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ABSTRACT

The intrinsic inability of the central nervous system to efficiently repair traumatic injuries renders transplantation of neural stem/precursor cells (NPCs) a promising approach towards repair of brain lesions. In this study, NPCs derived from embryonic day 14.5 mouse cortex were genetically modified via transduction with a lentiviral vector to overexpress the neuronal lineage-specific regulator BM88/Cend1 that coordinates cell cycle exit and differentiation of neuronal precursors. BM88/Cend1-overexpressing NPCs exhibiting enhanced differentiation into neurons in vitro were transplanted in a mouse model of acute cortical injury and analyzed in comparison with control NPCs. Immunohistochemical analysis revealed that a smaller proportion of BM88/Cend1-overexpressing NPCs, as compared with control NPCs, expressed the neural stem cell marker nestin 1 day after transplantation, while the percentage of nestin-positive cells was significantly reduced thereafter in both types of cells, being almost extinct 1 week post-grafting. Both types of cells did not proliferate up to 4 weeks in vivo, thus minimizing the risk of tumorigenesis. In comparison with control NPCs, Cend1-overexpressing NPCs generated more neurons and less glial cells 1 month after transplantation in the lesioned cortex whereas the majority of graft-derived neurons were identified as GABAergic interneurons. Furthermore, transplantation of Cend1-overexpressing NPCs resulted in a marked reduction of astrogliosis around the lesioned area as compared to grafts of control NPCs. Our results suggest that transplantation of Cend1-overexpressing NPCs exerts beneficial effects on tissue regeneration by enhancing the number of generated neurons and restricting the formation of astroglial scar, in a mouse model of cortical brain injury. STEM CELLS *2010;28:127–139*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The adult cerebral cortex has limited ability to regenerate lost neural tissue after brain damage, partly due to the lack of a resident population of neural stem/progenitor cells (NPCs) responsive to injury-derived signals. Limited compensatory cortical neurogenesis has been reported following stroke [1] or induced apoptotic degeneration [2], but the number of neurons produced is insufficient to replenish neuronal loss after injury and restore cortical function [3, 4]. To overcome this limitation, efforts have been made to stimulate the endogenous NPC population residing in the neighboring subventricular zone (SVZ) with growth factors, in order to recruit a population of NPCs to the lesioned cortex. Adequate recruitment of SVZ NPCs to successfully replace damaged cortical neurons has so far not been achieved [5–7]. Transplantation of suitable cell types into the adult central nervous system (CNS) has also attracted considerable interest as an alternative strategy to overcome the regenerative limitations of the lesioned brain [8–10].

To this end, the development of improved vector systems for gene delivery of regeneration-promoting molecules in conjunction with the exploitation of NPC transplantation has opened new venues for potential therapeutic interventions for neurodegenerative diseases and brain injury [11]. NPCs have been isolated from embryonic, postnatal, or adult brain tissue of different species [12–14]. It is well established that these cells consist of a heterogeneous population of mitotically active, self-renewing progenitor or immature precursor cells that can be expanded in vitro and, under specific conditions, give rise to neurons, astrocytes, and oligodendrocytes [15, 16]. This dynamic cell population has been extensively used as a cellular transplantation source [17, 18]. However, the yield of NPC-derived neurons is generally low, thus hampering their potential for neuronal replacement.

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We have previously reported the neuronal protein BM88/Cend1 (hereafter Cend1 for cell cycle exit and neuronal differentiation 1, NCBI nomenclature at www.ncbi.nih.gov) as a neuronal-lineage specific regulator coordinating cell cycle exit and differentiation of neuronal progenitors [19–22]. Cend1 is a marker for cells committed to the neuronal lineage during embryonic neurogenesis in the cortex and spinal cord [19, 20]. During embryonic development, Cend1 is detectable in NPCs when they generate neurons, whereasit is not detectable in progenitor cells during gliogenesis [19]. Interestingly, Cend1 expression is upregulated in differentiated neurons [19], and this appears to be functionally relevant. Gain- and loss-of-function approaches in vitro and in vivo demonstrated that Cend1 participates in the molecular networks activated by proneural genes to induce cell cycle exit and differentiation of neuronal progenitors both during embryonic spinal cord development and postnatal SVZ neurogenesis [21, 22]. In this study, we were interested to explore whether Cend1 would influence the differentiation of embryonic mouse cortical NPCs into neurons after transplantation in a mouse model of brain injury. We report that genetically modified NPCs overexpressing Cend1 with the help of a lentiviral vector show increased neuronal differentiation in vitro and in vivo and generate a larger proportion of neuronal cells after grafting, as compared to control NPCs. Additionally Cend1 overexpressing NPCs reduce astroglial scar formation around the injury site. The observed effects of Cend1 overexpression in grafted NPCs may be beneficial for repair of brain lesions.

Materials and Methods

Embryonic Neurosphere Cultures and Lentiviral Transduction

Embryonic neurospheres (NPCs) were derived from the cortical ventricular zone (VZ) of E14.5 wild-type C57BL/6J mice or transgenic C57BL/6J mice expressing the green fluorescent protein GFP (GFP-NPCs) under the control of the ubiquitous β-actin promoter (actin-GFP mice; Jackson Laboratories, Bar Harbor, ME, http://www.jax.org) as previously described [23]. The VZ was dissected and mechanically dissociated, and the resulting single-cell suspension was placed in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with N2 (500 μl/50 ml; Gibco, Grand Island, NY, http://www.invitrogen.com), insulin (20 μg/ml; Sigma, St. Louis, http://www.sigmaaldrich.com), recombinant human basic fibroblast growth factor (bFGF, 20 ng/ml; R&D Systems Inc., Minneapolis, http://www.rndsystems.com), and epidermal growth factor (EGF, 20 ng/ml, R&D Systems). After 7 days in culture, free-floating neurospheres were redissociated and allowed to reform spheres at least four times before further use. For proliferation/differentiation studies, whole-mount or dissociated neurospheres were plated onto poly-L-lysine coated coverslips and further cultured for 3 days in the presence (proliferation assessment) or absence (differentiation assessment) of bFGF/EGF.

The lentiviral vector Trip.Cend1 used for Cend1 expression under the control of the cytomegalovirus (CMV) promoter and the respective control Trip.GFP vector driving GFP expression have been previously described [22]. Vector particles were produced and propagated as previously [22]. Viral titer of nonconcentrated supernatant collected from the producer cells ($4\text{-}5 \times 10^6$ TU/ml) was estimated in 3T3 cells as described [24]. For lentiviral transduction, fourth-passage neurospheres were single-cell dissociated, grown in suspension for 2-3 days, and then exposed for 7 h to nonconcentrated Trip.Cend1 or control Trip.GFP viral supernatants. Transduction efficiency was estimated by quantitative polymerase chain reaction (qPCR) (described in supplemental online Materials & Methods) as the number of vector copies present in transduced cells before and after serial passages of neurosphere cultures, as well as by immunocytochemistry as the percentage of cells with transgene Cend1 expression. Transgene Cend1 expression was found in $72.0 \pm 2.5\%$ of cells 3 days after transduction and persisted in $52.0 \pm 8.0\%$ of cells in the following two passages. Estimation of viral copy number using a unique sequence within the pTrip vector indicated that 4-5 viral copies per cell were present 3 days after transduction, while viral integrations were stabilized to 1-2 copies per cell after 1 and 2 subsequent passages.

Traumatic Brain Injury and Transplantation

All animals were handled according to European Union regulations and ethical policies along with the Institutional guidelines. Female C57BL/6J mice 2-3 months old were deeply anesthetized intraperitoneally with Imalgene (100 mg/ml, 0.03 ml/20g of body weight; Merial, Harlow, Essex, U.K., http://www.merial.com) and Rompun (20 mg/ml, 0.005 ml/20 g of body weight; Bayer Health Care, Leverkusen, Germany, http://www.bayerhealthcare.com) and positioned in a stereotaxic frame. A burr hole was drilled at coordinates AP 1.0, L 1.75 (relative to Bregma = 0) using a dental drill, and stab wound injury was caused to the right hemisphere of the cerebral cortex by inserting a 26-gauge needle 1.0 mm deep from the brain surface (DV 1.0 relative to Bregma = 0) taking care not to injure the subcortical white matter. The needle was then retracted and reinserted twice. Immediately after injury, 1 μl of freshly dissociated GFP-NPCs from actin-GFP mice (10^5 cells), either transduced with Trip.Cend1 (Trip.Cend1-GFP-NPCs) or nontransduced (GFP-NPCs), was injected 0.3 mm ventrally to the injury site using a 1-μl syringe with a 26-gauge needle (SGE; Syringe perfection). Cells were injected slowly over 5 minutes, the syringe was left in place for an extra 5 minutes and then withdrawn gently, and the skin was sutured. After surgery, mice were held on a heated cushion before being returned to their home cages. Lesioned animals received 5-bromo-2-deoxyuridine (BrdU, 1 mg/ml; Sigma) in their drinking water during survival time [25] and were sacrificed 1 or 4 weeks after the operation. Each group of mice grafted with either Trip.Cend1-GFP-NPCs (Trip.Cend1-GFP group) or GFP-NPCs (control-GFP group) consisted of two series of six animals (six animals per time point). Another series consisting of four animals per group was sacrificed 1 day after the operation. Finally, an extra series consisting of three animals per group was sacrificed 4 weeks after the operation and was used to determine the extent of astrogliosis around the injury site.

