SPINAL CORD INJURY

GDNF rescues the fate of neural progenitor grafts by attenuating Notch signals in the injured spinal cord in rodents

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Neural progenitor cell (NPC) transplantation is a promising strategy for the treatment of spinal cord injury (SCI). In this study, we show that injury-induced Notch activation in the spinal cord microenvironment biases the fate of transplanted NPCs toward astrocytes in rodents. In a screen for potential clinically relevant factors to modulate Notch signaling, we identified glial cell–derived neurotrophic factor (GDNF). GDNF attenuates Notch signaling by mediating delta-like 1 homolog (DLK1) expression, which is independent of GDNF's effect on cell survival. When transplanted into a rodent model of cervical SCI, GDNF-expressing human-induced pluripotent stem cell–derived NPCs (hiPSC-NPCs) demonstrated higher differentiation toward a neuronal fate compared to control cells. In addition, expression of GDNF promoted endogenous tissue sparing and enhanced electrical integration of transplanted cells, which collectively resulted in improved neurobehavioral recovery. CRISPR-induced knockouts of the DLK1 gene in GDNF-expressing hiPSC-NPCs attenuated the effect on functional recovery, demonstrating that this effect is partially mediated through DLK1 expression. These results represent a mechanistically driven optimization of hiPSC-NPC therapy to redirect transplanted cells toward a neuronal fate and enhance their integration.

INTRODUCTION

Traumatic spinal cord injury (SCI) produces devastating neurological deficits. SCI results in the loss of critical cell populations required to effectively transmit signals between the brain and body. More than half of all traumatic SCIs occur at the cervical level (C1 to C7). Those with cervical injuries suffer the most devastating neurological impairments, including complete dependency for self-care, and have the highest mortality rates. The few treatment options currently available demonstrate only modest functional improvements (*1*). However, emerging data suggests that cell transplantation therapies represent a potentially effective therapeutic intervention for SCI by repairing and regenerating injured neurons (*2*). Transplantation of neural progenitor cells (NPCs) is an exciting approach to regenerating the traumatically injured spinal cord (*3*–*5*), particularly when derived from translationally relevant human-induced pluripotent stem cells (hiPSC-NPCs), which hold the potential to be autologously derived for clinical trials (*2*). Despite recent progress in promoting the regeneration of the spinal cord through transplantation of different NPC types after injury, the degree of functional recovery obtained has been modest. This is due to several parameters that need to be optimized to improve the effect of NPC transplantation on functional recovery. One of these parameters is the hostile microenvironment of the injured spinal cord, which is not permissive for grafted NPCs. The perturbation of factors and extracellular matrix proteins in the injured spinal cord niche (*6*–*8*) not only reduces the survival of transplanted cells but also affects their differentiation, synaptic connectivity, and integration with host tissue (*9*).

In the current study, we demonstrate that Notch ligands are upregulated in the spinal cord microenvironment after injury, which biases differentiation of transplanted NPCs toward more astrocytes. The Notch signaling pathway is a highly conserved signal transduction pathway that controls cell fate decisions and the differentiation of NPCs. Notch signaling requires direct cell-cell contact, where upon activation by the ligands [delta-like protein 1 (Dll1) or Jagged1] expressed on neighboring cells, Notch is cleaved and releases a Notch intracellular domain (NICD) that translocates to the nucleus and regulates gene expression (*10*).

Although differentiation to astrocytes is important for regeneration and to improve functional recovery after transplantation (*11*–*13*), differentiation to myelinating oligodendrocytes (*3*) and different neuronal subtypes (*14*, *15*) that make synaptic connections with local neural networks is also needed to form new relay circuits and consequently to improve function (*14*, *16*). Graft-derived neurons can act as new interneurons between the injured axons and the denervated neurons located downstream of the lesion.

To enhance functional recovery, we examined the effect of modulating Notch signaling on transplanted cells to bias differentiation toward a neuronal lineage to promote neuronal relay formation and the integration of transplanted cells. In a screen for translationally relevant factors to be used to modulate elevated Notch signaling in the SCI niche and bias cells toward a more neuronal fate, we identified glial cell–derived neurotrophic factor (GDNF). To trigger downstream signaling events in the cells, GDNF binds to GDNF family receptor α1 (GFRα1), and then, the GDNF/GFRα1 complex recruits transmembrane receptors such as the receptor tyrosine kinase Ret or the neural cell adhesion molecule (NCAM) (*17*). GDNF has been shown to increase the survival of NPCs and regulate their differentiation

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toward neurons (*18*). GDNF is also involved in the development of different neuronal subtypes from progenitors during development (*19*, *20*). Although the mechanisms underlying the survival-promoting effect of GDNF on NPCs have been studied, the precise role of GDNF on NPC differentiation remains poorly understood.

We engineered hiPSC-NPCs to express and secrete GDNF and examined the effects of this expression on restoring their differentiation profile after transplantation into the injured spinal cord. We also assessed the effects of GDNF expression and the involvement of delta-like 1 homolog (DLK1) signaling on the functional recovery of rodents after a cervical level C6/C7 SCI. This model of SCI was chosen because it produces both the most common type of injury, a bilateral cervical contusion compression, and the severe upper and lower limb neurological deficits associated with human SCI (*21*). This model closely mimics the pathophysiology of human SCI and the harsh postinjury milieu. Phenotypically, these animals demonstrate impaired locomotor kinetics, sensory deficits, and profound forelimb/paw dysfunction, similar to patient presentation (*21*). The findings from this work support the role of GDNF in biasing the fate of NPC transplants to appropriately differentiate into cell lineages required to enhance spinal cord regeneration.

RESULTS

The injured spinal cord microenvironment biases the differentiation of transplanted NPCs toward more astrocytes

The injured spinal cord microenvironment is a hostile niche in which the expression of several differentiation factors is changed as compared to the naïve spinal cord niche (*8*). To examine the effect of microenvironment on the differentiation profile of hiPSC-NPCs, we compared the differentiation profile of transplanted hiPSC-NPCs in the spinal cord parenchyma of naïve rats at cervical level C6/C7 to hiPSC-NPCs that were transplanted into the clip-contusion SCI rat model injured at cervical level C6/C7. NPCs were differentiated from hiPSCs using dual SMAD inhibition method (*22*). Intraparenchymal transplant (5×10^4 cells/μl) was completed at 2 weeks after injury at two rostral and two caudal perilesional sites, each 2 mm from the epicenter and from midline. We used immunodeficient Rowett nude (RNU) rats to limit host rejection of xenografted human cells. All hiPSC-NPCs were green fluorescent protein (GFP) tagged for identification in vivo. When we transplanted hiPSC-NPCs into the spinal cord parenchyma of naïve rats, hiPSC-NPCs were able to differentiate to more neurons ($Fox3^+$ cells) and fewer astrocytes [glial fibrillary acidic protein–positive ($GFAP^+$) cells] at 8 weeks after transplant as compared to hiPSC-NPCs transplanted into the injured cervical spinal cord (neurons: 36.2 ± 6.5% in naïve as compared to 16.8 ± 5.1% in SCI; $P < 0.05$; astrocytes: 18.7 ± 5.1% in naïve as compared to 36.5 ± 5.7% in SCI; $P < 0.05$; Fig. 1, A and B). This effect is independent of the NPC line (fig. S1). This differentiation profile shift has been previously shown to perturb the expression of microenvironmental cell fate determinants after SCI (*8*), such as bone morphogenetic proteins (BMPs) (*23*, *24*), transforming growth factor–β (TGF-β) (*25*, *26*), and Notch-activating ligand Jagged1 (*27*), which have been shown to drive differentiation of NPCs toward an astrocytic fate. In the present study, we detected an increase in the mRNA expression of BMP4, TGF-β, and Jagged1 in the cervical spinal cord at 2 weeks after injury, which is equivalent to the time point for cell transplantation during the subacute phase of injury (Fig. 1C). However, these protein expression changes did not attain significance for BMP4 or TGF-β (fig. S2). We also observed an increase in immunoreactivity for Jagged1 in rat and human SCI tissue compared to noninjured spinal cord tissue (Fig. 1, D and E).

GDNF counteracts injury-induced Notch activation in NPCs

Next, we sought to determine the effect of microenvironmental cell fate determinants after SCI on the expression of transcription factors (TFs) involved in the differentiation of astrocytes and neurons. The cultures of different lines of hiPSC- or human Embryonic Stem Cell (hESC)–derived NPCs, as well as human fetal NPCs, were treated with homogenate (100 μg/ml) from the injured (SCI-h) or naïve (Naïve-h) spinal cord for 1 week, and expression of TFs was evaluated using quantitative real-time polymerase chain reaction (PCR). Treatment with SCI-h resulted in up-regulation of TFs involved in differentiation to astrocytes, including Sox9, NFIA, and NFIB, and down-regulation of proneuronal TFs, including Ascl1, Atoh1, Ngn1, and Ngn2 (Fig. 2A). A portion of these changes in TF expression may be attributable to elevated amounts of Jagged1 in SCI-h and activation of Notch signaling. Hes1 is considered to be the primary target of Notch signaling (*28*) and is involved in the differentiation switch from neurons to astrocytes (*29*–*31*). Intracellular staining flow cytometric analysis showed that treating hiPSC-NPCs in vitro with Naïve-h (100 μg/ml) or SCI-h for 1 week resulted in activation of Notch signaling as indicated by increased expression of the downstream gene Hes1 (Fig. 2B).

