 days the cells remained adherent, began to form small aggregates and developed elongated spindle processes, accompanied by reduced proliferation and some cell death. These cells presented a neuronal morphology with the absence of Tuj1 and MAP2 expression, suggesting that these cells were immature neuronal progenitors. For further differentiation and maturation, FGF2 was withdrawn and cultures were maintained for another 2–3 weeks. By the end of fourth week of neuronal differentiation, many cells became Tuj1 positive and exhibited thin elongated processes (Fig. 3A and C), resembling primary neuronal cultures with extensive neurite networks. The expression of the stem cell and progenitor marker Nestin was greatly reduced in these differentiated cultures (Fig. 3C and Suppl. Figs. 2–4). To identify specific neuronal cell types generated from human NS cells *in vitro*, differentiated cultures were stained with different neuronal subtype markers. In both, brain and spinal cord cell cultures, a high percentage of cells were Tuj1$^+$ neurons with the majority displaying GABA expression. A few serotonin and TH positive neurons were observed from some, but not all of the lines (Fig. 3A and C and Suppl. Figs. 2–4). Several other antibodies were tested (Table 1). For example, no O4 oligodendroglial marker expression could be detected in these basic differentiation conditions, whose induction requires a prolonged cytokine treatment protocol comprising several stages of factor combinations (Sun et al., 2008; Glaser et al., 2007). These analyses demonstrate that Cortex04 and Hind05 human NS cell lines retain stable and robust neurogenic capacity after extensive expansion for more than 35 passages consistent with earlier observations on fetal forebrain hNS (Sun et al., 2008).

Microarray analysis of differentiated Cortex04 cells produced a list of genes that are suitable targets for drug development (“drugable” genes), including ion channels and neurotransmitter receptors, which displayed a consistent increase in their expression along the differentiation process at the analysed time points from 0 to 32 days of neuronal differentiation (Fig. 4). On a molecular level, the data show that neuronal physiological maturation is taking place over time in the cultures and offers a new route for drug discovery with target genes expressed at physiological levels in the appropriate cell type, as an alternative to standard technologies based on the over-expression of drugable targets in non-physiological transformed cell lines. A few of such genes are: adrenergic beta-1 receptor (ADRB1), solute carrier family 26 (sulfate transporter) member 1 (SLC26A1) transcript variant 2, solute carrier family 35 member E3 (SLC35E3), G-protein coupled 2 (P2RY2) transcript variant 1, glutamate dehydrogenase (GLUD2), purinergic receptor P2Y G-protein coupled 12 (P2RY12) transcript variant 1 and calcium channel voltage-dependent gamma subunit 5 (CACNG5) transcript variant 1.

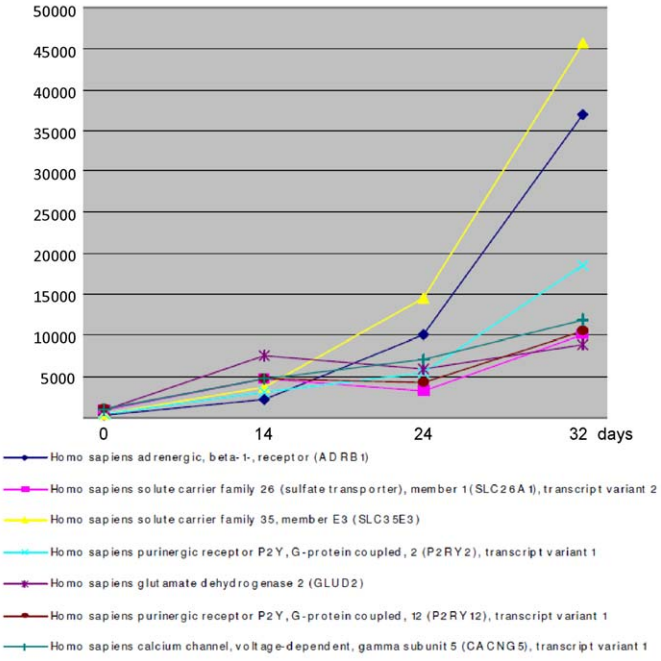

Fig. 4. List of druggable genes upregulated with differentiation of hNS. Microarray (Agilent 4 × 44K whole human genome array) gene expression analysis of RNA extracted from Cortex04 hNS cells at days 0, 14, 24 and 32 of neuronal differentiation showing examples of genes that are upregulated concurrent with early neuronal differentiation displayed in arbitrary units from normalized sample intensity signal data.