Quantification and Statistical Analysis

Using a Zeiss Axiophot photomicroscope, the degree of neuronal or glial differentiation and the proportion of proliferating cells in vitro was determined by calculating the percentage of cell-type-specific marker-positive cells out of the total number of cells per visual field at 40× magnification. At least three independent experiments carried out in duplicate coverslips and at least 1,000 cells per marker and per coverslip were analyzed, followed by statistical analysis using the two-tail Student's t-test with $p < .05$ for significance.

For quantification of the degree of proliferation or apoptosis as well as neuronal or glial differentiation of grafted NPCs in vivo, immunostained sections were analyzed using a Leica TCS SP confocal laser scanning microscope. Confocal images of immunofluorescence labeling were obtained using a 40× lens and constant image acquisition procedures. The red or blue channel of the images, corresponding to cell type-specific marker immunolabeling, was used for computer-assisted analysis in combination with the green channel corresponding to GFP-fluorescence of the grafted cells, utilizing the Image-Pro Plus image analysis software. Only the marker-positive fluorescence overlapping with the green GFP signal was taken into account. The ratio of marker fluorescence overlapping with the GFP signal over the total GFP signal was calculated and used as an estimate of the percentage of marker$^+$/GFP$^+$ grafted cells out of the total number of GFP$^+$ grafted cells.

The extent of astrogliosis in the lesioned hemisphere was measured 4 weeks after transplantation in the two groups of animals (Trip.Cend1-GFP and control-GFP groups) as follows. All sections of the right hemisphere encompassing the lesioned area along the rostro-caudal axis (in total nine vibratome sections 40-μm thick per animal), for a total number of three animals per group, were immunostained using antibodies to glial fibrillary acidic protein (GFAP). Confocal images of all GFAP-immunostained sections were obtained using a 10× lens and constant image-acquisition procedures. The red channel, corresponding to GFAP immunofluorescence, was then used for computer-assisted analysis with the help of the Image-Pro Plus image-analysis software. The GFAP$^+$ area around the injury site was marked, taking care not to include the subcortical white matter, and GFAP-fluorescence was quantified within this area. In all transplantation experiments, statistical analysis was performed by the Student's *t*-test between the two groups, with $p < .05$ for significance.

Immunohistochemistry, Western Blot Detection, qPCR, Astrocyte /Neurosphere Co-Culture Assay, and Rotarod Test

Detailed methods are presented in supplemental online Materials and Methods.

Results

Effect of Cend1 Overexpression in NPCs in Culture

Cend1 Attenuates Proliferation and Enhances Neuronal Differentiation of NPCs In Vitro. To investigate the effect of Cend1 overexpression on embryonic NPC proliferation and differentiation in vitro, we used neurosphere cultures derived from E14.5 mouse cortex. These cells self-renew when cultured in the presence of bFGF and EGF, either as free-floating neurospheres or after plating as single cells, and can differentiate into neurons, astrocytes, and oligodendrocytes upon growth factor withdrawal. For conferring Cend1 overexpression, fourth passage neurosphere cultures prepared from C57BL/6J mice were dissociated, grown in suspension for another two days, and transduced with the Trip.Cend1 lentiviral vector, driving the expression of the full length porcine Cend1 cDNA under the control of the CMV promoter [22]. Control cultures were transduced with the respective Trip.GFP vector driving GFP expression. Cend1 overexpression in embryonic neurospheres was verified by immunofluorescence labeling (Fig. 1A) and Western blot analysis (Fig. 1B). Quantification of the intensity of the produced Cend1 protein bands, relative to β-tubulin, revealed a 6.5-fold increase in Cend1 protein levels in Trip.Cend1- as compared to control Trip.GFP-transduced spheres (Fig. 1B). For transplantation experiments, we prepared neurosphere cultures from transgenic C57BL/6J actin-GFP mice, in which GFP is constitutively expressed under the β-actin promoter. Neurospheres from actin-GFP mice were transduced with the Trip.Cend1 vector, whereas nontransduced GFP-spheres served as their respective controls (Fig. 1C, 1D). The proliferation/differentiation properties of Trip.Cend1-transduced neurospheres from wild-type and actin-GFP mice were indistinguishable, as were the properties of the two control neurosphere types (data not shown).

Assessment of cell proliferation in Trip.Cend1- and Trip.GFP-transduced cultures was performed in neurospheres dissociated to single cells, plated onto poly-L-lysine coated coverslips, and cultured for a further 3 days in the presence of growth factors. Estimation of the percentage of BrdU$^+$ cells out of the total number of DAPI$^+$ nuclei revealed a significant reduction in BrdU incorporation in Trip.Cend1-transduced cultures as compared to Trip.GFP-transduced cultures (21.1 ± 0.2% vs. 28.9 ± 1.8%, $p < .05$; Fig. 1E). This observation was verified using antibodies to the mitosis marker pH3 (Fig. 1C) and measuring the percentage of pH3$^+$ cells out of the total number of DAPI$^+$ nuclei (1.1 ± 0.4% in Trip.Cend1-transduced vs. 2.9 ± 0.3% in Trip.GFP-transduced cultures, $p < .05$, Fig. 1E). Additionally, the percentage of nestin$^+$ cells was decreased by 1.8-fold (Fig. 1D, 1E, $p < .05$) in Trip.Cend1- as compared to Trip.GFP-transduced cultures, indicating that, upon Cend1 overexpression, a larger fraction of cells lost their stem/precursor cell properties. To assess if apoptosis contributed to the observed effects of Cend1 overexpression in NPCs, we performed immunofluorescence labeling for activated Caspase-3. We found a significant decrease in the fraction of cells undergoing apoptosis in Trip.Cend1 cultures as compared to Trip.GFP cultures (4.6 ± 0.5% vs. 7.2 ± 0.6%, $p < .01$). The reduced susceptibility of Cend1-overexpressing NPCs to apoptosis is in agreement with the already documented anti-apoptotic function of Cend1 in neuroblastoma cells [26].

We next investigated the implication of Cend1 in the differentiation program of embryonic cortical NPCs by analyzing lentivirally transduced NPCs with regard to their fate choice. Immunofluorescence labeling of whole-mount neurospheres allowed to differentiate for 3 days in the absence of growth factors revealed that, upon forced expression of Cend1, the neuronal marker βIII-tubulin was qualitatively upregulated (Fig. 2A), whereas the astrocytic and oligodendroglial markers GFAP and O4, respectively, were downregulated as compared to control spheres (Fig. 2C, 2D). For quantification, measurements were performed in dissociated neurospheres cultured for 3 days and 1 week without growth factors. Immunofluorescence analysis after 3 days in culture demonstrated a statistically significant 2.5-fold increase in the percentage of βIII-tubulin$^+$ cells in cultures transduced with Trip.Cend1 as compared to Trip.GFP-transduced cultures (12.8 ± 1.6% vs. 5.1 ± 0.7%, $p < .01$, Fig. 2B). On the other hand, the percentage of GFAP$^+$ cells was significantly decreased from 26.1 ± 1.1% in Trip.GFP-transduced cultures to 17.4 ± 2.2% in Trip.Cend1-transduced cultures ($p < .05$) (Fig. 2E), whereasthe decrease in O4$^+$ cells was not significant (Fig. 2F). Similar results were obtained when cultures were analyzed after 1 week in differentiating conditions (supplemental online Fig. 1). In this case also, a statistically significant 2.0-fold increase in the percentage of βIII-tubulin$^+$ cells was noted in Trip.Cend1- as compared to Trip.GFP-transduced cultures (13.9 ± 0.9% vs. 6.8 ± 1.7%, $p < .01$, supplemental online Fig. 1A, 1B), whereasthe levels of O4$^+$ cells remained similar in both groups of cells (supplemental online Fig. 1E, 1F). Although overall a larger percentage of cells had differentiated to GFAP$^+$ astrocytes after 1 week in culture as compared to 3 days, the difference between Trip.Cend1- and Trip.GFP-transduced cultures was maintained (42.9 ± 2.8% vs. 63.8 ± 3.5%; Supplemental Fig. 1C, 1D). These experiments indicate that, similarly to early postnatal age [22], in vitro Cend1 overexpression in embryonic NPCs can enhance their differentiation towards a neuronal phenotype.