Next, we decided to determine whether the unfavorable effects of injury-induced factors on the differentiation profile of transplanted cells can be counterbalanced by growth factors or cytokines. To assess this, we cotreated NPCs with Naïve-h or SCI-h, as well as with different growth factors and cytokines (see table S3 for list and concentration), and then quantified the expression levels of key TFs involved in the differentiation of NPCs. Supplementing SCI-h–treated hiPSC-NPCs with BMP and TGF-β antagonist peptides, Noggin and Follistatin, down-regulated proastrocytic TFs (NFIA and NFIB) but did not have a detectable effect on proneuronal TFs. Wnt-1 is an indirect inhibitor of the Notch pathway and had a modest effect on the expression of both proneuronal and proastrocytic TFs. Among growth factors, only supplementing hiPSC-NPCs with brain-derived neurotrophic factor (BDNF) and GDNF resulted in up-regulation of proneuronal TFs and down-regulation of proastrocytic TFs (Fig. 2C and table S3). However, only GDNF was able to reduce $Hes1^+$ NPCs after treatment with SCI-h (Fig. 2D). Therefore, we selected GDNF as the candidate to counteract Notch activation in the injured spinal cord niche.

To determine whether the Notch signaling pathway was affected by GDNF, we analyzed the presence of the active form of Notch receptor, NICD, by Western blotting (Fig. 2E). Notch is a plasma transmembrane receptor, which undergoes cleavage to NICD upon activation by Notch signaling ligands, like Jagged1. NICD contains nuclear localization domains and is translocated to the cell nucleus. NICD was detectable in hiPSC-NPCs treated with SCI-h for 1 week, but cotreatment with GDNF resulted in a reduction in the NICD signal, indicating that activation of Notch signaling was reduced (Fig. 2E).

Detailed gene expression analysis of GDNF-treated NPCs showed that the expression of genes such as Pax3, Ngn1, Ascl1, DLK1, and Atoh1 were up-regulated after GDNF treatment, whereas the expression of genes such as Sox9, Hes1, NFIA, NFIB, and STAT3 were down-regulated (fig. S3). DLK1 is a noncanonical ligand of Notch and antagonizes the activity of Notch ligands Dll1 and Jagged1 and

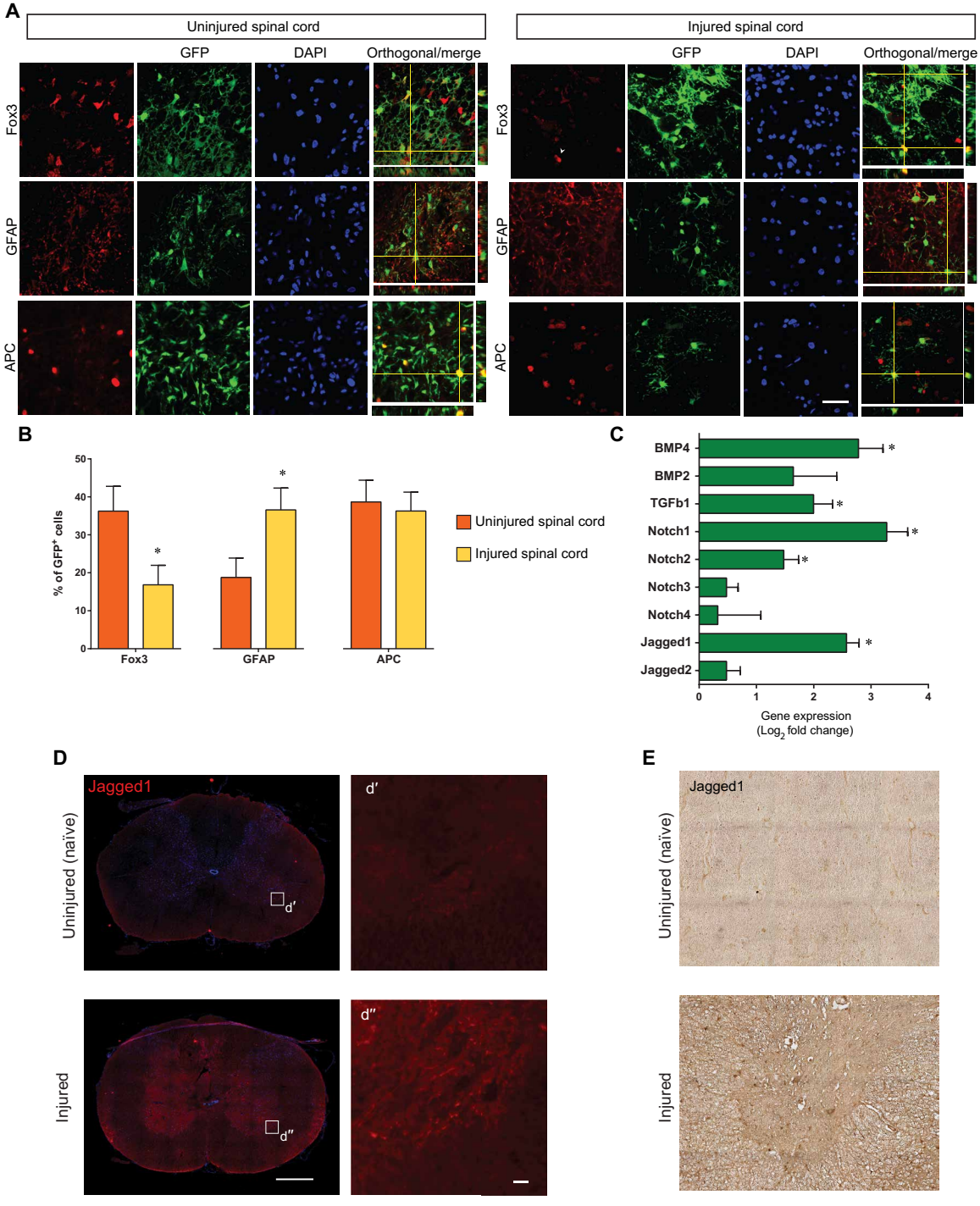

Fig. 1. Transplanted NPCs preferentially differentiate to astrocytes in the injured spinal cord microenvironment. (**A**) In vivo differentiation profiles of hiPSC-NPCs were compared after transplantation into naïve or cervical level C6/C7 injured spinal cords. Representative images show that transplanted GFP$^+$ cells in the spinal cord tissue differentiate into neuronal (Fox3$^+$), astrocytic (GFAP$^+$), and oligodendrocytic (APC$^+$) fates at 8 weeks after transplantation. Scale bar, 20 μm. DAPI, 4′,6-diamidino-2-phenylindole. (**B**) Quantification of the in vivo differentiation profile (means ± SEM, $n = 5$; *$P < 0.05$, t test). (**C**) Quantitative real-time PCR analysis of gene expression in the cervical spinal cord 2 weeks after injury relative to the uninjured (naïve) cervical spinal cord. Data represent the mean $\log_2$ fold change ± SEM, ($n = 3$; *$P < 0.05$, one sample t test compared to baseline uninjured). (**D**) Representative immunostaining for Jagged1 in cervical spinal cord sections 2 weeks after injury. The right panel represents the white box in the left panel at a higher magnification. Scale bars, 500 μm (left) and 50 μm (right). (**E**) Immunohistochemical staining using anti-Jagged1 antibody on cross sections of an uninjured human cervical spinal cord obtained from a 48-year-old female donor (top) and an injured human cervical spinal cord obtained 8 months after injury from a 45-year-old female patient with a C5/C6 level injury as a result of a motor vehicle accident (bottom).