3.4. Human NS-derived neurons are physiologically mature after prolonged culture

In order to examine whether human NS cells generate mature neurons, we investigated the functionality of differentiated cells by calcium-response assays performed under physiological ionic conditions. This analysis showed that prolonged neuronal maturation was required for normal neuronal physiological responses. In initial experiments with two human NS cell lines (Cortex04 and Hind05); differentiation for four and eight weeks gave mainly non-neuronal responses. At these time points, no spontaneous activity was detected and cells did not respond substantially to K^+ depolarization or to glutamate. However, glial-like responses to carbachol were clearly detected (Fig. 5C). Upon further maturation in Neurobasal medium plus B27 and BDNF, and by the fourth month we were able to detect much larger responses to K^+ and glutamate than in younger cultures. Although such responses were still quite heterogeneous, there were a few cells with large responses that resembled much more neuronal physiological

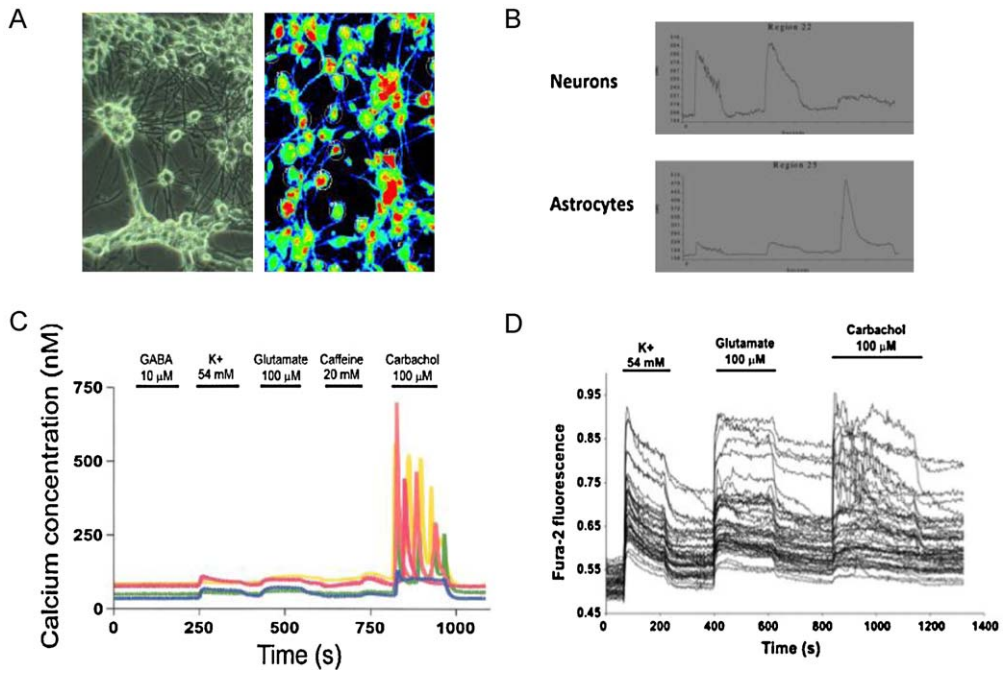

Fig. 5. Neuropharmacological calcium response neuronal maturation studies. (A) Phase contrast picture of a >5 months differentiated Hind05 culture (left); emission image of a representative field of differentiated hNS loaded with Fura 2. (B) Graphic representation of the ratio of emission intensity of two single cell traces in 4 months differentiated Hind05 cultures, one displaying a neuronal (upper graph) and the other a glial response (lower graph) (left peak: K^+ response; middle peak: glutamate response; right peak: carbachol response). (C) Diagram of calcium imaging (fluorimetric analysis) of Hind05 cultures after two month of exposure to differentiating conditions showing no neuronal-like GABA, K^+, glutamate or Caffeine responses. Only glial-like carbachol responses can be observed. (D) Diagram of calcium imaging (fluorimetric analysis) of Hind05 cultures after five month of exposure to differentiating conditions showing robust neuronal-like K^+ and glutamate responses and diminished carbachol responses.