Transplantation of Cend1-Overexpressing NPCs in the Injured Mouse Cortex

Experimental Model of Traumatic Brain Injury. We next proceeded to investigate whether NPCs transduced to overexpress Cend1 retain their ability for enhanced neuronal differentiation in the complex environment of the lesioned brain. To this end, we generated a stab wound injury in the motor

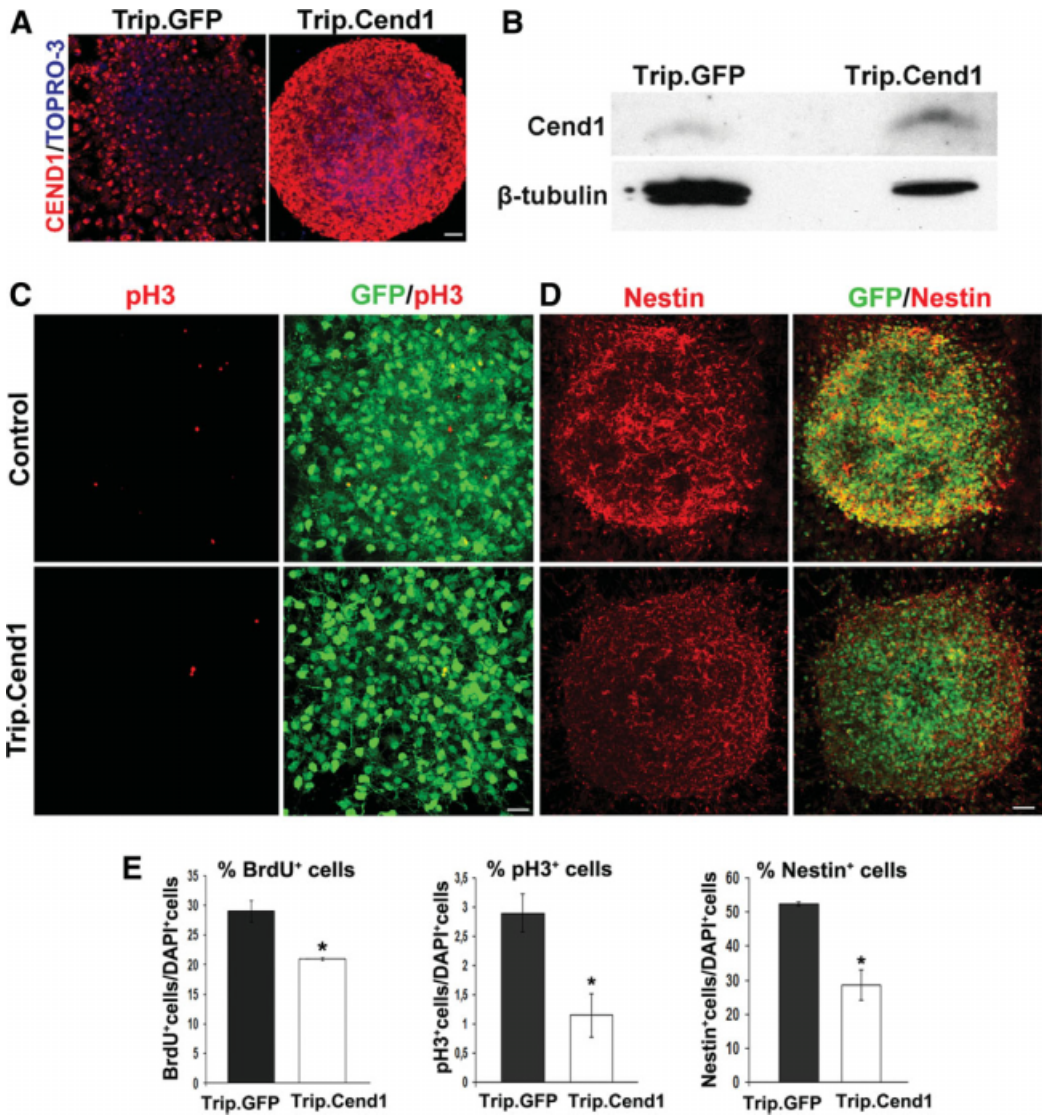

Figure 1. Effect of Cend1 overexpression on proliferating embryonic NPCs. (**A**): Immunofluoresence labeling and confocal analysis of whole-mount embryonic cortical neurospheres showing expression of Cend1 protein (red), 3 days after transduction with the Trip.GFP or Trip.Cend1 lentiviral vector, as indicated. TOPRO-3 (blue) was used for nuclear staining. Overexpression of Cend1 is evident in the Trip.Cend1-transduced spheres. Scale bar = 40 μm. (**B**): Western blot analysis of Cend1 protein expression in Trip.GFP-transduced neurospheres, as well as in neurospheres transduced with Trip.Cend1. β-tubulin was used for normalization of protein levels. (**C**): Expression of the mitotic marker pH3 (red) in whole-mount control and Trip.Cend1-transduced neurospheres derived from actin-GFP mice, cultured in the presence of growth factors. The number of pH3$^+$ cells is decreased upon Cend1 overexpression. Scale bar = 20 μm. (**D**): Immunofluorescence labeling for the NPC marker nestin (red) in whole-mount GFP-neurospheres prepared from actin-GFP mice, either nontransduced (control) or transduced with the Trip.Cend1 vector, and cultured under proliferative conditions in the presence of growth factors. Qualitatively, a decrease in nestin-immunofluorescence (red) is apparent upon Cend1 overexpression. GFP fluorescence is in green. Scale bar = 40 μm. (**E**): Graphs presenting the percentage of BrdU$^+$, pH3$^+$, and nestin$^+$ cells in Trip.GFP- or Trip.Cend1-transduced neurospheres prepared from wild-type C57BL/6J mice. Measurements were effectuated in dissociated neurospheres cultured for 3 days in the presence of growth factors. Estimation of the percentage of BrdU$^+$, pH3$^+$, or nestin$^+$ cells out of the total number of DAPI$^+$ nuclei revealed a statistically significant decrease in all three markers in Trip.Cend1-transduced as compared to Trip.GFP-transduced cells. Error bars represent SEM; *, $p < .05$; $n = 3$. Abbreviations: BrdU, 5-bromo-2-deoxyuridine; GFP, green fluorescent protein.

cortex of adult mice (Fig. 3A). Immediately after injury, NPCs derived from actin-GFP mice were transplanted 0.3 mm ventrally to the traumatized area just above the subcortical white matter. Examination of the lesioned area 1 day after injury revealed extensive cellular loss within the injury site as shown by lack of NeuN$^+$ neurons, in contrast to the widespread NeuN$^+$ immunoreactivity detected in the cortical parenchyma outside the lesion site (Fig. 3B). At this early time point, the expression of GFAP—an astrocytic marker highly expressed by reactive astrocytes invading the area around the injury site—was only evident in the host parenchyma surrounding the lesioned area, indicating an early response of astrocytes to the injury. Such GFAP$^+$ cells were absent in the contralateral uninjured cortex (Fig. 3E).

One week after injury, the expression of GFAP was markedly increased in the cortex of the traumatized hemisphere (Fig. 3D). Four weeks post-injury, GFAP$^+$ reactive astrocytes exhibited stronger immunoreactivity and a higher cellular density

