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Genetic selection of sox1GFP-expressing neural precursors removes residual tumorigenic pluripotent stem cells and attenuates tumor formation after transplantation

S. Chung[*,†], **B.-S. Shin**[†], **E. Hedlund**[*,†,‡], **J. Pruszak**[*,‡], **A. Ferree**[*,‡], **Un Jung Kang**[§], **Ole Isacson**[*,‡], and **Kwang-Soo Kim**[*,†]

[*]*Udall Parkinson's Disease Research Center of Excellence, McLean Hospital, Belmont, MA, USA*

[†]*Molecular Neurobiology Laboratories, Harvard Medical School, Belmont, MA, USA*

[‡]*Neuroregeneration Laboratories, McLean Hospital/Harvard Medical School, Belmont, Massachusetts, USA*

[§]*Department of Neurology, The University of Chicago, Chicago, Illinois, USA*

Abstract

Because of their ability to proliferate and to differentiate into diverse cell types, embryonic stem (ES) cells are a potential source of cells for transplantation therapy of various diseases, including Parkinson's disease. A critical issue for this potential therapy is the elimination of undifferentiated cells that, even in low numbers, could result in teratoma formation in the host brain. We hypothesize that an efficient solution would consist of purifying the desired cell types, such as neural precursors, prior to transplantation. To test this hypothesis, we differentiated sox1-green fluorescent protein (GFP) knock-in ES cells *in vitro*, purified neural precursor cells by fluorescence-activated cell sorting (FACS), and characterized the purified cells *in vitro* as well as *in vivo*. Immunocytofluorescence and RT-PCR analyses showed that this genetic purification procedure efficiently removed undifferentiated pluripotent stem cells. Furthermore, when differentiated into mature neurons *in vitro*, the purified GFP^+ cell population generated enriched neuronal populations, whereas the GFP^- population generated much fewer neurons. When treated with dopaminergic inducing signals such as sonic hedgehog (SHH) and fibroblast growth factor-8 (FGF8), FACS-purified neural precursor cells responded to these molecules and generated dopaminergic neurons as well as other neural subtypes. When transplanted, the GFP^+ cell population generated well contained grafts containing dopaminergic neurons, whereas the GFP^- population generated significantly larger grafts (about 20-fold) and frequent tumor-related deaths in the transplanted animals. Taken together, our results demonstrate that genetic purification of neural precursor cells using FACS isolation can effectively remove unwanted proliferating cell types and avoid tumor formation after transplantation.

Keywords

embryonic stem cell; fluorescence-activated cell sorting; neural precursors; sox1; transplantation

Embryonic stem (ES) cells are derived from the inner cell mass of pre-implantation mouse embryos (Evans and Kaufman 1981; Martin 1981) and represent pluripotent cells that can give rise to most cell types (Nagy *et al*. 1990, 1993). ES cells can be permanently maintained *in vitro* as pluripotent cells and, upon exposure to appropriate differentiation signals, can

Address correspondence and reprint requests to Kwang-Soo Kim, Ph.D., MRC 216, McLean Hospital, Harvard Medical School, 115 Mill Street, Belmont, MA 02178, USA. E-mail: kskim@mclean.harvard.edu.

differentiate into a vast range of cell lineages (Smith 1991; Desbaillets *et al*. 2000). These unique properties make ES cells a useful tool for analyzing critical steps of cell development using both animal models and *in vitro* differentiation culture systems (Hooper *et al*. 1987; Thomas and Capecchi 1987; Nagy *et al*. 1990, 1993; Dinsmore *et al*. 1996; Wutz and Jaenisch 2000). In addition, the capacity of ES cells to generate terminally-differentiated cell types provides a potentially unlimited resource for cell replacement therapy (Dinsmore *et al*. 1996; Brustle *et al*. 1999; Lumelsky *et al*. 2001; Bjorklund *et al*. 2002).

We have previously shown that *in vivo* grafting of mouse ES (mES) cells could ameliorate behavioral deficits in rodent models of Parkinson's disease (PD) by generating mature dopaminergic (DA) neurons in the brain (Bjorklund *et al*. 2002). In addition, we and others have shown that mature DA neurons can be efficiently generated *in vitro* by genetic modification of mES cells with the transcription factor, Nurr1 (Chung *et al*. 2002; Kim *et al*. 2002). However, we and others observed that grafting *in vitro*-differentiated ES cells does not eliminate abnormal and disruptive growth post-transplantation (data not shown; Arnhold *et al*. 2004). Thus, for clinical application of ES-derived cells for transplantation therapy, it is essential to safeguard from any potential tumor formation derived from the grafted cells. It is therefore important to establish a procedure that can purify only the desired cell type and thus avoid post-grafting tumor formation.

Sox1 is a member of a family of transcription factors containing the HMG-box DNA binding domain (Kamachi *et al*. 2000). Three members of the Sox family (Sox1, Sox2 and Sox3) are expressed in the neuroectoderm. While expression of Sox2 and Sox3 starts at the pre-implantation and the epiblast stage, respectively (Pevny *et al*. 1998), the onset of Sox1 expression correlates with the formation of neural plate, and its expression is down-regulated as neural cells exit the cell cycle and differentiate (Uwanogho *et al*. 1995; Pevny *et al*. 1998; Wood and Episkopou 1999). These observations mean that Sox1 is an ideal marker for neural precursor (NP) cells. In addition, Sox1 has been shown to be critical in maintaining NPs at the undifferentiated state by counteracting the activity of proneural proteins and inhibiting neurogenesis (Bylund *et al*. 2003). In Sox1-green fluorescent protein (GFP) knock-in ES cells, GFP is specifically expressed by NP cells both *in vivo* and *in vitro* (Ying *et al*. 2003), thus making fluorescence-activated cell sorting (FACS) purification of NPs possible.

To test whether purification of NP cells removes tumor-forming cells, we purified ES cell-derived NPs using sox1-GFP knock-in ES cells by FACS, and characterized GFP^+ versus GFP^- cells both *in vitro* and *in vivo*. Here, we show that sorting GFP^+ cells greatly enriched the NP population and efficiently removed stage specific embryonic antigen-1 ($SSEA1^+$) pluripotent stem cells from the NP population. Furthermore, transplantation of sorted cells did not generate tumors, strongly suggesting that this genetic procedure could play an important role in future cell therapy by efficiently removing tumor-forming cells.

Materials and methods

ES cell culture and *in vitro* differentiation

Mouse ES cell lines 46C (sox1-GFP knock-in ES cells, a kind gift from Dr Smith) (Ying *et al*. 2003) and J1 were maintained as described previously (Deacon *et al*. 1998). Briefly, undifferentiated ES cells were cultured on gelatin-coated dishes in Dulbecco's modified minimal essential medium (DMEM; Life Technologies, Rockville, MD, USA) supplemented with 2 mM L-glutamine (Life Technologies), 0.001% β-mercaptoethanol (Life Technologies), 1× non-essential amino acids (Life Technologies), 10% donor horse serum (Sigma, St. Louis, MO, USA) and 2000 U/mL human recombinant leukemia inhibitory factor (LIF; R & D Systems, Minneapolis, MN, USA).

ES cells were differentiated into embryoid bodies (EBs) on non-adherent bacterial dishes (Fisher Scientific, Pittsburgh, PA, USA) for 4 days in the above medium without LIF and exchanging horse serum with 10% fetal bovine serum (Hyclone, Logan, UT, USA). EBs were then plated onto an adhesive tissue culture surface (Fisher Scientific). After 24 h in culture, selection of neuronal precursor cells was initiated in serum-free insulin, transferin, selenium and fibronectin (ITSFn) media (Okabe *et al*. 1996). After 10 days of selection, cells were trypsinized and nestin$^+$ neuronal precursors were plated onto poly L-ornithine- (PLO; 15 μg/mL; Sigma) and fibronectin (FN; 1 μg/mL; Sigma)-coated plates in NP medium [NP medium; N2 medium (Johe *et al*. 1996) supplemented with 1 μg/mL laminin (Sigma) and 10 ng/mL basic fibroblast growth factor (bFGF) (R & D Systems)]. After 2 days' expansion of nestin$^+$ neuronal precursors, cells were trypsinized and subjected to FACS. Subsequently, 1.5×10^6 sorted cells/cm^2 were plated onto PLO/FN-coated 6 wells, expanded in the presence of 500 ng/mL N-terminal fragment of sonic hedgehog (R & D Systems) and 100 ng/mL fibroblast growth factor-8 (FGF-8) (R & D Systems) for 4 days. Cells were either harvested for transplantation or induced to differentiate by removal of bFGF in the presence of 200 μM ascorbic acid (Sigma) (Lee *et al*. 2000; Chung *et al*. 2002). Cells were eventually fixed 10 days after starting neuronal differentiation.

