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In Vitro Characterization of a Human Neural Progenitor Cell Coexpressing SSEA4 and CD133

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The stage-specific embryonic antigen 4 (SSEA4) is commonly used as a cell surface marker to identify the pluripotent human embryonic stem (ES) cells. Immunohistochemistry on human embryonic central nervous system revealed that SSEA4 is detectable in the early neuroepithelium, and its expression decreases as development proceeds. Flow cytometry analysis of forebrain-derived cells demonstrated that the SSEA4-expressing cells are enriched in the neural stem/progenitor cell fraction ($CD133^+$), but are rarely codetected with the neural stem cell (NSC) marker CD15. Using a sphere-forming assay, we showed that both subfractions $CD133^+/SSEA4^+$ and $CD133^+/CD15^+$ isolated from the embryonic forebrain are enriched in neurosphere-initiating cells. In addition CD133, SSEA4, and CD15 expression is sustained in the expanded neurosphere cells and also mark subfractions of neurosphere-initiating cells. Therefore, we propose that SSEA4 associated with CD133 can be used for both the positive selection and the enrichment of neural stem/progenitor cells from human embryonic forebrain.

Key words: forebrain; flow cytometry; neurosphere; sphere-forming assay

Neural stem cells (NSC) have been the focus of much research, not only for understanding their role in building the nervous system but also for their prospective use in cell replacement therapy of neurodegenerative disease (Bjorklund and Lindvall, 2000). Despite their therapeutic value, the lack of universal NSC surface markers constitutes the major limitation for their identification and isolation via fluorescence-activated cell sorting (FACS). To date, only one cell surface marker, CD133, has been used for the positive selection of neural stem and progenitor cells from the central nervous system (CNS) of human fetuses (Uchida et al., 2000). In contrast, several cell surface markers have been used to enrich for NSCs in the rodent CNS. Notch1, peanut-agglutinin, and CD15 (also named LewisX/SSEA1) have been used to enrich for NSCs from the adult mouse CNS (Johansson et al., 1999; Rietze et al., 2001; Capela and Temple, 2002), whereas β1 integrin was used to isolate the NSCs from neonatal mice and rats (Campos et al., 2004; Leone et al., 2005). Notch1 and syndecan-1 have been used for positive selection of NSCs from embryonic mice (Nagato et al., 2005). Finally, negative selection strategies have been also developed as an alternative method to enrich for NSCs from both adult and developing CNS in rodents (Rietze et al., 2001; Maric et al., 2003). Although rapid progression has been made recently in the discovery of NSC markers in rodents, very few human-specific NSC markers have been identified.

Several genes that are expressed by embryonic stem (ES) cells are known to be sustained during CNS development, including the transcription factor Sox2 (Zappone et al., 2000; Ferri et al., 2004), the ABC transporter Bcrp1/ABCG2 (Hulspas et al., 1997; Zhou et al., 2001; Kim and Morshead, 2003), and the fibroblast growth factor (FGF) receptor 4 (Cai et al., 2002; Limke et al., 2003; Ginis et al., 2004).

The stage-specific embryonic antigen 4 (SSEA4) is a glycolipid antigen with globo-series carbohydrate core structures (Kannagi et al., 1983). SSEA4, but not SSEA1 (also named CD15), is expressed by undifferentiated human ES cells and teratocarcinoma stem cells, and it is down-regulated while SSEA1 is up-regulated during their differentiation (Solter and Knowles, 1979; Henderson et al., 2002). In humans, SSEA4 is expressed by nonneural cells such as the erythrocytes (Kannagi et al., 1983) and the multipotent progenitor cells from fetal

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liver (Dan et al., 2006). SSEA4 is also expressed by neural cells; it has been used to identify a subset of dorsal root ganglion sensory neurons from both rat and adult human (Jessell and Dodd, 1985; Holford et al., 1994) and a subset of immature neural cells from the human forebrain and spinal cord and from the forebrain- and spinal cord-derived neurosphere cells (Piao et al., 2006).

In the present study, we show that SSEA4 is expressed in proliferative areas of the developing brain but is rarely codetected with the neural stem cell marker CD15. Using flow cytometry, we also show that SSEA4 is associated with the neural stem/progenitor marker CD133 and that SSEA4 is enriched in the CD133-positive subset of neuroepithelial cells known to contain most neurosphere-initiating cells (Uchida et al., 2000). These results suggest that SSEA4, in combination with CD133, can be used for identification and positive selection of neural stem/progenitor cell from both developing forebrain and expanded as neurosphere cultures.

MATERIALS AND METHODS

Human Tissue Dissection

Human embryos used for in vitro studies and flow cytometry were obtained from elective abortion with consent given from the mother. Tissue collection was acquired under compliance with Swedish government guidelines and by approval of the Lund University Research Ethical Committee and the Swedish National Board of Health and Welfare. Eight human embryos aged from week 7 to week 9 of development [age estimated from crown-rump length (CRL)] were used for this study. The forebrains (telencephalic vesicles and the rostral half of the diencephalons) were dissected out after removal of the covering ectoderm and meninges. Two choroid plexi were taken as control tissue.

Cell Preparation for FACS

The tissues were mechanically dissociated and the neurospheres were dissociated with Accutase (Sigma, St. Louis, MO). The cells were resuspended in a phosphate-buffered saline (PBS) containing 2 mM EDTA, 0.5% bovine serum albumin, and were incubated for 30 min on ice with the monoclonal anti-SSEA4 (Chemicon, Temecula, CA). Then, the cells were incubated for 30 min on ice with the fluorescein isothiocyanate (FITC)-conjugated anti-mouse (Jackson Immunoresearch, West Grove, PA) and for 10 min on ice with the allophycocyanin (APC)-conjugated anti-CD34 (CD34-APC; BD Biosciences. San Jose, CA), the anti-CD45-FITC (BD Biosciences), CD133/1-APC (Miltenyi), and R-phycoerythrin (PE)-CD15 (CD15-PE; BD Biosciences). As negative control, some cells were incubated only with the secondary antibody (FITC-conjugated anti-mouse). The samples were filtrated through a 70-μm filter cup (Falcon, Oxnard, CA) to eliminate cell aggregates, and 7-amino-actinomycin D (7AAD; Sigma) was added to label the dead cells. Cell sorting was performed on a FACS Vantage TS flow cytometer (Becton Dickinson, San Jose, CA).