phenotypes. This was confirmed in samples from sister plates, for both neuronal and glial-like (Fig. 5B) responses. In these experiments, neuronal-like activity was considered as a response to K^+ and glutamate, and a faint response to carbachol. For glial-like activity, the opposite response is expected. In cultures differentiated for >5 months, more robust neuronal-like K^+ and glutamate responses and diminished carbachol responses were observed (Fig. 5D). At this later time point, the cultures were still healthy and nearly all the cells showed a fully differentiated neuronal morphology resembling primary neuronal cultures (Fig. 5A, left). Furthermore, cultures showed a K^+ response equivalent in magnitude to primary hippocampal neurons and the glutamate response approximately half of primary rodent hippocampal neuronal cultures used as controls (data not shown). Nevertheless, spontaneous network activity in the form of oscillations was still absent. In order to stimulate the appearance of spontaneous electric activity, cultures were exposed to treatment with GABA and valproate, with no apparent effect after two months. After >5 months the glial-like carbachol response was reduced to a greater degree in valproate-treated than in control cultures, although this was not significant (data not shown). Therefore, human neurons derived from hNS cells achieve electrophysiologically mature excitability to relevant known agonists, although further characterization will be needed in order to define the signature of specific subtypes of neuronal populations present in such cultures.

3.5. Human neural stem cells can be genetically modified

For the development of rapid, quantitative assays for drug screening, it is useful to have genetically modified neural stem cells lines that can express specific proteins (i.e. GFP or specific cell surface proteins). To develop such lines, three methods for gene delivery were tested on human neural stem cell lines. Both lipofection and electroporation gave very poor levels of transfection efficiency. However, the nucleofection technology gave high yields of transfected cells and enabled the possibility of transient expression studies and the generation of stable cell lines. Once optimal conditions for nucleofection were determined, we tested both transient and stable expression applications. The second application was tested by generating a stable cell line expressing either GFP (Fig. 6A) or hCD2 (Fig. 6B). For GFP, human NS cells with stable and readily visualised GFP expression were established by repeated cell sorting at 3, 6, and 9 weeks after transfection. After the third sort, cells were homogeneously GFP positive and could be expanded for at least 3 months with retention of GFP expression (Fig. 6A). They also exhibited GFP expression after differentiation into neurons or glia (data not shown). For hCD2, the antigen could be detected both by LacZ expression and by flow cytometry for CD2 expression on the cell surface (Fig. 6B). Finally, we also performed transient gene expression and knockdown studies with GFP and co-transfection with either siRNA against GFP (Fig. 6C and D) or the human NS cell marker Nestin (Fig. 6C). Results were analysed by qRT-PCR and flow cytometry. We observed, in all cases, a decrease in the expression levels of the messengers targeted (Fig. 6C and D), proving the efficacy of this system and the possibility of using human NS cells in siRNA screening campaigns.

3.6. The characterized in vitro properties of human neural stem cells are reflected by their engraftment and differentiation potential in vivo

For further validation and to ensure the predictability of results from human neural stem cell *in vitro* assays for preclinical safety studies, it will be important to characterize the *in vivo* performance of these cells by transplantation into the rodent brain of immunologically compromised animals. hCNS-SCns have been characterized extensively *in vivo*, using the neonatal NOD-scid mouse model (Uchida et al., 2000; Tamaki et al., 2002; Guzman et al., 2007) as well as various preclinical models (Kelly et al., 2004;

