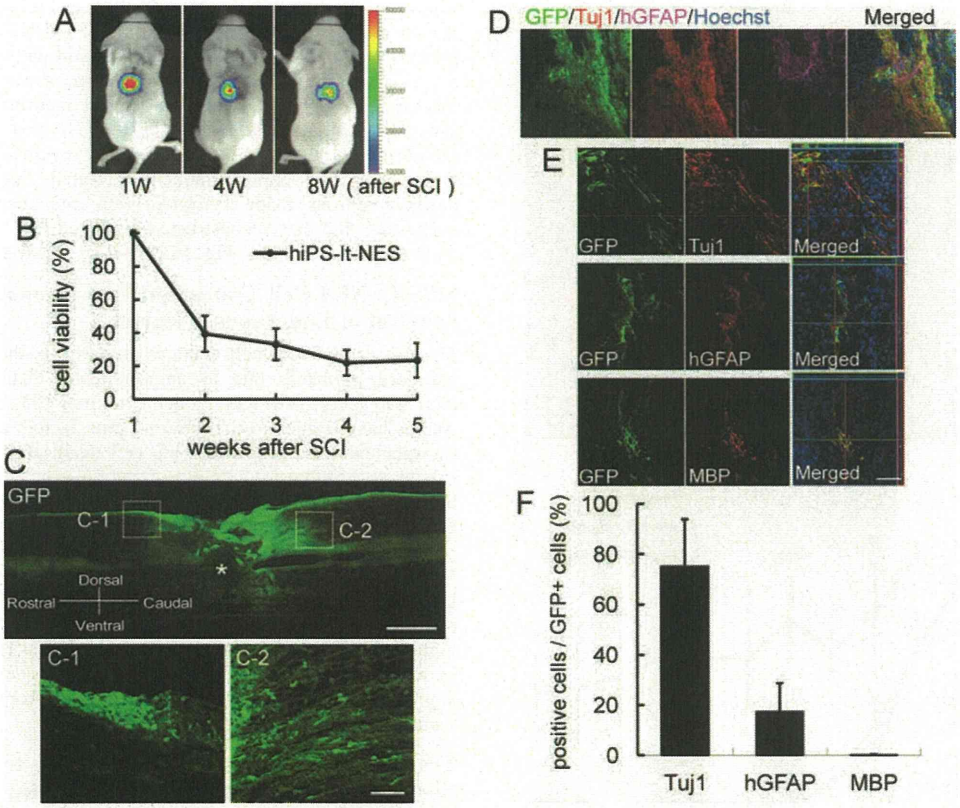

Figure 3. Transplanted human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem (hiPS-lt-NES) cells survive and differentiate in the injured spinal cord of nonobese diabetic-severe combined immunodeficient mice. **(A):** Survival of transplanted cells was checked every week using a bioluminescence imaging system; left, 2 hours after transplantation; middle, 4 weeks after SCI; and right, 8 weeks after SCI. **(B):** Time course of transplanted hiPS-lt-NES cell survival in SCI model mice. Optical signal intensity was measured using the bioluminescence imaging system. Quantification of the photon intensity revealed that approximately 20% of the transplanted cells survived 5 weeks after SCI; thereafter, the photon signals remained stable. Data are means ± SEM ($n = 6$). **(C):** Sagittal sections of SCI model mice treated with hiPS-lt-NES cells at 8 weeks after transplantation. Sections were stained with anti-GFP antibody (green). The epicenter of the SCI is indicated (*). Higher-magnification images of the white dotted boxes (C-1 and C-2) show GFP-positive transplant-derived cells extending their processes into gray and white matter. **(D):** Immunostaining images, 8 weeks after injury, of hiPS-lt-NES cells grafted into spinal cord. Spinal cord sections were stained with anti-GFP (green), hGFAP (magenta) and Tuj1 (red) antibodies and with Hoechst (blue). **(E):** Confocal images, 8 weeks after injury, of hiPS-lt-NES cells transplanted into spinal cord, revealing transplanted cells which were double-positive for GFP and markers of neural lineages. **(F):** Quantitative analyses of Tuj1-positive neurons, hGFAP-positive astrocytes, and MBP-positive oligodendrocytes as in E. Data are means ± SD ($n = 3$). Scale bars = 500 μm in C, 100 μm in C-1, C-2, D, and E. Abbreviations: GFP, green fluorescent protein; hGFAP, anti-human-specific glial fibrillary acidic protein; MBP, and SCI, spinal cord injury.

differentiation into MBP- or APC-positive oligodendrocytes was less than 1% (Fig. 3E, 3F, Supporting Information Fig. 2). These data indicate that transplanted hiPS-lt-NES cells differentiate preferentially into neurons in the injured spinal cord.

hiPS-Lt-NES Cell Transplantation Does Not Promote CST Axon Re-Extension After SCI

Since transplanted NSCs have been reported to play a supportive role in the re-extension of injured axons [29], we examined the effect of the transplanted hiPS-lt-NES cells on CST first-order axonal regeneration. We injected BDA into the bilateral motor cortices and labeled CST first-order neuron axons by anterograde tracing [21, 22]. Because BDA is not transported from first- to second-order neurons across the synapse, only the axons of first-order neurons in the CST are visualized by this method.

