Environmental signals regulate lineage choice and temporal maturation of neural stem cells from human embryonic stem cells

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Human embryonic stem cells (hESCs) are a potential source of defined tissue for cell-based therapies in regenerative neurology. In order for this potential to be realized, there is a need for the evaluation of the behaviour of human embryonic stem cell-derived neural stem cells (hES-NSCs) both in the normal and the injured CNS. Using normal tissue and two experimental models, we examined the response of clinically compatible hES-NSCs to physiological and pathological signals. We demonstrate that the phenotypic potential of a multipotent population of hES-NSCs is influenced by these cues both in vitro and in vivo. hES-NSCs display a temporal profile of neurogenic and gliogenic differentiation, with the generation of mature neurons and glia over 4 weeks in vitro, and 20 weeks in the uninjured rodent brain. However, transplantation into the pathological CNS accelerates maturation and polarizes hES-NSC differentiation potential. This study highlights the role of environmental signals in determining both lineage commitment and temporal maturation of human neural stem cells. Controlled manipulation of environmental signals appropriate to the pathological specificity of the targeted disease will be necessary in the design of therapeutic stem cell-based strategies.

Keywords: human embryonic stem cells; neural stem cells; temporal maturation; phenotypic potential; cell transplantation

Abbreviations: DG = dentate gyrus; hES-NSCs = human embryonic stem cell-derived neural stem cells; HNM = human neuralizing medium; NPCs = neural precursor cells; BMP = bone morphogenetic protein

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Introduction

Cell implantation therapy potentially offers a realistic strategy for the management of a wide range of neurological diseases, that involve both degenerative and inflammatory mechanisms of tissue injury (Peschanski and Dunnett, 2002; Bjorklund et al., 2003; Tai and Svendsen, 2004; Lindvall and Kokaia, 2006; Martino and Pluchino, 2006). But it is likely however, that functional neural repair will require approaches that are individually tailored to each specific pathological process or clinical condition. Therefore, a fundamental requirement for therapies is the ability to replace a diverse range of neural cell types. Human embryonic stem cells (hESCs) have the potential to generate unlimited numbers of neural cells and, due to their developmental plasticity, have the capacity to differentiate into the large variety of cell types needed in order for functional repair of the diseased CNS. Recent advances in the generation of good manufacturing practice (GMP) standard methods for hESC derivation, cell propagation and neural differentiation bring forward these possibilities (Joannides et al., 2007; Lu et al., 2006; Ludwig et al., 2006; Yao et al., 2006). However, functional evaluation of hESC-derived neural stem cells (hES-NSCs) is necessary before applications can reasonably be considered. Specifically, hES-NSCs must at a minimum differentiate into mature and functional neural progeny, and demonstrate appropriate integration in tissue rendered abnormal by the pathological process. Thus, the modulation of their normal developmental properties needs to be evaluated, initially in the context of experimental models of neurological disease.
A number of studies have reported the engraftment potential of hESC-derived neural derivatives. Although encouraging, several important variables preclude direct comparison and extrapolation of these studies. Several hESC lines, derived and propagated under distinct conditions, have been studied. Variations between these lines with respect to genomic stability and differentiation potential are well recognized (Cai et al., 2006; Hoffman and Carpenter, 2005; Loring and Rao, 2006; Rao and Cavin, 2005). The methods used to generate transplanted neural cells are also diverse, ranging from exposure to undefined signals, such as serum and stromal cell lines, to the application of known developmental cues (Schulz et al., 2004; Nistor et al., 2005; Keirstead et al., 2005; Park et al., 2005; Tabar et al., 2005; Brederlau et al., 2006; Guillaume et al., 2006; Roy et al., 2006). Other variables encountered prior to transplantation depend on the method and extent of ex vivo propagation and/or pre-differentiation. Recent studies highlight the importance of methods used to propagate hESCs in determining karyotypic stability (Buzzard et al., 2004; Draper et al., 2004; Mitalipova et al., 2005), and suggest non-equivalence of phenotypic neural stem cell potential upon extended manipulation in vitro (Itsykson et al., 2005; Li et al., 2005; Bouhon et al., 2006; Wright et al., 2006).

Aside from these features, there are considerable differences with respect to the nature of the recipient host environment. Specifically, developmental stage and details of the pathological injury provide distinct micro-environmental conditions for transplanted cells. In view of the well-established role of extrinsic signals on phenotypic potential of neural stem cells (Gross et al., 1996; Caldwell et al., 2001; Hsieh et al., 2004), the differential behaviour of a given population of hES-NSCs in response to contrasting in vitro and in vivo environments is important, yet understudied. Against this background, we sought to examine the behaviour of hES-NSCs derived under defined conditions in the normal and injured brain with respect to phenotypic potential and temporal maturation. Such insights will begin to provide a pre-clinical substrate for understanding the issues that must be resolved if the therapeutic potential of human stem cell biology is to be realized for neurological medicine.

**Materials and methods**

**Cell culture**

The hESC lines H9 (WiCell Research Institute; Madison, WI, USA) and HUES9 (hES facility; Harvard University, Cambridge, MA, USA) derived in accordance with local and national guidelines were used, between passages 30–60. hESCs were cultured and passed on mouse embryonic fibroblasts as previously described (Joannides et al., 2006). hES-NSCs were generated by culturing hESC colony fragments in an optimized and fully defined human neuralizing medium (HNM) under substrate-free conditions, and passaged mechanically (Joannides et al., 2006b). For clonal analysis, dissociated hES-NSCs were plated at limiting dilution in 96-well plates, and resulting spheres were transfected and cultured under similar conditions to expanding hES-NSC cultures. For terminal differentiation, hES-NSCs were plated onto pol-y-lysine/laminin coated coverslips and cultured in DMEM/2% B27 (Invitrogen; Carlsbad, CA, USA), supplemented with different growth factors and/or fetal bovine serum (FBS) as indicated in the results, for up to 4 weeks.