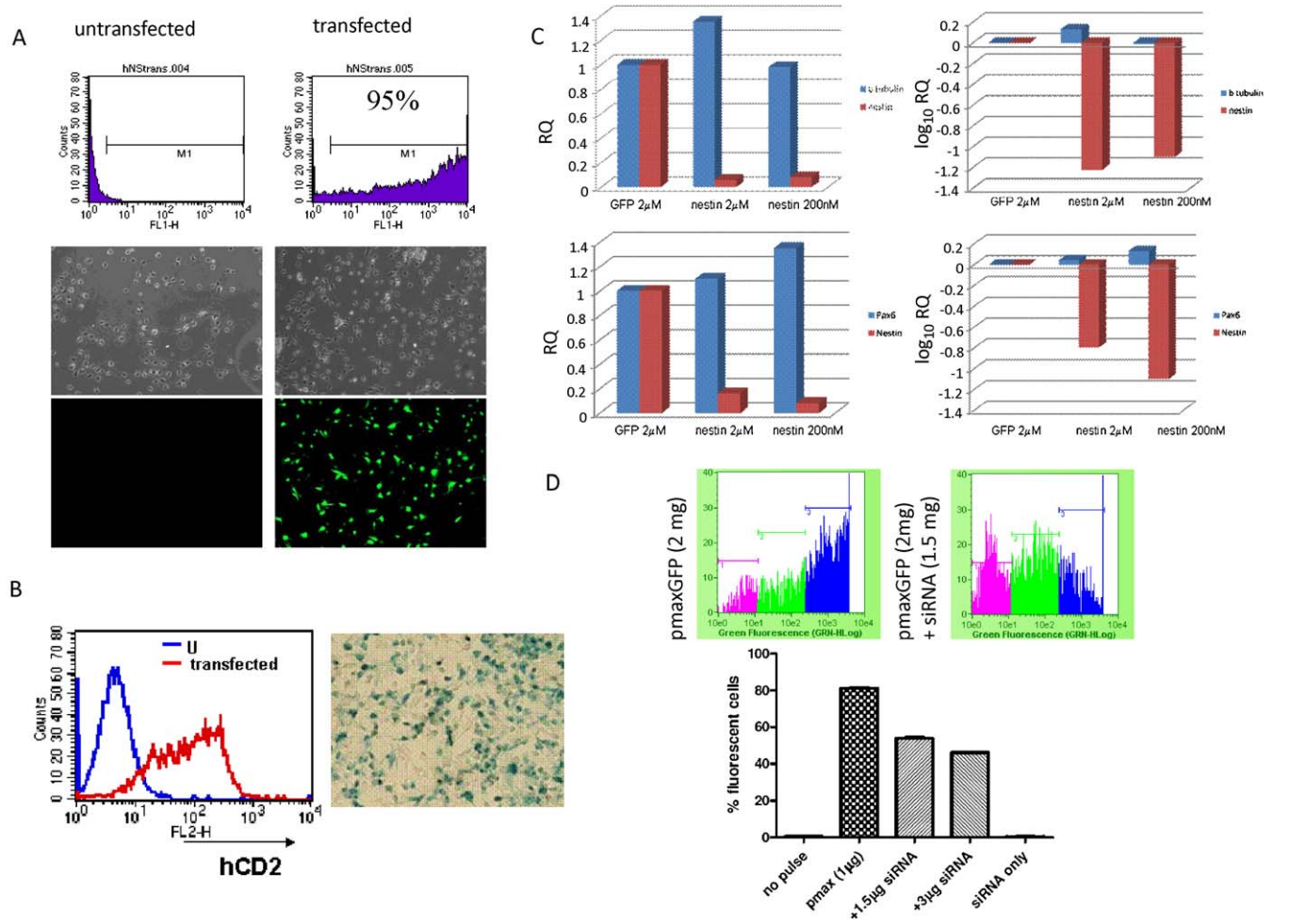

Fig. 6. Gene delivery in hNS. High efficiency of gene delivery is achieved using nucleofection technology in hNS Cortex04 cells (A) using a GFP reporter plasmid, which displayed high levels of GFP expression 24 h post-nucleofection; (B) stable transfection with CAG-βgeo-hCD2 fusion construct, demonstrating the potential of generating stable genetically modified human NS cell lines; (C) quantitative RT-PCR on cDNA isolated from human NS cells treated with Nestin siRNA or GFP siRNA (values are normalized to GAPDH and relative to sample treated with GFP siRNA) also demonstrated the utility of siRNA technology on human NS cell lines and its specificity as the expression level of β-tubulinIII or Pax6 was not altered; and (D) GFP knockdown in double transiently transfected human NS cell cultures detected using flow cytometry (GUAVA, Millipore) analysis gating for low (left), medium (middle) and high (right) intensity levels of GFP expression and percentage of GFP positive cells (graph).

Cummings et al., 2005; Tamaki et al., 2009; Hooshmand et al., 2009; Salazar et al., 2010). Upon NOD-scid transplantation, hCNS-SCns engraft and migrate via white matter tracts including the corpus callosum, the rostral migratory stream (RMS; Uchida et al., 2000) or intraparenchymally into basal ganglia and cortex of mouse brains. Human-specific SC121 immunoreactivity revealed the morphology of immature neural progenitor cells, neurons, astrocytes, and oligodendrocytes. SC121 stained the cytoplasm in the soma and processes of engrafted human neural cells, displaying distinct morphological features of differentiated hCNS-SC-derived neurosphere cells, which aids in their classification into neurons, astrocytes and oligodendrocytes (Fig. 7B), demonstrating that these cells engraft with long-term survival, migrate and differentiate into the three principal neural lineages after transplantation into neonatal NOD-scid mouse brains. Additional studies will be required to further characterize the neurotransmitter phenotype and maturation state, e.g. synapse formation of engrafted neuronal cells.