In the region caudal to the injury site, >50% of the labeled fibers observed at 5 mm anterior to the lesion site were detected in the intact mice, whereas almost no BDA-traced CST fibers could be seen in the SCI control or hiPS-lt-NES cell-transplanted mice (Fig. 4A). Quantitative analysis revealed that there was no significant difference in the number of BDA-labeled fibers between the SCI control and hiPS-lt-NES cell-treated groups at any position up to 5 mm on either side of the lesion site (Fig. 4B). Although we cannot exclude possibilities such as the re-extension of descending raphespinal axons, which are also important for the motor functional recovery of hind limbs [30, 31], these results suggest that the regeneration of CST axons, if it occurs, cannot be a major contributing factor in the recovery induced by hiPS-lt-NES cell transplantation in our SCI model.

Local Neurons Reconstruct Disrupted CST Neuronal Circuits in a Relay Manner

Recent studies have revealed that local endogenous and transplant-derived neurons can form new neuronal circuits and make synaptic connections after SCI [32–34]. To determine

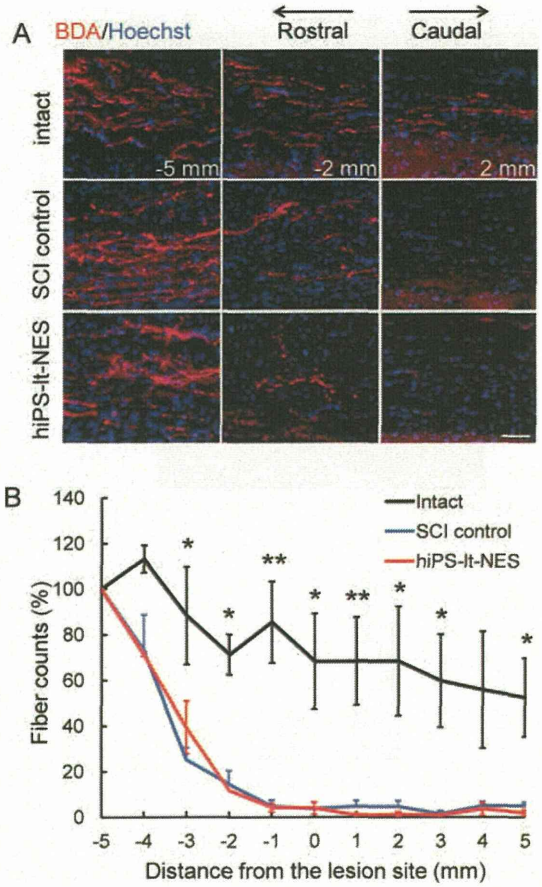

Figure 4. hiPS-lt-NES cell transplantation does not promote corticospinal tract (CST) axon re-extension after SCI. **(A):** Representative pictures of BDA-labeled CST fibers (red) at 5 and 2 mm rostral and 2 mm caudal to the lesion site at 12 weeks after SCI. Hoechst (blue) shows nuclear staining. Scale bar = 50 μm. **(B):** Quantification of the labeled CST fibers in the spinal cords of untreated, SCI control, and hiPS-lt-NES cell-transplanted mice. The x-axis indicates specific locations along the rostro-caudal axis of the spinal cord, and the y-axis indicates the ratio of the mean number of BDA-labeled fibers at the indicated site to that at 5 mm rostral to the lesion site (thoracic vertebra [Th] 9). Intact mice were compared with SCI control mice. *, $p < .05$; **, $p < .01$. There was no significant difference in the number of BDA-labeled fibers between hiPS-lt-NES cell-treated (blue line) and SCI control groups (red line). Data are means ± SEM ($n = 5$). Abbreviations: BDA, biotinylated dextran amine; hiPS-lt-NES, human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem; and SCI, spinal cord injury.

whether disrupted CSTs were reconstructed by forming a local neuronal relay, we injected WGA-expressing adenoviruses into the motor cortex of the hind limb area at 12 weeks after SCI. WGA, a plant lectin, has been widely used as a tracer of neuronal pathways [24, 25] because it is transported in axons and dendrites, and across synapses, to second- and even third-order neurons.

After injection, we could detect WGA-immunoreactive intracellular granule-like structures in MAP2ab-positive neurons (Fig. 5A). Furthermore, we found that hiPS-lt-NES cell-treated mice had more WGA/Map2ab double-positive cells than SCI control mice in the caudal region below the injured site (Fig. 5A, 5B). Taking these data and the results of the BDA experiment into consideration, it is suggested that WGA was transferred to the caudal area through the lesion site via new synaptic connections, and that hiPS-lt-NES cell transplantation promoted the CST reconstruction without CST axonal re-extension. In support of this proposition, we could observe GFP-positive transplant-derived neurons adjacent to synaptophysin-positive patches (Fig. 5C-1 and 5C-1′). Furthermore, immunohistochemistry using species-specific antibodies for presynaptic markers revealed that transplant-derived neurons made synapses with endogenous neurons, suggesting that they reconstructed disrupted CST neuronal circuits in a relay fashion (Fig. 5C-2, 5C-2′, 5C-3, and 5C-3′).

hiPS-Lt-NES Cell Transplantation Promotes the Survival of Endogenous Neurons

Massive endogenous cell death is observed in the injured spinal cord, probably due to environmental changes such as increased levels of inflammatory cytokines [35, 36]. Previous studies have suggested that neurotrophic factors secreted from transplanted cells could alleviate cell death and thereby contribute to improved locomotor function after SCI [37]. We therefore wanted to examine whether transplanted hiPS-lt-NES cells influence the survival of endogenous neurons. We performed immunohistochemistry and counted the number of NeuN-positive/GFP-negative endogenous neurons around the lesion site (Fig. 6A) and found that the number of endogenous neurons in the hiPS-lt-NES cell-treated mice was significantly higher than that in the SCI control (Fig. 6B). These findings indicate that transplantation of hiPS-lt-NES cells alters the environment of the injured spinal cord and promotes the survival of endogenous neurons.