Neurosphere-Forming Assay

Neurosphere-forming assays were performed from five freshly dissected forebrains (two from 7/7.5-, one from 8.5-, and two from 9-week-old embryos) and from bulk neurosphere cultures at passages 2 and 3, generated from an 8-week-old forebrain. Cells were sorted at a density of 10 cells/μl (clonal density; see Barraud et al., 2005) with an automated cell deposition unit device (Becton Dickinson) into a 96-well plate containing growth medium. The growth medium is composed of a defined DMEM/F12-based medium (Gibco Life Technologies, Grand Island, NY) containing 0.6% glucose, 25 μg/ml human insulin, 100 μg/ml human transferrin, 20 nM progesterone, 60 μM putresceine, 30 nM selenium chloride, 2 nM glutamine, 3 mM sodium bicarbonate, 5 mM HEPES, and 2 μg/ml heparin (Sigma), supplemented with 20 ng/ml epidermal growth factor (EGF), 20 ng/ml basic fibroblast growth factor (bFGF), and 10 ng/ml leukemia inhibitory factor (LIF; all are human recombinant factors from R&D Systems, Minneapolis, MN). Cultures were maintained at 37°C in a humid atmosphere with 95% oxygen and 5% CO_2, and cells were fed every third day by adding fresh medium. After 7 days in vitro, the number of single-cell-generated spheres was counted in each well containing 1,000 cells. For in vitro differentiation, 10 day-old spheres were plated onto poly-L-lysine (PLL)-coated chamber slides in a medium in which growth factors were replaced by 1% fetal bovine serum. After 7 days in vitro, cells were fixed for 15 min with 4% paraformaldehyde (PFA) at room temperature (RT), rinsed three times in PBS, and processed for immunocytochemistry.

Immunocytochemistry

Cultures were preincubated in 5% normal serum and 0.025% Triton-X in potassium-buffered PBS (KPBS) for 1 hr and overnight at RT with the primary antibodies: mouse monoclonal anti-2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNPase; 1:100; Sigma), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; 1:1,000; Dako, Carpinteria, CA), and rabbit polyclonal anti-βIII-tubulin (1:1,000; Covance). After three rinses in KPBS, cultures were incubated with FITC- or cyanin 3 (Cy3)-conjugated secondary antibodies to mouse and rabbit IgG (1:200; Jackson Immunoresearch) for 2 hr at RT. Cultures were mounted in PVA-DABCO.

Immunohistochemistry of Intact Paraffin-Embedded Embryonic Forebrain

The specimens used for immunohistochemistry were obtained according to Danish guidelines. Informed consent from women undergoing elective abortions was obtained according to the guidelines of the Helsinki Declaration II. The specimens were fixed for 12–24 hr at 4°C in one of the following fixatives: 10% buffered formalin, 4% formol-calcium, Lillie's AAF, or Bouin's fixatives. The specimens were dehydrated with graded alcohols, cleared in xylene, and embedded in paraffin wax (Merck). Serial sections 3–5 μm thick were cut in frontal or horizontal planes and placed on silanized slides. The paraffin sections were dewaxed, rehydrated, and washed in Tris-buffered saline (TBS; 0.05 M Tris, 0.15 M NaCl, pH 7.6) with 0.01% Nonidet P-40 (TBS/Nonidet).

Antigen retrieval was performed in a microwave oven with Tris-EGTA buffer (0.01 M Tris base + 5 mM EGTA, pH 9) and a boiling time of 10 min. After heat treatment, the sections rested for 20 min at RT. They were incubated in 0.45% H_2O_2 in TBS/Nonidet for 15 min to block endogenous peroxidase activity and then in 10% normal goat serum in TBS/Nonidet for 30 min at RT to block nonspecific binding. Sections were incubated overnight at 4°C with the monoclonal mouse anti-SSEA4 (1:100; Chemicon) or the monoclonal mouse anti-human CD15 (1:150; BD Pharmingen) diluted in 10% goat serum. The primary antibodies were detected by using the peroxidase revelation method (DakoCytomation EnVision + DualLink System), used at the manufacturer's recommendation. As negative controls, sections were incubated with only the secondary antibody.

Bulk Neurosphere Cultures

The neurosphere cultures were generated from the forebrain of an 8-week-old (50-day-old) human embryo. The forebrain was mechanically dissociated into a single–cell suspension. The cells were plated at a density 2.5×10^5 cells/ml in a serum-free medium (see composition above). After 10 days, the neurospheres were dissociated (passage) using Accutase (Sigma) for a single-cell suspension and then resuspended at density 10^5 cells/ml. The neurosphere cultures used in this study have been passaged two or three times.