3.7. Human NS cell culture can be automated in a robotic cell culture system

The use of RHB-A/RHB-Basal in combination with FGF-2 and EGF ensures the maintenance of NS cells in an undifferentiated state and is fully compatible with standard cell culture methodology as well as scaling up strategies in bioreactors and automated cell culture systems, as demonstrated for mouse NS cells using microcarriers in stirred bioreactors and the CompacT SelecT cell culture robotic system (unpublished data). The requirement of laminin coating of all tissue culture plasticware used with human NS cells can also be automated. Instead of applying Accutase, human NS cells were easily non-enzymatically dissociated using cell dissociation buffer (since CompacT SelecT, Suppl. Fig. 5, does not include a centrifuge in its system), displaying normal morphology and expression of undifferentiated markers. Doubling time is 4–5 days (Fig. 7A), and cells can be expanded continuously and scaled-out to multiwell plates for medium to high-throughput screening. Cells produced this way were successfully used in differentiation experiments and calcium-response studies, thus validating the capacity of automated NS cell cultures to retain stem cell properties and differentiation potential.

4. Discussion

Current methods of drug screening rely mostly on recombinant mammalian cell lines engineered to express the target gene of interest, e.g. the NMDA receptor (Kemp and McKernan, 2002), but otherwise are not directly relevant to the disease or condition