Transplanted Cells Contribute Directly to Functional Recovery of Hind Limb Movement in SCI Model Mice

Ablation of specific cells is a useful method to evaluate their functional contribution in animal models. Mouse and rat cells are less sensitive to DT than human cells, which express human heparin-binding epidermal growth factor-like growth factor (hHB-EGF) as a DT receptor [26, 27]. We administered DT to hiPS-lt-NES cell-transplanted mice at 7 weeks after SCI. Using an in vivo imaging system, we were able to trace the survival of transplanted cells while evaluating hind limb function. Almost all the transplanted hiPS-lt-NES cells were specifically ablated following DT administration (Fig. 7A). After ablation of the transplanted cells, the BMS score of hiPS-lt-NES cell-treated mice dropped to a level similar to that of SCI control mice, although the reduction was not large enough to make the score differ significantly from that attained by hiPS-lt-NES cell-treated mice without DT administration (Fig. 7B). This may be because transplanted hiPS-lt-NES cells promoted the survival of endogenous neurons (Fig. 6) that participated in the reconstruction of disrupted neuronal circuitry. Nevertheless, these results suggest that transplanted hiPS-lt-NES cells play direct and important roles in the functional recovery of the injured spinal cord.

Discussion

The advent of cell reprogramming and the establishment of iPS cells have provided new prospects for stem cell transplantation therapy [7, 8], and a large number of different types of iPSCs have already been generated by various methods [38–40]. Transplantation of NSCs into the injured spinal cord has been shown to be effective, and with recent advances in stem cell biology, particularly in human ES/iPS cells, we anticipate

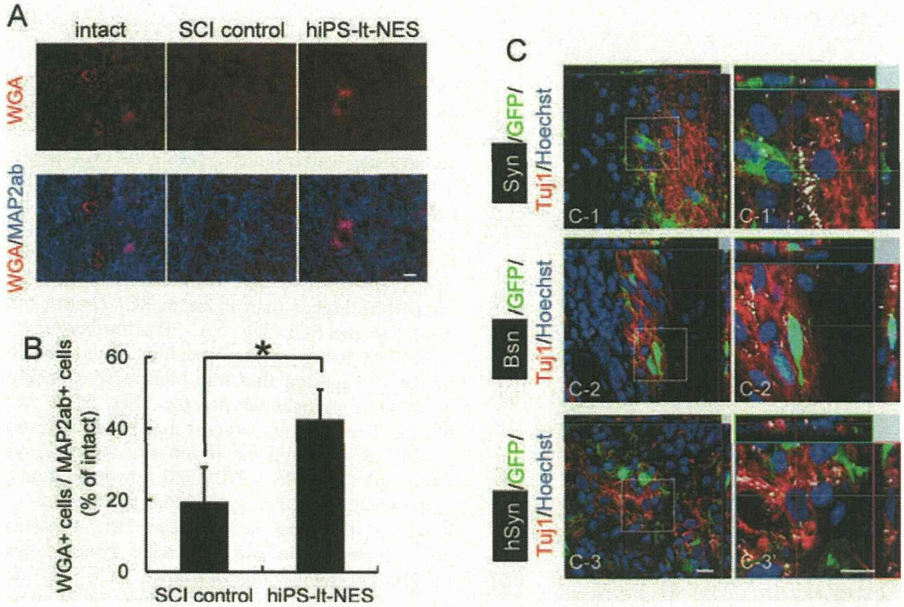

Figure 5. Local neurons reconstruct disrupted corticospinal tract neuronal circuits in a relay manner. **(A):** Representative pictures of WGA-labeled neuronal cell bodies located in the caudal area (Th11 to lumbar vertebra [L] 1) at 12 weeks after spinal cord injury (SCI; left, intact mice; middle, SCI control mice; and right, hiPS-lt-NES cell-treated mice). Spinal cord sections were stained with anti-WGA (red) and anti-MAP2ab (neuronal marker, blue) antibodies. WGA immunoreactivity was observed as intracellular granule-like structures. **(B):** WGA-positive cells/MAP2ab-positive neurons in the caudal area were quantified. Values are expressed as percentages of those in intact mice. The percentage of WGA-positive cells in hiPS-lt-NES cell-treated mice was significantly higher than that in SCI control mice. *, $p < .05$. Data are means ± SD ($n = 3$). **(C):** Spinal cord sections were stained with anti-GFP and Tuj1 antibodies, Hoechst (blue), and Syn, Bsn, or hSyn antibodies. (C-1) Representative confocal image showing that GFP (green) and Tuj1 (red) double-positive transplant-derived neurons were adjacent to synaptophysin-positive patches (white). (C-1′) High-magnification view of boxed area in (C-1). (C-2) Host and graft synapses were distinguished using a monoclonal antibody for the presynaptic marker Bsn that selectively recognizes rat and mouse, but not human, epitopes. Confocal image showing that GFP (green) and Tuj1 (red) double-positive transplant-derived neurons were in contact with Bsn-positive host synapses (white). (C-2′) High-magnification view of boxed area in (C-2). (C-3) Confocal images showing that GFP (green)-negative and Tuj1 (red)-positive endogenous neurons were in contact with hSyn-positive synapses (white). (C-3′) High-magnification view of boxed area in (C-3). Scale bars = 10 μm. Abbreviations: Bsn, anti-Bassoon; GFP, green fluorescent protein; hiPS-lt-NES, human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem; hSyn, anti-human-specific synaptophysin; Syn, anti-synaptophysin; and WGA, wheat germ agglutinin.