**Transcriptional analysis**

RT-PCR analysis was performed as previously described (Joannides et al., 2006a). Briefly, total RNA was extracted using a RNeasy Mini Kit (Qiagen; Valencia, CA, USA; http://www.qiagen.com) and cDNA was synthesized from 2.5 µg of RNA using Moloney Murine Leukaemia Virus reverse transcriptase (Invitrogen) and oligo-dT primers. PCR was carried out using Taq polymerase (Invitrogen). PCR products were separated on a 2% agarose gel and visualized with SYBR-Green (Invitrogen). The expression of the housekeeping gene GAPDH was used to normalize PCR reactions. Forward and reverse primer sequences, annealing temperature and PCR cycles were as follows: Nestin, 5'-CAGCTGGGCCACCTACAGATG-3', 5'-AGGAGATGTTGGGCTCAGGACTTG-3', 60°C, 20 cycles; Musashi1, 5'-GAGACTGACGGGCCCCAGCGG-3', 5'-GCCTGTTGCTCAGGAGAG-3', 65°C, 25 cycles; SOX1, 5'-TACACGCGATCATCTCAACTCT-3', 5'-GCTTCCACTCTACGGAGAG-3', 65°C, 30 cycles; SOX2, 5'-ATGAGCGGTTCTACGACGTGCA-3', 5'-TCTTTGGAACCCCTCCTCCAATTT-3', 60°C, 35 cycles; PAX6, 5'-AACAGACACGCGCTCACAAAAGCT-3', 5'-GGGGAACGTTGGAACCTGGAC-3', 60°C, 25 cycles; NCAM, 5'-AGGAGACAGAAAGGAGCCACAGCAC-3', 5'-GTTGTGAGTATGGCTGTG-3', 60°C, 25 cycles; GAPDH, 5'-AGGCCATGTGGTCTAGACCG-3', 5'-GTACTCAGGGGCCAGCATCG-3', 60°C, 30 cycles.

**Electrophysiological analysis**

Terminally differentiated hES-NSCs on glass coverslips were transferred into a perfusion chamber mounted on the stage of an Olympus CK40 inverted microscope equipped with phase-contrast optics. Cells were continuously perfused at room temperature using a gravity fed system at a rate of 2–4 ml/min with a bath solution containing: 135 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 5 mM HEPES and 10 mM glucose with the pH adjusted to 7.4 with NaOH. Somae of cells which displayed a neuronal phenotype (cell bodies of 8–12 µm with long branching projections) were voltage- or current-clamped following the voltage step. All currents were normalized to cell membrane resistance compensation was employed at 80–85%. The standard voltage-clamp protocol consisted of holding cells at −60 mV using a P/N 4 on-line subtraction protocol. Inward currents were measured as the peak current between 1 and 20 ms into the voltage step whilst outward currents were measured as the mean current between 180 and 195 ms into the voltage step. All currents were normalized to cell membrane area by dividing cell capacitance to yield current density (pA/pF). Further, cells were current-clamped at 0 pA and two protocols employed: (i) continuous recording of resting membrane potential
interspersed with current injection to bring membrane potential about threshold for action potential generation (typically 50–100 pA), and (ii) stepped current injection of 100 ms duration from 0 pA to that which elicited action potential generation and beyond (typically from between 10 to 200 pA). All recordings were obtained using an Axopatch 700A amplifier (Axon Instruments; Forster City, CA, USA) and a DigiData 1320 A/D interface. Current and voltage protocols were generated by PClamp 9 software and recordings were obtained at a digitization rate of 10 kHz through a 4-pole Bessel low pass filter set at 2 kHz.

Organotypic hippocampal slice cultures

Hippocampal slice cultures were produced at a thickness of 300 μm from 5-day-old mice as described previously (Gahwiler, 1981; Raineteau et al., 2004). Cultures were initially kept in a serum-based medium [25% horse serum, 50% basal medium and 25% Hank’s balanced salt solution (Invitrogen), enriched with glucose to a concentration of 5.6 mM] for 3 days before being transferred to a serum-free medium (Neurobasal-A medium with B27 supplement, glucose 5 mM, L-glutamine 2.5 mM). hES-NSCs, dissociated using 0.1% trypsin and prepared as a single-cell suspension at a density of 5 × 10^5 cells/ml, and 2 μl of suspension (i.e. about 1000 cells) were plated on top of each hippocampal slice, previously grown for 10 days in vitro. Cells were allowed to adhere for 5 minutes before placing the slices back onto the roller-drum, with all cells showing insufficient adhesion being washed away in the culture medium. Medium was changed 2 days following cell plating. Hippocampal slices were subsequently cultured and processed at 2, 14 and 35 days following hES-NSC plating.

Cell transplantation

All animal experiments were performed in full compliance of local ethical guidelines and approved animal care according to the UK Animals (Scientific Procedures) Act 1986. All animals were obtained from Harlan, UK. hES-NSCs were dissociated using accutase and prepared as a single-cell suspension at a density of 1 × 10^5–2.5 × 10^5 cells/μl prior to transplantation.