RESULTS

SSEA4 Is Expressed During Human CNS Development

SSEA4 expression was analyzed by immunohistochemistry on sections through the CNS of 6–9-week-old human embryos. The data are summarized in Table I. At the sixth week of development (37 days postconception), most neuroepithelial cells in the midline and the medial regions of the forebrain strongly express SSEA4. It is localized in both apical and basolateral cell membranes and also in the cytoplasm (Fig. 1D). At the seventh week, SSEA4 becomes restricted to the roofplate of the rhombencephalon, the dorsal midline of the mesencephalon, the ventral midline of the diencephalon, the medial telencephalic wall, and the lamina terminalis (Fig. 1A,C). The SSEA4 reactivity continues along the ventral raphe in the floorplate of the brainstem (Fig. 1A). In the lateral telencephalon, a weak SSEA4 staining is confined to the apical and basal cell membranes at the pial surface (Fig. 1G). At the end of week 7 (49 days postconception), SSEA4 is localized in the ventricular (VZ) and the subventricular zones (SVZ) in the telencephalon, particularly in the septum and the medial telencephalic wall (Fig. 1G). At week 9, no evident SSEA4 staining appears in the VZ of the pallium, whereas a strong SSEA4 staining is present in the cortical plate/marginal zone region (Fig. 1B,H). The VZ in the dorsolateral telencephalic wall appears only weakly immunopositive for CD15 (Fig. 1I). In addition, many choroid plexus (CP) epithelial cells start to express SSEA4 and to a lesser extent CD15 (Fig. 1E,F, respectively).

TABLE I. Distribution of SSEA4 in the Early Developing Human Forebrain⋆

Region	6 Weeks	7 Weeks	8 And 9 weeks
Telencephalon			
Dorsolateral wall			
Ventricular zone	++	–	–
Cortical plate/marginal zone	n.p.	++	++
Medial wall			
Ventricular zone	++	++	+
Cortical plate/marginal zone	n.p.	+++	++
Ganglionic eminence			
Ventricular zone	++	–	–
Subventricular zone	++	+	+
Diencephalon			
Lamina terminalis	+++	++	+
Thalamus	++	–	–
Hypothalamus (median eminence)	+	+	+
Choroid plexus			
Lateral ventricle	n.p.	+ to ++	+++
Third ventricle	n.p.	n.p.	++

⋆Level of SSEA4, immunoreactivity; +++, intense; ++, moderate; +, week; –, not detected, n.p., not present.

According to these data, the human ES marker SSEA4 is expressed during CNS development and is present at higher levels of expression during the early stages. To estimate the proportion of SSEA4-expressing cells during forebrain development, we labeled with the anti-SSEA4 antibody the forebrain cells isolated from three developmental stages: 7/7.5 weeks (n = 2 forebrains analyzed), 8/8.5 weeks (n = 3), and 9 weeks (n = 3). These cells were then analyzed via FACS. At 7/7.5 week, 7.3% ± 0.5% of all forebrain cells were $SSEA4^+$, and only 3.2% ± 1.3% and 1.6% ± 0.9% expressed SSEA4 at 8/8.5 and 9 weeks, respectively.

The SSEA4-Expressing Cells Are Enriched in the Neural Stem and Progenitor Cell Fraction

The CD133 and the CD15 markers have been used to isolate the neural stem/progenitor cells from the human embryonic forebrain and the adult mouse SVZ, respectively (Uchida et al., 2000; Capela and Temple, 2002). To characterize the SSEA4-expressing cells further, we labeled the forebrain cells with the CD133, SSEA4, and CD15 antibodies for FACS analysis. We checked possible contamination with blood and endothelial cells by staining a cell sample with the anti-CD45 and the anti-CD34, respectively. Under 0.1% of the live cells stained for these markers (data not shown), suggesting a low contamination with nonneural cells. FACS analysis was acquired on four acquisition dot plots for the forward scatter (FSC) and side scatter (SSC); for the dead ($7AAD^+$) and live ($7AAD^-$) cells; and for APC, FITC, and PE fluorescence. No nonspecific labeling with the secondary antibody mouse anti-IgG-FITC was observed (Fig. 2B). After gating out on high and low FSC/SSC corresponding to cell aggregates and cell de-