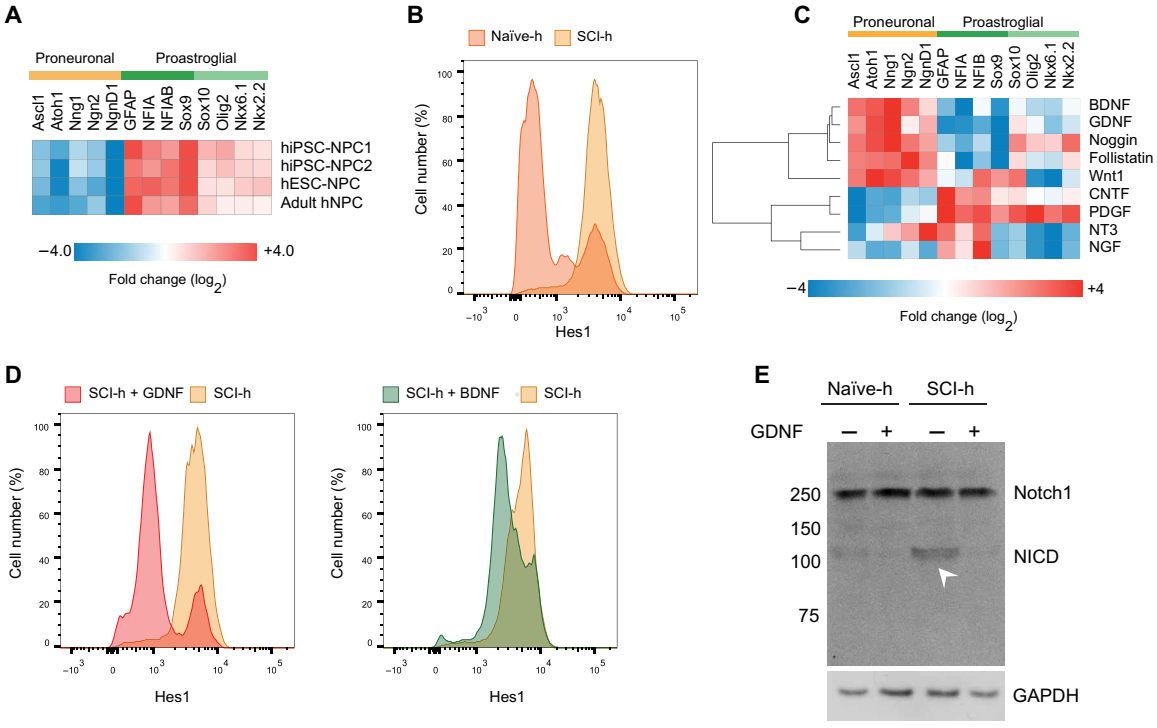

Fig. 2. GDNF counteracts injury-induced Notch activation. (**A**) Quantitative real-time PCR analysis of gene expression of different lines of hiPSC- or hESC-derived NPCs, as well as human fetal NPCs, treated with cleared SCI-h. Samples from each line were compared to corresponding NPC lines treated with Naïve-h. Gene expression values are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and presented as $\log_2$-transformed fold-change color-coded values. (**B**) Overlay histogram of intracellular staining flow cytometric analysis of Hes1 in hiPSC-NPCs treated with Naïve-h or SCI-h (total protein, 100 μg/ml). (**C**) Quantitative real-time PCR analysis of gene expression of hiPSC-NPCs treated with SCI-h and cotreated with different morphogens and differentiation factors for 1 week (rows). Samples were compared to no growth factor treatment. Gene expression values are normalized to GAPDH expression and presented as $\log_2$-transformed fold-change color-coded values. (**D**) Overlay histogram of flow cytometric analysis of intracellular Hes1 in hiPSC-NPCs treated with SCI-h and cotreated with GDNF (10 ng/ml; red) or BDNF (10 ng/ml; green). (**E**) Western blot analysis hiPSC-NPCs treated with Naïve-h or SCI-h for 1 week. Cleavage of Notch1 to Notch intracellular domain (NICD; arrowhead) fragments represents an indication for activation of Notch signaling. GAPDH was used as loading control.

inhibits Hes1-mediated Notch signaling (*32*). It has been shown that DLK1 promotes neurogenesis of human and mouse PSC derived neural progenitors via modulation of Notch and BMP signaling (*33*). Therefore, up-regulation of DLK1 in GDNF-treated NPCs suggests that GDNF exerts an effect on Notch activity via DLK1.

GDNF-expression does not affect self-renewal of NPCs but biases NPC differentiation toward a neuronal cell fate

To study the effect of GDNF on transplanted NPC fate determination and functional recovery in the challenging SCI environment, we genetically engineered hiPSC-NPCs to express GDNF. GDNF-expressing NPCs have previously been used to successfully treat neurological disorders (*18*, *34*–*39*). Stable hiPSC-NPC lines expressing GDNF-GFP or GFP alone (control) were generated by stable genomic integration of transgenes using nonviral piggyBac transposon vectors (*40*, *41*). GDNF-hiPSC-NPCs expressed about 3.5-fold more GDNF than control NPCs (Fig. 3A).

To assess the effect of GDNF expression on proliferation and self-renewal of NPCs, we used the neurosphere assay at clonal densities (*42*, *43*). Neurospheres are free-floating three-dimensional spherical colonies whose diameter and numbers reflect the ability of NPCs to self-renew and proliferate. GDNF expression did not have an effect on the self-renewal ability of hiPSC-NPCs (Fig. 3C). Self-renewal is usually measured on the basis of secondary or tertiary neurosphere formation to show that this ability is maintained for at least three passages. The average size of neurospheres did not change after GDNF expression, suggesting no effect of GDNF on the proliferation rate of the NPCs (Fig. 3C), as was further confirmed by 5-bromo-2′-deoxyuridine (BrdU) assay (Fig. 3D).

Next, we examined the effect of GDNF expression on the differentiation potential of hiPSC-NPCs by assessing their capacity to differentiate into the three main neuroglial lineages. Supplementing NPCs with fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF) is necessary for their continuous proliferation by symmetrical division, whereas withdrawal of FGF-2 and EGF from their culture media is anticipated to induce their differentiation to neurons, oligodendrocytes, and astrocytes (*44*).The differentiation of hiPSC-NPCs was induced by withdrawing FGF and EGF, as well as exposure to 0.1% fetal bovine serum for 3 weeks, which yielded cells with neuron (β-III-tubulin$^+$), astrocyte (GFAP$^+$), and oligodendrocyte (O1$^+$) morphology (Fig. 3E) (*44*, *45*).

Differentiation to a neuronal fate was increased in the GDNF-expressing group compared to control cells (30.7 ± 2.4% GDNF versus 14.1 ± 2.2% control; $P < 0.01$; Fig. 3F). However, GDNF expression significantly reduced the number of GFAP$^+$ cells as compared to the control (43.9 ± 2.7% GDNF versus 59.9.2 ± 1.5% control; $P < 0.01$; Fig. 3F). The whole-cell patch-clamp recordings of neurons differentiated from control hiPSC-NPCs and GDNF-hiPSC-NPCs at 5 weeks

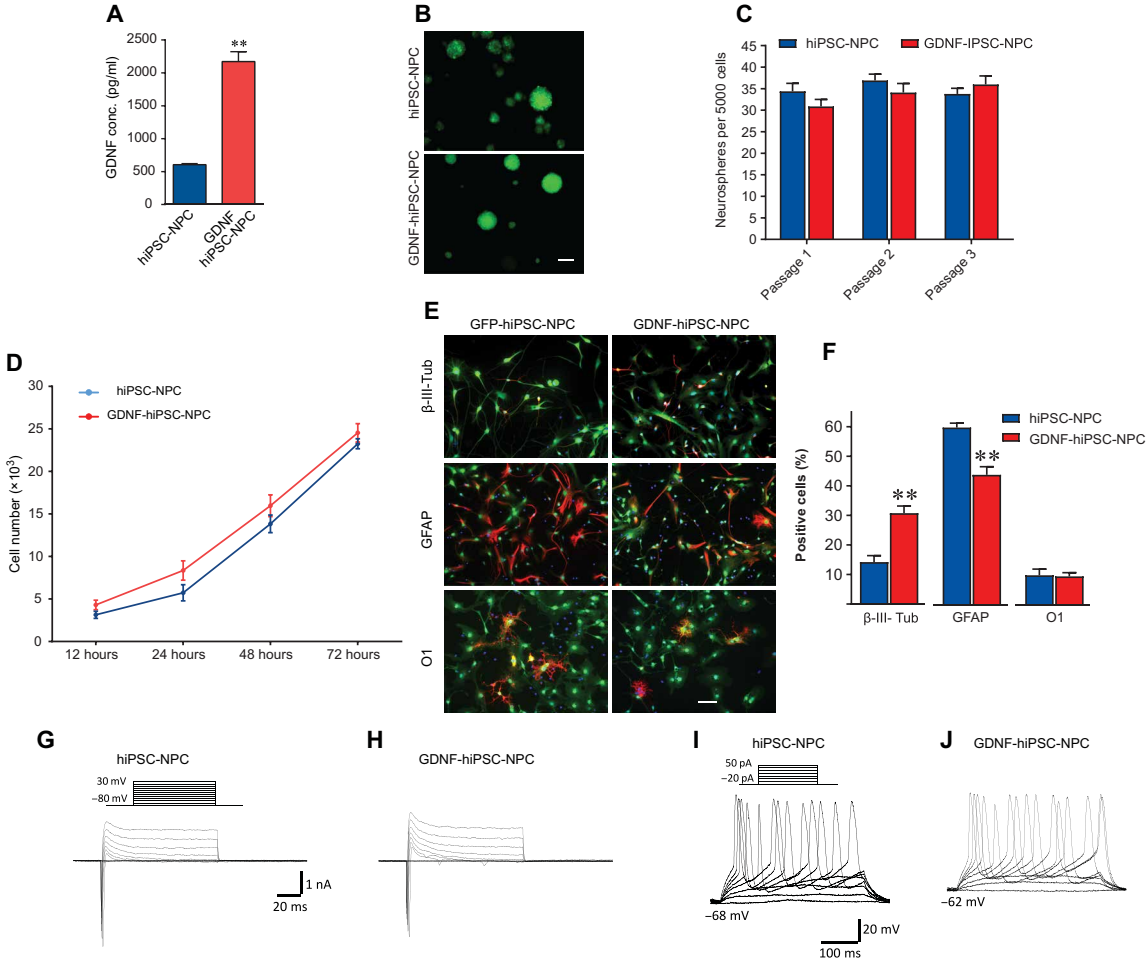

Fig. 3. GDNF expression does not affect self-renewal of NPCs but biases NPC differentiation toward a neuronal cell fate. (**A**) The purified, monoclonal lines were expanded, and expression of GDNF was confirmed by a GDNF immunoassay ($n = 3$; $^{**}P < 0.01$, t test). (**B** and **C**) Neurosphere forming assay for GDNF- and control-hiPSC-NPCs; cells were plated at a clonal density of 10 cells/μl. Scale bar, 200 μm (means ± SEM, $n = 3$; two-way ANOVA). (**D**) Effects of GDNF expression on the in vitro proliferation rate of hiPSC-NPCs. At each time point, a BrdU proliferation assay was performed to determine cell number (means ± SEM, $n = 3$; two-way repeated-measures ANOVA). (**E** and **F**) Representative image of in vitro differentiated hiPSC-NPCs and GDNF-hiPSC-NPCs that were stained for β-III-tubulin (β-III-Tub) (neuron marker), GFAP (astrocyte marker), or O1 (oligodendrocyte marker) and corresponding quantification (percentage of means ± SEM, $n = 5$; $^{**}P < 0.01$, t test). Scale bar, 20 μm. (**G** to **J**) Patch-clamp analysis showing the Na^+ current (G and H) and action potential firing (I and J) in neurons differentiated from hiPSC-NPCs and GDNF-hiPSC-NPCs.

after neuronal induction demonstrated that these neurons are able to generate inward sodium currents and action potentials, showing that they are electrically functional (Fig. 3, G to J).