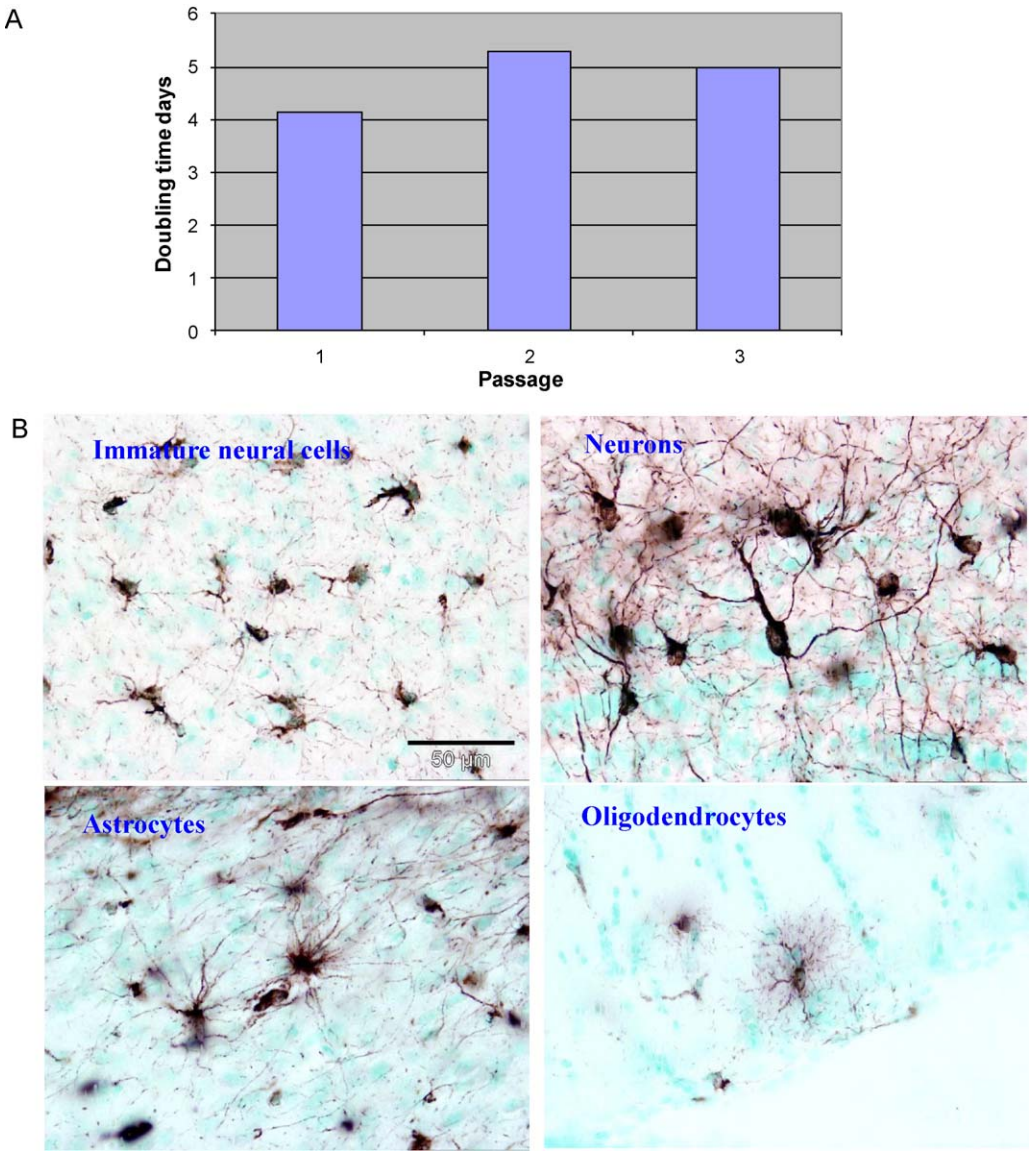

Fig. 7. Automation of hNS culture and differentiation of hCNS-SCns after engraftment in the neonatal mouse brain. Serial fully automated passage and expansion of hNS, Hind05 line with a uniform doubling time was achieved on the CompacT SelecT cell culture robotic system. (A) Human fetal NS maintained on the robot had a doubling time of 4–5 days and were routinely passaged every fifth day. The graph shows the example of three serial passages. (B) Migration and differentiation of transplanted hCNS-SCns progeny, immunoperoxidase stained with human-specific mAb SC121. The SC121 immunostaining detected human cells that morphologically resemble: immature neural cells in the cortex, neurons in the granule layer of the olfactory bulb, astrocytes near the subventricular zone and oligodendrocytes in the corpus callosum.

being studied. The use of primary cells is desirable since they are physiologically relevant and contain all the endogenous signalling pathways and factors at physiological levels. However, cells from primary human origin are very limited in their availability and it is time consuming and technically challenging to obtain sufficient numbers for high-throughput screening applications. Additionally, variability between donors and in the preparation of cell batches can lead to inconsistent results. Stem cells can be a valuable alternative to recombinant cell lines and primary cells in drug discovery high-content screening assays and toxicology studies, in that they can produce differentiated cell types with well-defined characteristics, but can be propagated for prolonged periods of time and scaled-up on demand from cryopreserved banks. Therefore, stem cell-based technology offers great potential for understanding disease mechanisms and identifying targets by means of small molecule screening and functional genomics. In this study, we set out to validate fetal human NS cells, derived from either the whole brain or from different regions of the CNS, which lend themselves to be attractive to drug screening application because of their adherent and homogenous nature as a screening platform, in terms of their physiological relevance and functionality, ease of genetic modification for functional genomics studies and robustness of processes for scale-up and differentiation.

In total, we successfully derived 29 adherent human NS cell lines from different CNS regions from six out of seven fetuses at 8–9 weeks gestation from aborted samples with ethical approval and fully informed consent. We have also established more than 50 hCNS-SCns lines from 16 to 22 gestational week brain tissues. Some of these lines have been adapted to long-term adherent culture, and while more extensive characterization is required, their properties are consistent with a neurogenic radial glia fate. That neurogenic radial glia can still be derived *in vitro* from 16 to 17 weeks gestation human fetal cortex was shown recently (Hansen et al., 2010). The high level of success in human NS cell derivation demonstrates the robustness of the protocol and quality of the reagents developed for this purpose, including animal free media

formulations. The phenotype and multilineage differentiation potential of newly generated human NS cell lines is consistent in different media formulations tested (including animal component free conditions) and the automated cell culture process. Moreover, differentiated cultures displayed mature features such as neuronal and glial calcium responses to known agonists. Additional work will be required to provide a more detailed signature of the cells obtained in such differentiated cultures and their practical application as disease model, toxicology or drug discovery tools.

We have developed a fully defined serum and animal component free medium in which hNS cells can be derived, propagated and differentiated. Cells have similar characteristics to those derived in standard RHB-A medium in that they express markers of undifferentiated NS cells when propagated in FGF2 and EGF and are competent for differentiation into neurons and glia. Future work will determine whether it is possible to grow the cells long-term with functional and genomic stability, and whether the medium is compatible with the scale-up and automation processes that will be required to manufacture sufficient quantities of hNS.