Fluorescence-activated cell sorting

In vitro-differentiated NP cells derived from 46C ES cells were trypsinized after expansion for 2 days as described above, suspended in phosphate-buffered saline (PBS) and subjected to FACS using the FACSAria (BD Biosciences, San Hose, CA, USA) to purify GFP$^+$ and GFP$^-$ cell populations. The samples were first gated on forward and side-light scatter, and subsequently, within this population based on GFP expression. Non-GFP-expressing J1 cells that had been similarly differentiated were used as negative control for background fluorescence.

Immunocytochemistry and cell counting

For immunofluorescence staining, cells were fixed in 4% formaldehyde (Electron Microscopy Sciences, Ft. Washington, PA, USA) for 30 min, rinsed with PBS and then incubated with blocking buffer (PBS, 10% normal donkey serum; NDS) for 10 min. Cells were then incubated overnight at 4°C with primary antibodies diluted in PBS containing 2% NDS. The following primary antibodies were used: mouse anti-nestin (Rat401; Developmental Studies Hybridoma Bank, Iowa City, IA, USA; 1 μg/mL), rabbit anti-β-tubulin (Covance, Princeton, NJ, USA; 1 : 2000), mouse anti-Engrailed-1 (En-1; 4G11; Developmental Studies Hybridoma Bank; 1 : 40), sheep anti-tyrosine hydroxylase (TH) (Pel-Freeze, Rogers, AR, USA; 1 : 200), sheep anti-aromatic L-amino acid decarboxylase (AADC; Chemicon, Temecula, CA, USA; 1 : 200), rat anti-dopamine transporter (DAT; Chemicon; 1 : 2000), mouse anti-SSEA1 (Developmental Studies Hybridoma Bank; 1 μg/mL), mouse anti-neuron-specific nuclear (NeuN) (Chemicon; 1 : 100), rabbit anti-serotonin (5-HT; DiaSorin, Stillwater, MN, USA; 1 : 2500), rabbit anti-gamma aminobutyric acid (GABA; Sigma; 1 : 1000), rabbit anti-glutamate (Sigma; 1 : 200), rabbit anti-glial fibrillary acidic protein (GFAP; Dako, Denmark; 1 : 500), rabbit anti-Ki67 (Novocastra, Newcastle upon Tyne, UK; 1 : 2000) and mouse anti-galactocerebroside (galC) antibody (Chemicon; 1 : 200). After additional rinsing in PBS, the coverslips were incubated in fluorescent-labeled secondary antibodies (Cy2- or rhodamine red-X-labeled donkey IgG; Jackson Immunoresearch, West Grove, PA, USA) in PBS with 2% NDS for 30 min at 21°C. After rinsing for 3 × 10 min in PBS, sections were counterstained using 5 μg/mL Hoechst, then mounted onto slides in Gel/Mount (Biomeda Corp., Foster City, CA, USA). Coverslips were examined using a Leica (Wetzlar, Germany) TCS/NT confocal microscope equipped with krypton, krypton/argon and helium lasers.

Cell density was determined by counting the numbers of cells with marker gene expression per field at 40× magnification using a Zeiss (Thornwood, NY, USA) Axioplan I fluorescent microscope. Ten visual fields were randomly selected and counted for each sample. Numbers presented in figures represent the average percentage and SEM from three samples from independent experiments. For statistical analysis, we used STATVIEW software (SAS Institute, Cary, NC, USA) and performed ANOVA with an alpha level of 0.05 to determine possible statistical differences between group means. When significant differences were found, post-hoc analysis was performed using Fisher's protected least significant difference (PLSD) (alpha = 0.05).

Semi-quantitative RT-PCR analysis

Total RNA from plated cells at different stages during the differentiation protocol was prepared using TriReagent (Sigma) followed by treatment with DNase I (Ambion, Austin, TX, USA). For RT-PCR analysis, we transcribed 5 μg RNA into cDNA using oligo (dT) primers, according to the SuperScript Preamplification Kit (Life Technologies). The cDNA was then analyzed by PCR using the following primers: β-actin: 5′-GGCATTGTGATGGACTCCGG-3′, 5′-TGCCACAGGATTCCATACCC-3′ (358 bp); Oct4: 5′-CTGAGGGCCAGGCAGGAGCACGAG-3′, 5′-CTGTAGGGAGGGCTTCGGGCACTT-3′ (462 bp; Mitsui *et al*. 2003); nanog: 5′-AGGGTCTGCTACTGAGATGCTCTG-3′, 5′-CAACCACTGGTTTTTCTGCCACCG-3′ (Mitsui *et al*. 2003); ERas: 5′-ACTGCCCCTCATCAGACTGCTACT-3′, 5′-CACTGCCTTGTACTCGGGTAGCTG-3′ (Takahashi *et al*. 2003); Nestin: 5′-GGAGTGTCGCTTAGAGGTGC-3′, 5′-TCCAGAAAGCCAAGAGAAGC-3′ (327 bp; Lee *et al*. 2000); Sox 1: 5′-GCCCAGGAAAACCCCAAGATG-3′, 5′-CCGTTAGCCCAGCCGTTGAC-3′; Bmi1: 5′-TTGCTGCTGGGCATCGTAAG-3′, 5′-CCAATGGCTCCAATGAAGACC-3′ (Molofsky *et al*. 2003).

PCR reactions were carried out in 1 × IN Reaction Buffer (Epicentre Technologies, Madison, WI, USA) containing 1.4 nM each primer and 2.5 U Taq I DNA polymerase (Promega, Madison, WI, USA). Samples were amplified in an Eppendorf Thermocycler (Brinkmann Instruments, Westbury, NY, USA) under the following conditions: denaturing step at 95°C, 40 s; annealing step at 60°C, 30 s; amplification step at 72°C, 1 min for 20–28 cycles and a final amplification step at 72°C, 10 min. For semi-quantitative PCR, cDNA templates were normalized by amplifying actin-specific transcripts.

Analysis of catecholamines

Differentiated ES cells or E12.5 ventral mesencephalon (VM) cells (stage 5 day 10) in 12-well plates were treated with 200 μL N3 medium supplemented with 50 mM KCl; the medium was collected after 30 min, followed by addition of perchloric acid (PCA) to a final concentration of 0.1 M PCA/0.1 mM EDTA. These de-proteinated samples were centrifuged and supernatant fluids were kept at − 80° until further analysis. Samples were filtered through a 0.22 μm nylon filter (Osmonics, Inc., Trevose, PA, USA) and analyzed for their catecholamine content by reverse-phase HPLC using a Velosep RP-18 column (100 × 3.2 mm; Brownlee Laboratories, Wellesley, MA, USA) and an ESA Coulochem II electrochemical detector equipped with a 5014 analytical cell (ESA Biosciences, Inc., Chelmsford, MA, USA) as described (Wachtel *et al*. 1997). The mobile phase consisted of 0.1 M sodium phosphate buffer (pH 2.65), 0.1 mM EDTA, 0.4 mM sodium octyl sulfate and 9% (v/v) methanol. The flow rate of the mobile phase through the system was 0.8 mL/min. The guard cell potential was set at 330 mV. The potential of the first electrode in the analytical cell was set at 0 mV, the second at 310 mV. L-DOPA, dopamine, dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) were identified by retention time, and quantified based on peak height using the EZChrom Chromatography Data System (ESA Biosciences, Inc.). The limit of detection for all compounds was < 1 pg. DA content of each sample was normalized with the amount of total

cellular proteins. For protein measurement, after harvesting cells in 0.1 M PCA/0.1 mM EDTA, precipitates were resuspended in 10 mM potassium phosphate buffer with 0.2% triton-X, pH 7 and sonicated. The protein content was measured using the Bio-Rad Assay (Bio-Rad Laboratories, Hercules, CA, USA).