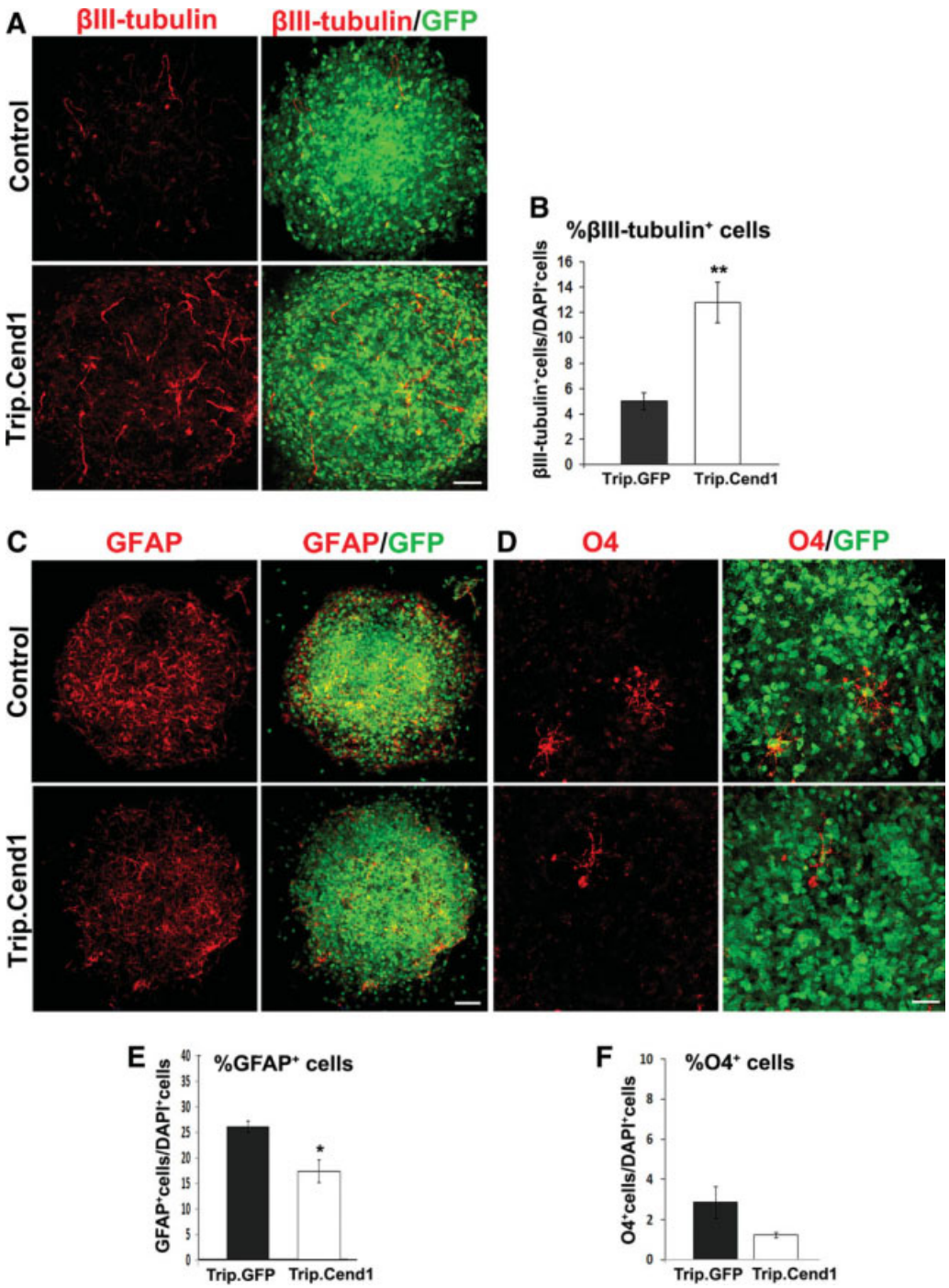

Figure 2. Qualitative and quantitative molecular phenotypic analysis of embryonic neural stem/precursor cells upon Cend1 overexpression, after 3 days of differentiation. (**A**), (**C**), and (**D**): Confocal images of whole-mount differentiating nontransduced (control) or Trip.Cend1-transduced GFP-neurospheres prepared from actin-GFP mice, immunostained for the indicated differentiation markers (red). GFP fluorescence is in green. Qualitatively, a marked increase in the number of βIII-tubulin$^+$ neurons (**A**) and a decrease in GFAP$^+$ astrocytes (**C**) was evident upon Cend1 overexpression whereas significant differences in O4$^+$ cells were not noticeable (**D**). Scale bar in (**A**), (**C**) = 40 μm and in (**D**) = 20 μm. (**B**), (**E**), and (**F**): Graphs presenting the quantitative changes in the percentage of βIII-tubulin$^+$ (**B**), GFAP$^+$ (**E**), and O4$^+$ (F) cells out of the total number of DAPI$^+$ nuclei, for each lineage marker, respectively. Measurements are from dissociated neurospheres derived from wild-type C57BL6/J mice transduced with either the Trip.GFP or Trip.Cend1 vector and cultured in the absence of growth factors. **, $p < 0.01$, *, $p < 0.05$; $n = 3$. The difference in the percentage of O4$^+$ cells was not significant. Abbreviations: GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein.

around the lesion site, allowing clear identification of the glial scar delimitating the traumatized area (Fig. 3D, 3E). Strong GFAP-immunoreactivity was also detected in the subcortical white matter of both the injured and uninjured hemispheres due to the presence of white matter astrocytes (Fig. 3E). At the same time, neuronal cells in the host cortex appeared immunopositive for NeuN (Fig. 3E) whereas endogenous expression of neuronal Cend1 was also detected (Fig. 3C).

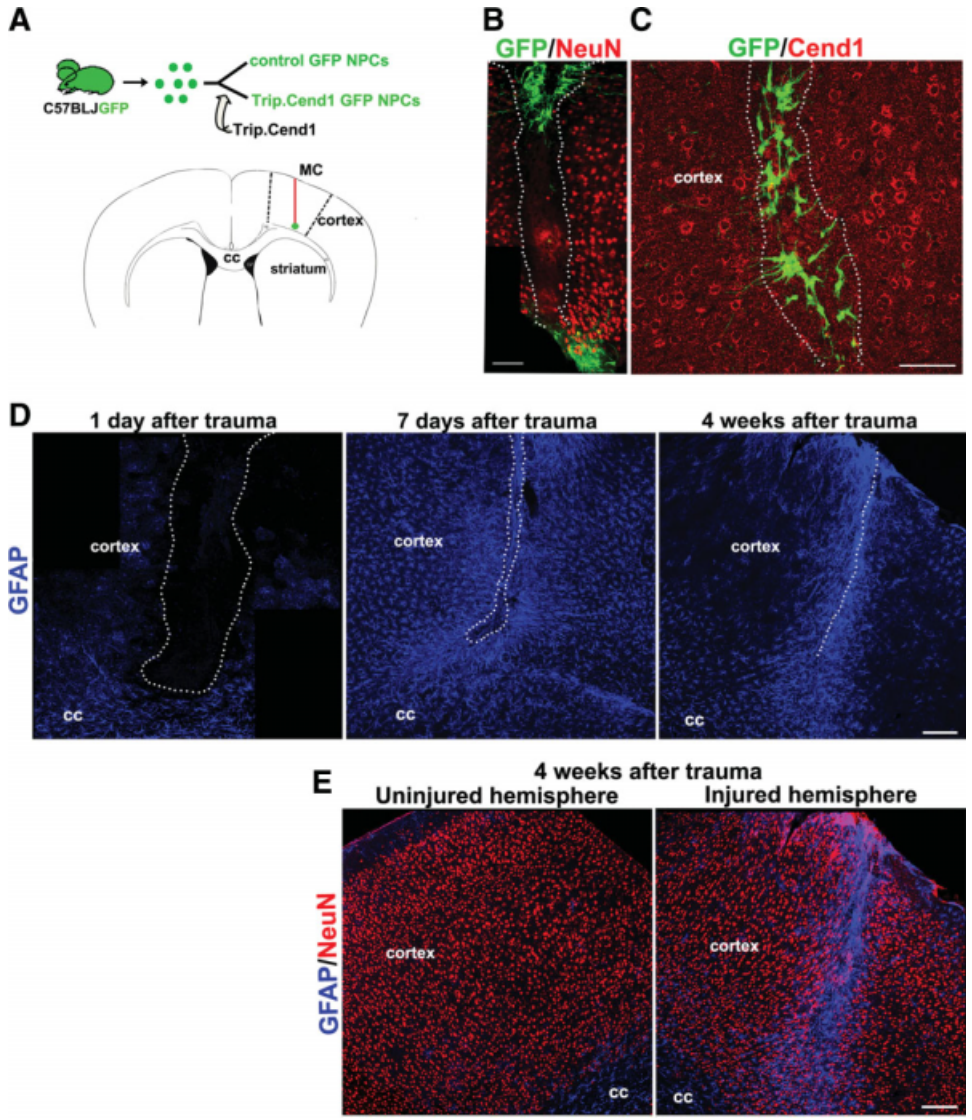

Figure 3. Traumatic brain injury and neural stem/precursor cell (NPC) transplantation. (**A**): Schematic representation of the production and viral transduction of NPCs from actin-GFP mice and diagram illustrating the injury site (red line) in the MC and NPC grafting (green dot). (**B**): Confocal image of a coronal section presenting the site of injury (between the white dotted lines) and grafted control GFP-NPCs (green), 1 day after injury. Note the lack of $NeuN^+$ neurons within the injury site whereas $NeuN^+$ immunoreactivity (red) is evident outside the lesion in the host cortical parenchyma. Scale bar = 80 μm. (**C**): Widespread expression of endogenous Cend1 (red) in the host neuronal cells outside the lesion site, 4 weeks after injury and transplantation of control GFP-NPCs (green). Scale bar = 40 μm. (**D**): GFAP expression levels (blue) around the injury site over time: 1 day, 7 days, and 4 weeks after injury. One day after injury, GFAP expression begins to be noticeable around the injury site, while 7 days post-injury, $GFAP^+$ cells are widely dispersed in the traumatized hemisphere; 4 weeks after injury, reactive astrocytes have formed an astrogliotic scar surrounding the injury site. Scale bar = 100 μm. (**E**): Confocal images of coronal sections of the injured and uninjured hemispheres 4 weeks after injury, immunostained with the astroglial marker GFAP (blue) and the neuronal marker NeuN (red). In the injured hemisphere, the lesioned site is clearly identified by the high expression of GFAP in reactive astrocytes forming the glial scar. Resident mature astrocytes in the cortical gray of the uninjured hemisphere do not express GFAP, whereas white matter astrocytes of both the injured and uninjured hemispheres are also $GFAP^+$. Note the loss of host $NeuN^+$ neurons in the traumatized area. Scale bar = 100 μm. Abbreviations: cc, corpus callosum; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; MC, motor cortex area.