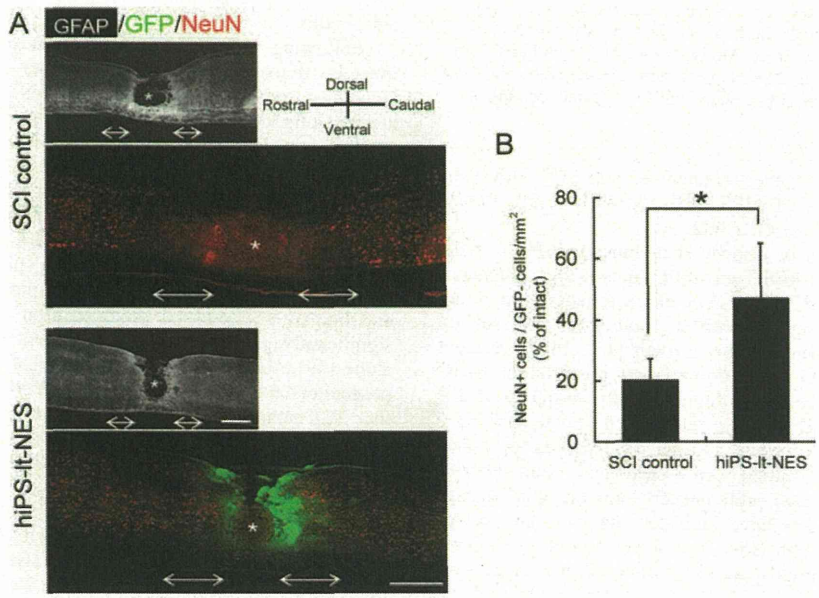

Figure 6. Transplanted hiPS-lt-NES cells promote the survival of endogenous neurons. **(A):** Representative sagittal sections of SCI control and hiPS-lt-NES cell-transplanted mice at 12 weeks after SCI. Sections were stained with anti-GFAP (white), anti-NeuN (mature neuronal marker, red) and anti-GFP (green) antibodies. Two 500-μm regions, at the rostral and caudal edge of the lesioned site (double-headed arrows), were selected for the assessment. The epicenter of the SCI is indicated (*). Scale bars = 500 μm. **(B):** Quantification of NeuN-positive/GFP-negative endogenous neurons. Values are expressed as percentages of those in intact mice. The number of NeuN-positive/GFP-negative endogenous neurons in hiPS-lt-NES-treated mice was significantly higher than that in SCI control mice. *, $p < .05$. Data are means ± SD ($n = 3$). Abbreviations: GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; hiPS-lt-NES, human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem; and SCI, spinal cord injury.

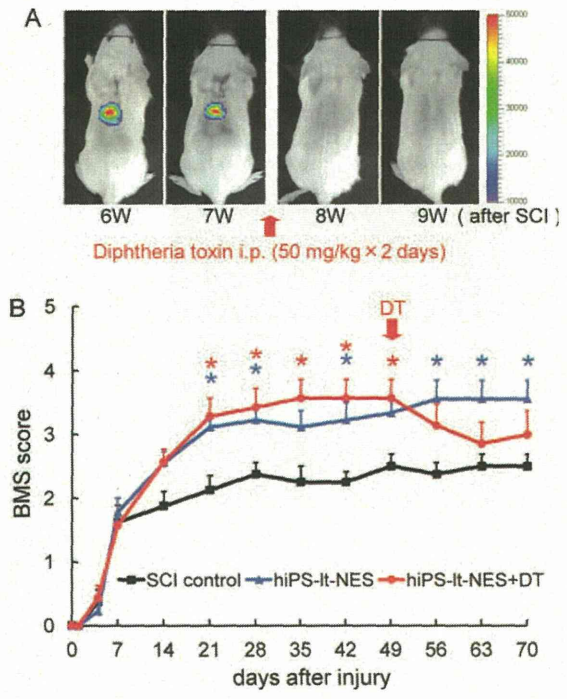

Figure 7. Transplanted cells contribute directly to the functional recovery of hind limb movement in SCI model mice. **(A):** Survival of transplanted cells was monitored using a bioluminescence imaging system. Seven weeks after SCI (6 weeks after transplantation), each mouse was administered DT. One week later, luciferase activity had completely disappeared in hiPS-lt-NES cell-transplanted mice. **(B):** Time course of functional recovery of hind limbs after SCI and DT administration. Data are means ± SEM (SCI control, $n = 8$; hiPS-lt-NES, $n = 9$; and hiPS-lt-NES+DT, $n = 7$). Hind limb function of hiPS-lt-NES cell-transplanted mice deteriorated after DT administration (red line). BMS values of the hiPS-lt-NES and hiPS-lt-NES+DT groups were compared with those of the SCI control group. *, $p < .05$. Abbreviations: BMS, Basso Mouse Scale; DT, diphtheria toxin; hiPS-lt-NES, human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem; and SCI, spinal cord injury.

further improvements in the treatment of SCI [41]. However, transplantation studies of hiPS-NSCs, initially using mouse models of SCI [9], are in their infancy.

We have previously shown that human lt-NES cells, derived from different hESC and iPSC lines in two independent laboratories, exhibit consistent characteristics and retain their ability to proliferate and differentiate even after several passages and long-term in vitro growth [11]. In the present study, we investigated the differentiation potential of transplanted hiPS-lt-NES cells and found that the majority of differentiated hiPS-lt-NES cells in the injured spinal cord were neurons (Fig. 3D–3F), even though endogenous or transplanted NSCs in the injured spinal cord have been reported by others to differentiate preferentially into the glial lineage [42, 43]. Recent studies have indicated that neurons derived from transplanted human NSCs can integrate into the injured spinal cord and form synaptic connections with host neurons [33, 34]. We therefore took advantage of the tendency of our hiPS-lt-NES cells to differentiate into neurons and tried to apply these cells to reconstruction of the injured spinal cord.