GDNF counteracts the fate alteration of NPCs in the injured spinal cord niche via up-regulation of DLK1

We sought to determine whether GDNF could counter the NPC astrocyte differentiation bias that is typically observed after exposure to the injured spinal cord niche. hiPSC-NPCs were cultured in the absence of FGF/EGF and treated with cleared homogenate (100 μg/ml) from the SCI-h or Naïve-h spinal cord for 1 week. Treating control hiPSC-NPCs with SCI-h resulted in a significant increase in the number of $GFAP^+$ cells (70.4 ± 9.3% as compared to cells treated with Naïve-h, 28.4 ± 4%; $P < 0.01$). Furthermore, a significant decrease in the number of β-III-$tubulin^+$ neurons (4.6 ± 1.1% as compared to cells treated with Naïve-h, 8.8 ± 1.4%; $P < 0.01$) was observed (Fig. 4, A and B). In contrast, GDNF expression mitigated this effect and kept the differentiation profile of hiPSC-NPCs exposed to SCI-h similar to cells that were exposed to Naïve-h for both astrocyte differentiation [28 ± 2% for control NPCs in the Naïve-h group versus 31.8 ± 2% for GDNF-NPCs in the SCI-h group; not siginificant (n.s.); Fig. 4, A and B] and neuronal differentiation (8.8 ± 1.3% for control NPCs in the Naïve-h group versus 8.6 ± 2% for GDNF-NPCs in the SCI-h group; n.s.). This effect may occur through DLK1 because its expression is up-regulated with GDNF exposure (Fig. 4C). To test whether DLK1 is involved in this process, we used CRISPR-Cas9 editing to knock out DLK1 in hiPSC-NPCs (fig. S4). Up-regulation of proastrocytic TFs, NFIA and NFIB, in SCI-h–treated NPCs was mitigated by GDNF expression (Fig. 4D). However, in DLK1–knockout (KO) NPCs, GDNF did not have a significant effect on NFIA and NFIB expression ($P < 0.05$; Fig. 4D). In accordance with this, GDNF expression did not mitigate the differentiation profile of DLK1-KO NPCs exposed to SCI-h (60.0 ± 4.5% $GFAP^+$ cells in GDNF-hiPSC-DLK1-KO versus 71.8 ± 4.3% in control hiPSC-NPCs; n.s.; Fig. 4, E and F).

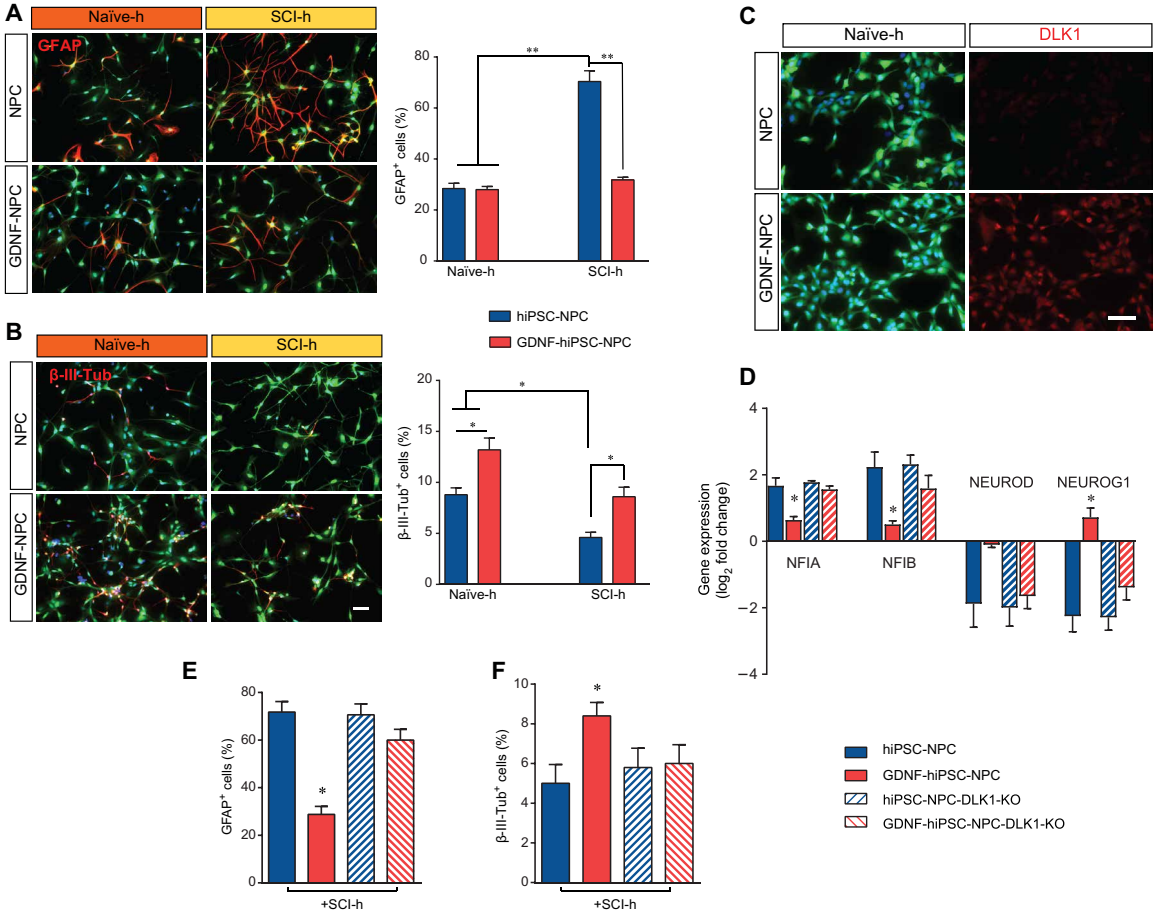

Fig. 4. GDNF counteracts the effect of SCI-h on hiPSC-NPC fate determination via the DLK1 pathway. (**A** and **B**) hiPSC-NPC cultures were treated with a final concentration of homogenate (100 μg/ml) from injured (SCI-h) or naïve spinal cords (Naïve-h) in the absence of FGF and EGF to allow differentiation. Differentiated cells were stained for β-III-tubulin and GFAP, and the percentage of β-III-tubulin$^+$ and GFAP$^+$ cells was determined (means ± SEM, $n = 5$; *$P < 0.05$, **$P < 0.01$, one-way ANOVA). Scale bar, 20 μm. (**C**) Immunofluorescence staining of hiPSC-NPCs and GDNF-hiPSC-NPCs with anti-DLK1 antibody (red). Cell nuclei are labeled with DAPI. Scale bar, 20 μm. (**D**) Quantitative real-time PCR analysis of the expression profile of TFs involved in the differentiation of NPCs to neurons or astrocytes in cells treated with SCI-h relative to control hiPSC-NPCs treated with Naïve-h. Data represent the mean $\log_2$ fold change in gene expression relative to GAPDH ± SEM ($n = 3$; one-way ANOVA, *$P < 0.05$ compared to hiPSC-NPCs). (**E** and **F**) The CRISPR DLK1-KOs of hiPSC-NPCs and GDNF-hiPSC-NPCs were treated with SCI-h, and the differentiation profile was determined (means ± SEM, $n = 5$; one-way ANOVA, *$P < 0.05$ compared to the vehicle control group).

GDNF-expressing NPCs demonstrate enhanced survival in the injured spinal cord

After confirming the effectiveness of GDNF expression in vitro, we sought to determine whether GDNF expression can offer a beneficial effect for transplanted cells. To investigate the effect of GDNF-hiPSC-NPCs in vivo, T cell–deficient RNU rats received a clip-contusion SCI at cervical level C6/C7, followed by cell transplantation at 2 weeks after injury. Histological assessment was performed at 8 weeks after transplant. The concentration of detectable GDNF in spinal cord tissue was significantly higher in the rats transplanted with GDNF-expressing NPCs as compared to vehicle (647 ± 45 pg/mg in GDNF-hiPSC-NPC–transplanted rats as compared to 494 ± 31 pg/mg in vehicle-transplanted rats; $P < 0.05$; fig. S5). Transplanted cells (GFP$^+$) were found in both the white and gray matter (Fig. 5A). For both control and GDNF groups, engraftment was largely around the site of injection, with some migration toward the injury epicenter and as far as 4-mm rostral and caudal (fig. S5). Quantification of the number of transplanted cells (GFP$^+$) revealed that survival of GDNF-expressing cells was significantly enhanced ($81.5 \times 10^3 \pm 8 \times 10^3$ cells) as compared to the control group ($44.5 \times 10^3 \pm 3 \times 10^3$ cells; $P < 0.05$) (Fig. 5, A and B). This does not, however, preclude the possibility of cell proliferation after transplantation in the spinal cord. GDNF is also a survival factor and helps NPCs to better survive both in vitro and in vivo in the harsh postinjury spinal cord microenvironment (fig. S6). There is a possibility that GDNF simply increases the survival of neurons but does not have an effect on the survival of astrocytes or oligodendrocytes, but our data suggested that GDNF is more involved in committing cells to a neuronal fate than to neuronal survival (fig. S6). The effect of GDNF on transplanted cell survival was independent of DLK1 because the cell survival in DLK1-KO lines was equivalent to their wild-type counterparts (Fig. 5, A and B). Similar to in vitro results, GDNF expression resulted in up-regulation of DLK1 in transplanted cells (Fig. 5C and fig. S7) and reduction of Hes1 immunoreactivity in the nuclei of grafted cells and surrounding cells (Fig. 5D and fig. S7). At 8 weeks after transplantation, most of the transplanted cells were in a postmitotic state, and less than 4% of them were positive for the proliferation marker, Ki67 (3.2 ± 0.45 for hiPSC-NPC as compared to 3.9 ± 0.34 GDNF-hiPSC-NPC; n.s.; fig. S7).