Transplantation analysis

GFP^+ or GFP^- cell populations were trypsinized after 4 days' induction with SHH and FGF8, and resuspended at a density of 200 000 cells/μL. A 1 μL volume of cell suspension was grafted into the right striatum (from the bregma: AP + 0.05, L − 0.18, V − 0.30, IB 9) of C57/BL6 mice ($n = 11$) (Charles River Breeding Laboratories, Wilmington, MA, USA). Prior to surgery, mice received an i.p. injection of acepromazine (3.3 mg/kg, PromAce, Fort Dodge, IA, USA) and atropine sulfate (0.2 mg/kg, Phoenix Pharmaceuticals, St. Joseph, MO, USA), followed by anesthesia with an i.p. injection of ketamine (60 mg/kg, PromAce) and xylazine (3 mg/kg, Phoenix Pharmaceuticals). Transplantation was performed using a 22-gauge, 10 μL Hamilton syringe and a Kopf stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Post-operative analgesia consisted of two s.c. injections of buprenorphine (0.032 mg/kg, Sigma) over 24 h. Eight weeks after transplantation, mice were killed with an i.p. overdose of pentobarbital (150 mg/kg, Sigma). Subsequently, mice were perfused intracardially with 100 mL heparin saline (0.1% heparin in 0.9% saline) followed by 200 mL paraformaldehyde (4% in PBS). Brains were post-fixed for 8 h, equilibrated in sucrose (20% in PBS), sectioned at 40 μm on a freezing microtome (Microm, Waldorf, Germany) and collected in PBS. For histological analysis, sections were stained with antibodies against TH and NeuN (see above). Graft volumes were measured using an integrated Axioskop 2 microscope (Carl Zeiss, Thornwood, NY, USA) and StereoInvestigator image capture equipment and software (Microbright Field, Williston, VT, USA). To determine the total TH^+ cell number within the GFP^+ grafts, every sixth section was stained and counted.

Results

Sox1-GFP knock-in ES cells generate GFP^+ cells that co-localize with nestin expression after *in vitro* differentiation and can generate dopaminergic neurons

Several lines (e.g. D3, J1 and R1) of mES cells have been shown to differentiate into NPs and then into dopaminergic neurons, using the five-stage *in vitro* differentiation method (Lee *et al*. 2000). We first tested whether the same method could be used for *in vitro* differentiation of Sox1-GFP knock-in ES cells. In brief, ES cells were differentiated *in vitro* as embryoid body (EB) cells for 4 days, then transferred to tissue culture plates and serum-free medium for selection of NP cells. At the ES and EB stages, neither endogenous Sox1 mRNA nor GFP expression was detected (data not shown). After 10 days of selection, cells were trypsinized and transferred into N2 medium containing bFGF for expansion of NP cells. At this stage, numerous GFP^+ cells were generated (Fig. 1a). In addition to GFP expression, these GFP^+ cells expressed another NP marker, nestin (Figs 1b and c). However, most of GFP^+ cells did not overlap with more mature neural cell markers such as NeuN and GFAP, demonstrating the transient nature of GFP expression (Figs 1d–i). Overlap between GFP and mature neural cell markers in a few cells is likely due to the longer half life of GFP compared with endogenous sox1 (Pevny *et al*. 1998). When bFGF was removed from the medium to further differentiate these NP cells, a great number of $Tuj1^+$ neurons were generated (Fig. 1j) that contained many dopaminergic neurons, as examined by TH immunocytofluorescence (Fig. 1j–l).

FACS efficiently enriches NP population and removes pluripotent stem cells

Next, we purified GFP^+ NPs derived from sox1-GFP ES cells. Prior to FACS, sox1-GFP ES cells were differentiated to the NP stage and expanded in bFGF for 2 days. Cells were first gated using side and forward scatter to remove any cell debris and doublets. Then, cells were

sorted and collected into GFP^+ and GFP^- populations, as shown in Fig. 2(a). GFP non-expressing J1 cells were similarly differentiated and used as negative control to set up the gating. Sorted cells were immediately re-analyzed by FACS scan (Fig. 2b), which showed that 93.9% of sorted GFP^+ cells fell within the GFP^+ gate. Analysis by inverted microscope immediately after sorting (Figs 2c and d) also showed that most of the cells sorted were GFP^+. These analyses of purified cells demonstrated that the FACS procedure effectively purified NP cells expressing GFP. In addition, sorted cells were plated back on tissue culture plates and recovered/expanded for 4 more days in NP medium. Here, a recovery/expansion step after FACS was included to increase survival of the NPs to be used for further *in vitro* analyses, as well as for transplantation. The proportion of GFP^+ cells was decreased during the *in vitro* differentiation of sorted cells. After 4 days of expansion (NP stage day 4), there were 68.02 ± 6.56% GFP^+ cells out of the total cells. After induction of final differentiation by mitogen removal, the proportion of GFP^+ cells further decreased to 27.18 ± 3.54% at early ND stage (ND day 5) and to 7.36 ± 0.71% GFP^+ at later ND stage (ND stage day 10) (Fig. 2e).

As shown in Figs 3(a–p), in the GFP^+ cell population after 4 days' expansion, GFP^+ cells that co-express the neural precursor marker, nestin, were greatly enriched. These GFP^+ cells showed a wide range of nestin expression, suggesting different stages of neural precursor and/or cellular variability. In contrast, the GFP^- population contained few GFP^+ and $nestin^+$ cells following the 4 days' *in vitro* recovery/expansion step after FACS sorting. Further immunocytofluorescence analysis showed that FACS efficiently removed cells expressing the pluripotent cell marker, SSEA1 (Figs 3q–v). These $SSEA1^+$ cells, due to their avid proliferation capacity and potential to generate different cell types, are likely to generate large and disruptive grafts in the host brain. We next performed RT-PCR analysis to assess differential gene expression of these marker proteins. Consistent with our immunocytofluorescence analysis, mRNAs encoding NP markers sox1 and nestin were highly enriched in the GFP^+ cell population, while those encoding pluripotent stem cell markers (e.g. Oct4, Eras and nanog) were significantly enriched in the GFP^- cell population (Fig. 3w).

We further differentiated FACS-purified GFP^+ and GFP^- cells *in vitro*, in serum-free medium, and analyzed them by immunocytofluorescence (Fig. 4). This analysis showed that the GFP^- population generated very few neuronal or astocytic cells (Figs 4a–d). In sharp contrast, FACS-purified GFP^+ cell populations efficiently generated $Tuj1^+$ neuronal cells and $GFAP^+$ astrocytes (Figs 4e–h). Cell counting analysis showed that GFP^- and GFP^+ cells generated 2.90 ± 0.13% and 44.29 ± 7.93% $\beta III\text{-tubulin}^+$ neurons per total cells, respectively (Fig. 4i). In addition, GFP^- and GFP^+ cells generated 1.09 ± 0.27% and 22.94 ± 2.07% $GFAP^+$ astrocytes per total cells, respectively (Fig. 4i). These results demonstrate that our procedure can effectively select ES-derived NP cells that have differential potential to generate neural cell populations. We also performed immunocytofluorescence using GalC antibody. This analysis showed that a small number of oligodendrocytes was generated from sox1-GFP^+ cells, but not at all from sox1-GFP^- cells (data not shown). Modification of our spontaneous differentiation procedure may be needed to for optimal generation of oligodendrocytes.

FACS-purified NPs can generate dopaminergic neurons as well as other neural subtypes

To test the potential of FACS-purified NPs to generate midbrain dopaminergic lineages, we differentiated them *in vitro* and further analyzed them by immunocytofluorescence. As shown in Fig. 5(a–c), we found that many GFP^+ cells also expressed the early midbrain marker, Engrailed 1 (En-1). After full differentiation (day 10 of the ND stage), some of these cells expressed the dopaminergic marker, TH (Figs 5d–f). These TH^+ neurons, generated from FACS-purified NPs, also expressed other dopaminergic markers such as dopa decarboxylase (DDC) (Figs 5g–i) and dopamine transporter (DAT) (Figs 5j–l). Cell counting analysis showed that sox1-GFP^- and sox1-GFP^+ cells generated 0.69 ± 0.22% and 2.42 ± 0.36% TH^+ neurons

per total cells, respectively (Fig. 5m). Unexpectedly, when analyzed for DA neuronal proportion among neurons, sox1-GFP$^-$ cells generated a higher proportion of DA neurons compared with sox1-GFP$^+$ cells, with sox1-GFP$^-$ and sox1-GFP$^+$ cells generating 19.44 ± 6.02% and 6.43 ± 0.95% TH$^+$ neurons per total neurons, respectively (Fig. 5m). To test the functionality of these DA neurons, *in vitro*-differentiated cells were treated with 50 m$_M$ KCl for 30 min and the dopamine content of the medium was analyzed by HPLC. As shown in Fig. 5(n), these dopaminergic neurons originating from GFP$^-$ and GFP$^+$ cells released significant amounts of dopamine in response to membrane depolarization, with 2.96 ± 0.10 and 5.03 ± 0.72 pg/μg cellular proteins, respectively. Because of the unexpected observation that a large proportion of GFP$^-$ cell-derived neurons are dopaminergic, even though very small numbers of neurons are generated from the GFP$^-$ cell population, it might be possible that the GFP$^-$ population may contain a DA inducing activity/signal. Thus, we mixed the GFP$^+$ and GFP$^-$ population after FACS and fully differentiated them *in vitro*. However, we could not detect any additive effect by mixing the GFP$^-$ population with the GFP$^+$ population, in terms of DA differentiation (4.69 ± 0.50 pg/μg cellular proteins; Fig. 5n). Primary dopaminergic neurons, derived from E12.5 mouse embryo VM, were cultured in the same way as the FACS sorted cells and used as a control for DA release; these released 3.06 ± 0.28 pg/μg cellular proteins in response to membrane depolarization (Fig. 5n).