Multiple therapeutic effects of NSC transplantation have been reported, and many different types of stem cells have been grafted into the injured spinal cord, yielding improved functional recovery in animal models [44, 45]. However, the growth of axons through the lesion to reinnervate the side caudal to the injury has so far been very limited. Our experiments using anterograde labeling of CST fibers revealed that this was also the case after transplantation of hiPS-lt-NES cells (Fig. 4). We have previously shown that neurons derived from mouse NSCs could restore disrupted neuronal circuits [15]. Consistent with that observation, we demonstrated here that WGA-positive cell numbers in hiPS-lt-NES cell-treated mice were higher than those in SCI control mice in the area caudal to the SCI (Fig. 5A, 5B). Furthermore, immunohistochemistry using species-specific antibodies for presynaptic markers suggested that transplant-derived neurons made synapses with endogenous neurons (Fig. 5C-2, 5C-2′, 5C-3, and 5C-3′). These results suggest that WGA was transferred to the caudal area through the lesion site via new synaptic connections, and that hiPS-lt-NES cell transplantation promoted CST reconstruction in a relay fashion. Other studies have provided evidence that local neurons can form intraspinal neural circuits in the lesion site and make synaptic connections with descending-tract collaterals after SCI [32, 46], and human NSCs transplanted into the injured rat or mouse spinal cord differentiated into neurons that formed axons and synapses, and also established contacts with host neurons [4, 34].

As well as their roles in cell replacement, NSCs reportedly exert other effects through the secretion of neurotrophic factors [37], which, by means such as suppressing myelin inhibitors, prevent neuronal cell death, enhance remyelination and regenerate axons [47, 48]. We found that transplantation of hiPS-lt-NES cells supported the survival of endogenous neurons (Fig. 6). Conversely, ablation of transplanted cells decreased previously attained functional recovery (Fig. 7), suggesting that transplanted cells contribute directly to functional recovery of hind limb movement in SCI model mice. In summary, we suggest that not only neurons derived from transplanted hiPS-lt-NES cells but also surviving endogenous neurons contribute to functional improvement by forming multiple synaptic connections which restore disrupted neuronal circuits.

Following injury, demyelinated and reconstructed axons must be remyelinated to ensure proper functional recovery. Generally, endogenous or transplanted NSCs which differentiate into oligodendrocytes have been reported to contribute to remyelination of axons and thereby to help in recovery after SCI. Transplanted oligodendrocyte progenitor cells (OPCs) derived from human ESCs have been shown to differentiate into oligodendrocytes and enhance remyelination in the moderate rat model of contusion spinal cord injury [5, 6]. A further study reported that in complete spinal cord transection rats, locomotor recovery after transplantation with both OPCs and human ESC-derived motor neuron progenitor cells was significantly greater than after treatment with either cell type alone [49]. Given that the application of stem cells and their progenitors for transplantation is currently in its infancy, and that SCI pathology differs between contusion and transection [50], these findings indicate that neural cells in an appropriate lineage should be transplanted according to the degree and/or type of SCI.

When ES and iPS cells are used for transplantation treatment, a key consideration is the likelihood of tumor formation, since the transplanted cell population may include undifferentiated cells [51, 52]. Indeed, the sources and methods of induction of NSCs are critical for differentiation and tumor formation [33, 53]. Neurosphere cultures are heterogeneous and sensitive to variations in methodological procedures [12], whereas monolayer cell cultures give rise to more homogeneous population [13, 14]. In this study, we detected no tumor

formation in more than 40 hiPS-lt-NES cell-treated mice up to 12 weeks after SCI. This is probably attributable to complete differentiation of hiPS cells into NES cells in our robust and stable monolayer cell cultures, since the NES cells were established by initially purifying neural rosette structures from differentiating cultures [11]. Furthermore, we transplanted hiPS-lt-NES cells which had been expanded in the presence of growth factors and passaged more than 20 times. Before human iPS cell-based therapies can be implemented for clinical application, the cells' proliferation and tumor formation after transplantation must be strictly evaluated.

Conclusion

In this study, we have demonstrated that hiPS-lt-NES cells survived and differentiated in the injured spinal cord of NOD-SCID mice, and promoted functional recovery of hind limbs. Moreover, we have shown that transplanted hiPS-lt-NES cells support the reconstruction of CST pathways, promote endogenous neuron survival, and directly contribute to improved hind limb movement. To achieve a more efficient treatment for SCI, detailed investigations of hiPS-lt-NES cells and SCI pathology will be necessary. Nevertheless, our study raises the possibility that hiPS-based therapy can be applied in the near future to SCI patients who currently have few or no therapeutic options.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Insulin biosynthesis in neuronal progenitors derived from adult hippocampus and the olfactory bulb

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In the present study, we demonstrated that insulin is produced not only in the mammalian pancreas but also in adult neuronal cells derived from the hippocampus and olfactory bulb (OB). Paracrine Wnt3 plays an essential role in promoting the active expression of insulin in both hippocampal and OB-derived neural stem cells. Our analysis indicated that the balance between Wnt3, which triggers the expression of insulin via NeuroD1, and IGFBP-4, which inhibits the original Wnt3 action, is regulated depending on diabetic (DB) status. We also show that adult neural progenitors derived from DB animals retain the ability to give rise to insulin-producing cells and that grafting neuronal progenitors into the pancreas of DB animals reduces glucose levels. This study provides an example of a simple and direct use of adult stem cells from one organ to another, without introducing additional inductive genes.