Neonatal rodent hippocampal grafting

Neonatal pups were anaesthetized by cooling on ice and placed in a support to immobilize the head and permit visualization of bregma. One microlitre of cell suspension was injected over 1 min to the right hippocampus at the following co-ordinates relative to bregma: lateral (L) –1 mm, anterior (A) –1 mm and vertical (V) –2 mm from the skin surface. The needle was left in situ for 2 min following grafting to minimise reflux of grafted cells along the needle tract. Cells were implanted under direct vision using a standard operating microscope (Leica). Six animals were sacrificed at each timepoint following transplantation (1, 3, 6 and 20 weeks) for histological processing.

Striatal lesioning and grafting

Adult rats were immunosuppressed with daily intraperitoneal injections of cyclosporin A (10 mg/kg) for the duration of the experiment, commencing the day prior to transplantation. All surgery was performed under isoflurane anaesthesia. One week prior to cell transplantation, animals received a unilateral 45 mmol quinolinic acid lesion of the right striatum. A volume of 0.75 μl was infused to two sites over a 3 min period using a 1 μl Hamilton syringe at the following co-ordinates relative to bregma: L –3.2/–2.4 mm, A +0.4/+1.4 mm, V –5.0/–4.5 mm below dura, with the incisor bar set to 2.3 mm below the interaural line. The syringe needle was left in situ for 3 min following the infusion. Two microlitres of cell suspension was delivered over a 2 min period to the striatum ipsilateral to the side of the lesion (A +1.0 mm, L –2.8 mm, V –5.0/–4.5 mm, incisor bar set 2.3 mm below the interaural line). Animals were sacrificed at 6 weeks after transplantation for histological processing.

Ethidium bromide spinal cord lesioning

X-irradiated ethidium bromide lesions were induced as described previously (Blakemore and Crang, 1992; Chandran et al., 2004) in the spinal cord of six adult male nude (RNU/RNU) rats. Three days after lesioning, 1 μl of cell suspension was injected into the lesion site. Animals were sacrificed at 6 weeks following transplantation.

Immunocytochemistry and quantification

For immunocytochemical analysis, primary antibodies against the following antigens were used: β-III-tubulin (1 : 200; Sigma–Aldrich, St Louis, MO, USA), BMP2/4 (1 : 250; R&D Systems, MN, USA), Doublecortin (DCX – 1 : 400; Santa Cruz Biotechnology, CA, USA), DARPP-32 (1 : 3000; Chemicon, Temecula, CA, USA), GABA (1 : 500; Sigma), GFAP (1 : 100; DakoCytomation, Glostrup, Denmark), Glutamate (1 : 500; Sigma), human nuclear antigen (1 : 100; Chemicon), Nestin (1 : 500; Chemicon), MAP2 (1 : 200; Sigma), Musashi1 (1 : 500; Chemicon), NG2 (1 : 400; kind gift from William Stallcup), O4 [supernatant diluted 1 : 5; (Sommer and Schachner, 1981)], Olig2 (1 : 40,000, kind gift from David Rowitch), NeuN (1 : 1000; Chemicon), Pax6 (1 : 50; DSHB), Sox1 (Chemicon, 1 : 1000), Synapsin1 (1 : 500; Calbiochem; Darmstadt, Germany) and Vimentin (1 : 100; Chemicon).

In vitro hES-NSC characterization

Immunocytochemistry was carried out using standard procedures, as previously described (Joannides et al., 2007). Fixed cells and embedded sections were stained with primary antibody and appropriate conjugated secondary antibody (1 : 1000; Invitrogen) was applied. Stained cells were mounted and viewed under a Leitz microscope for cell identification and counting. For each coverslip, five consecutive random fields were counted using a grid; all cells from at least 4–6 cryostat sections for each experiment were counted. The number of positive cells was expressed as a mean ± SEM in each experiment, and each experiment was repeated at least three times. Statistical analysis was carried out using GraphPad Prism 3.03 (GraphPad Software; San Diego, CA, USA).

Organotypic slice cultures

Slices were fixed in 4% paraformaldehyde overnight, washed in 0.1 M phosphate buffer (PB), and subsequently removed from their support matrix. Subsequent processing was performed in free-floating conditions. Fixed slices were blocked and permeabilized with PB/0.4% Triton X-100/10% normal horse serum (NHS) for 24 h at 4°C. Primary antibodies were applied sequentially for 4 days at 4°C in PB/0.4% Triton X-100/5% NHS. Cultures were...
rinsed and secondary antibodies applied in PB/0.4% Triton X-100/5% NHS overnight at 4°C (1:350, Invitrogen, and Jackson Immunoresearch, West Grove, PA, USA). Cultures were subsequently counterstained with 4',6-Diamidino-2-phenylindole (DAPI) and mounted using the ProLong® Antifade Kit (Invitrogen). For confocal analysis, stacks of images were captured on a Leica TCS-NT-UV confocal laser scanning microscope using a 40 x 1.25 N.A. Leica lens. Typical stacks were composed of 10–20 optical sections of 1 μm thickness taken at 0.8 μm intervals. Sequential acquisitions were performed in the different channels in order to avoid any misinterpretation of the results due to signal cross-talk.