We next tested whether sorted GFP$^+$ cells could generate other neuronal subtypes. Immunocytofluorescent analyses of *in vitro*-differentiated GFP$^+$ cells demonstrated that serotonergic (Figs 6a–c), GABAergic (Figs 6d–f) and glutamatergic neurons (Figs 6g–i) could be efficiently generated from these cell populations. Cell-counting analysis showed that the proportion of each subtype was 5.06 ± 1.38%, 51.12 ± 4.91% and 26.77 ± 2.04% for serotonergic, GABAergic and glutamatergic neurons, respectively (Fig. 6j). Taken together, these FACS-purified GFP$^+$ cells appear to have the full developmental potential of authentic NP cells to differentiate into various neuronal subtypes and glial cell types.

Transplantation analysis of FACS-purified NPs

Based on the above *in vitro* data, we speculated that FACS-purified GFP$^+$ or GFP$^-$ cells may behave quite differently after grafting into the host brain. To address this hypothesis, we transplanted 200 000 GFP$^+$ or GFP$^-$ FACS-purified cells into the striatum of normal mice (n = 11). Grafts were analyzed 8 weeks post-transplantation. Six out of 11 mice transplanted with GFP$^-$ cells succumbed to tumors before 8 weeks. Some of these mice were post-fixed and included in the histological analysis. GFP$^-$ cells generated large and disruptive grafts, whereas GFP$^+$ cells generated well contained grafts (Figs 7a and b). The presence of TH$^+$ neurons in grafts from GFP$^+$ cells was established by TH immunohistochemistry (Figs 7c and d). Sham-treated sides (contralateral to the grafted side) had only TH fibers in the striatum, but never TH cell bodies (Fig. 7e).

When total graft volume was measured, there was a significant difference between the sizes of GFP$^-$ grafts and those of GFP$^+$ grafts (43.262 ± 10.757 mm^3 vs. 2.758 ± 1.962 mm^3; Fig. 7f). However, DA neuronal density within the grafts was significantly higher in GFP$^+$ grafts compared with GFP$^-$ grafts, with 103.22 ± 23.28 DA neurons/mm^3 and 19.15 ± 3.18 DA neurons/mm^3 for GFP$^+$ and GFP− grafts, respectively. Based on the high occurrence of SSEA$^+$ cells only in the GFP$^-$ population (Fig. 3), we speculate that the graft size differences may be due to the presence of highly proliferative pluripotent stem cells within the GFP$^-$ cell population. Indeed, immunohistochemistry analysis confirmed the presence of SSEA1$^+$ cell clusters in some GFP$^-$ grafts even 8 weeks after transplantation (Fig. 7h). In contrast, no SSEA1$^+$ cells were detected in the GFP$^+$ grafts (Fig. 7i). Analysis of the presence of proliferating cells in the grafts showed that GFP$^-$ grafts contained numerous Ki67$^+$ cells 8 weeks after transplantation (Fig. 7j), whereas few such cells were found in GFP$^+$ grafts (Fig.

7k). Cell-counting analysis of GFP^+ grafts showed that they contained 198 ± 75.6 TH^+ neurons per graft, and these TH neurons also co-expressed DDC and DAT (Fig. 7l–o).

Discussion

Neural stem/precursor cells can self-renew and maintain their potential to generate differentiated progenies such as neurons, astrocytes and oligodendrocytes (Seaberg and van der Kooy 2003). Thus, they represent both an excellent tool to study neural cells *in vitro* and a potential source of unlimited cells for cell replacement therapy of neurodegenerative diseases. In order to use these differentiated neural cells therapeutically, however, they must be separated from the undifferentiated cell population to prevent teratoma formation. Efforts to purify neural stem/precursor cells from embryonic or adult brain, using cell surface markers or transgene expression driven by NP-specific promoters, have been reported (Keyoung *et al.* 2001; Rietze *et al*. 2001; Capela and Temple 2002; Murayama *et al*. 2002; Tamaki *et al*. 2002; Nagato *et al*. 2005). Of the markers that have been investigated, sox1 has shown the most specific expression pattern for NP populations during development (Pevny *et al*. 1998; Aubert *et al*. 2003; Pevny and Placzek 2005). Aubert and co-workers generated a sox1-GFP knock-in mouse and demonstrated that GFP expression overlaps well with endogenous sox1 expression, thus providing a good handle for purification of NPs using FACS. In addition to the embryonic or adult brain, ES cells represent another source of NP cells due to their unlimited proliferation capacity and their potential to generate most cell types *in vitro* (Lang *et al*. 2004a). Ying *et al*., using sox1-GFP knock-in ES cells, demonstrated that differentiated ES cells expressing sox1-GFP acquired neuroepithelial morphology (Ying *et al*. 2003). Lang *et al*. (2004b) generated sox knock-in ES cells harboring the neomycin resistance gene, allowing enrichment of NPs by drug selection. These drug-selected ES cell-derived NP cells generated electrophysiologically functional neurons *in vitro*.

In this study, we used florescence-activated cell sorting (FACS) to purify ES cell-derived NPs using sox1-GFP knock-in reporter expression (Ying *et al*. 2003) and characterized them *in vitro* and *in vivo.* FACS purification of sox1-GFP^+ cells efficiently yielded an enriched neural cell population while effectively removing teratoma-causing pluripotent cells. The resulting neural cell population can generate multiple subtypes of neural cells (Figs 4 and 6), demonstrating the value of these purified NPs as a tool for studying various neural cells *in vitro* and as a possible cell source for cell replacement therapy of various neurodegenerative diseases. In addition, we demonstrated that purifying NP prior to transplantation efficiently reduced tumor formation in the host brain. The heterogeneous nature of *in vitro* differentiated ES cell preparations may raise concerns about their safe usage for therapeutic application and thus, it is essential to establish their safety before applying them to human diseases (Odorico *et al*. 2001). One way to prevent teratoma formation is to remove possible tumor-forming cells from the ES cell-derived neural cell preparation. We achieved this by using FACS purification of genetically marked ES cells.

The therapeutic use of ES cell-derived neural populations in animal models of various neurodegenerative diseases, such as intracerebral hemorrhage, Parkinson's disease, Huntington's disease, ischemia and myelin disease, has been reported (Dinsmore *et al*. 1996; Brustle *et al*. 1999; Kim *et al*. 2002; Barberi *et al*. 2003; Nonaka *et al*. 2004; Wei *et al*. 2005). Since the NPs purified in this study can be used to generate the various neural cells that are dying in these diseases, such as dopaminergic neurons, glutamatergic neurons and GABAergic neurons (Fig. 6; Lang *et al*. 2004b), the procedure described in this paper can be applied to these fields with a high degree of confidence that potentially tumor-forming pluripotent stem cells can be removed prior to transplantation.

Another way to purify and prepare cells for transplantation is to isolate specific neuronal cell types from *in vitro*-differentiated ES cells rather than NPs. Zhao *et al.* generated Pitx3 knock-in ES cells and purified midbrain dopaminergic neurons (Zhao *et al*. 2004). Transplantation of these cells has not been reported and thus, no evaluation of their safety and efficacy is available. Another group used the TH promoter to drive GFP expression, and transplanted the purified GFP^+ cells into rat brain (Yoshizaki *et al*. 2004), but they reported very low graft survival. Unlike the NP population that we purified, mature neurons are more vulnerable to manipulations such as trypsination and FACS. When we transplanted FACS-sorted cells that had reached post-neuronal differentiation stages, we similarly observed lower graft survival compared with cells at the NP stage (data not shown). Thus, purification of specific types of neurons offers the advantage of a unique cell population for transplantation, whereas transplanting NPs has the advantage that these cells have better survival over the course of the manipulations. Additionally, in some cases, co-transplantation with astrocytes enhances neuronal differentiation and/or survival (Song *et al*. 2002; Dhandapani *et al*. 2003). By transplanting NPs that can generate both neurons and astrocytes (Fig. 6; Lang *et al*. 2004b), we may be able to achieve such a neuroprotective effect. Eventually, it will be even better if marker gene expression specific for each cell type can be used, and then single cell types can be purified and used alone or in combination for complete control of the phenotype of the cell being transplanted.