It was shown previously that human neurospheres derived from different fetal CNS regions can retain region-specific molecular specification and neuronal differentiation potential to a certain extent (Horiguchi et al., 2004; Kim et al., 2006), and for mouse fetal-derived adherent radial glia-like NS cells the preservation of positional identity was also reported (Onorati et al., 2010b). Here we have demonstrated, by RT-PCR and Taqman array analysis, the long-term (~20 passages) maintenance of a stable rostrocaudal identity, indicated by the differential expression of FoxG1, Emx2, Otx2, Dlx1 + 2, Mef2C, Slc1A1 aka. EAAC1/EAAT3 and Sox6 (Tao and Lai, 1992; Kimura et al., 2005; Anderson et al., 1997; Li et al., 2008; Torp et al., 1997; Batista-Brito et al., 2009) in anterior-derived hNS cells and by En1 + 2, Irx2 + 3 and HoxB2 (Joyner, 1996; Gomez-Skarmeta and Modollel, 2002; Davenne et al., 1999) in posterior-derived hNS cell lines. With respect to the less extensively described positional identity genes identified in the Taqman array: Mef2C regulates neuronal differentiation and layer formation in the neocortex (Li et al., 2008), Slc1A1is expressed exclusively in glutamatergic projection neurons (Torp et al., 1997) and Sox6 is required for cortical interneuron development (Batista-Brito et al., 2009). Sun et al. (2008) had already demonstrated that fetal hNS cells derived from forebrain and spinal cord maintain differential expression of regional markers in culture. However, there is a small discrepancy to our findings in the current study, in that the expression of Emx2 and Dlx2 was not detected in forebrain-derived cultures. This difference might be explained by the fact that these cells were derived from a slightly earlier developmental stage (50–55 days vs. 60–65 days) and the observation by Onorati et al. (2010b), that in the mouse system only NS cells derived from E14.5 cortex and not from E12.5 cortex carried a consistent transcription factor profile. However, it remains to be determined whether hNS cell lines isolated from different brain regions can indeed be induced to easier generate neuronal subtypes particular to their region of origin *in vitro* or would adapt such phenotypes after transplantation into the rodent brain.

We went on to characterize the neuronal neurotransmitter subtype and functional maturation of hNS cultures in differentiation conditions. Other than the predominantly GABAergic neurons previously reported (Conti et al., 2005; Sun et al., 2008) we were able to consistently generate a small number of dopaminergic and serotonergic neurons from hNS cells of different regional identities. Like Sun et al. (2008), we observed that a gradual and sequential withdrawal of the mitogenic growth factors aids to obtain a greater percentage of neurons from hNS cells, consistent with the observation that a slow exit from the cell cycle promotes neuro- vs. gliogenesis in neural stem cells (Götz and Huttner, 2005). We also conducted neuronal differentiation experiments with hCNS-SCns and examined the neurotransmitter identity that the neuronal progeny acquired and found adoption of GABAergic, cholinergic and dopaminergic neuronal cell fates (unpublished results). As far as physiological maturation is concerned, this was studied in greater detail in mouse NS cells (Biella et al., 2007; Goffredo et al., 2008). hNS were also shown to mature in ion channel composition by their ability to fire overshooting action potentials in response to current injection (Sun et al., 2008), similar to hCNS-SCns co-cultured on an astrocyte feeder layer (Guzman et al., 2007). We chose to conduct calcium-imaging neuropharmacology studies on maturing hNS cultures and monitor their increasing, or decreasing responsiveness to known agonists, and observed a shift from mostly glial to predominantly neuronal-like agonist profile responses, even though in the most mature cultures there was still no sign of spontaneous activity, indicated by the lack of network oscillations in unstimulated cultures. A possible explanation might be that differentiated hNS predominantly express the inhibitory neurotransmitter GABA. The spontaneous release of GABA in this neuronal network could lead to hyperpolarization of neurons and prevent action potential firing. This could explain the absence of spontaneous spikes or synchronous activity. Nevertheless, the electrophysiological maturation of differentiating cultures was also demonstrated in a microarray study, which revealed upregulation of drugable ion channel and neurotransmitter target genes.