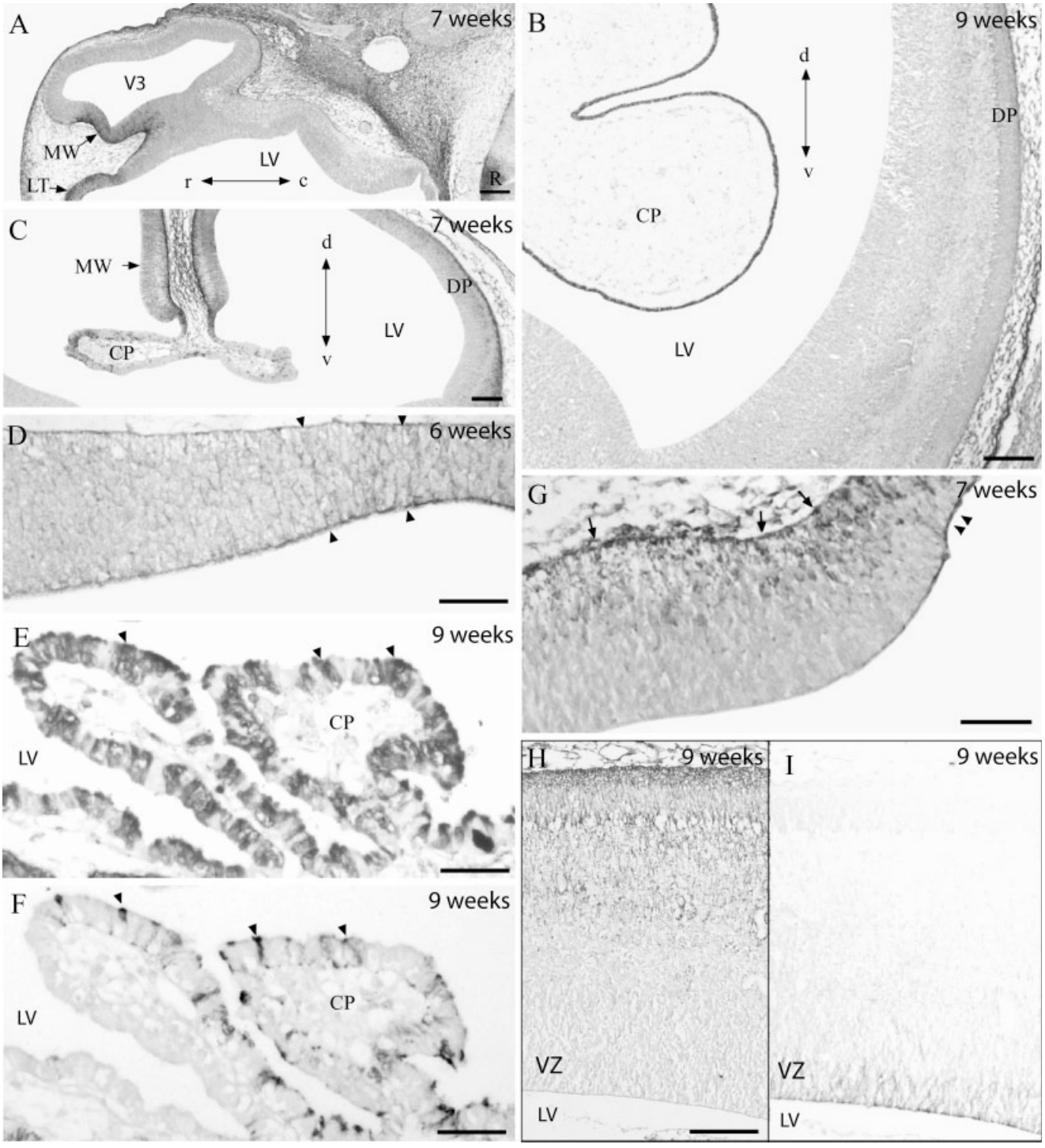

Fig. 1. SSEA4 and CD15 expression during human forebrain development. Low-power micrographs of human embryo sections at weeks 7 (44 days postconception in **A**, 49 days postconception in **C**) and 9 (57 days postconception in **B**) of development immunolabeled for SSEA4, demonstrating the regional distribution. High-power micrographs of human embryo sections at weeks 6 (37 days postconception in **D**), 7 (44 days postconception in **G**), and 9 (57 days postconception in **E,F,H,I**) of development, showing the cellular distribution of SSEA4 in D,E,G,H, and CD15 in F,I. MW, medial telencephalic wall; LT, lamina terminalis; CP, choroid plexus; R, raphe; V3, third ventricle; LV, lateral ventricle; VZ, ventricular zone. The rostrocaudal (r-c) axis is indicated in the horizontal section (A), and the dorsoventral (d-v) axis is shown in the frontal sections (B,C). Note the decreasing staining in the dorsal pallium (DP) from the end of week 7 to week 9 (C and B, respectively). The arrowheads in D and G show reactivity in neuroepithelial cells from ventricular to pial surface (D), at the medial-most ventricular surface (G) in contrast to the pial surface, which is strongly stained (arrows). Strongly stained epithelial cells in the choroid plexus are indicated by arrowheads (E,F). Scale bars = 200 μm in A–C; 25 μm in D–I.

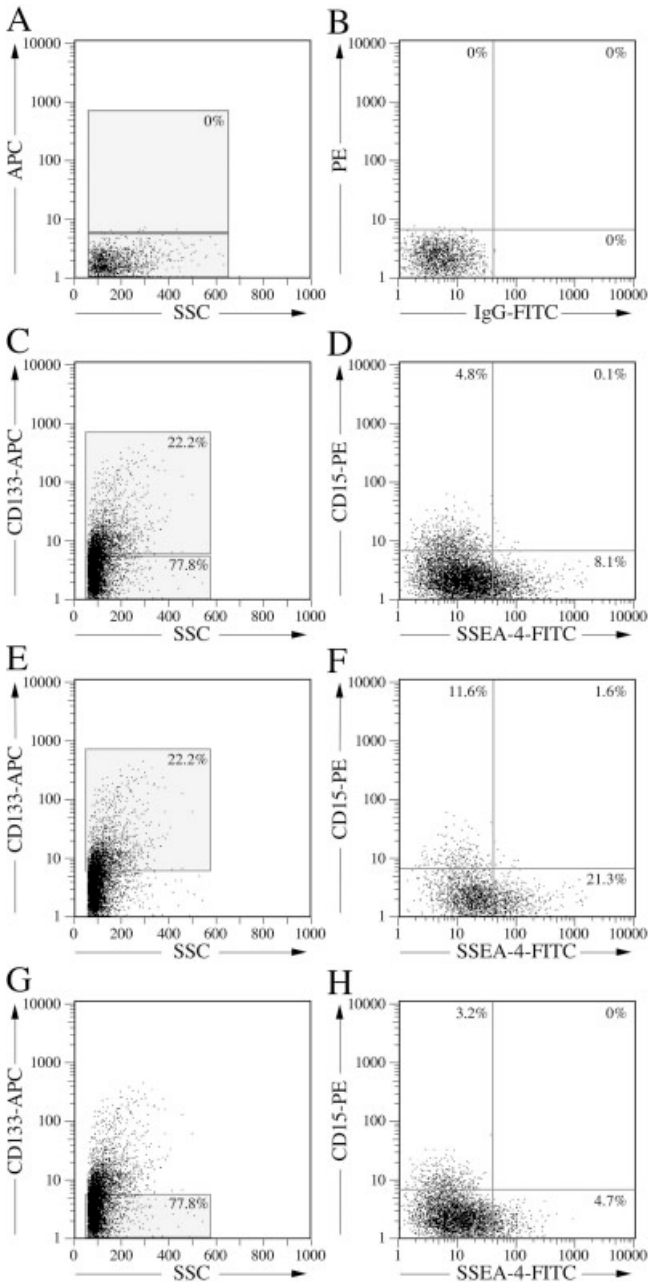

Fig. 2. Flow cytometry analysis of human embryonic forebrain-derived cells to analyze the expression of CD133, CD15, and SSEA4 at the seventh week of development. Some cells were incubated with the secondary antibody only (mouse anti-IgG-FITC) used as a control to show the absence of nonspecific labeling with the human forebrain-derived cells (**A,B**). FACS dot plots showing all live (7AAD$^-$) cells expressing CD133 (**C**), CD15, and SSEA4 (**D**). The cells were gated on the CD133$^+$ subfraction (**E**) and the CD133$^-$ cells (**G**) and were analyzed for the coexpression of SSEA4 and CD15 (**F,H**).