Trip.Cend1-Transduced NPCs Generate More Neurons and Less Glial Cells After Transplantation in the Lesioned Cortex. NPCs destined for transplantation were prepared from actin-GFP mice and transduced with the Trip.Cend1 lentiviral vector (Trip.Cend1-NPCs) while nontransduced GFP-NPCs, possessing the same in vitro characteristics as the Trip.GFP-transduced cells, served as controls. Two groups of animals were generated and analyzed: the Trip.Cend1-GFP group and the control GFP-group. Survival and migration of grafted cells was similar in the two groups. Transplanted cells migrated towards the lesion and were found within the injury site 1 week post-injury (Fig. 4B). Practically no grafted cells were detected outside the lesioned area up to 4 weeks. At this time point, the lesion extended over a distance of approximately 360 μm along the rostro-caudal axis, and this value was highly reproducible among operated animals. Cend1 overexpression was evident by immunofluorescence in the transplanted Trip.Cend1-GFP-NPCs as compared to GFP-NPCs (Fig. 4A). To check the proliferation potential of the grafted cells, lesioned animals received BrdU in their drinking

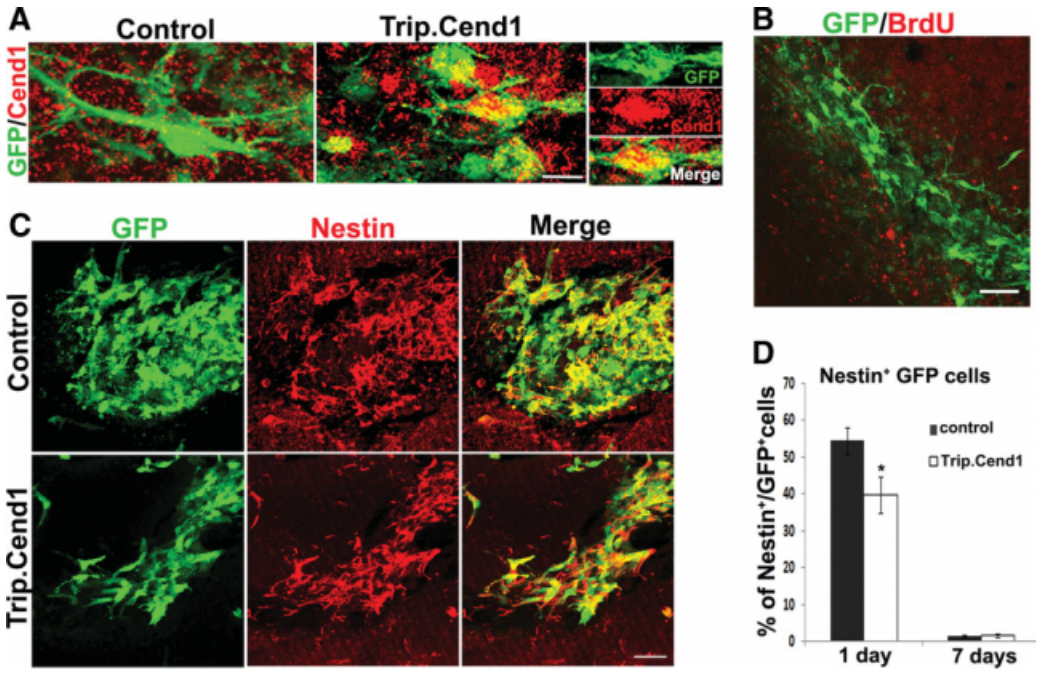

Figure 4. Cend1 expression, BrdU incorporation, and nestin immunoreactivity in grafted neural stem/precursor cells (NPCs). (**A**): Confocal images of coronal sections immunostained for Cend1 (red) 7 days post-injury and transplantation. Cend1 is barely detectable in control GFP-NPCs, whereas transgene Cend1 expression is discernible in Trip.Cend1-GFP NPCs. Scale bar = 20 μm. (**B**): Illustration of BrdU incorporation (red) in a coronal section of the adult mouse cortex 7 days after injury and transplantation of control GFP-NPCs (green). No BrdU incorporation was observed in the grafted cells, in contrast to the BrdU immunolabeling of host cells around the lesioned area. Scale bar = 40 μm. (**C**): Immunofluorescence labeling of coronal sections of the injured cortex for the NPC marker nestin (red), 1 day after transplantation of either control GFP-NPCs or Trip.Cend1-GFP-NPCs (green), as indicated. Both types of grafted cells express nestin at this time point. Scale bar = 40 μm. (**D**): Quantification of the percentage of nestin$^+$ cells out of all grafted GFP$^+$ cells, 1 and 7 days after transplantation. At 1 day, more than half of control GFP-NPCs and a significant smaller fraction of Trip.Cend1-GFP NPCs expressed nestin (*, $p < 0.05$). Seven days after transplantation, the expression of nestin in the transplanted cells was drastically reduced with no statistically significant differences between the two groups. Abbreviations: BrdU, 5-bromo-2-deoxyuridine; GFP, green fluorescent protein.

water during the whole period of their survival after transplantation. No GFP-NPCs were found to incorporate BrdU in either the control (Fig. 4B) or the Cend1-overexpressing group (not shown) at any time point tested (1 day, 1 week, or 4 weeks after grafting). However, BrdU incorporation was evident in the host parenchyma, especially within the glial scar formed around the lesion site (Fig. 4B). Therefore, transplanted NPCs did not proliferate, at least for the first 4 weeks, thus excluding the possibility that these cells could form tumors.

To investigate whether transplanted NPCs retained their neural stem/precursor cell identity, the expression of the NPC marker nestin was determined in the two groups. One day after transplantation both Trip.Cend1-GFP-NPCs and control GFP-NPCs largely expressed nestin (Fig. 4C), but its expression was almost extinct at 1 week, and by 4 weeks nestin immunoreactivity was no longer detectable in the transplanted cells in either group. In agreement with the in vitro data, quantification at 1 day postgrafting showed a lower percentage of nestin$^+$ cells among all GFP$^+$ cells in the Trip.Cend1-GFP group as compared to the control GFP-group (37.2 $\pm$ 3.5% vs. 54.4 $\pm$ 3.8%, $p < .05$; Fig. 4D). By 7 days after transplantation, the percentage of nestin$^+$ cells among all GFP$^+$ cells was dramatically reduced with no significant difference between the two groups (Fig. 4D). These results indicate that, after engraftment in the lesioned cortex, NPCs cease to proliferate and gradually lose their immature neural stem/precursor cell state.

We then compared the differentiation properties of the grafted cells in the two groups of animals. Immunofluorescence analysis revealed that transplanted cells in both groups did not express neuronal or glial differentiation markers 1 day or 7 days postgrafting (data not shown). By 4 weeks, the grafted cells appeared largely differentiated with well developed processes. A fraction of grafted cells in both animal groups expressed the astrocytic marker GFAP (Fig. 5A), whereasa smaller fraction expressed the oligodendrocyte precursor marker NG2 (Fig. 5C). Quantification revealed a significantly lower percentage of GFAP$^+$ cells out of all GFP$^+$ cells in the Trip.Cend1-GFP group as compared to the control GFP-group (25.7 $\pm$ 2.6% vs. 42.5 $\pm$ 3.3%, $p < .01$; Fig. 5B). Similarly, the proportion of NG2$^+$ cells was smaller in the Trip.Cend1-GFP group as compared to the control GFP-group (6.0 $\pm$ 0.2% vs. 15.0 $\pm$ 1.7%, $p < .05$; Fig. 5D), whereas no grafted cells in either group expressed markers of more mature oligodendrocytes, such as O4 or proteolipid protein (PLP) (not shown). Interestingly, analysis of the expression of the neuronal marker NeuN revealed that the percentage of grafted cells that differentiated into neurons was significantly higher in the Trip.Cend1-GFP group as compared to control (Fig. 6A, 6B). Quantification indicated a 2.3-fold increase in the percentage of NeuN$^+$ cells out of all GFP$^+$ cells in the Trip.Cend1-GFP group as compared to the control GFP-group (19.5 $\pm$ 3.7% vs. 8.5 $\pm$ 2.8%, $p < .05$; Fig. 6E). We then asked if the grafted neurons differentiated into specific neuronal sub-types. Immunofluorescence analysis revealed that, upon Cend1 overexpression, a large fraction of the transplanted cells differentiated into interneurons expressing the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Fig. 6C). Quantification indicated a marked increase of GABA$^+$