Using the five-stage procedure (Lee *et al*. 2000; Chung *et al*. 2002) to differentiate sox1-GFP ES cells, we have shown that some of the sox1-GFP^+ cells express the early midbrain marker Engrailed-1 and could generate DA neurons. We observed a rather low proportion of DA neurons being generated from sox1-GFP^+ cells compared with the number generated from unsorted cells (Figs 1j–l and 5d–f). Interestingly, even though the GFP^- population generated far fewer neurons (Fig. 4), a majority of these neurons was dopaminergic (Fig. 5m; data not shown). We have postulated two explanations for this. First, it is possible that some NP cells that can generate DA neurons are not expressing sox1 and thus, are sox1-GFP^-. Secondly, it is possible that there are DA-inducing, factor-releasing cells in the GFP^- populations. Our mixing experiment (Fig. 5m) supports the possibility that at least some of the DA precursors may be GFP^-. Further studies will be needed to understand clearly this unexpected observation.

While we were preparing this manuscript, Fukuda *et al*. also reported reduction of teratoma formation by purification of ES-derived NPs using FACS (Fukuda *et al*. 2005). Using different methods of *in vitro* differentiation (PA6 co-culture procedure), they also showed enrichment of NPs and removal of pluripotent cells by FACS. Taken together, using two different *in vitro* differentiation methods, both studies demonstrate that NP cells derived from FACS purification of the sox1-GFP^+ population can prevent tumor formation even 8 weeks post-transplantation. The PA6 co-culture procedure is less time-consuming and more efficient for DA differentiation than the five-stage method. However, the reason we used the five-stage procedure was so that we could compare the GFP^+ versus GFP^- population without contaminating feeder cells. By using the five-stage procedure instead of the PA6 method, we could compare differentiation and proliferative potentials of GFP^+ and GFP^- populations more clearly, which could not be done in the study of Fukuda *et al*. Thus, even though there are many advantages to using the PA6 system, we believe that the five-stage procedure is more beneficial for comparative studies of different cell populations. Furthermore, our study demonstrates that these purified NP cells can differentiate into various subtypes of neurons and glial cells and thus, can potentially be applied to various disease models. One striking difference in these two studies is the extremely small graft size generated by sox-GFP^+ cells and the small number of DA neurons in the Fukuda *et al*. study, possibly because these cells were transplanted immediately after FACS. In contrast, we incorporated a recovery/expansion stage after FACS and before transplantation, resulting in increased graft size and more than 15-fold total DA neurons compared with their study. Taken together, whereas it can be a powerful method of

removing unwanted cell types before transplantation, FACS could, at the same time, lower cell survival by imposing stress on the cells sorted. Our studies demonstrate that this pitfall can be largely resolved by providing for a recovery/expansion stage after FACS purification of the desired cell type.

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Abbreviations used

bFGF
basic fibroblast growth factor

DAT
dopamine transporter

EB
embryoid body

ES
embryonic stem cell

FACS
fluorescence-activated cell sorting

FN fibronectin

GFP green fluorescent protein

5-HT serotonin

NDS normal donkey serum

NP neural precursor

PBS phosphate-buffered saline

PCA perchloric acid

PD Parkinson's disease

PLO poly L-ornithine

VM ventral mesencephalon

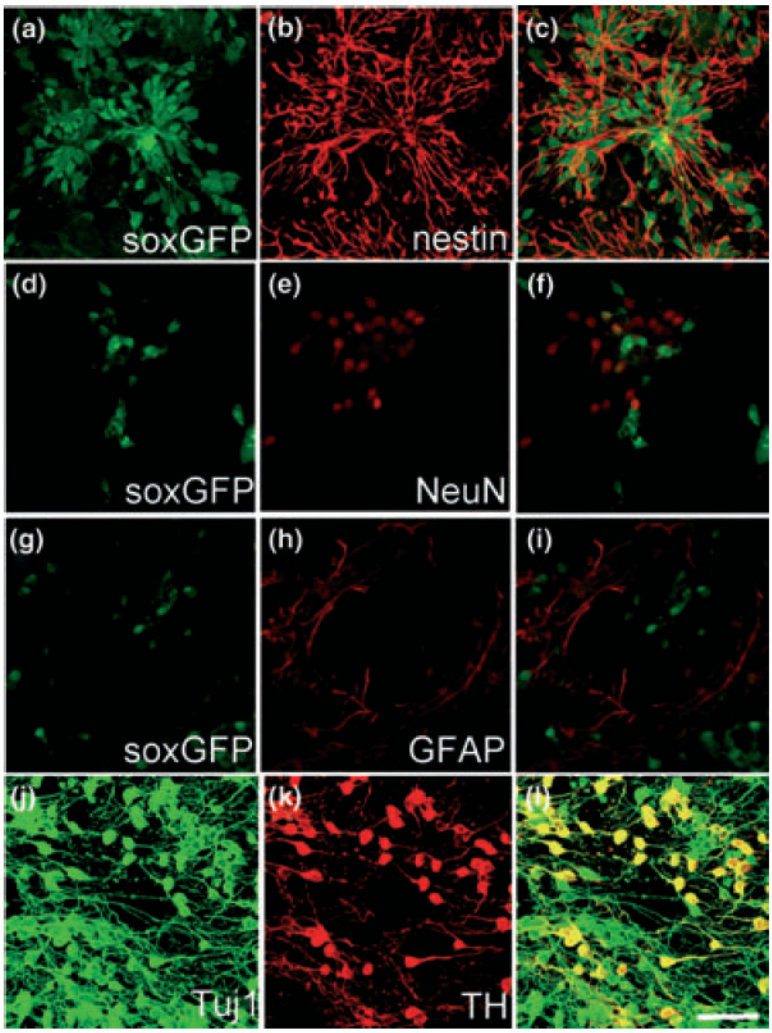

Fig. 1.

In vitro differentiation of sox1-GFP knock-in ES cells. Sox1-GFP ES cells were differentiated as described in Materials and methods, fixed at the NP stage (a–i) or ND stage (j–l) and analyzed by immunocytofluorescence. Confocal microscopy at 40× magnification of representative fields is shown. Scale bar = 50 μm.