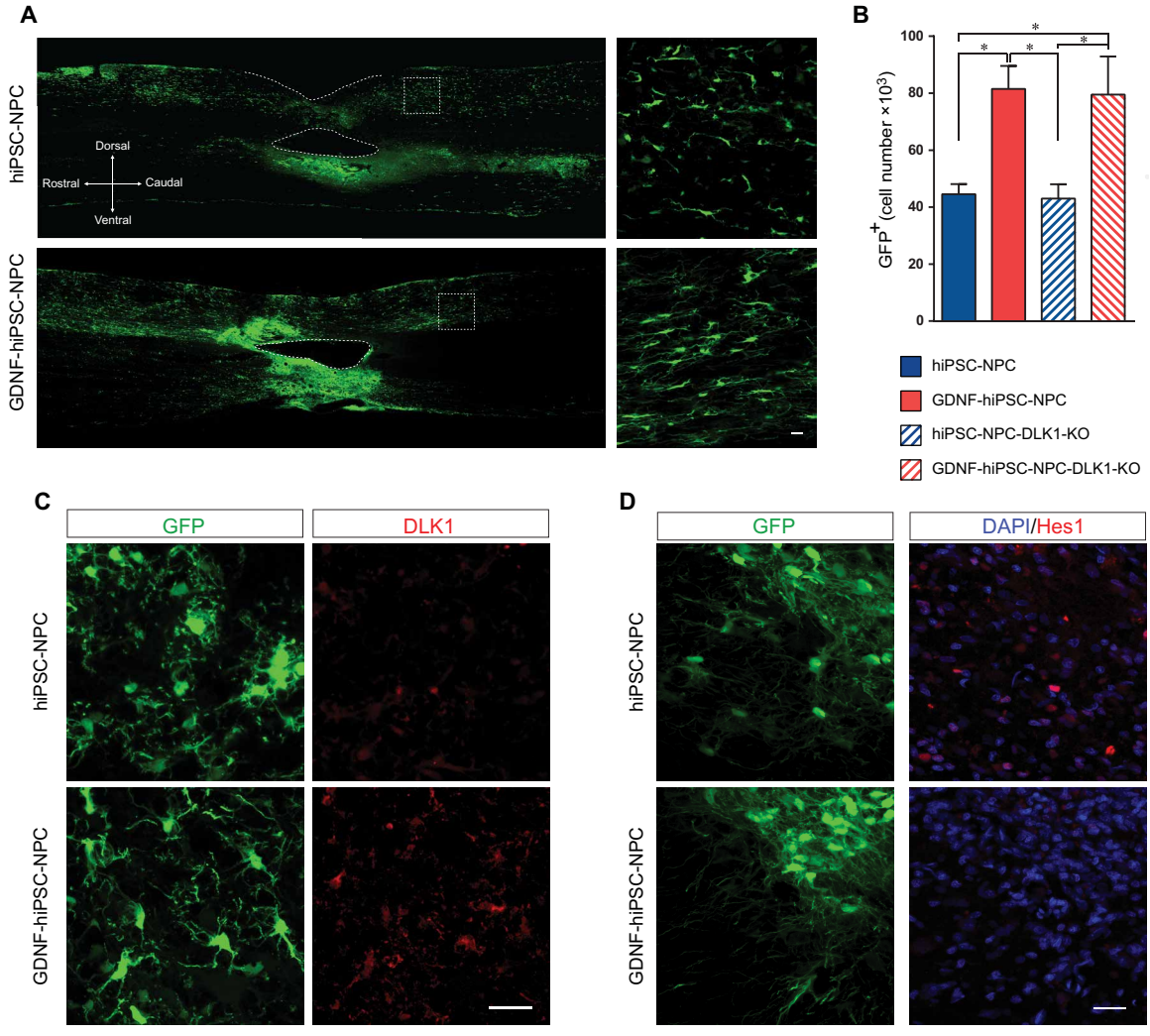

Fig. 5. GDNF-expressing NPCs demonstrate enhanced survival and increased DLK1 expression. (**A**) Representative images of GFP^+-transplanted cells in longitudinal sections of the injured spinal cord. (**B**) Quantification of the survival or GFP^+ cells at 8 weeks after transplantation into SCI (means ± SEM, $n = 6$; $^*P < 0.05$, one-way ANOVA). (**C**) Representative confocal images of cervical spinal cord sections of rats transplanted with hiPSC-NPCs and GDNF-NPCs (green) at 8 weeks after transplantation stained with anti-DLK1 antibody (red). Scale bar, 20 μm. (**D**) Representative confocal images of cervical spinal cord sections of rats transplanted with hiPSC-NPCs and GDNF-hiPSC-NPCs (green) at 8 weeks after transplantation stained with anti-Hes1 antibody (red). Cell nuclei are labeled with DAPI. Scale bar, 20 μm.

GDNF influences the in vivo fate determination of transplanted NPCs

Next, we assessed the effect of GDNF on the in vivo differentiation profile of transplanted hiPSC-NPCs. GFP colocalization with the neuronal marker Fox3 (NeuN) was significantly more abundant in the GDNF group (33.2 ± 4.2% in $GDNF^-$ versus 18.5 ± 3.2% in control hiPSC-NPCs; $P < 0.05$; Fig. 6, A and B). $GFP^+/\beta\text{-III-tubulin}^+$ colocalization was observed in 36.8 ± 3.5% of GDNF-expressing cells versus 20.7 ± 2.5% of control NPCs (Fig. 6, A and B). Conversely, $GFAP^+$ astrocytes were more frequently observed in the control hiPSC-NPC group than the GDNF-hiPSC-NPC–transplanted rats ($GFAP^+$ transplanted; 33.1 ± 3.1% versus 16.7 ± 3.3%, respectively; $P < 0.05$; Fig. 6, A and C). The changes in population of transplanted cells that were positive for an early astrocytic differentiation marker (Aldh1L1) (*46*, *47*) did not attain significance ($Aldh1L1^+$ transplanted; 27.4 ± 3.2% versus 16.8 ± 3.1%; n.s.), suggesting that most of the astrocytes derived from transplanted cells were in a mature state 8 weeks after transplantation. These data suggest that control hiPSC-NPCs differentiate primarily into cells of an astroglial lineage in vivo, whereas GDNF-hiPSC-NPCs have a higher propensity to terminally differentiate into neurons.

In DLK1-KO groups, GDNF expression did not have a significant effect on the differentiation of NPCs to neurons and astrocytes. The percentage of transplanted cells that differentiated to mature oligodendrocytes was comparable in both groups [GFP^+/adenomatous polyposis coli (APC^+); 39.6 ± 4.5% in the control hiPSC-NPC–transplanted group and 34.5 ± 5.6% in the GDNF-hiPSC-NPC–transplanted group; n.s.; Fig. 6, A and D]. Few transplanted cells remained as immature oligodendrocytes ($GFP^+/Olig2^+$; 10.3 ± 2.1% and 8.9 ± 3.3%, respectively; Fig. 6, A and D). In several animals, no detectable $GFP^+/Olig2^+$ cells could be found, indicating that after 8 weeks, most human NPC-derived oligodendrocytes had reached a mature state. Past immunoelectron microscopy work has demonstrated that mature NPC-derived oligodendrocytes are able to myelinate denuded axons (fig. S8).