The neuronal subtype lineages, which can be derived from neural stem cells, could be restricted over time by the conditions of their *in vitro* culture, including exposure to the growth factors required to expand them. FGF2, which is universally used for the proliferation of NSC, is well known to exert a posteriorizing effect during nervous system development (Stern, 2001). This issue should be considered in the light of a landmark publication by Ying et al. (2008), which led to a paradigm shift of thought in the field of chemical screening. Whereas previously such screens with neuronal and stem cells focused on influencing cell survival or directed differentiation towards a specific lineage, this study demonstrated that it was possible, for mouse embryonic stem cells and with knowledge of the pathways involved, to use small molecules to replace the use of protein growth factors with promiscuous effects (and thought to be essential for cell expansion for decades) thereby leading to a dramatic improvement of the quality, homogeneity and stem cell 'ground state' of the cultures. It is therefore an attractive proposition to pursue specific small molecule compounds that can replace the requirement for mitogenic protein growth factors for the expansion of neural stem cells to circumvent the utility limiting long-term effect of restricted differentiation potential exerted by these factors. Small molecule screens conducted with mouse NS cells gave us a first glimpse of pathways and structures for this purpose and warrant further examination with human NS cells (Gorba et al., 2010).

The understanding of gene function is paramount to identifying and validating drug targets. Therefore, another important aspect of hNS cells is their amenability to genetic alteration by nucleofection. This has important implications for the use of hNS cells in disease modelling, functional genomics with gain and loss of function experiments and RNAi library and drug screening, for which this study presents proof of principle. The example of the CD2 construct further demonstrates how this technology can be utilized for lineage selection strategies to obtain purer populations of desired neural subtypes. The CD2 cell surface receptor is exclusively expressed by certain populations of lymphocytes and not by neural cells. If its expression is artificially driven by promoter sequences of intracellularly expressed specific lineage markers, these populations could be isolated by antibody-coupled magnetic bead selection for CD2.

We had previously demonstrated that hCNS-SCns can be efficiently genetically modified using lentiviral vectors, examples being GFP expression under the control of an ubiquitous, constitutively active promoter for *in vivo* tracking of all transplanted cells and the expression of GFP under the control of lineage-specific myelin basic protein (MBP) promoter sequences to trace myelinating human oligodendrocytes in the rodent brain (Tamaki et al., 2002; Capela et al., 2007).

Provision of the pharmaceutical industry with a sufficient supply of stem cells for drug discovery requires the production of cells on a large scale, preferably in batches for several drug screens. As described in this and previous manuscripts (Conti et al., 2005; Sun et al., 2008) manual neural stem cell culture is well understood, with established and robust methods. However, traditional large-scale parallel manual or stationary culture methods are not suitable for the generation of large numbers of cells, proving too expensive and labor intensive. Therefore, we have opted for the path of cell automation for the maintenance and expansion of our adherent fetal hNS cultures. While there are a number of automated platforms capable of cell culture, we chose the CompacT SelecT, manufactured by The Automation Partnership, because it has become the currently adopted pharmaceutical industry standard for cell production (Monotoute et al., 2003). The use of automated cell culture robots is more suited to the production of cells in small well format for drug screening applications, where the whole process from scale-up, plating out of cells in multiwell plates and screening can be automated, and therefore preferable over expansion in bioreactors, which maybe more suitable for clinical applications (Kallos et al., 2003). Further, each flask can be processed with its own unique set of parameters. We report here that hNS expanded in the CompacT SelecT robot faithfully maintain their stem cell self-renewal phenotype and multilineage differentiation potential. Our calculations show that with uniform expansion and a single CompacT robot working at maximum capacity we could theoretically produce 5000 million cells within a week, which is sufficient for a 40,000 compound screening campaign.

It is reasonable to assume that the predictability of human NS cell *in vitro* assays for drug discovery and toxicology screening will correlate with the ultimate test for their validity and functionality – the ability to properly engraft and differentiate after transplantation in the CNS. This is also a pre-requisite for following up *in vitro* screening results, with a second level *in vivo* validation of the test compound effects by either pre-treating human NS cells *in vitro* prior to their transplantation into experimental animals, or *in vivo* drug application to animals previously transplanted after established engraftment. We report here, as we had demonstrated previously (Uchida et al., 2000; Tamaki et al., 2002; Guzman et al., 2007), that hCNS-SCns engraft efficiently into the neonatal NOD-scid mouse brain and respond to host environmental cues by cell type differentiation in a site-specific manner.

In summary, adherent radial glia-like human neural stem cells (hNS and hCNS-SC) have promise for a variety of biopharmaceutical and regenerative medicine applications. They can be grown and differentiated at scale in automated culture, in small well format and can be genetically modified and therefore represent a stable physiologically relevant cell resource for disease modelling and drug screening.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuint.2011.06.024.

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