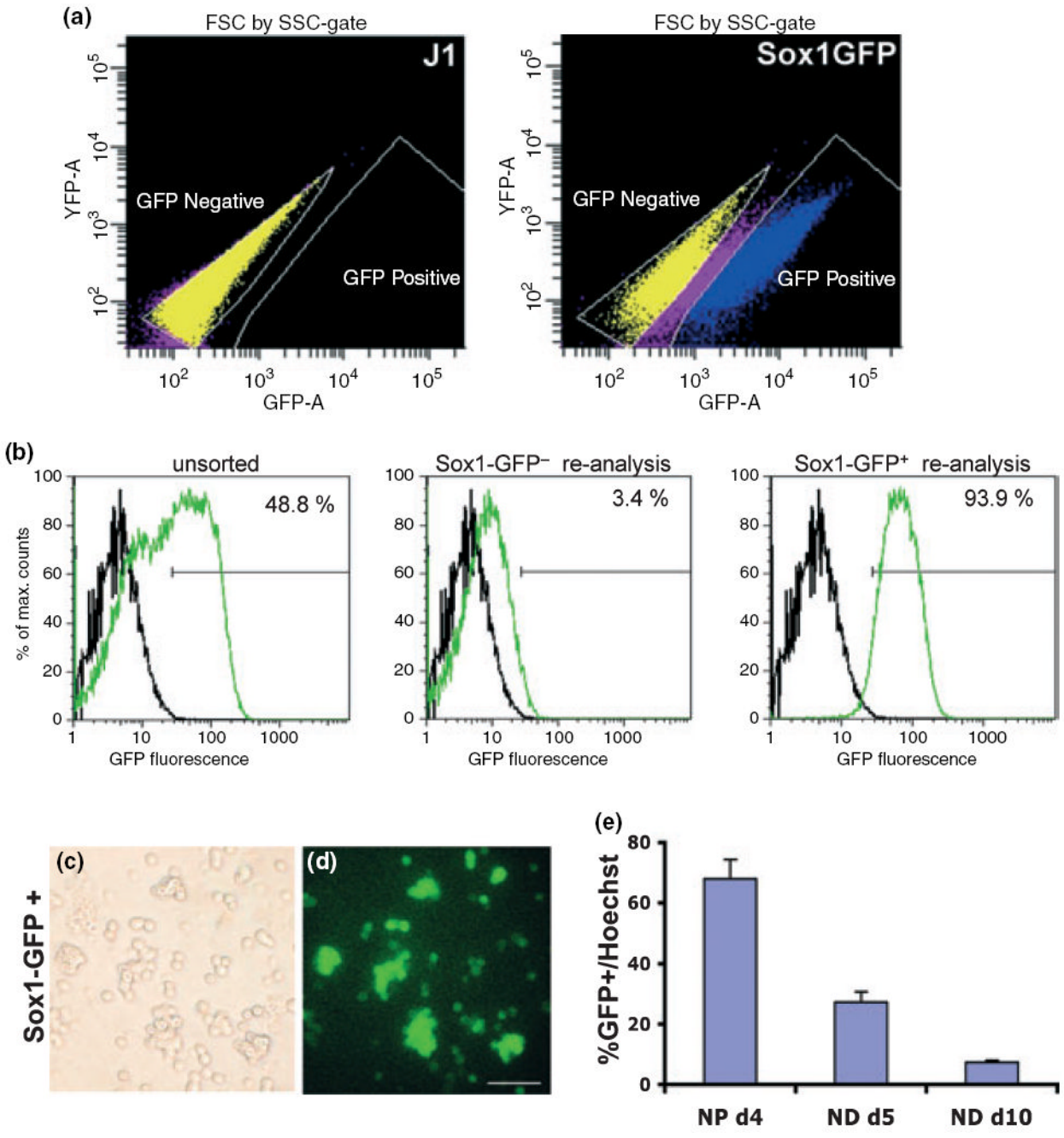

Fig. 2.

Isolation of sox1-GFP-expressing NPs using FACS. (a) Sox1-GFP ES cells were differentiated to the NP stage, and GFP^+ and GFP^- cell populations were isolated by FACS. J1 ES cells differentiated the same way were used as negative control. (b) FACS purification of Sox1-GFP precursors was re-analyzed after FACS purification to confirm purity. The black line represents FACS profile of J1 cells as a negative control and the green line represents the samples analyzed. Sox1-GFP^+ cells were fixed 2 h after sorting and analyzed by bright field and fluorescent microscopy (c, d) for validation of sorting efficiency. Scale bar = 50 μm. (e) Sox1GFP^+ cells were fixed at different time points during *in vitro* differentiation [4 days after FACS (NP d4), 5 days of neuronal differentiation stage (ND d5) and 10 days of neuronal

differentiation stage (ND d10)]. Numbers of GFP^+ cells and $Hoechst^+$ cells were counted on multiple random fields for each sample, and the proportion of GFP^+ cells was calculated by dividing the number of GFP^+ cells by the number of $Hoechst^+$ cells. Each group represents an average of three samples from each independent experiment.

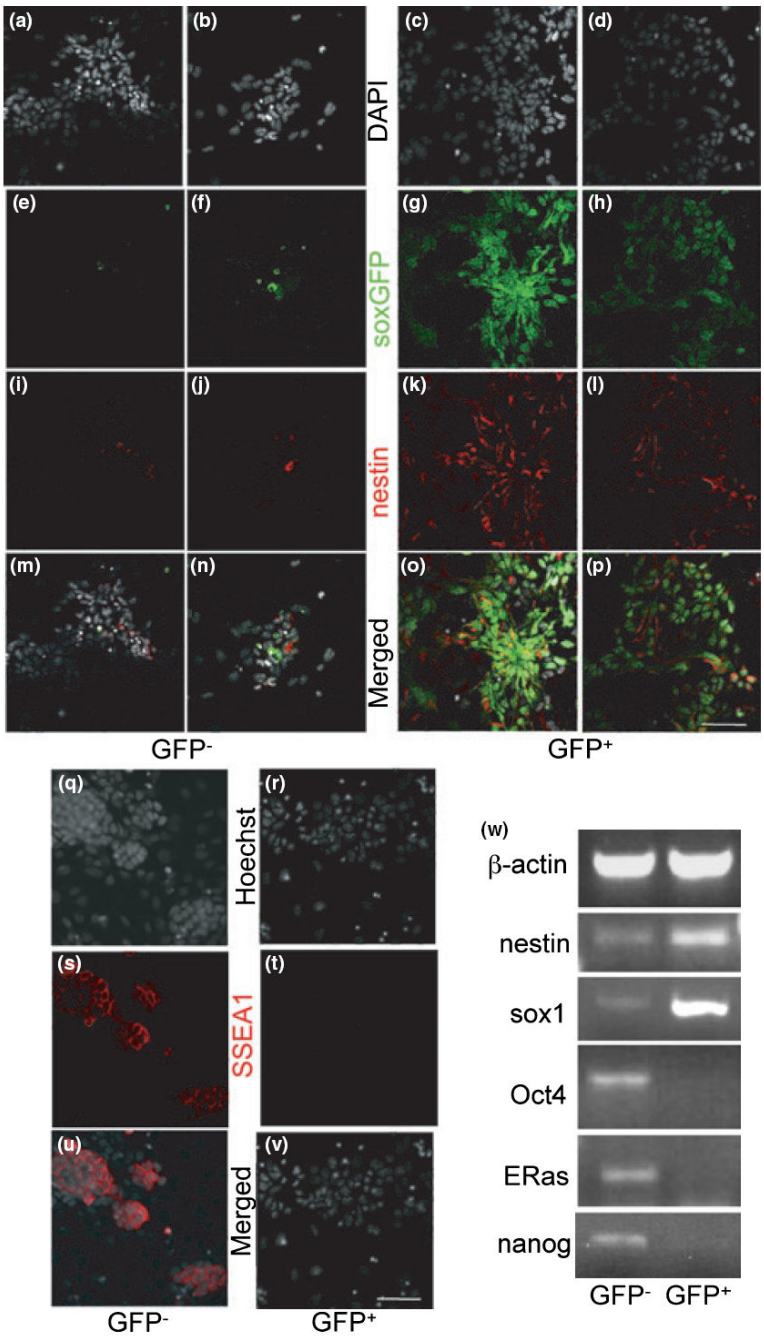

Fig. 3.

NPs can be efficiently purified and pluripotent stem cells removed by FACS. GFP^+ and GFP^- cells were fixed 4 days after FACS and analyzed by immunocytofluorescence using antibodies against nestin (a–p) or SSEA1 (q–v). Shown are representative fields using confocal microscopy at 40×. Scale bar = 50 μm. (w) Total RNAs were prepared from GFP^- and GFP^+ cells at the NP stage after FACS and analyzed by RT-PCR analysis.

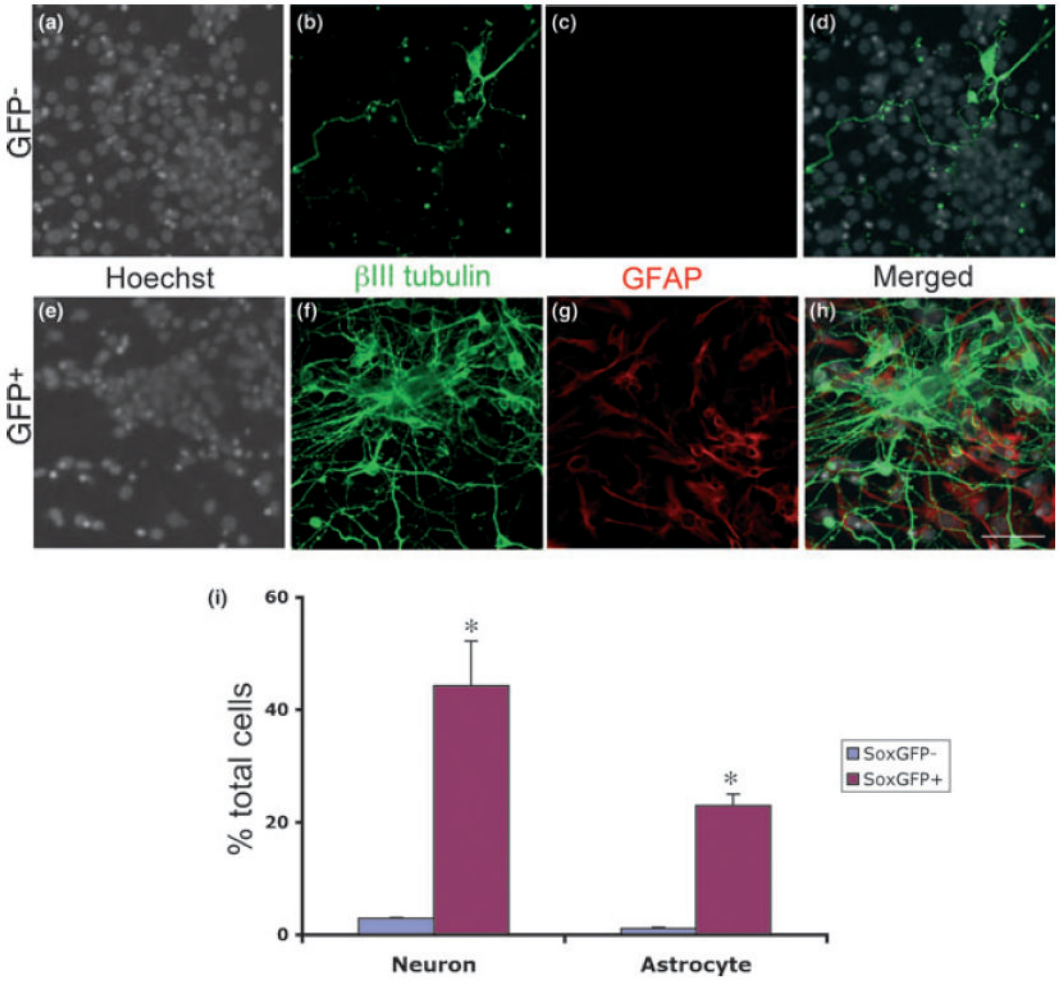

Fig. 4.