**Transplanted CNS tissue**

For light microscopic examination, fixed spinal cord tissue was cut into 1 mm coronal slices, embedded in resin, and 1 μm sections were cut and stained for toluidine blue. Immunohistochemistry was carried out using standard procedures as previously described (Burnstein et al., 2004; Le Belle et al., 2004). Fixed brain and spinal cord tissue was cryoprotected by transferring to 30% sucrose, and sectioned coronally at 40 μm using freezing microtome (Leica). A one in six series of sections was processed for each stain unless otherwise stated. The Nissl stain cresyl violet was used to identify surviving grafts. Sections were quenched with 10% hydrogen peroxide in 10% methanol for 5 min. For human nuclear antigen (hNA) staining, sections were then treated with 2 M HCl at 37°C for 20 min, neutralized with borate buffer. Tissue was subsequently blocked in Tris buffered saline (TBS)/0.2% Triton X-100/5% normal donkey serum for 2 h prior to overnight incubation at 4°C with primary antibody. Tissue sections were subsequently washed and appropriate biotinylated secondary antibody (1:200; Invitrogen) applied, before transfer to an avidin–biotin complex (streptavidin ABC kit; Dako) in TBS with 1% serum for 2 h. Positive staining was visualized using dianinobenzidine (DAB). Sections were counterstained with Vector SG and mounted on gelatinized slides in DPX mountant. Quenching and avidin–biotin complexing was omitted for Vector SG and mounted on gelatinized slides in DPX mountant.

**Results**

**In vitro lineage commitment of hES-NSCs is dependent on both intrinsic determinants and extrinsic cues**

In order to examine the behaviour of hESC-derived neurons, neural cultures were first established from hESCs under defined conditions that avoid the use of animal products, proprietary formulation or genetic manipulation (Joannides et al., 2007). Serial gene expression and phenotypic analysis revealed a temporal course in differentiation potential. Early hES-NSCs (D25) showed expression of the immature neural markers Nestin, Musashi1, SOX1, SOX2, PAX6 and NCAM (Fig. 1A), and generated a highly neuronal population when plated for 14 days in 2% B27/1% FBS supplemented medium, with no detectable O4 or GFAP+ glial cells (see table in Fig. 1). Analysis at 28 days post plating confirmed the absence of glial markers excluding delayed maturation as an explanation for the lack of glia.

Unlike earlier cultures, hES-NSCs from D45 onwards showed expression of Olig2, a transcription factor necessary and sufficient for oligodendrogenesis (Lu et al., 2000; Zhou et al., 2000; Takebayashi et al., 2000), while maintaining expression of all other undifferentiated markers (Fig. 1A). In contrast to previous time points, Olig2 was present in the majority of cells at D45 (83.9 ± 7.5%) (Fig. 1B–C), and was maintained at comparable levels at later time points analysed (D60, D85; data not shown). Consistent with this observation, hES-NSCs were now able to generate glial progeny, whilst maintaining an immature phenotype. Late hES-NSCs (D70) were highly proliferative, with BrdU incorporation in 43.1 ± 4.4% of cells over 72 h, and expressed Nestin (93.6 ± 1.7%), Musashi1 (85.0 ± 4.1%), Pax6 (72.5 ± 6.6%) and Sox1 (77.1 ± 7.3%), consistent with a neural stem cell phenotype (Fig. 2A–E). Upon terminal differentiation, hES-NSCs were now able to generate, in addition to neurons, significant numbers of GFAP+ astrocytes and O4+ oligodendrocytes following plating (see table in Fig. 1, and Fig. 2F–I). Interestingly, GFAP+ cells were first identified from D45-50 and preceded the emergence of O4+ cells by approximately 2-3 weeks. Therefore, in order to examine the influence of extrinsic cues on hES-NSC multipotent differentiation, further analysis was restricted to hES-NSCs between 50 and 70 days following neuralization from four hES-NSC lines.

Single cell analysis using limiting dilution was next undertaken to confirm neural stem cell potential (Reynolds and Rietze, 2005; Singec et al., 2006). Resulting neurospheres were generated in response to EGF and FGF2 under substrate and serum free conditions, and assessed for phenotypic potential. Clonal efficiency was 1.5%, with six
clones generated out of a total of 400 wells. All clones generated β-tubulin⁺ neurons and GFAP⁺ astrocytes, and four clones generated O4⁺ oligodendrocytes in addition to neurons and astrocytes (Fig. 2J). These findings demonstrate that hESC derived neural cultures exhibit neural stem cell properties following extended propagation in vitro.

Having established multipotency of hES-NSCs, we next sought to examine whether they could be predictably manipulated by extrinsic signals to promote a neuronal or glial fate. Differentiation in serum free conditions and in the presence of the neurotrophins BDNF and GDNF resulted in a significant increase in cells expressing β-tubulin (78.7 ± 3.8%; P < 0.05). Addition of BMP4 to plating medium resulted in a significant increase in GFAP⁺ astrocytes (86.2 ± 4.8%, P < 0.0001) with a concomitant reduction in β-tubulin⁺ neurons.

hES-NSCs differentiate in vitro into mature and functional neurons by 4 weeks

In order to examine in greater detail the temporal and functional acquisition of neuronal fate, plated hESC-NSCs were examined at serial time points following mitogen withdrawal in the presence of neurotrophins. At 12 h, cells remained positive for nestin, with few β-tubulin⁺ cells detectable within the sphere (Fig. 3A–C). Long processes positive for nestin, vimentin and occasionally GFAP, extending from adherent spheres were evident by 36 h, consistent with a radial glial phenotype (Fig. 3D–F)(Caldwell et al., 2001). In addition to nestin expression in the majority of cells, the presence of the immature neuroblast marker doublecortin (DCX) was now evident, co-labelling β-tubulin⁺ cells having an immature bipolar morphology and not staining for nestin (Fig. 3G–J).