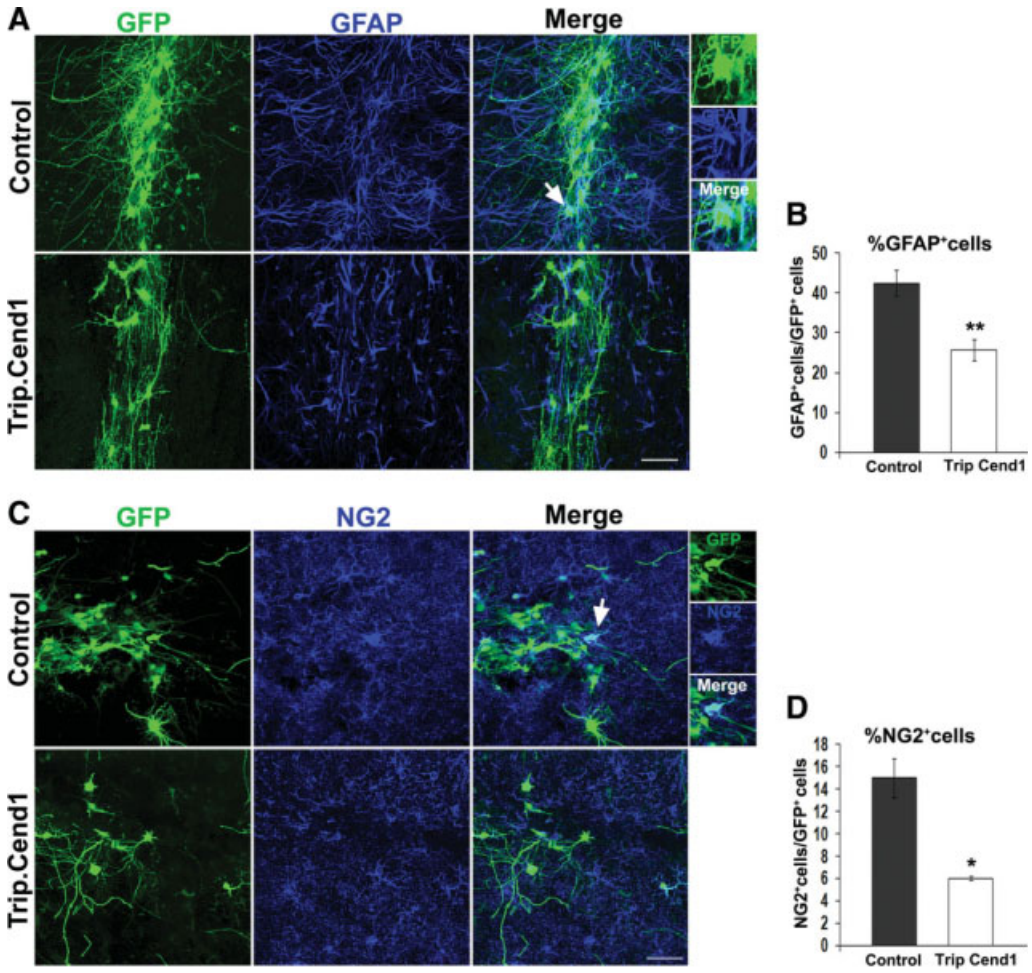

Figure 5. Cend1 overexpression interferes with glial differentiation of grafted neural stem/precursor cells. (**A**): Astroglial differentiation of grafted control GFP cells and Trip.Cend1-GFP cells (green) assessed by immunolabeling for the astrocyte marker GFAP (blue) followed by confocal analysis, 4 weeks after injury and transplantation. Arrow indicates a $GFAP^+/GFP^+$ cell also shown at higher magnification. Scale bar = 40 μm. (**B**): Quantification of the percentage of $GFAP^+$ cells out of all GFP^+ cells revealed a marked reduction in the fraction of graft-derived astrocytes upon Cend1 overexpression, 4 weeks after injury (**, $p < .01$). Notably, $GFAP^+$ host cells around the lesion site forming the astroglial scar were also more numerous in the control group as compared to the Trip.Cend1-GFP group. (**C**): Oligodendroglial differentiation of grafted control GFP cells and Trip.Cend1-GFP cells (green) assessed by immunolabeling for the oligodendroglial precursor marker NG2 (blue), 4 weeks after injury and transplantation. Arrow indicates a $NG2^+/GFP^+$ cell also shown at higher magnification. Host cells were also immunoreactive for NG2 with no obvious differences between the two groups. Scale bar = 40 μm. (D) Quantification of the percentage of $NG2^+$ cells out of all GFP^+ cells demonstrated a 2.5-fold reduction in the fraction of graft-derived oligodendroglial precursors upon Cend1 overexpression, 4 weeks after injury (**, $p < .01$). Error bars represent SEM. Abbreviations: GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein.

cells out of all GFP^+ cells in the Trip.Cend1-GFP group as compared to control (14.9 ± 1.8% vs. 2.7 ± 0.5%, $p < .01$; Fig. 6E). To estimate the percentage of $NeuN^+$ grafted cells that expressed GABA in both groups of animals, we performed double immunofluorescence labeling using antibodies to NeuN and GABA. Our results demonstrate that Cend1 overexpression drives 80.2 ± 2.3% of $NeuN^+$ cells to differentiate specifically to the GABAergic phenotype, whereas only 48.8 ± 7.7% of $NeuN^+$ grafted cells expressed GABA in the control group (Fig. 6D, 6F). On the other hand, no grafted cells in either group had differentiated to glutamatergic neurons as revealed by glutamate immunofluorescence (not shown), suggesting that the remaining $NeuN^+/GABA^-$ grafted cells probably represent neurons that have remained in a more immature stage. These results indicate that Cend1 overexpression efficiently increases by 5.5-fold the percentage of GABAergic neurons generated after NPC transplantation in the lesioned cortex.

Cend1 Overexpression Reduces Astrogliosis After Brain Injury. Traumatic brain injury is accompanied by the emergence of large numbers of reactive astrocytes around the lesion site. Astrocytes react to brain injury by hypertrophy of their somata and processes, increasing the synthesis of GFAP. To check the potential effect of Cend1 overexpression on astrogliosis, we measured the levels of GFAP expression around the injury site, 4 weeks after transplantation. To obtain reliable results representing the whole cortical area affected, we performed immunostaining using anti-GFAP antibodies in all sections along the rostro-caudal axis encompassing the traumatized area in three animals from each group. Quantification of the GFAP immunfluorescence signal in the astrogliotic area marked in each section revealed that astrogliosis was reduced by 40% when Cend1 overexpressing cells were transplanted as compared to control cells (47,568 ± 4,898 fluorescence arbitrary units/μm^2 vs. 80,038 ± 11,083, $p < 0.01$, Fig. 7A, 7C). Additionally, we observed that host astrocytes