FACS-purified NPs generate neural cell-enriched cell populations. (a–h) FACS-isolated GFP$^-$ and GFP$^+$ cells were fully differentiated, fixed at the ND stage and analyzed by immunocytofluorescence using antibodies against βIII-tubulin and GFAP. Slides were counterstained using Hoechst. Shown are representative fields using confocal microscopy at 40× magnification. Scale bar = 50 μm. (i) The numbers of βIII-tubulin$^+$, GFAP$^+$ and Hoechst$^+$ cells from sox1-GFP$^-$ and sox1-GFP$^+$ cells were counted on multiple random fields per each sample, and the proportions of neurons and astrocytes were calculated by dividing the numbers by the total number of Hoechst$^+$ cells. Each group represents an average of three samples from each independent experiment. For neuronal phenotype, ANOVA revealed $F = 27.240$, $p < 0.05$. Fisher's PLSD post-hoc analysis was performed with a significance level of 0.05; *indicates significant difference from sox-1GFP$^-$ cells. For astrocytic phenotype, ANOVA revealed $F = 99.536$, $p < 0.05$. Fisher's PLSD post hoc analysis was performed with a significance level of 0.05 ($n = 3$); *indicates significant difference from sox1-GFP$^-$ cells.

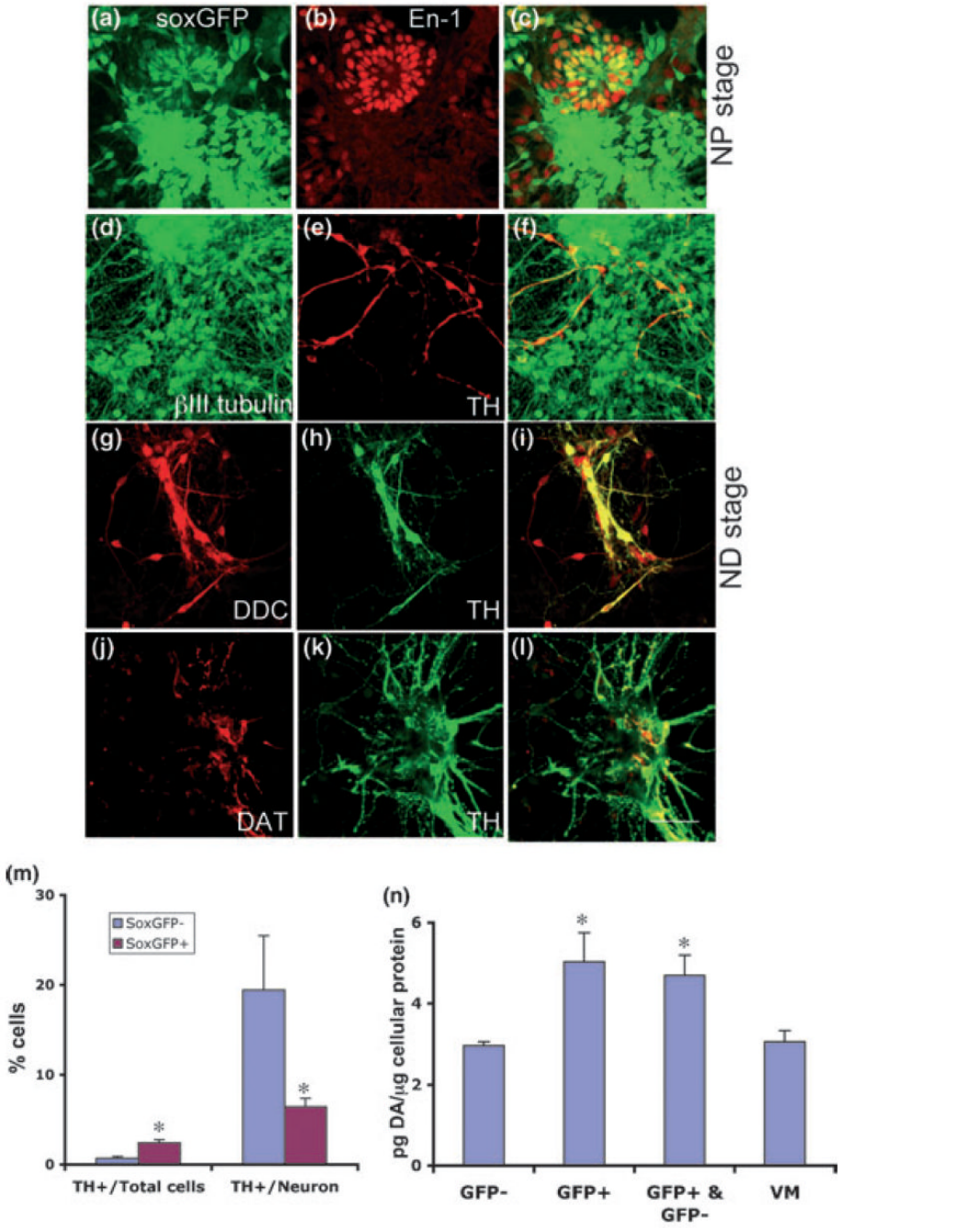

Fig. 5.

FACS-purified NPs can generate dopaminergic neurons. FACS-purified GFP$^+$ cells were analyzed at the NP stage by immunocytofluorescence using an antibody against the early midbrain marker En-1 (a–c). FACS-purified GFP$^+$ cells were fully differentiated and analyzed by immunocytofluorescence using antibodies against dopaminergic markers such as TH (d–f), DDC (g–i) and DAT (j–l). Shown are representative fields using confocal microscopy at 40× magnification. Scale bar = 50 μm. (m) Fully differentiated sox1-GFP$^+$ cells and sox1-GFP$^-$ cells were stained, using antibodies against βIII-tubulin and TH, and counterstained with Hoechst. The numbers of TH$^+$, βIII-tubulin$^+$ and Hoechst$^+$ cells from sox1-GFP$^-$ and sox1-GFP$^+$ cells were counted in multiple random fields for each sample, and the proportions of DA

neurons among the total number of neurons and total cells were calculated by dividing the numbers of DA neurons by the number of Hoechst$^+$ cells. Each group represents an average of three samples from each independent experiment. For proportion of TH$^+$ cells among neurons, ANOVA revealed $F = 4.566$, $p < 0.05$. Fisher's PLSD post-hoc analysis was performed with a significance level of 0.05; *indicates significant difference from sox1-GFP$^-$ cells. For the proportion of TH$^+$ cells among total cells, ANOVA revealed $F = 17.364$, $p < 0.05$. Fisher's PLSD post-hoc analysis was performed with a significance level of 0.05; *indicates significant difference from sox1-GFP$^-$ cells. (n) sox1-GFP$^+$ cells and sox1-GFP$^-$ cells, after full differentiation, released dopamine in response to membrane depolarization. GFP$^+$ cells, GFP$^-$ cells, a mixture of GFP$^+$/GFP$^-$ cells and mouse E12.5-derived primary DA cells (VM) were differentiated (stage 5 day 10) and treated with 50 mM KCl for 30 min, then the medium was collected and analyzed for dopamine content by HPLC. ANOVA revealed $F = 5.459$, $p < 0.05$. Fisher's PLSD post-hoc analysis was performed with a significance level of 0.05; *indicates significant difference from GFP$^-$ and VM cells.