Following 2 weeks differentiation, neuronal cells exhibited a mature polarized morphology with multiple processes and bouton-like structures positive for SynapsinI, along with expression of mature cytoskeletal (MAP2ab 73.3 ± 8.9% of β-tubulin⁺ cells) and neurotransmitter (GABA 55.9 ± 12.1%; Glu 34.8 ± 6.3% of β-tubulin⁺ cells) markers, and concomitant loss of nestin and DCX expression (Fig. 4A–F). Neurotransmitter staining was most evident in the soma and boutons of neuronal cells. By 4 weeks, NSC-derived neurons were fully mature, with whole-cell currents demonstrating robust neuronal characteristics including fast, voltage-activated and rapidly inactivating inward currents followed by slowly activated but sustained outward currents (Fig. 4G). Current density-voltage relationships of these two components (Fig. 4H) were indicative of voltage-activated sodium currents (filled circles) and delayed-rectifier potassium currents (filled squares). In order to demonstrate that these currents were able to contribute to neuronal spiking, current was injected to evoke a change in membrane potential to a level above the threshold for voltage-activated sodium current activation. This resulted in the generation of action potentials, thus confirming the contribution of the observed currents to neuronal spiking (Fig. 4I–J).

hES-NSCs show site specific differentiation upon engraftment into organotypic hippocampal slices

Having established phenotypic plasticity and preferential neuronal maturation of hES-NSCs in vitro, we next...
examined their behaviour in response to hippocampal signals following dissociation and plating upon normal postnatal hippocampal organotypic slice cultures. Analysis at 2, 14 and 35 days revealed successful engraftment with evidence for migration, and site-specific neuronal and astrocytic differentiation. At 2 days post-plating, cells were mainly found on top of the slice cultures, an area composed largely of astrocytes (Del Rio et al., 1991). An average of 96.7 ± 17 hNA+ cells could be detected which were homogeneously distributed over the entire slice, indicating that ∼10% of plated cells successfully attached to the cultures (see table in Fig. 5). Engrafted hNA+ cells started to show expression of DCX at day 2, which increased substantially by 14 days post-plating. At this stage, cells could be found throughout the slice (Fig. 5A) indicating migration from the astrocytic top border to deeper regions. The cell distribution remained homogeneous between the dentate gyrus (DG) and pyramidal cell (CA1 and CA3) regions (see table in Fig. 5). The vast majority of cells both at the top and within the slice were seen to express the neuronal marker DCX.

DCX+ cells showed a polarized morphology with several dendrites extending in a single orientation from the cell body (Fig. 5B–C). Neurotransmitter subtype analysis revealed that, although both glutamate and GABA expression in hNA+ engrafted cells were detectable, the majority of cells expressed a glutamatergic phenotype, in contrast to in vitro differentiation (Fig. 5D). Further analysis at 35 days confirmed maintenance of the neuronal phenotype, and partial down-regulation of DCX expression. Although the number of cells decreased after 2 weeks, numerous hNA+ cells were still detectable at 35 days with a similar distribution to previous timepoints, suggesting long-term engraftment of surviving cells (see table in Fig. 5). It was of interest that at 14 and 35 days post-plating, some hNA+ cells expressing GFAP were also evident. Such co-positive cells were only identified at the surface (astrocytic region) of the slice, but not in deeper layers where exclusive neuronal differentiation occurred (Fig. 5E). Together, these results provide evidence that hES-NSCs can appropriately respond to the hippocampal micro-environment.
the normal neonatal rodent hippocampus. Analysis was undertaken at four time points (1, 3, 6 and 20 weeks). hNA+ cells were apparent within the hippocampus with evidence of migration within the hippocampus, cortex and corpus callosum (Fig. 6). In contrast to the in vitro findings, neuroglial differentiation was markedly delayed (see table in Fig. 6). Nestin expression, predominantly evident at 1 week (Fig. 5A), was still detected on up to 50% of cells even at 20 weeks. DCX, absent at 1 week, became evident in the majority of cells by three weeks (Fig. 6B–C), and persisted in >25% of cells at 20 weeks. Consistent with these findings, mature neuronal markers (human neurofilament, NeuN) were first detected at 20 weeks within a small minority of engrafted cells (Fig. 6D–F). In addition, a fraction of neuronal cells also expressed synaptophysin, suggestive of maturation and integration (Fig. 6G–H). No graft-derived glial cells were present at the early time points. GFAP+ glia were first identified in small numbers at 20 weeks (Fig. 6I). The remaining cells at 20 weeks retained an immature progenitor (Nestin+) or neuroblast (DCX+) phenotype. Importantly, there was no evidence of uncontrollable growth and tumour formation within the transplanted tissue.

**Fig. 4** Functional in vitro maturation of hESC-derived neurons. (A–D) Phase and immune micrographs for β-tubulin, MAP2ab and SynapsinI of NSC-derived neurons after 14 days of terminal differentiation in the presence of neurotrophins. (E, F) Transmitter subtype analysis of NSC-derived neurons, demonstrating glutamergic and GABAergic differentiation. (G) Exemplar family of superimposed whole-cell currents obtained under voltage-clamp conditions showing voltage-activated, rapidly inactivating inward currents (marked as filled circle) followed by sustained outward currents (marked as filled square). Cells were held at −90 mV and stepped in 10 mV increments for 200 ms from −100 to +60 mV at a frequency of 0.2 Hz. (H) Mean (n = 4) current-density versus voltage relationships for inward (filled circle) and outward currents (filled square). (I) Membrane potential recording from the same cell as in (G) and (J) under fast current-clamp conditions. At the points indicated by the arrows, 60 pA of current was injected to elicit rapid depolarization and action potential generation. (J) Family of superimposed current-clamp recordings of membrane potential. Cells were held at 0 pA and stepped in 10 pA increments from −10 pA to +180 pA for 100 ms at a frequency of 0.2 Hz. Scale bar, 50 μm.