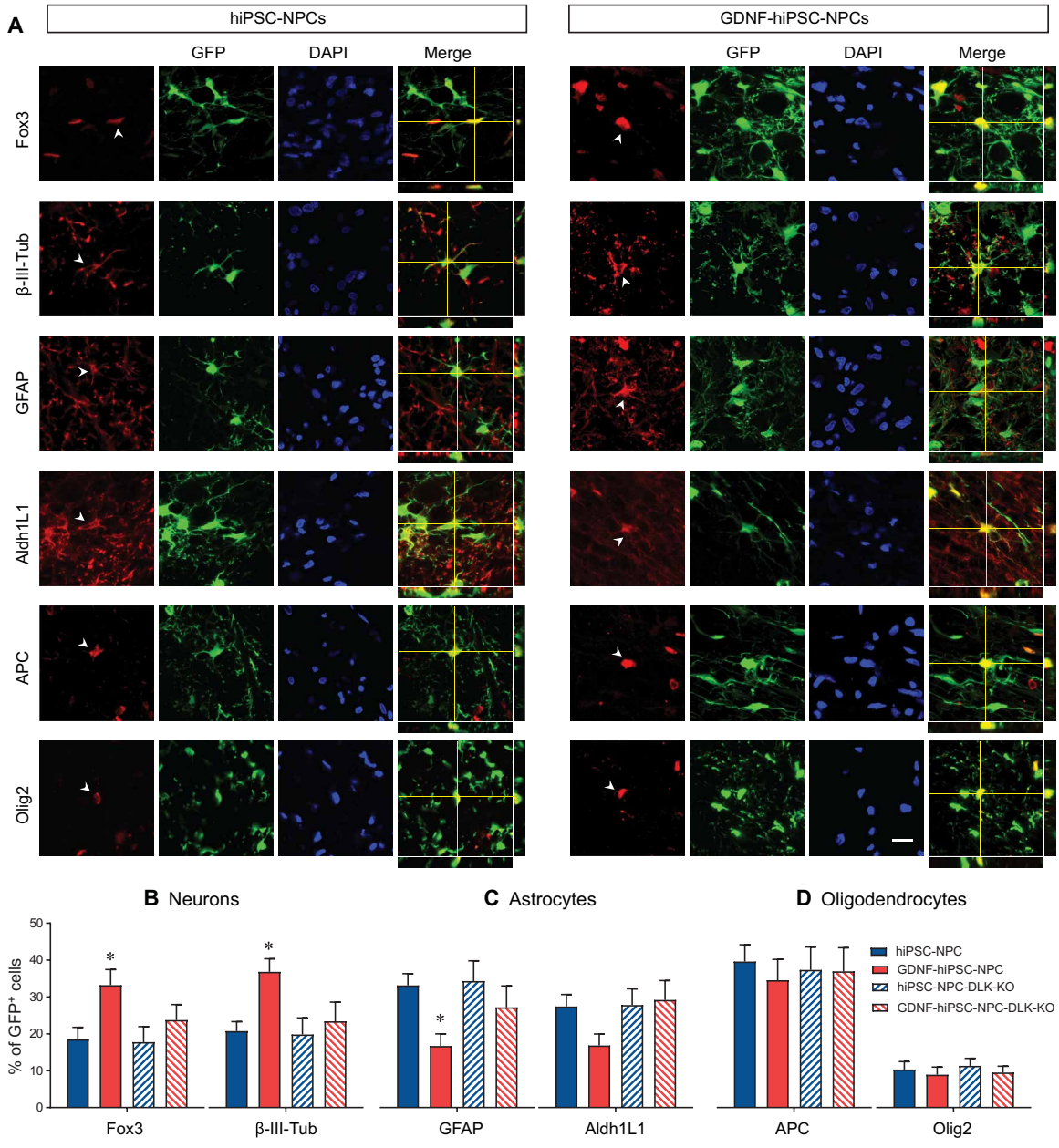

Fig. 6. GDNF-expressing NPCs preferentially differentiate toward more neurons in vivo. (**A**) Representative images of transplanted NPCs differentiated to express markers of neurons (β-III-tubulin and Fox3), astrocytes (GFAP), early astrocytic differentiation (Aldh1L1), mature oligodendrocytes (APC), and immature oligodendrocytes (Olig2). Scale bar, 20 μm. (**B** to **D**) Quantitative analysis of trilineage differentiation profiles with specific markers (means ± SEM, $n = 10$; one-way ANOVA, $^*P < 0.05$ compared to hiPSC-NPCs).

Graft-derived neuronal subtypes make synaptic connections with endogenous cells and enhance electric conduction

Next, we assessed whether transplanted hiPSC-NPCs are able to differentiate to subtype-specific neurons. Neurons differentiated from graft cells expressed different neuronal subtype–specific TFs 12 weeks after transplantation, including Isl1, Hb9 (for motor neurons), FoxP1, Lhx1, Chx10 (for premotoneuron interneurons), Pax2, and Gata3 (for inhibitory interneurons) (Fig. 7A and fig. S9). These neurons must form synaptic connections with endogenous cells and integrate into local networks to promote functional recovery. Using immunotransmission electron microscopy, we assessed whether gold-labeled GFP$^+$ cells formed synaptic connections with label-negative endogenous cells (Fig. 7B). Synapses can be identified by the apparent thickening of the apposed membranes of two cytoplasmic profiles. The inhibitory and excitatory synapses were identified on the basis of their symmetrical or asymmetric morphology, respectively (*48*, *49*). The total density of synapses was not significantly different between control NPCs and GDNF-NPCs (Fig. 7C). However, the ratio of asymmetric/symmetric synapses was significantly higher in the GDNF group (0.42 ± 0.03) compared to control hiPSC-NPCs (0.28 ± 0.03; $P < 0.05$), indicating that GDNF cells made more excitatory connections (Fig. 7D). Because GDNF-NPCs produced more neurons

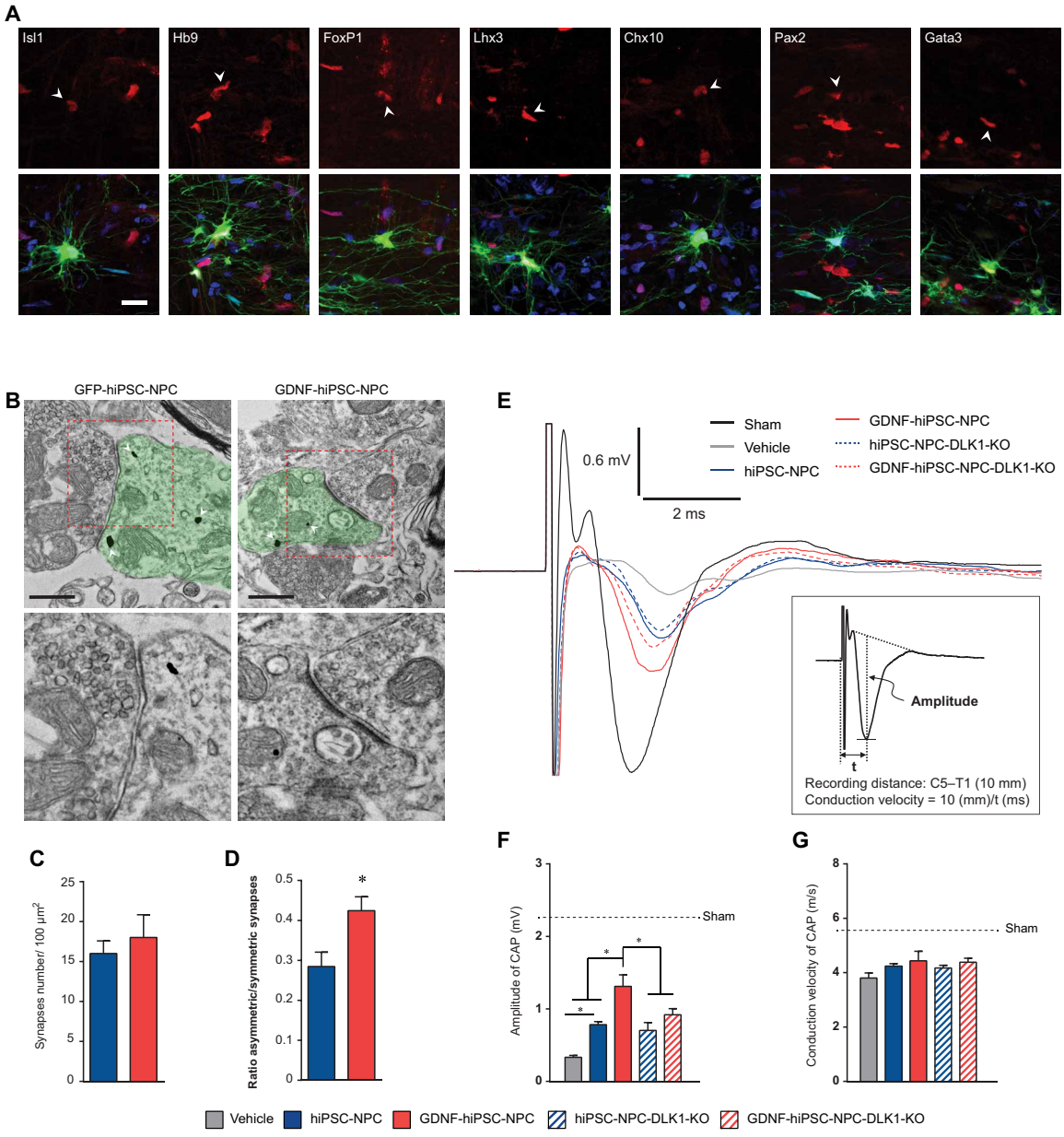

Fig. 7. Graft-derived neuronal subtypes make synaptic connections with endogenous cells and enhance electric conduction. (**A**) Representative confocal images of cervical spinal cord sections of rats transplanted with GDNF-hiPSC-NPCs (green) at 8 weeks after transplantation stained with antibodies against neuronal subtype–specific TFs for motor neurons (Isl1 and Hb9), premotoneuron interneurons (FoxP1, Lhx3, and Chx10) and inhibitory interneurons (Pax2 and Gata3) 12 weeks after transplantation. Scale bar, 20 μm. (see fig. S9 for non–GDNF-expressing NPCs). (**B**) Transmission electron micrographs of spinal cord sections at the site of transplantation to show the formation of synapses between anti-GFP immunogold (black dots, white arrowheads)–labeled cells (pseudocolored to green) and endogenous axon terminals. Insets identify postsynaptic densities. The average diameter of gold particles was around 42.2 nm. Scale bars, 500 nm. (**C**) The number of synaptic densities per 100 μm^2 of labeled cells and (**D**) the ratio of asymmetric/symmetric connections were quantified (means ± SEM, $n = 4$; *$P < 0.05$, t test). (**E**) To test the effect of GDNF on electrical transmission, we analyzed electrically evoked compound action potential (CAP) transmission across the injury site (C5 to T1). Traces represent the average of six animals per group. (**F**). Quantification of CAP amplitude in animals translated hiPSC-NPCs at 8 weeks after transplantation (means ± SEM, $n = 6$; *$P < 0.05$, one-way ANOVA). (**G**) The conduction velocity was calculated as the recording distance (10 mm) divided by latency (t) (means ± SEM, $n = 6$; Kruskal-Wallis test and Dunn's multiple comparisons test).