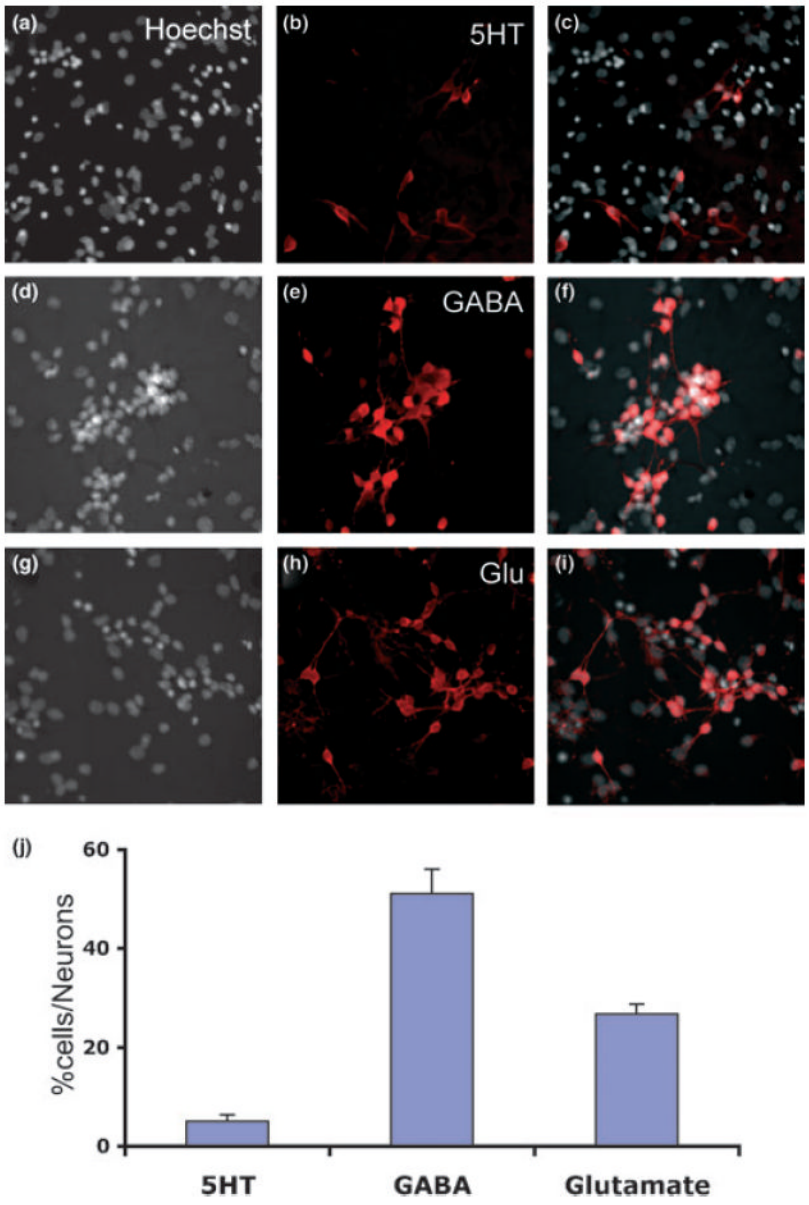

Fig. 6.

FACS-purified NPs can generate other neural subtypes. FACS-purified GFP$^+$ cells were fully differentiated and analyzed by immunocytofluorescence using antibodies against serotonin (a–c), GABA (d–f) and glutamate (g–i). Shown are representative fields using confocal microscopy at 40× magnification. Scale bar = 50 μm. (j) sox1-GFP$^+$ cells were fixed after neuronal differentiation stage (ND d10) and numbers of 5HT$^+$, GABA$^+$, glutamate$^+$ and Hoechst$^+$ cells were counted in multiple random fields per each sample. The proportion of each phenotype was calculated by dividing the cell numbers by the number of Hoechst$^+$ cells. Each group represents an average of three samples from each independent experiment.

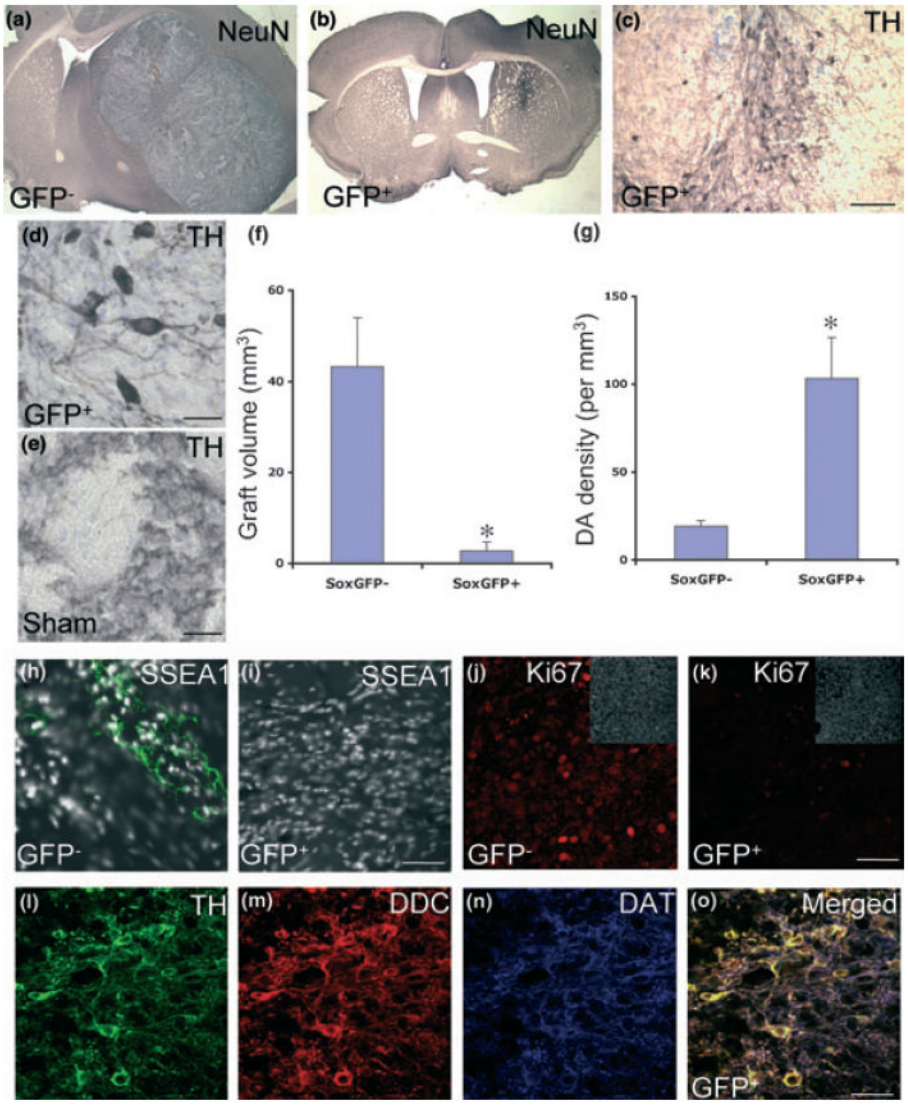

Fig. 7.

Transplantation analysis of FACS-purified cells. GFP$^+$ and GFP$^-$ cells were transplanted into mice striatum 4 days after FACS (NP stage), and the animals were killed 8 weeks after transplantation for histological analysis. Representative low power images of NeuN immunohistochemistry of grafts from GFP$^+$ (a) and GFP$^-$ (b) cells in naïve mouse striatum. TH immunohistochemistry on grafts from GFP$^+$ cells using 20× objective (c; scale bar = 50 μm) and 63× objective (d; scale bar = 20 μm). (e) TH immunohistochemistry on contralateral side of the graft. Scale bar = 20 μm. (f) Total graft volume in GFP$^+$ versus GFP$^-$ grafts. ANOVA revealed $F = 18.457$, $p < 0.005$. Fisher's PLSD post-hoc analysis was performed with a significance level of 0.005; *indicates significant difference from GFP$^-$ cells. (g) DA neuronal density expressed by DA neuronal number per mm^3 graft volume in GFP$^+$ versus GFP$^-$ grafts. ANOVA revealed $F = 4.914$, $p < 0.05$. Fisher's PLSD post-hoc analysis was performed with a significance level of 0.05; *indicates significant difference from sox1-GFP$^-$ cells. (h, i) Representative confocal images of SSEA1 immunohistochem-istry in graft from GFP$^-$ cells. Scale bar = 50 μm. (j, k) Representative confocal images of Ki67 immunohistochemistry in graft from GFP$^-$ cells. Scale bar = 50 μm. (l-o) Representative confocal images of TH/DDC/DAT immunohistochemistry in grafts of GFP$^+$ cells. Scale bar = 50 μm.