**hES-NSC maturation is delayed upon engraftment into the uninjured neonatal rodent hippocampus**

In order to study the capacity of hES-NSCs to respond to hippocampal cues in vivo, we first transplanted NSCs into the normal neonatal rodent hippocampus. Analysis was undertaken at four time points (1, 3, 6 and 20 weeks). hNA+ cells were apparent within the hippocampus with evidence of migration within the hippocampus, cortex and corpus callosum (Fig. 6). In contrast to the in vitro findings, neuroglial differentiation was markedly delayed (see table in Fig. 6). Nestin expression, predominantly evident at 1 week (Fig. 5A), was still detected on up to 50% of cells even at 20 weeks. DCX, absent at 1 week, became evident in the majority of cells by three weeks (Fig. 6B–C), and persisted in >25% of cells at 20 weeks. Consistent with these findings, mature neuronal markers (human neurofilament, NeuN) were first detected at 20 weeks within a small minority of engrafted cells (Fig. 6D–F). In addition, a fraction of neuronal cells also expressed synaptophysin, suggestive of maturation and integration (Fig. 6G–H). No graft-derived glial cells were present at the early time points. GFAP+ glia were first identified in small numbers at 20 weeks (Fig. 6I). The remaining cells at 20 weeks retained an immature progenitor (Nestin+) or neuroblast (DCX+) phenotype. Importantly, there was no evidence of uncontrollable growth and tumour formation within the transplanted tissue.

**Signals from the injured adult CNS accelerate and polarize phenotypic fate of transplanted hES-NSCs**

In order to investigate whether the injured adult environment influences both lineage choice and temporal maturation of transplanted NSCs, we next examined the behaviour of hES-NSCs in two animal injury models chosen to represent potentially different clinical contexts for eventual application.

Four animals received unilateral quinolinic acid to create neurodegenerative striatal lesions 1 week prior to hES-NSC transplantation (Beal et al., 1986). All animals showed good graft survival at six weeks following transplantation (Fig. 7A). The grafts contained hNA+ cells confined to the lesion area (Fig. 7B). In contrast to the response seen in the unlesioned brain, engrafted hES-NSCs demonstrated, in addition to immature (hNA+/DCX+) (Fig. 7C and F), evidence of mature (hNA+/NeuN+) (Fig. 7D and G) neuronal differentiation at 6 weeks. No evidence of striatal neuron differentiation (DARPP-32+) was detectable. Furthermore, glial differentiation (GFAP+) was observed at 6 weeks (Fig. 7E). Although the majority of GFAP+ cells were hNA+ consistent with host gliosis, double-positive GFAP+/hNA+ cells were also identified, indicating limited astrocytic differentiation of engrafted hES-NSCs (Fig. 7H).

We next examined the behaviour of hES-NSCs in the focal X-irradiated ethidium bromide spinal cord (X-EB) lesion that produces gliotoxic demyelination with axononal and neuronal preservation. Non-transplanted X-EB lesions contained demyelinated axons and myelin debris-filled...
macrophages, with no astrocytes, oligodendrocytes or NG2+ progenitors present within the demyelinated tissue (Fig. 8A). hES-NSCs were transplanted 3 days after lesion induction with analysis at 6 weeks. In all the transplanted animals, hNA+ cells were present within the area of demyelination, with no evidence of migration in the surrounding normal tissue, despite the X-irradiation induced absence of endogenous NG2+ cells. In contrast to both the uninjured hippocampal and the lesioned striatal environment, no neuronal differentiation was evident, as determined by the absence of DCX and neurofilament (data not shown). The majority of hNA+ cells (>95%) co-expressed GFAP, with the remainder staining positive for nestin, consistent with predominantly astrocytic differentiation of transplanted cells (Fig. 8B–E). Confocal analysis confirmed co-localization of GFAP with hNA+ cells (Fig. 8F–H). In view of the BMP-mediated astrocytic differentiation in vitro, it was of interest that BMP2/4 was detectable within the lesion site (Fig. 8I–J). There was no evidence of graft-derived oligodendrocyte remyelination.

Discussion

We have studied the fate of hES-NSCs in a physiological context, both in vitro and in vivo, and in experimental situations that model neurodegenerative and demyelinating disease processes, respectively. Our results highlight the role of micro-environmental signals in determining both lineage commitment and temporal maturation of potentially clinical grade neural stem cells derived from hESCs.
The ability to derive a defined and characterized hES-NSC population is an important step in beginning to realize the therapeutic promise of human neural stem cells. Although the hESC lines used in this study were initially propagated using mouse feeder cells, recent advances in hESC line derivation and propagation raise the prospect of generating definitive clinical grade hES-NSC lines (Joannides et al., 2007; Lu et al., 2006; Ludwig et al., 2006; Yao et al., 2006). The demonstration of multipotency at the clonal level, and electrophysiological function of derived neurons from hESCs that have been neuralized under defined and humanized conditions raise the prospect of generating clinical grade NSCs. The detection of synapse formation of transplanted hESC-derived cells within the host brain provides further evidence for their potential utility as therapeutic agents. Cell fusion of non-neural stem cells has also been implicated as an alternative explanation for apparent in vivo neural differentiation (Terada et al., 2002; Ying et al., 2002).