(~1.7× more), the overall number of exogenous-endogenous synaptic connections, particularly excitatory ones, is likely higher in the GDNF group. These new connections could potentially contribute to greater electrical transmission across the injury site. To test this, we analyzed electrically evoked compound action potential (CAP) transmission across the injury site (C5 to T1). The CAP amplitude was significantly higher in the GDNF-NPC transplant group (1.31 ± 0.51 mV) compared to the control NPC group (0.78 ± 0.04 mV; $P < 0.05$). The changes in CAP amplitude were reduced for DLK1-KO cells and control NPCs (Fig. 7, E to G). This could reflect a lower number of neurons and therefore fewer new synaptic connections in DLK1-KO cells and control NPCs, as compared to GDNF-NPCs.

GDNF-hiPSC-NPC transplantation improves forelimb motor function after injury

To assess the effect of GDNF on functional recovery after SCI, we performed a series of sensorimotor tests during the 8-week post-transplant period. GDNF expressed from transplanted cells can affect functional recovery through changing the fate of NPCs and affecting endogenous tissue. Our data indicate that transplantation of hiPSC-NPCs resulted in tissue sparing, although this was more prevalent in the GDNF group (fig. S10). The compression-contusion SCI in our model caused severe damage to the spinal cord gray matter, with cystic cavitation at the injury epicenter obliterating the central canal. A reduction in cavity volume was confirmed in live animals using very high-resolution ultrasound, with smaller cavity sizes observed in GDNF-hiPSC-NPC animals (fig. S10). To delineate the involvement of these mechanisms in functional recovery, we performed neurobehavioral assessments in hiPSC-NPCs, GDNF-hiPSC-NPCs, and their DLK1-KO groups. Forelimb strength and trunk stability were assessed with grip strength and inclined plane behavioral tasks, respectively (*50*). All injured animals consistently recovered forelimb grip strength over the assessment period, although recovery trajectories diverged at about 4 weeks after transplantation. There was a significant improvement in forelimb grip strength in the GDNF-hiPSC-NPC group compared to the vehicle control group ($P < 0.01$; Fig. 8A). The recovery in the control NPC and in the DLK-KO groups was not different to vehicle. There was a significant improvement in inclined plane performance in the GDNF-hiPS-NPC group compared to the vehicle control group ($P < 0.05$; Fig. 8B). Similar to grip strength data, there was no difference between the other cell-transplanted groups and vehicle in inclined plane test (Fig. 8B). This indicates that knocking down the DLK1 gene in GDNF-hiPSC-NPCs blocked the effect of GDNF on functional recovery.

Using the CatWalk (Noldus Inc.) digital gait analysis system, we quantified several static and dynamic parameters of locomotion

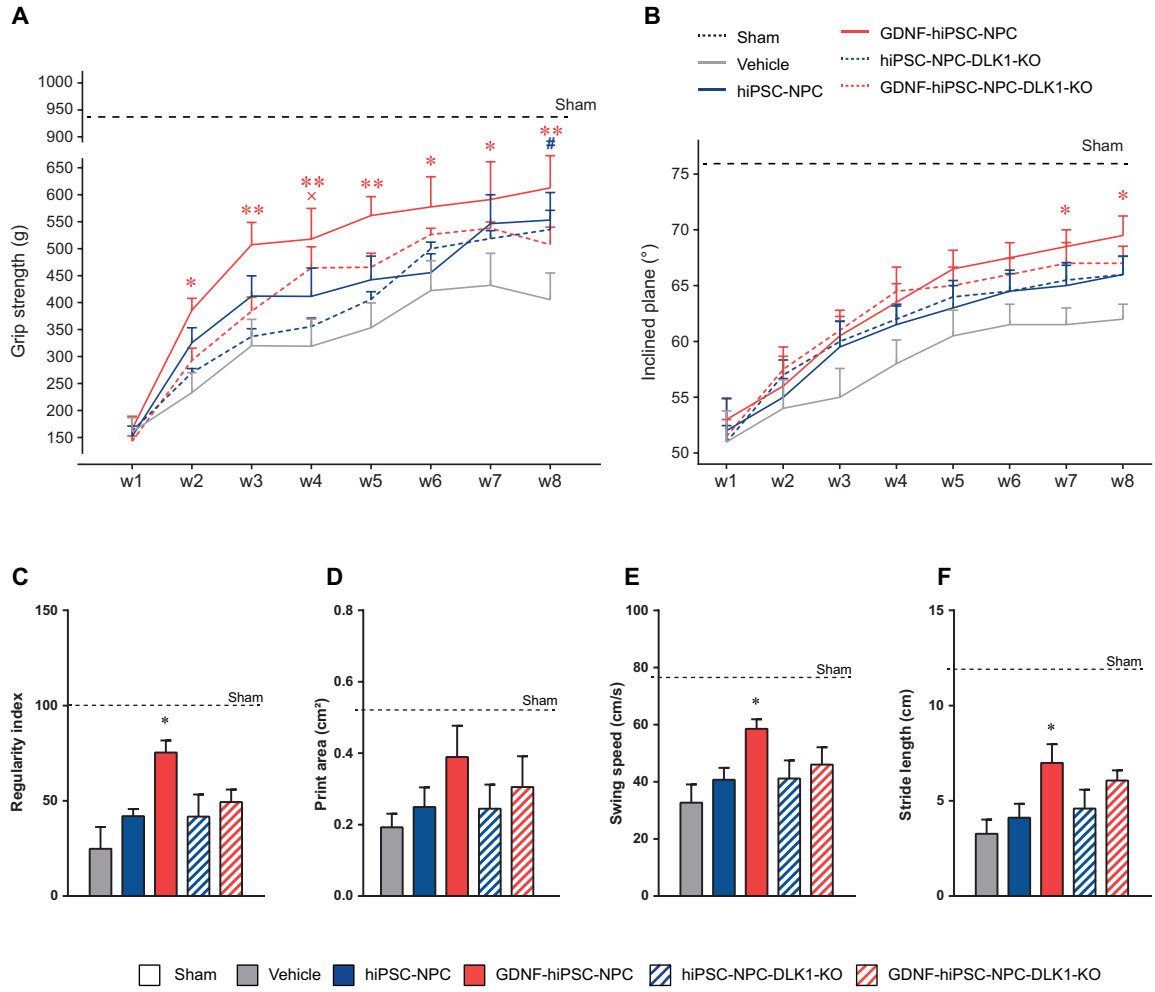

Fig. 8. GDNF-expressing NPCs contributed to better functional recovery than control NPCs. (**A**) The grip force exerted by the forelimbs was measured using a grip strength meter during the 8 weeks after transplantation. Each graph represents the force (in grams). (**B**) Global motor function was tested by each animal's ability to sustain increasing degrees of incline with platforms ranging from 30° to 90°. An animal's ability to tolerate larger incline angles is associated with better functional recovery. [for (A) and (B): means ± SEM, $n = 10$; two-way ANOVA with repeated measures, $^*P < 0.05$ and $^{**}P < 0.01$ for GDNF-hiPSC-NPCs compared to vehicle group, and # and × indicate $P < 0.05$ significance difference for GDNF-hiPSC-NPC-DLK1-KO and hiPSC-NPC groups, respectively, compared to vehicle group]. (**C** to **F**) Quantification of different forelimb gait parameters at 8 weeks after transplantation [means ± SEM, $n = 6$; Kruskal-Wallis test for (C), (D), and (F) and one-way ANOVA for (E), $^*P < 0.05$ compared to vehicle group].

relevant to cervical SCI at 8 weeks after transplantation. All injured groups exhibited abnormal walking patterns, slow rates of locomotion, and abnormal paw prints (Fig. 8, C to F). The regularity index, which is an indicator of coordination between all four limbs, increased by 50.59% in the GDNF group compared to vehicle (Fig. 8C). Forelimb print area was not significantly improved in cell-transplanted animals [hiPSC-NPC (0.24 ± 0.05) and GDNF-hiPSC-NPC (0.38 ± 0.08) compared to vehicle (0.19 ± 0.03); n.s.; Fig. 8D]. However, forelimb swing speed and forelimb stride length were significantly improved in rats transplanted with GDNF-expressing cells versus the vehicle control [GDNF-hiPSC-NPC (58.56 ± 3.31) compared to vehicle (32.7 ± 6.381) (Fig. 8E) and GDNF-hiPSC-NPC (6.9 ± 0.9) compared to vehicle (3.2 ± 0.7) (Fig. 8F); $P < 0.05$].

Increased neuropathic pain after cell-based treatment is a potential concern (*51*). Hence, we assessed thermal and mechanical allodynia. Latency times for the rat to remove its tail from a focal heat source (fig. S11) were not different at any time point among the groups. In addition, there was no difference in the responses to von Frey filament application to the plantar surface of fore or hind paws at 8 and 10 weeks after injury (fig. S11).