bris, respectively, and then the dead cells (7AAD$^+$), we analyzed live cells (7AAD$^-$) for their CD133, CD15, and SSEA4 expression (Fig. 2). At the seventh week of development 22.2% of all live cells were CD133$^+$ (Fig. 2C), 8.2% were SSEA4$^+$, and 4.9% were CD15$^+$ (Fig. 2D). In addition 22.9% of the CD133$^+$ cells were SSEA4$^+$ and 13.2% were CD15$^+$ (Fig. 2F). However, only 4.7% of the CD133$^-$ cells express SSEA4 and 3.2%, CD15 (Fig. 2H). A small subset of cells representing 1.6% of the CD133$^+$ cells (under 0.1% of all live cells) express all three markers, CD133, CD15, and SSEA4 (Fig. 2F). With regard to this last subset (CD133$^+$/SSEA4$^+$/CD15$^+$), it is interesting to note that the intensity of fluorescence for SSEA4 and CD15, which reflect the level of expression of the cell surface antigens, is very low in comparison with the intensity of fluorescence of the CD133$^+$/SSEA4$^+$/CD15$^-$ and CD133$^+$/SSEA4$^-$/CD15$^+$ subsets (Fig. 2D,F).

SSEA4 and CD15 are both enriched in the neural stem and progenitor subset of CD133$^+$ cells (representing approximately a threefold enrichment in both subpopulations in comparison with the control). Interestingly, SSEA4 and CD15 marked two distinct cell subpopulations in the neural stem and progenitor cell fraction (CD133$^+$).

SSEA4 in Association With CD133 Allows Enrichment for Neural Progenitor Cells

The neurosphere-forming assay has been the most commonly used in vitro assays to study the clonogenic property of the putative NSCs (Hulspas et al., 1997; Reynolds and Weiss, 1996). To test for this property, we labeled the forebrain-derived cells (two forebrains from 7/7.5-, one forebrain from 8.5-, and two forebrains from 9-week-old embryos) with all three antibodies (CD133, SSEA4, and CD15) and isolated subpopulations by FACS. It has previously been shown that virtually all the neurosphere-forming cells are found in the CD133$^+$ subpopulation (Uchida et al., 2000). Thus, SSEA4$^+$ and CD15$^+$ cells were isolated from the CD133$^+$ subpopulation. Four subpopulations were isolated from the CD133$^+$ subset (Fig. 3G): CD133$^+$/CD15$^+$/SSEA4$^+$ (representing under 0.1% of all live cells), CD133$^+$/CD15$^+$/SSEA4$^-$ (2.6% of all live cells), CD133$^+$/CD15$^-$/SSEA4$^+$ (4.7% of all live cells), and CD133$^+$/CD15$^-$/SSEA4$^-$ (14.5% of all live cells), and one subpopulation was isolated from the CD133$^-$ subset: CD133$^-$/CD15$^-$/SSEA4$^-$ (71.6% of all live cells). A control corresponding to all live cells was passed through the FACS and isolated. Cells were collected in 96-well plates at clonal density (Barraud et al. 2005). After 7 days in vitro, we counted the number of clones generated in each subpopulation (n = 5 individual experiments; Fig. 3C,D). Because of the very low number of CD133$^+$/CD15$^+$/SSEA4$^+$ cells in all live cells (less than 0.1%), we were unable to isolate enough cells to test for their clonogenic property. Clonal analysis of the other subpopulations revealed that the CD133$^+$/CD15$^-$/SSEA4$^+$ subpopulation contained the majority of neurosphere-initiating cells (1.6% ± 0.1%; see Fig. 3F). In addition, some neurosphere-initiating cells were found

in the $CD133^+/CD15^+/SSEA4^-$ subset (0.7% ± 0.07%; see Fig. 3F). However, very few spheres were generated by the $CD133^+/CD15^-/SSEA4^-$ and the $CD133^-/CD15^-/SSEA4^-$ subfractions (0.2% ± 0.04% and 0.04% ± 0.01%, respectively). In the control group, under 0.2% ± 0.06% of the live cells generated neurospheres. Thus, the number of spheres formed by the $CD133^+/CD15^-/SSEA4^+$ and the $CD133^+/CD15^+/SSEA4^-$ subsets represented an eightfold and a fourfold enrichment, respectively, compared with the control cells.

To demonstrate multipotency, individual spheres generated from each subpopulation were picked and plated under differentiation conditions (Fig. 3E). After 7 days in vitro, the $CD133^+/CD15^-/SSEA4^+$-derived spheres generated both astrocytes (GFAP-positive) and neurons (βIII-tubulin-positive), but no oligodendrocytes were identified with the anti-CNPase antibody. However, we could not follow the lineage differentiation of the spheres generated by $CD133^+/CD15^+/SSEA4^-$ subset because of limited adhesion of the spheres.

SSEA4 is also expressed by a population of CP epithelial cells, so we dissected the CP, dissociated into a single-cell suspension, and plated the cell at 10 and 100 cells/μl. After 7 days in vitro, very small CP-derived cell clusters were generated. However, their growth rate was extremely slow, and upon differentiation they gave rise to fibroblast-like cells ($GFAP^-$ data not shown). This control experiment confirms that the clones generated by the $SSEA4^+$ cells that we analyzed after FACS sorting of the forebrain-derived cells were not derived from the CP. Therefore, SSEA4 and CD15 allow the identification and the enrichment of the neurosphere-initiating cells in the $CD133^+$ subfraction of the human developing forebrain.