**Fig. 6** Delayed neuronal differentiation of hES-NSCs in the neonatal hippocampus. Representative immune micrographs demonstrating co-localization of hNA with (A) nestin adjacent to the CA2 hippocampal region at 1 week, and (B, C) DCX within the dentate gyrus at 3 weeks. (D, E) Low and high power immune micrographs of engrafted cells within the DG with typical neuronal morphology expressing human-specific neurofilament at 20 weeks. (F) Immune micrograph demonstrating NeuN expression in the cortex at 20 weeks, and co-localizing with hNA (arrowhead). (G, H) Low power view showing evidence of human-specific synaptophysin expression (inset), with high power micrograph demonstrating a typical punctate pattern of staining. (I) Immune micrograph of human-specific GFAP expression at 20 weeks, consistent with astrocytic differentiation. Scale bar: A–F, H–I: 50 μm; G: 200 μm. Table illustrates the temporal timecourse of hES-NSC maturation over 20 weeks following hippocampal transplantation (−: <1%, +: <25%, ++: 25−50%, +++: 50−75%, ++++: >75% of hNA− cells).
However, several lines of evidence make this an unlikely possibility in this study; the combination of confocal analysis, expression of human-specific filamentous and nuclear proteins, and a correlation between in vitro and in vivo differentiation potential is consistent with graft-derived neural differentiation. Importantly, no teratomas were detected after transplantation. This is consistent with recent observations reporting no neoplastic transformation following transplantation of hES-NSCs (Tabar et al., 2005; Guillaume et al., 2006). However, there is conflicting data of teratoma development following transplantation of both undifferentiated hESCs and their neural derivatives (Muotri et al., 2005; Brederlau et al., 2006; Roy et al., 2006). The reasons for these differences are unclear; plausible explanations include the use of different hESC lines, neuralization protocols, and NSC propagation times.

The migratory capacity of hES-NSCs is a further consideration for clinical application. Following seeding onto organotypic slice cultures, hES-NSCs migrated within the hippocampal tissue and subsequently differentiated appropriately into glutamatergic neurons. After transplantation into the uninjured neonatal hippocampus, hNA⁺/DCX⁺ cells were found to have migrated not only to the granular layer of the dentate gyrus but also into regions rich in interneurons such as the molecular and hilar layers. This pattern is similar to that reported for human fetal-derived neural precursors (NPCs) (Englund et al., 2002b). Interestingly, the majority of hNA⁺ cells stained positive for DCX and as such are identified as migrating neuroblasts. Interestingly, there were no hNA⁺ cells migrating outside the core of the striatal transplant which could be due to the inherently glial nature of the quinolinic acid lesion (Watts and Dunnett, 1998). The relationship between host gliosis and the absence of migration is unclear. Reactive glia may provide the transplanted cells with a

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**Fig. 7** Accelerated neuroglial differentiation of hES-NSCs into the adult lesioned striatum. (A) Series of cresyl violet-stained brain sections 6 weeks after transplantation, demonstrating graft integration within the striatum. (B) Low power overview of hNA⁺ cells distributed throughout the graft site. (C–E) Low power micrographs demonstrating expression of (C) DCX, (D) NeuN and (E) GFAP co-expression within transplanted tissue counterstained with human nuclear antigen. High power micrographs demonstrating co-localization of hNA with (F) DCX, and (G) NeuN (arrowheads). (H) High power immune micrographs demonstrating GFAP expression by host (upper panel) and engrafted hNA⁺ (lower panel) cells. Scale bar: B: 500 μm; C–E: 100 μm; F–H: 25 μm.

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**Fig. 8** Astrocytic hES-NSC differentiation into the irradiated ethidium bromide lesioned spinal cord. (A) Phase micrograph of the lesion site demonstrating axonal demyelination and macrophage infiltration. (B) Low power immune micrograph of GFAP and hNA expression within the grafted lesion site. (C–E) Immune micrograph demonstrating co-localization of GFAP and hNA⁺ cells, consistent with astrocytic differentiation of transplanted hES-NSCs. (F–H) Confocal micrographs demonstrating co-localization of GFAP and hNA in engrafted cells. (I, J) Low and high power immune micrographs demonstrating BMP2/4 expression within the demyelinated lesion site (DC: dorsal column; DH: dorsal horn). Scale bar: A, C–J: 50 μm; B: 200 μm.
Extrinsic signals determine lineage choice in hES-NSCs