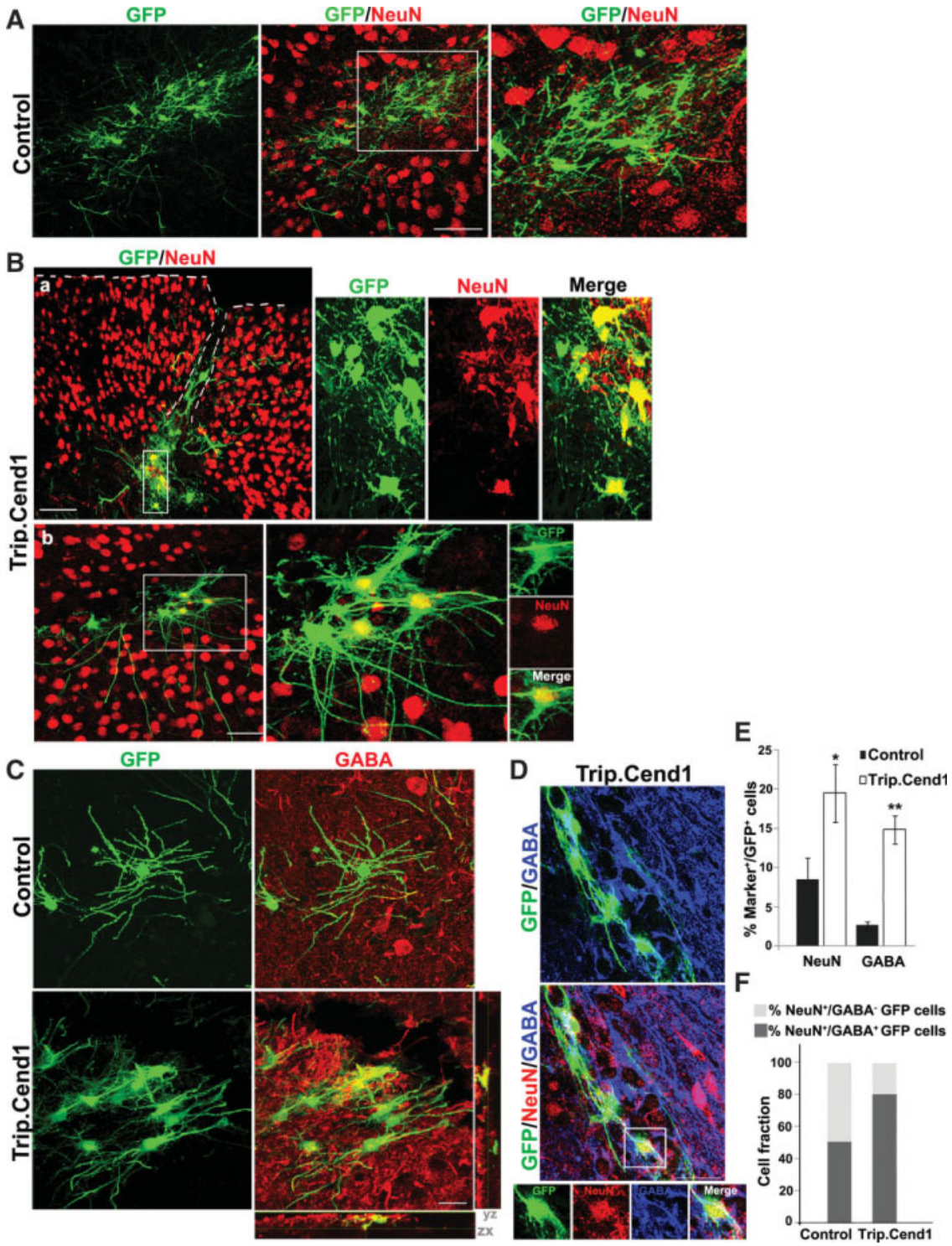

Figure 6. Cend1 overexpression promotes neuronal differentiation of grafted neural stem/precursor cells. (**A**): Expression of the neuronal marker NeuN (red) in grafted control GFP cells (green), 4 weeks after injury and transplantation, shows that hardly any grafted cells in the field differentiated into a neuronal phenotype. On the other hand, numerous host NeuN$^+$ neuronal cells were detected around the cellular graft. Scale bar = 40 μm. (**Ba, Bb**): Expression of NeuN in transplanted Trip.Cend1-GFP cells, 4 weeks after injury, demonstrating that a significant fraction of the grafted cells differentiated into neurons. Coronal sections from two animals immunostained for NeuN are presented. The framed areas in (**A**) and (**Ba, Bb**) are also shown at higher magnification. Scale bar = 80 μm for (**Ba**); 40 μm for (**Bb**). (**C**): Expression of the neurotransmitter GABA (red) in grafted control GFP cells and Trip.Cend1-GFP cells (green), 4 weeks after injury and transplantation. Extensive differentiation of the grafted cells towards GABAergic neurons was noted upon Cend1 overexpression. Colocalization is verified in orthogonal sections. Scale bar = 20 μm. (**D**): Double immunofluorescence labeling of grafted control GFP cells and Trip.Cend1-GFP cells (green), 4 weeks after injury and transplantation, using antibodies to NeuN (red) and GABA (blue). Cend1 overexpression leads to extensive differentiation of NeuN$^+$ grafted cells to GABAergic neurons. The framed area is presented at higher magnification. (**E**): Quantification of the percentage of NeuN$^+$ or GABA$^+$ cells out of all GFP$^+$ cells 4 weeks after injury. (**F**): Quantification of the percentage of GABA$^+$ and GABA$^-$ cells out of NeuN$^+$ grafted cells 4 weeks after injury. (*, $p < 0.05$, **, $p < 0.01$). Abbreviations: GABA, γ-aminobutyric acid; GFP, green fluorescent protein.

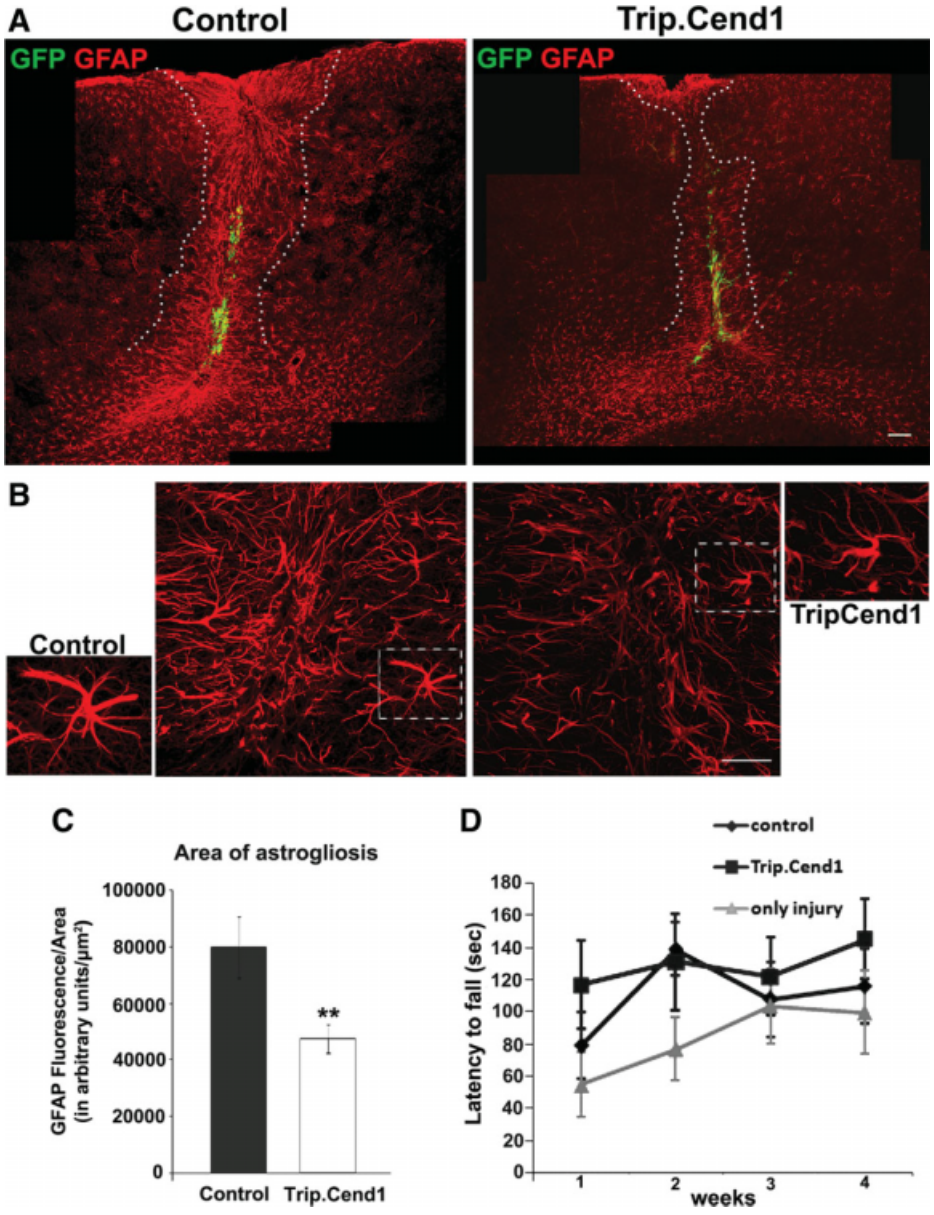

Figure 7. Cend1 overexpression in grafted neural stem/precursor cells reduces astrogliosis. (**A**): Coronal sections of the traumatized hemisphere immunostained for GFAP (red), 4 weeks after transplantation of either control GFP cells or Trip.Cend1-GFP cells (green), followed by confocal analysis. The GFAP$^+$ area contained within the white dotted lines corresponds to the astroglial scar. Note that both the area of astrogliosis as well as fluorescence intensity of the red channel corresponding to GFAP immunoreactivity are reduced upon Cend1 overexpression, as shown in representative sections. (**B**): Larger magnification of GFAP expression within the glial scar formed in the injured hemisphere after transplantation of either control GFP cells or Trip.Cend1-GFP cells. The GFAP$^+$ cells formed a denser network and were more hypertrophic in the control as compared to the Trip.Cend1 group. (**C**): Quantification of astrogliosis, as measured by GFAP fluorescence intensity/μm^2, deduced from all nine coronal sections (40-μm thick) containing the injury site along the rostro-caudal axis in three animals from each group. A significant decrease in the extent of astrogliosis upon Cend1 overexpression was noted (**, $p < 0.01$). (**D**): Motor coordination assessed on the rotarod behavioral task revealed no statistically significant differences among mice that received control GFP cells or Trip.Cend1-GFP cells or a third group that received no cell transplantation. Abbreviations: GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein.

forming the astroglial scar in the control group were present at a higher cellular density around the lesion site and appeared more hypertrophic as compared to the Trip.Cend1-GFP group (Fig. 7B; also compare the density of GFAP-labeled cells in Fig. 5A between the control GFP group and the Trip.Cend1-GFP group). However, no difference in the density or morphological characteristics of the host NG2$^+$-expressing glial progenitors around the injury site or within the subcortical white matter was observed between the injured and uninjured hemisphere (supplemental online Fig. 2), as well as between the two groups of transplanted NPCs (not shown). These results indicate that Cend1 restricts the transformation of host astrocytes to reactive astrocytes and, therefore, limits glial scar formation around the traumatized area.

As a first step to explore astroglial scar reduction following Cend1 overexpression, we used an in vitro co-culture assay, where whole mount neurospheres from actin-GFP mice transduced with either Trip.Cend1 or Trip.GFP (supplemental online Fig. 3) or nontransduced spheres (not shown) were placed onto a monolayer of astrocytes and were further co-cultured for 3 days. Control spheres either transduced with Trip.GFP or nontransduced behaved identically. Interestingly, we observed an obvious reduction in the density of GFAP$^+$ astrocytes surrounding Cend1-overexpressing neurospheres as compared to control spheres (supplemental online Fig. 3A). This was accompanied by a reduction in the percentage of Cyclin D1$^+$ cells out of all DAPI$^+$ cells within the astrocytic monolayer surrounding Cend1-overexpressing spheres (supplemental online Fig. 3B, 3C). These results point to a non-cell autonomous Cend1 function that impacts on astrocyte proliferation.