INTRODUCTION

There are considerable similarities between the genes expressed in the developing brain and pancreas in mammals (Edlund, 2002; Habener et al, 2005; Hori et al, 2005). In *Drosophila*, insulin is produced by neurons within the brain (Brogiolo et al, 2001; Rulifson et al, 2002). Clusters of these insulin-producing cells in flies and vertebrate pancreatic islet β cells were considered functional analogues and evolved from a common ancestral insulin-producing neuron (Rulifson et al, 2002). Insulin signalling plays important roles in the proliferation and differentiation of cells in both developmental and adult stages (Hsieh et al, 2004; McMorris and Dubois-Delcq, 1986). Insulin, IGF-1 and IGF-2 provide instructive signals for regulating cell fate choice of adult neural stem cells (NSCs) in the hippocampus (HPC) (Hsieh et al, 2004). Interestingly, diabetes impairs hippocampal learning and memory in diabetic (DB) rats and mice, thus indicating its detrimental effects on neurogenesis and synaptic neuronal plasticity (Stranahan et al, 2008). Moreover, diabetes increases the risk of clinical depression and dementia (Greenwood and Winocur, 2005; Messier 2005).

Mammalian neurogenesis occurs throughout adulthood in the subventricular zone (SVZ) of the forebrain lateral ventricles and the HPC. In the SVZ, NSCs migrate within the rostral extension of the SVZ along the rostral migratory stream, eventually reaching the olfactory bulb (OB). Multipotent NSCs can be isolated from the easily accessible OB (Bédard and Parent, 2004; Gritti et al, 2002; Hayakawa et al, 2007; Pagano et al, 2000). Extensive studies on adult hippocampal neurogenesis have revealed that it can be modulated by physiological and behavioural events, such as aging, stress, disease, seizures and learning. In the HPC, new neurons are continuously generated from self-renewing NSCs in the dentate gyrus (DG) (D'Amour and Gage, 2003; Gage, 2000; Suh et al, 2007), a process promoted by Wnt3 released from underlying astrocytes (Lie et al, 2005). Therefore, local inhibition of Wnt signalling in the DG reduces the number of newborn neurons (Jessberger et al, 2009; Kuwabara et al, 2009; Lie et al, 2005).

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We demonstrated that adult hippocampal Wnt/β-catenin signalling triggers NeuroD1 expression (Kuwabara et al, 2009). NeuroD1, a basic helix-loop-helix (bHLH) transcription factor, has been shown to play an important role in both developing pancreas and brain (Liu et al, 2000; Miyata et al, 1999; Naya et al, 1997, 1995). NeuroD1-deficiency in mice causes severe diabetes and perinatal lethality because NeuroD1 is required for insulin gene expression (Naya et al, 1997, 1995). Similar to the loss of Wnt/β-catenin signalling (Lee et al, 2000), NeuroD1 deficiency during hippocampal development leads to a complete loss of DG formation in mice (Liu et al, 2000; Miyata et al, 1999). Despite their separate developmental origins, the gene expression programs for developing neurons and β cells are remarkably similar (Edlund, 2002; Habener et al, 2005). However, insulin expression in the adult brain and the underlying regulatory mechanism, have not been demonstrated.

Here, we provide evidence that adult granule neurons natively express insulin. This insulin expression during neuronal differentiation was observed not only in hippocampal NSCs (HPC NSCs) but also in adult NSCs isolated from the OB (OB NSCs). Wnt3 played an important role in up-regulating endogenous NeuroD1 expression, which in turn activated insulin gene expression by the direct association of NeuroD1 with the insulin promoter in both HPC and OB NSCs. Although the contribution of Wnt3 to insulin expression was apparent, inhibitory mechanisms also played a role. Insulin-like growth factor-binding proteins (IGFBPs), which acted as inhibitory factors of Wnt-mediated promotion, were up-regulated upon neuronal differentiation. In DB animals, IGFBP-4 expression was further up-regulated, whereas, Wnt3 and NeuroD1 expression declined. Importantly, adult HPC and OB NSCs derived from DB animals retained the ability to generate insulin-producing cells. *Ex vivo* culture with Wnt3a ligand and a neutralizing antibody against IGFBP-4 significantly promoted insulin production, and grafted adult HPC and OB NSCs from DB animals became efficient sources of *de novo* insulin biosynthesis *in vivo*. Our results confirm the strong functional similarity between adult neurons and β cells and suggest possible approaches to cell replacement therapy for diabetes.

RESULTS

Hippocampal neurons express insulin and C-peptide

We first investigated *insulin-1* mRNA expression in adult HPC by *in situ* hybridization. Positive *insulin-1* mRNA signals were detected at granule cell layers (GCL) in DG (Fig 1A; negative controls are shown in Fig S1A of Supporting information). Strong signals were observed in neuronal layers but were not found in cells at the inner layer of the DG where astrocytes and undifferentiated NSCs reside. Signals were also detected in CA1 and CA3 pyramidal neuron layers (Fig S1B of Supporting information).