DISCUSSION

Traumatic SCI leads to the rapid necrotic death of neurons and glia due to mechanical compressive and shearing forces. This is followed by an inflammatory response and the activation of astrocytes (*27*). These events are accompanied by widespread changes to the expression of cell fate determinants (*8*, *52*, *53*) and extracellular matrix molecules in the microenvironment (*54*). The complex postinjury milieu that results from these changes has an important impact on the fate determination of transplanted multipotent cells, reducing their survival and integration. Several studies with rodent endogenous or transplanted NPCs have demonstrated that the postinjury microenvironment of the spinal cord is proastrocytic (*55*–*59*). These studies have been performed using rodent cells, which have inherent differences (*60*) from human NPCs. During neural tube development, human neural progenitor differentiation follows a more protracted time course than their rodent counterparts, which is also observed in vitro. Although there are some inconsistencies in the field regarding the differentiation percentage of human NPCs to neurons/astrocytes in the injured spinal cord niche (*16*, *61*), in our rodent model of SCI, different human NPCs from different sources bias more toward astrocytic differentiation. This inconsistency may arise from our use of a different injury model and method of assessment. It is possible that NPCs at different stages of development behave differently in the injured spinal cord microenvironment. Early (primitive) NPCs tend to differentiate toward neurons compared to (definitive) NPCs, which we have used in this study. However, in addition to the risk of tumor formation when using primitive NPCs, these cells also do not differentiate to myelinating oligodendrocyte cells in a sufficient proportion—limiting functional recovery. In this work, we showed that activation of Notch signaling in the postinjury microenvironment is one of the determinants that increase the differentiation of NPCs toward a more astrocytic fate through alterations in TF expression.

Notch receptors and their ligands participate in a wide variety of biological events and are best known for influencing cell fate decisions during development (*62*). Notch signaling induces the self-renewal of NPCs and also inhibits neuronal differentiation; however, its effect is highly dependent on the microenvironment (*63*, *64*). Upon ligand binding, NICD is released from the membrane and translocates into the nucleus, where it not only activates the expression of genes such as Hes1 that antagonize proneuronal genes (*63*) but also increases the expression of proastrocytic genes like NF1A (*31*). Activation of Notch signaling is necessary but not sufficient for NPC differentiation to astrocytes (*31*). Notch activation potentiates the process, but other signals from the microenvironment such as TGF-β, interleukin-6, and BMPs are required to induce $GFAP^+$ astrocytes (*31*, *65*, *66*). Notch1 activity is enhanced in the injured spinal cord tissue (*67*), and it is suggested that Notch signaling contributes to the apparent restriction of de novo neurogenesis in the adult spinal cord (*67*). Here, we showed that the Notch ligand Jagged1, which is produced by reactive astrocytes (*68*), is up-regulated in the injured spinal cord microenvironment. The increased expression of Jagged1 after SCI promotes the astrocytic differentiation of progenitors and increases astrogliosis (*27*, *69*).

In this work, we showed that treatment with GDNF, a proneuronal GF, can oppose Notch signaling and increases differentiation to a more neuronal fate in the proastrocytic postinjury microenvironment. Our gene expression analysis indicated that DLK1, an antagonist of Notch signaling, is one of the Notch signaling–related genes that is up-regulated in NPCs upon treatment with GDNF. Concurrently, it has been shown that GDNF increases the expression of DLK1 in midbrain progenitors (*70*) and DLK1 expression induces neurogenesis in the postnatal subventricular zone (*71*). Therefore, one potential mechanism of action of GDNF in modulating Notch signaling could be through DLK1. Expression of GDNF resulted in up-regulation of DLK1 and consequently in the elevated expression of proneuronal TFs Ngn1 and NeuroD. GDNF also down-regulated the expression of proastrocytic TFs, NFIA and NFIB, potentially through DLK1 inhibition of Notch, which is known to induce NFIA expression (*31*). The effect of GDNF on the fate determination of NPCs through modulation of Notch signaling was further confirmed using DLK1-KO cells. Once transplanted in vivo, GDNF expression influenced the fate determination of the transplanted cells toward a proneuronal lineage and substantially decreased the number of astrocytes generated compared to control hiPSC-NPCs.

GDNF not only increased the differentiation of grafted NPCs toward a neuronal fate but also improved the survival of transplanted cells, as has been described previously (*18*, *39*). Furthermore, GDNF overexpression by graft NPCs protected endogenous tissue and reduced cavity size. This provides further evidence for the role of GDNF to provide important trophic support in SCI (*72*–*75*).

As it has been shown before, human NPC transplantation improves functional recovery through trophic support, cell replacement, and remyelination. The combination of NPCs with GDNF can act synergistically as a potential treatment for SCI through two mechanisms: (i) as a cell fate determinant, mediated by DLK1, to increase differentiation toward neurons and (ii) as a trophic factor, independent to DLK1, to improve the survival of both transplanted cells and endogenous tissue. The functional recovery observed after GDNF-NPC transplantation could be the result of a combination of these two mechanisms. Graft-derived neurons can form synapses with endogenous circuits and relay the signal over the injury site to the target cells.

These data might represent a key step in optimizing hiPSC-NPC transplantation for SCI. The ability to alter the fate determination of transplanted NPCs toward a proneuronal lineage and also improving graft survival, integration, and functional recovery in rats moves us closer to translating an effective stem cell therapy for individuals

living with the sequelae of traumatic SCI. To advance these cells to clinical use, we need further investigation to identify safe harbor sites in the human genome for genetic manipulation and to develop closed loop feedback systems to regulate the expression of GDNF.

A potential limitation of this study was the transplantation of human NPCs into a rodent model of SCI. There are numerous differences in the size, anatomy and disease/developmental time scale, and physiology of the spinal cord between rodents and humans, which make translating the results of rodent research to human patients challenging. Specifically, the human spinal cord is larger and longer, which may require increased cell migration and growth after transplantation. In addition, the disease progression time scale is not the same between rodents and human, whereas the time scale for development of human NPCs is much longer compared to rodents NPCs.

MATERIALS AND METHODS

Study design

The aims of this study were to (i) assess whether it is possible to modulate the increased Notch signaling observed in the injured spinal cord microenvironment via expression of a protein to rescue the fate of NPCs toward neurons using a clinically realistic approach and (ii) determine the therapeutic potential as an SCI treatment. To this end, we screened several proteins and identified the role of GDNF expression in attenuating Notch signaling by mediating DLK1 expression. We first generated hiPSC-NPCs, which express GDNF and its CRISPR-Cas9 KO for the DLK1 gene, and then characterized their differentiation profile in vitro. Next, we transplanted these cells into a clinically relevant model of rodent cervical SCI and monitored rats for 8 weeks after transplantation for their functional recovery. Rats were block-randomized into control or treatment groups based on grip strength 1 day before transplantation to eliminate group variation bias. Electrically evoked CAPs were analyzed before euthanizing the animals. Subsequently, histology and staining of tissue sections and quantification of in vivo differentiation were performed. No outliers were excluded from data analysis. Study size for experiments was selected on the basis of power calculations of historical data from our laboratory. Operators were blinded to the treatment during acquisition and analysis of data.

Statistical analyses

All animals were randomized into either injury or treatment groups. All data were collected and quantified in a blinded fashion. Results are stated as means ± SEM, and statistical $P < 0.05$ was considered significant. The normality assumption was verified using the Shapiro-Wilk test. To assess significant differences between single measurements of two groups of normally distributed data, we used a two-tailed Student's *t* test. Gene expression data were analyzed using two-tailed one-sample Student's *t* tests when compared to baseline control group. To assess significant differences between more than two groups of normally distributed data, we performed one-way analysis of variance (ANOVA), followed by post hoc analyses. Comparison against a control group was performed using Dunnet's multiple comparisons test, and comparison of all pairs of datasets was performed using Tukey's multiple comparisons test. Kruskal-Wallis test was applied to not normally distributed data, and post hoc analyses were corrected for multiple comparisons using Dunn's multiple comparisons test. Grip strength and inclined plane data were analyzed using a two-way ANOVA with repeated measures and Dunnett's post hoc compared to vehicle control group. Statistical analyses were performed with Prism 8 (GraphPad Software).

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Differentiation bias of transplanted human NPCs to astrocytes.

Fig. S2. Increase in the amount proastrocytic differentiation factors after SCI.

Fig. S3. Functional interaction network for differentially expressed genes in hiPSC-NPCs after treatment with GDNF.

Fig. S4. Western blot confirmation of knocking out of DLK1 in hiPSC-NPCs.

Fig. S5. Expression and secretion of GDNF into tissue from transplanted cells.

Fig. S6. Reduced death in GDNF-expressing hiPSC-NPCs after exposure to injured spinal cord homogenate.

Fig. S7. Quantification of DLK1, Hes1, and Ki67 staining.

Fig. S8. Immunoelectron microscopy to analyze the myelination by transplanted cells.

Fig. S9. Staining for neuron-specific subtypes and human cell–specific marker.

Fig. S10. The effect of GDNF secretion from hiPSC-NPCs on host tissue preservation.

Fig. S11. Evaluation of thermal and mechanical allodynia.

Table S1. List of antibodies.

Table S2. List of Applied Biosystems TaqMan probes used for qPCR.

Table S3. List of growth factors and cytokines used for screening.

Data file S1. Raw data.

References (*76*–*85*)

View/request a protocol for this paper from *Bio-protocol*.

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GDNF rescues the fate of neural progenitor grafts by attenuating Notch signals in the injured spinal cord in rodents

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The fate-determining factor

Neural progenitor cells (NPCs) emerged as a potential therapeutic approach for repairing and regenerating neurons after spinal cord injury. However, the hostile microenvironment of the injured spinal cord contributes to the limited degree of recovery observed after NPC transplant in rodents. Now, Khazaei *et al.* have shown that activation of Notch signaling in the spinal cord after injury reduces the therapeutic potential of the transplanted NPCs. Counteracting Notch activation by expressing GDNF in transplanted NPCs promoted differentiation toward a neuronal cell fate and improved motor function after injury in rodents. The results suggest that modulating the injured microenvironment might improve recovery after stem cell transplant.