Motor Co-Ordination of the Operated Animals. Operated animals of both groups exhibited no obvious motor defects related to their walking, climbing, or feeding abilities, most probably due to the mild severity of the inflicted injury in the motor cortex area. We nevertheless explored the potential

effects of the injury and NPC transplantation in the more sophisticated task of motor coordination, using a rotarod behavioral test. Three groups of animals were analyzed: the Trip.-Cend1-GFP group, the control GFP-group, and an additional group of animals that received no cell transplantation. Before injury, all animals were pretrained and baseline measurements were determined at constant rates of 16 and 32 rpm. After injury, the performance of mice was recorded at 16 and 32 rpm once a week over 4 weeks. At 16 rpm, no difference was observed in the latency to fall among the three groups of mice (not shown). At 32 rpm, mice that received cell grafts of either type appeared to be doing slightly better than the group that received no cells, especially at 2 weeks; nevertheless, the differences in the values recorded were not statistically significant (Fig. 7D).

Discussion

In this study, we have used a lentivirus-based gene transfer system [22] to overexpress Cend1, a neuronal lineage specific regulator that coordinately induces cell cycle withdrawal and differentiation of neuronal progenitors, in embryonic cortical neural stem/precursor cells. We have shown that Cend1 enhances the differentiation of NPCs into neurons at the expense of astrocytes, most prominently in vivo after transplantation in a mouse model of cortical injury. The higher proportion of Cend1-overexpressing cells, as compared to control cells, differentiating into neurons in the lesioned cortex coincides with an overall increase in the generation of graft-derived GABAergic interneurons. Although Cend1 overexpression in vivo did not influence the proliferation and migration of the grafted cells, it exerted a non-cell autonomous effect on the surrounding cortical area, by restricting the extent of the astrogliotic scar formed around the injury site. This was an unexpected finding, suggesting for the first time that, in addition to its previously noted cell-autonomous function [21, 22], Cend1 can also act in a non-cell autonomous fashion. Consequently, the effect of Cend1 overexpression was not limited to the differentiation properties of the transplanted cells to generate a larger fraction of graft-derived neurons, but was extended to the surrounding host parenchyma to restrict astrogliosis. Because of the lack of an apparent functional deficit of the injured mice, we were not able to deduce whether transplantation of Cend1-overexpressing NPCs could have a potential benefit in functional recovery. Nevertheless, the unilateral cortical injury model that we generated proved to be a mild severity model that was particularly useful for anatomical analysis because of its simplicity and high reproducibility, allowing assessment of the effects of Cend1 overexpression by morphological criteria.

Cend1 Promotes Neuronal Differentiation of Grafted NPCs

Previous studies have shown that Cend1 is present in neural precursors during the time window in which they generate neurons, whereasit is downregulated when they shift from a neurogenic to a gliogenic potential [19, 20]. Cend1 is also expressed in young and mature neurons, thus marking the different neuronal lineage stages from precursor to mature cell [19, 20]. As Cend1 expression is low in neuronal precursors and higher in differentiated neurons [19, 20, 27], we have previously investigated whether this is a functionally relevant observation. Gain- and loss-of-function studies in the developing neural tube [21] and in postnatal SVZ-derived neural precursor cells [22], as well as our present studies in embryonic cortical neurospheres, demonstrate that Cend1 levels are important for controlling proliferation versus differentiation decisions in neural precursors. Previous studies have shown that the negative influence of Cend1 on cell proliferation is mediated through the p53/cyclin D1/pRb signaling pathway that controls the balance between cell cycle progression and exit, whereasits neuronal differentiation-promoting activity involves downregulation of Notch signaling and activation of the proneural genes network [21, 22, 28]. In agreement, Cend1-overexpressing neurospheres are primed to a more differentiated phenotype, as the NPC marker nestin is downregulated in a larger fraction of these cells when compared to their respective control cells. Altogether, the functional properties of Cend1 are consistent with observations in this study, showing that transplantation of embryonic NPCs overexpressing Cend1 enhances the generation of neuronal cells in the injured mouse cortex.

Cend1 Reduces Astrogliosis

An interesting, potentially beneficial, effect of Cend1 overexpression in grafted cells was the significant reduction in astroglial scar formation observed 4 weeks after NPC transplantation. Astrocytes, NG2-expressing glial precursors, and microglia all take part in the gliotic response [29, 30]. Astrocytes react to brain injury by hypertrophy of their cell bodies and processes, increased synthesis of GFAP, and reexpression of the progenitor markers nestin and vimentin [31]. Cend1 overexpression in the grafted NPCs influenced the extent of astrogliosis by reducing the density of activated astrocytes present within the glial scar as well as the degree of hypertrophy of the activated astroglial cells. It is noteworthy that the effect of Cend1 was specifically targeted to the $GFAP^+$ cells forming the gliotic scar, whereasno effect was noted on the host $NG2^+$ glial precursors participating in glial scar formation [32]. The effect on astrogliosis, as well as the astrocyte/neurosphere co-culture data from this study, suggests a previously unexplored non-cell autonomous function for Cend1, which probably impacts on Cyclin D1-dependent regulation of astrocyte proliferative properties and possibly also on their survival, which deserves further study. Recent findings indicating that Cend1 is involved in regulation of stimuli-induced intracellular calcium mobilization may provide a link to explain the propagation of Cend1 action to the host cortical tissue [26].

Glial scar formation around the traumatized area has both beneficial as well as detrimental consequences after CNS insults. Initially it is instrumental for sealing off the injured tissue and restricting inflammation and neuronal death [29, 33], but at later stages it inhibits regeneration [34, 35]. Although early manifested reactive astrocyte activities have been associated with restricting tissue damage [33, 36–38], the astroglial scar which is eventually formed renders the injured tissue hostile to regeneration and repair [29, 39–42]. Current evidence suggests that different effects of reactive astrogliosis on overall outcome after brain insults are likely to be time- and context-dependent and regulated by different intracellular signaling pathways. Our results demonstrate that Cend1-overexpressing grafts attenuate astrogliosis 1 month after injury when the adverse effects of activated astroglia are likely to outnumber their positive contributions, without impinging on lesion size. As such, the reduction in astrogliosis observed in this study by Cend1 overexpression should contribute in enhancing regenerative processes leading to repair.

NPC Transplantation for Repair of Brain Lesions

Damage to the adult motor cortex can lead to severe and frequently irreversible deficits in motor function. Transplantation of embryonic cortical neurons into the adult motor cortex has been previously shown to result in partial reestablishment of cortical circuitry in an acute cortical aspiration lesion model [43], suggesting that there is potential for neural cell transplantation to promote reconstruction after brain injury. This and other studies [44–46] have demonstrated the potential of using NPC for repair of the injured brain and have set the stage for additional approaches involving NPC transplantation in combination with ex vivo gene transfer for expression of regeneration-promoting molecules in the lesioned CNS. So far, several studies have addressed the efficacy of transplanting embryonic stem cells preconditioned to restrict their differentiation to neural lineages as well as neural stem/precursor cells from the embryonic or adult CNS into animal models of brain and spinal cord injury [47]. Additionally, a number of molecules have been used to enhance the capacity of embryonic stem cells and NPCs to repair CNS damage in animal models of traumatic brain injury. These include trophic factors such as NGF [48], GDNF [49], EGF [50], and FGF2 [7], cytokines such as erythropoietin [51], and also cell adhesion molecules [52–55] and extracellular matrix proteins including tenascin-R [56]. In these paradigms, introduction of each individual factor resulted in improvement of either cell survival or migration or an increase in the differentiation potential of the transplanted cells towards a desirable pathway. In some cases, stimulation of the endogenous precursor pool of the host was also noted. It is likely that a combination of molecules with complementary functions, capable of promoting integration and migration of the grafted cells, as well as differentiation of both grafted and endogenous NPCs to repopulate and reconnect neuron-depleted brain regions, should have additive effects on brain repair. Cend1 promotes recovery after injury, as seen in this study at least by morphologically detectable beneficial effects, such as enhanced neuronal differentiation of the transplanted cells and attenuation of astrogliosis, and therefore is a good candidate for cell replacement approaches, especially if used in combination with other regeneration-promoting molecules.

Conclusion

In summary, our data show that overexpression of Cend1 in NPCs enhances differentiation of the transplanted cells into neurons and increases the fraction of graft-derived GABAergic interneurons in a mouse model of cortical trauma. Moreover, Cend1 overexpression reduces astrogliosis and may thus potentially promote regenerative processes. As there is no apparent functional deficit in our model, further studies should establish whether Cend1 overexpression contributes to functional recovery using models of acute and/or progressive neurodegeneration. Nevertheless our data suggest that Cend1 is a good candidate for structural repair after brain damage. Therefore, its use may have important implications for directing controlled differentiation of neural stem/progenitor cells towards the neuronal lineage in cell-replacement approaches, especially in those using a combination of molecules with regeneration-promoting properties.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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