The expression of insulin-1 protein in adult HPC and pancreas was examined in parallel by enzyme-linked immunosorbent assay (sandwich enzyme-linked immunosorbent assay; ELISA). Although the insulin expression level was significantly higher in the pancreas than in the brain (>10-fold), adult brain and HPC contained released insulin (Fig S2 of Supporting information). Insulin expression was also analysed immunohistochemically (IHC). Insulin-producing cells were clearly detected in granule neurons in DG, and these cells expressed β-tubulin III (TUJ1, Fig 1B). Insulin was expressed in the cell body and extensively in neurites extending from neurons (Fig 1B; molecular layer). Neurons in the CA1 pyramidal region, cortex and substantia nigra were also positive (Fig S3 of Supporting information). Adult pancreatic islet β cells were TUJ1+ (Fig S3B of Supporting information), suggesting similar gene expression between β cells and neurons. In DG neurons, *de novo* insulin production was confirmed by the simultaneous detection of C-peptide (Fig 1C), similar to that in pancreatic islets (Fig S3C of Supporting information). From these *in situ* hybridization, ELISA and IHC data, we confirmed that adult hippocampal neurons endogenously expressed insulin to a lesser extent than pancreatic islets.

Pancreatic α cells release Wnt3, and its level is decreased in diabetic rats

Because neurons produced insulin (Fig 1), we were interested in the niches that supported neuronal differentiation. Astrocytes define the HPC niche (Song et al, 2002), and astrocyte-secreting Wnt3 factors (Fig S4A of Supporting information) have instructive effects in promoting adult neurogenesis (Lie et al, 2005). Glial fibrillary acidic protein (GFAP) is an astrocyte marker, and GFAP-expressing (GFAP+) cells were detected in pancreatic α cells (Fig S4B of Supporting information). Interestingly, IHC revealed that the pancreatic GFAP+ cells co-localized with Wnt3+ cells (Fig 2A), indicating that α cells release the neurogenic Wnt3 as do hippocampal astrocytes.

In diabetes, deficits in insulin secretion influence the pancreatic endocrine system and HPC function (Stranahan et al, 2008). To determine Wnt3 expression under physiological changes, we compared the Wnt3+ cell population between wild-type and DB rats. In both streptozotocin (STZ)-induced type 1 DB and type II DB Goto-Kakizaki (GK) rats, marked reductions in Wnt3+ cells were observed in the pancreas Fig 2B, left; $p < 0.001$, data represent +s.d. $n = 6$ *per* group), which was also observed in the HPCs of DB rats (Fig 2B, right). Following the reduction in Wnt3+ cells in DB rats, the expression of *Wnt3* mRNA was down-regulated consistently in both pancreatic islets and the HPC (Fig 2C and D). Insulin, IGF-1 and IGF-2 were also down-regulated. Interestingly, IGFBP-4 expression was up-regulated in DB rats (Fig 2C and D).

A family of IGF-binding proteins (Firth and Baxter, 2002) modulates the bioactivity of IGF and impairs Wnt/β-catenin signalling (Zhu et al, 2008). The most potent canonical Wnt inhibitor in the IGFBP family is IGFBP-4 (Zhu et al, 2008). In diabetes, potent competitors (IGF-1 and IGF-2) of IGFBP-4 were down-regulated and the Wnt3 inhibitor IGFBP-4 was present in higher levels; further, the HPC and pancreas expressed lower levels of Wnt3.

Adult neural progenitor cells from the HPC and OB

Manipulating endogenous NSCs or transplanting the progeny of exogenously expanded neural progenitors may lead to