We observed consistently that neuroglial lineage commitment of hES-NSCs is a function of both intrinsic temporal determinants and the external micro-environment. The observed temporal determination of differentiation with neurogenesis preceding gliogenesis is consistent with previous studies on mouse and human ESC-derived NSCs (Ibiskyson et al., 2005; Guillaume et al., 2006; Bouhon et al., 2006). The in vitro timing of appearance of glia in this study parallels that observed in normal human development, with astrocytes and oligodendrocytes initially emerging in small numbers from mid to late first trimester. Our initial in vitro studies suggest that, in the absence of extrinsic signals, multipotent hES-NSCs default largely to a neuronal fate. Addition of neurotrophins results in an increased proportion of neurons, an observation likely to be due to selective survival (Caldwell et al., 2001). However, treatment with BMP in vitro causes a marked fate switch to an astroglial phenotype. This is consistent with the known in vitro and effect of BMP signalling on neural stem cells (Gross et al., 1996; Rajan and McKay, 1998; Mabie et al., 1999; Rajan et al., 2003). Therefore, it is significant that in the pathological lesions, the same population of neural stem cells were able to differentiate into astrocytes. This was particularly pronounced in the irradiated demyelinated spinal cord, where phenotypic differentiation was excluded confined to the astrocytic lineage. Injury-induced upregulation of BMP, a well recognized finding, offers one explanation (Setoguchi et al., 2001, 2004). Indirect support for this is provided by the presence of BMP2/4 within the irradiated spinal cord lesion site. The absence of human oligodendrocytes within the X-EB lesion is consistent with previous observations that multipotent NPCs require ex vivo oligodendrocyte lineage commitment prior to transplantation (Smith and Blakemore, 2000; Chandran et al., 2004).

In contrast, very low numbers of astrocytes were detected in the uninjured hippocampal environment in vivo. This is not altogether surprising, given that the hippocampus is an established neurogenic region (Song et al., 2002), and the result is consistent with the predominantly neuronal differentiation that was observed within the hippocampal slices. Nonetheless, astrocytic differentiation in slices was observed in restricted superficial areas that correspond to astrocytic regions (Del Rio et al., 1991; Scheffler et al., 2003). These observations suggest site-specific differentiation of NSCs in response to the local micro-environment (Gage et al., 1995; Suhonen et al., 1996; Shin et al., 2000; Harkany et al., 2004; Guillaume et al., 2006). Further support for this interpretation is provided by the almost exclusive polarization of neuronal subtype specification to an appropriate glutamatergic phenotype found in organotypic hippocampal slice cultures. Analysis at later timepoints would be necessary to further define neuronal behaviour, as reported in previous studies using mouse ESC neural derivatives (Benninger et al., 2003). By comparison, under controlled in vitro conditions, both glutamatergic and GABAergic differentiation were present in approximately equal proportions.

Temporal maturation of hES-NSCs is dependent on the in vivo environment

Clinical application of neural stem cell population requires, in addition to appropriate neuroglial specification, functional maturation. Both explant and transplant data suggest a graded, environmentally-dependent, maturational delay compared to in vitro differentiation. Explant cultures demonstrated persistence of immature (DCX) markers at timepoints where no DCX is evident in vitro. Furthermore, there was a marked delay in neuronal maturation in the uninjured brain, even at the latest timepoint of 20 weeks. This was in marked contrast to in vitro acquisition of mature neuronal markers which was evident by 2 weeks, along with evidence for electrophysiological function by 4 weeks, the latter finding is consistent with a recent in vitro report of neuronal maturation from adult human CNS-derived NPCs (Moe et al., 2005). In support of delayed in vivo maturation, human fetal-derived NPCs have been shown to differentiate into neurofilament-expressing cells only at 20 weeks following neonatal hippocampal transplantation (Englund et al., 2002b). Furthermore, an additional study reports the persistence of large numbers of human fetal-derived NPCs in an immature state at 14 weeks post transplantation into the intact adult striatum (Englund et al., 2002a). In contrast, we report the presence of mature neurons in the injured brain by 6 weeks following transplantation. The reasons for these findings are unclear. One interpretation is the dynamic interplay between injury-mediated maturation signals, and those maintaining an immature neuroblast phenotype that are neurogenic and niche-derived (Lie et al., 2005). In addition, cell loss is recognized to promote neurogenesis and maturation (Magavi et al., 2000). Alternatively, this difference may be accounted for by the fact that the quinolinic acid lesioned environment is known to support cell survival of primary striatal grafts to a greater extent that its non-lesioned counterpart, and in so doing may enhance their maturity (Watts and Dunnett, 1998; Bresjanac and Antauer, 2000).

A comparable in vivo delay to that seen with neuronal differentiation was also observed with respect to astroglial differentiation. GFAP+ astrocytes were evident in vitro and in hippocampal slices by 2 weeks. In contrast, hGFAP+ cells were only detectable at 20 weeks following neonatal hippocampal transplantation, comparable with earlier reports using human fetal-derived NPCs (Englund et al., 2002b). Conversely, injury resulted in the emergence of graft-derived astrocytes by 6 weeks. In addition to injury, different host developmental stages offer a further explanation for this finding. However, it has been previously described that in the intact adult brain, small numbers of human fetal NPC-derived
astrocytes were detected at 14 weeks following transplantation (Englund et al., 2002a).

Conclusions

This study reports the differential behaviour of clinically compatible NSCs derived from hESCs in a range of experimental contexts relevant to clinical application. Our findings highlight the need for caution when extrapolating in vitro findings with respect both to fate and timing of maturation. Furthermore, it is clear that the in vivo environment exerts distinct effects consequent on developmental stage, region and pathological state. Further understanding of the effect of environmental influences on specification, migration and maturation of transplanted NSCs will inform the design of future pre-clinical studies. For example, the marked upregulation of graft astrogliosis in spinal cord demyelination suggests a role for BMP antagonism (Setoguchi et al., 2004) along with ex vivo oligodendrocyte lineage commitment of NSCs prior to transplantation (Smith and Blakemore, 2000) in order to enable exogenous myelin repair. Thus, the ability to derive potentially clinical grade neural stem cells that exhibit function and appropriately respond to environmental signals provides grounds for cautious optimism in the therapeutic application of hESCs in neurological disease.

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