SSEA4 Expression is Maintained in the Neurosphere Cultures

The neurosphere cultures represent a standard protocol for the expansion of NSCs (Reynolds and Weiss, 1992, 1996; Carpenter et al., 1999). We generated neurosphere cultures from the whole forebrain of a 8-week-old (50 days) human embryo (Carpenter et al., 1999; Englund et al., 2002a,b). We labeled the neurosphere cells (two or three passages) with the three antibodies

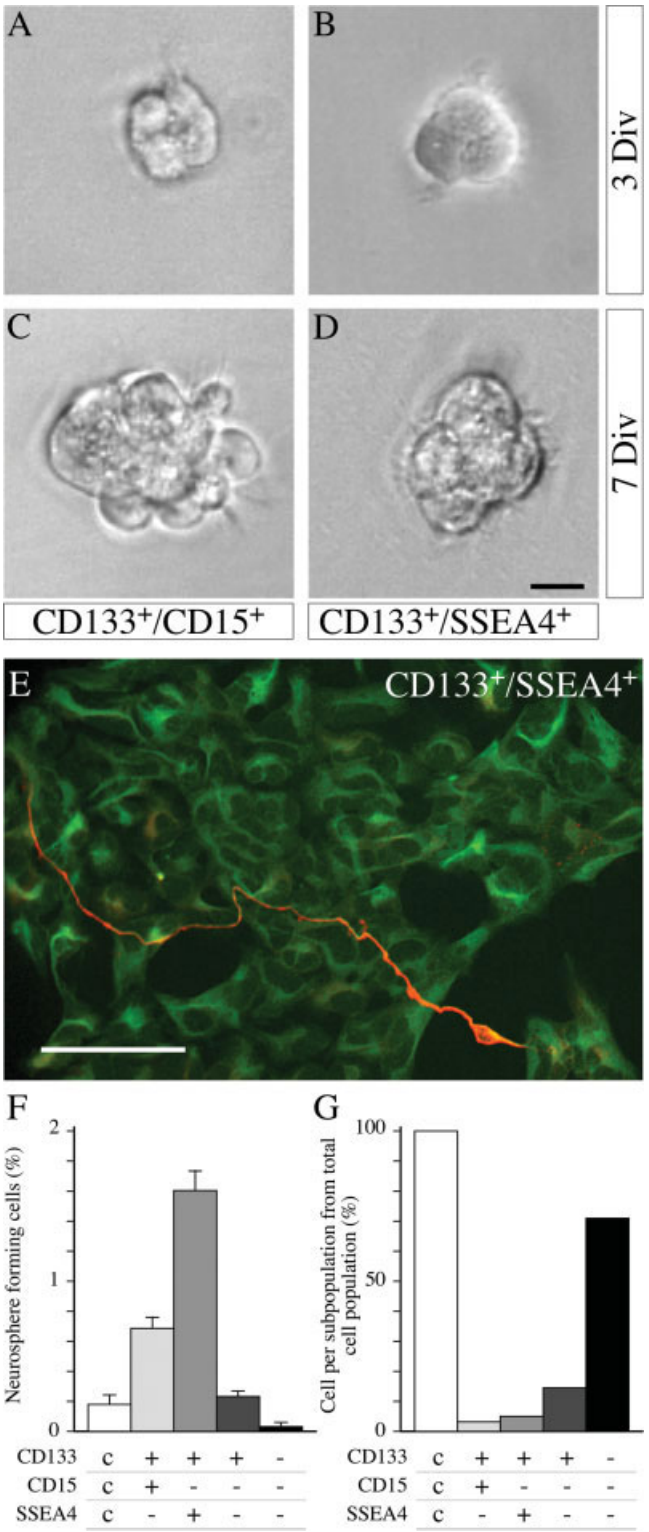

Fig. 3. Neurosphere-forming assay. **A–D:** Photomicrograph of the clones generated by the $CD133^+/CD15^+/SSEA4^-$ (**A,C**) and the $CD133^+/CD15^-/SSEA4^+$ (**B,D**) subpopulations 3 days (A,B) and 7 days (C,D) after FACS isolation. **E:** In vitro differentiation. The clones generated by the $CD133^+/CD15^-/SSEA4^+$ cells were transferred onto PLL-coated chamber slides and differentiated. Immunocytochemistry on differentiated spheres generated by the $CD133^+/CD15^-/SSEA4^+$ cells to identify neurons (βIII-tubulin, red) and astrocytes (GFAP; green). **F:** Histogram representing the neurosphere-forming assay. The cells were plated directly after FACS isolation into 96-well plates at clonal density (10 cells/μl). The bars represent the percentage of spheres generated per single sorted cell for each subpopulation after 7 days in vitro. **G:** Three-dimensional histogram showing the percentage of cells for each FACS-sorted populations in all live cells. Scale bars = 10 μm in D (applies to A–D); 50 μm in E. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

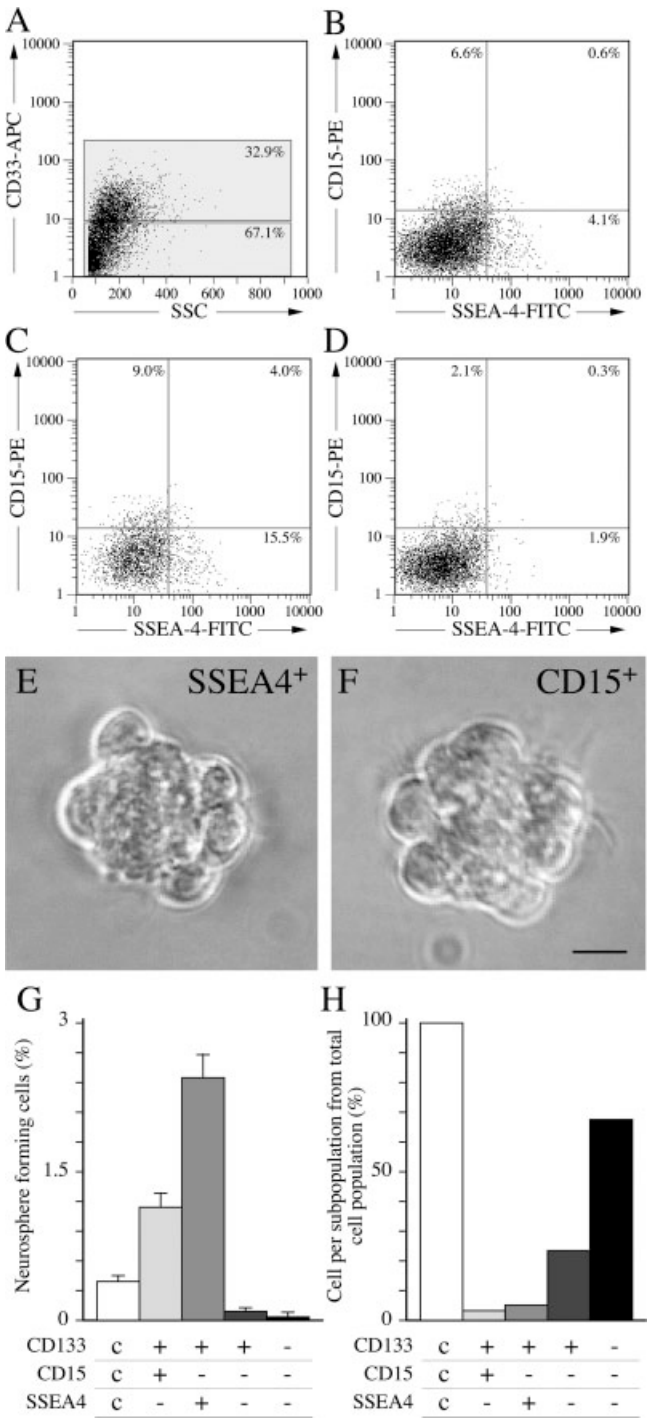

Fig. 4. Flow cytometry analysis of the human neurosphere cells generated from an 8-week-old human embryonic forebrain. **A–D:** Dot plots representing the CD133 expression in all live cells (A), and CD15/SSEA4-expression in all live cells (B), in the $CD133^+$ subpopulation (C) or in the $CD133^-$ subpopulation (D). **E,F:** Photomicrographs of the clones generated by the $CD133^+/CD15^-/SSEA4^+$ (E) and the $CD133^+/CD15^+/SSEA4^-$ (F) subpopulations 7 days after FACS isolation. **G:** Neurosphere-forming assay. The bars represent the percentage of spheres generated per single sorted cell for each subpopulation after 7 days in vitro. **H:** Three-dimensional histogram showing the percentage of cells for each subpopulation in all live cells. Scale bar = 50 μm.

against CD133, CD15, and SSEA4 and analyzed them by FACS. Two experiments were performed at passage 2, and one experiment was performed at passage 3. The percentages of $CD133^+$, $CD15^+$, and $SSEA4^+$ cells (in all live cells) were 32.9% (Fig. 4A), 7.2%, and 4.7% (Fig. 4B), respectively. In the $CD133^+$ subpopulation (Fig. 4C), 4% and 15.5% of the live cells were $CD133^+/CD15^+/SSEA4^+$ and $CD133^+/CD15^-/SSEA4^+$, respectively, representing a seven- and a fourfold enrichment, respectively, in the $CD133^+$ subpopulation in comparison with the control (Fig. 4B). We isolated subpopulations by FACS and plated the cells at a low density to test for their properties to form clones in a neurosphere-forming assay (Fig. 4E–G). In the $CD133^+/CD15^-/SSEA4^+$ subpopulation, which represented 5.1% of all live cells (Fig. 4H), 2.5% ± 0.2% of the cells generated spheres, whereas only 1.1% ± 0.1% of the $CD133^+/CD15^+/SSEA4^-$ cells (representing 2.9% of all live cells) generated spheres. These data demonstrate that the expressions of CD133, CD15, and SSEA4 are sustained by a subset of neurosphere cells and can also be used for the positive selection of the neurosphere-forming cells within the neurosphere cultures.

DISCUSSION

The globo-series glycolipid SSEA4 is expressed not only by undifferentiated human ES cells (Allendoerfer et al., 1995; Thomson et al., 1998; Henderson et al., 2002; Ginis et al., 2004) but also by a small subset of cells from the human fetal forebrain and the forebrain-derived neurosphere cells (Piao et al., 2006). Here, we first showed that the SSEA4 is expressed in the proliferative areas of the developing human brain (from week 6 to week 9 of development). Second, by using flow cytometry and sphere-forming assay, we demonstrated that 1) both SSEA4 and the NSC marker CD15 are enriched in the neural stem progenitor fraction ($CD133^+$), 2) SSEA4 and CD15 label distinct subsets of neural stem progenitor cells ($CD133^+$), and 3) SSEA4 allows for the enrichment of neurosphere-initiating cells within the neural stem progenitor cell subset ($CD133^+$), in that isolation of the $CD133^+/SSEA4^-/CD15^-$ cells generated few spheres at low density.

In a very recent study, immunocytochemistry on dissociated forebrain and spinal cord cells from first-trimester human fetuses has shown that SSEA4 is expressed by a small subset of immature neural cells (Piao et al., 2006). In the present study, we localized by immunohistochemistry the expression of SSEA4 through sections of human embryonic CNS from the sixth week to the ninth week of development. At week 6, SSEA4 is highly expressed in the neuroepithelium of the developing forebrain, with a strong expression at the apical and basal aspects. Interestingly most neuroepithelial cells at this developmental stage are immunopositive for the radial glial markers vimentin, the glutamate astrocyte-specific transporter (GLAST), and GFAP (Stagaard and Mollgard, 1989; Zecevic, 2004). At the seventh week, we found that

SSEA4 expression decreases and becomes restricted to the VZ cells, a developmental stage corresponding to a period when some cells in the developing pallium become immunopositive for some neuronal markers such as neurofilament and βIII-tubulin, in addition to the neuroepithelial marker nestin (Zecevic, 2004), although SSEA4 expression decreases in the VZ to levels undetectable by immunohistochemistry at week 9. Cells making up the CP are specialized cells in producing cerebrospinal fluid. They share the same origin as the ventricular ependymal cells (Spector and Johanson, 1989). Some human CP cells have been shown to be immunopositive for the marker vimentin (Kasper et al., 1986; Stagaard and Mollgard, 1989) and also neurofilaments (Kasper et al., 1986). A subset of CP cells in the postnatal rat brain expresses the neural stem and progenitor marker Musashi-1 in addition to vimentin (Itokazu et al., 2006). Therefore, it appears that some CP cells may share some common features with neural stem and progenitor cells. Interestingly, it has been reported that a very small fraction of CP cells (approximately 0.06–0.14% of the whole CP) isolated from the postnatal rat are expandable as free-floating spheres under bFGF and EGF stimulation (Itokazu et al., 2006). Upon differentiation, however, approximately 45% of the cultured cells generated non-neural cells, i.e., endothelial cells, fibroblasts, and macrophages (Itokazu et al., 2006). Although, we cannot exclude that our forebrain cell preparations were contaminated with some CP cells (even though efforts were made during the dissection to discard the CP), when we cultured the CP cells alone in the same manner as the forebrain cells, the CP cells formed very small cell clusters that grew very slowly and only gave rise to epithelial-like cells ($GFAP^-$) upon differentiation.

The neurosphere-forming assay has become a standard method either to expand under growth factor stimulation the neural stem and progenitor cells when plated at high density or to measure the stem cell frequency, i.e., the number of clones, when plated at low density or clonal density (for review see Reynolds and Rietze, 2005). In our laboratory, we found that use of lower cell densities than 10 cells/μl did not allow the human embryonic forebrain-derived cells to grow (unpublished data) like the mouse embryonic forebrain-derived cells (Barraud et al., 2005). However, the cell density 10 cells/μl was defined as the clonal density both for human and for mouse cells, a density that does not allow the cells to reaggregate.

The CD133 antibody has been used to select for most of the sphere-initiating cells from the human developing forebrain and therefore allows for the positive selection of most of the neural stem and precursor cells (Uchida et al., 2000). We found that SSEA4 and CD15 are both enriched in the neural stem and progenitor cell fraction ($CD133^+$), and SSEA4 and CD15 combined with CD133 allow for the isolation of most neurosphere-initiating cells not only from the primary embryonic forebrain tissue but also from the forebrain-derived neurosphere cultures. Interestingly, we found similar results from high-passage neurosphere cells (passage 27 times) established from a 9-week-old human forebrain, because the $CD133^+/SSEA4^+$ subset was 7 times enriched in sphere-initiating cells, whereas $CD133^+/SSEA4^-$ and $CD133^-/SSEA4^+$ were twofold enriched in sphere-initiating cells (P.B., unpublished data). Therefore, if the $CD133^+/SSEA4^+$ and the $CD133^+/CD15^+$ subpopulations from the forebrain and the forebrain-derived neurosphere cultures (at passage 2–3) are both enriched in sphere-initiating cells, these data tend to demonstrate that the neurosphere cultures are renewed from these two subsets of cells. However, although the $CD133^+/SSEA4^+$ subset gives rise to both glial and neuronal cells upon differentiation, the $CD133^+/CD15^+$ subpopulation remains uncharacterized for lineage differentiation potential because of its limited adhesion capacity onto a PLL substrate. Furthermore, this would suggest the presence of a heterogeneous population of neural progenitor cells that is $CD133^+$. The heterogeneity in these two subpopulations within the $CD133^+$ subpopulation has been demonstrated in this study based on their expression of either SSEA4 or CD15, insofar as SSEA4 or CD15 appears to label two distinct neural stem and progenitor cell subpopulations regardless of the age of the embryo (from week 7 to week 9 of development). However, we found that, upon differentiation of the spheres generated from a single $CD133^+/SSEA4^+$ cell, no oligodendrocytes were identified (using an antibody against CNPase). It has been shown elsewhere that oligodendrocytes are rarely generated from the differentiation of the primary as well as from neurosphere cultures derived from human fetal CNS tissue (Carpenter et al., 1999; Uchida et al., 2000; Barami et al., 2001). The authors of those studies suspected that either the antibodies failed to recognize human oligodendrocytes or the neural stem and progenitor cells do not differentiate toward this lineage under these differentiation conditions using 1% serum.

The exact role of SSEA4 and also CD15 during development remains unknown. Because SSEA4 and CD15 belong to the globo-series cell surface molecules associated with proteoglycans, we could suspect that both cell surface epitopes may play a role in modifying the nature of the extracellular environment, influencing cell migration and interactions with other cells or with the extracellular matrix. For example, the proteoglycans can bind to several key molecules, such as bFGF (Rapraeger et al., 1991; Yayon et al., 1991) and the neural cell adhesion molecule NCAM (Reyes et al., 1990).

In summary, the results show that the human ES cell marker SSEA4 is expressed in the developing human CNS and that the $SSEA4^+$ cells are enriched in the $CD133^+$ subfraction, which is known to contain the neurosphere-initiating cells. By using these two cell surface markers in combination, we have been able to enrich the proportion of neurosphere-initiating cells, within the $CD133^+$ subfraction, both from human embryonic forebrain tissue and from forebrain-derived neurospheres. The prospective isolation of NSCs provides a

tool for studies of the genetic and epigenetic events that drive the NSCs toward a specific cell fate.

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