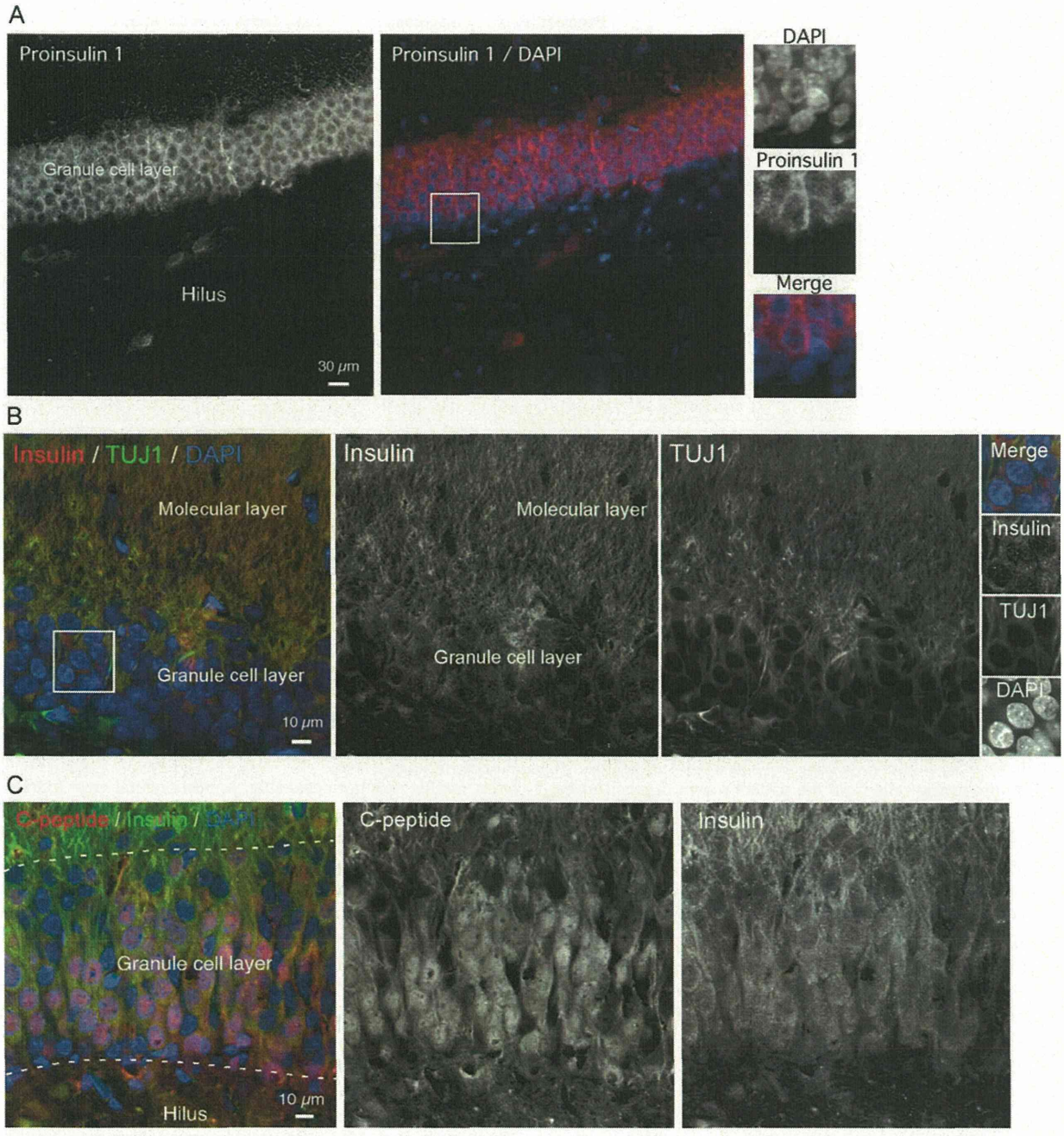

Figure 1. Adult hippocampal neurons express insulin.

A. *In situ* hybridization of proinsulin 1 mRNA in adult rat HPC. *In situ* hybridization of insulin and DAPI staining in the DG region are shown. Cells in the white square region are magnified and shown in separate panels at right. Proinsulin 1, red; DAPI, blue.

B. Immunohistochemistry analysis of insulin in DG of adult HPC. Insulin-immunoreactive cells (red) were detected in both the GCL and molecular layer of HPC, and they also expressed β tubulin III (TUJ1, green). Cells in the white square region are magnified and shown in separate panels at right. Insulin, red; TUJ1, green; DAPI, blue.

C. Detection of C-peptide in adult granule neurons in DG of HPC. C-peptide is generated when proinsulin is split into insulin and C-peptide. C-peptide, red; Insulin, green; DAPI, blue.

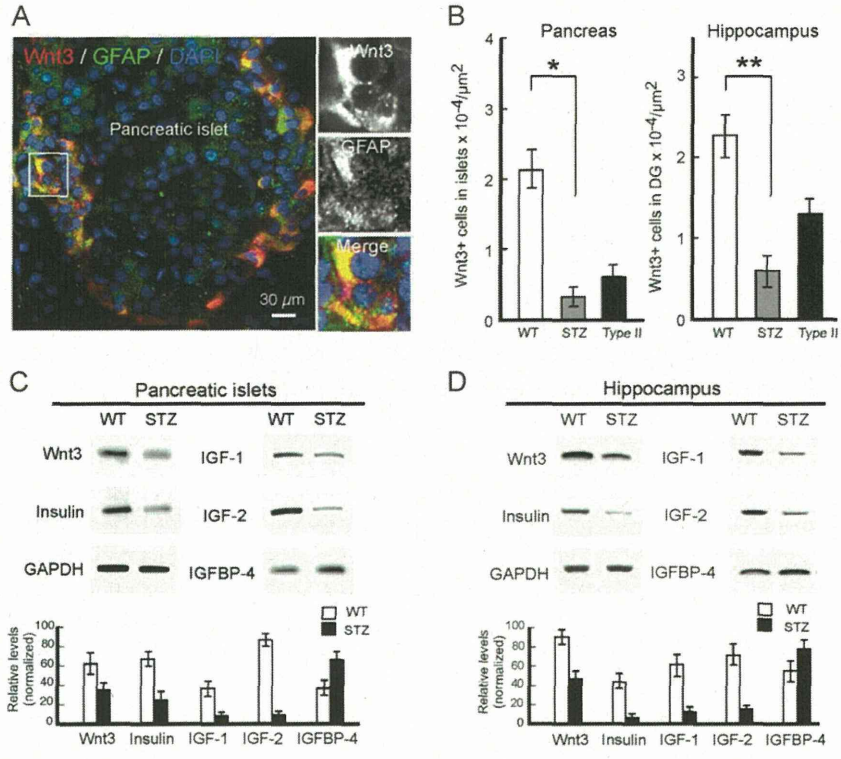

Figure 2. Wnt3, released from pancreatic α cells, decreases in diabetes.

A. Detection of Wnt3 in the pancreatic α cells expressing GFAP. A confocal image of IHC of Wnt3 and GFAP in adult pancreatic islet is shown. Cells in the white square region are magnified and shown in separate panels at right. Wnt3, red; GFAP, green; DAPI, blue.

B. Comparison of the number of Wnt3-positive cells in pancreas (left) and in DG of HPC (right) obtained from wild-type and diabetes rats. White bars, wild-type Fisher 344 rats (10-week old, male); grey bars, STZ-induced type I DB rats (10-week old, male); type II DB GK rats (10-week old, male). $^{*}p < 0.01$ and $^{**}p < 0.001$.

C. Comparison of mRNA levels of Wnt3, insulin, IGFs and IGFBP-4 between wild-type and DB animals (STZ-induced DB rats) in the pancreas.

D. Comparison of mRNA levels of Wnt3, insulin, IGFs and IGFBP-4 between wild-type and DB animals (STZ-induced DB rats) in the HPC. Relative levels of mRNA were normalized to GAPDH in the following Q-PCR analysis and are plotted at bottom.

successful cell replacement therapies for various diseases. The discovery of insulin expression in neurons prompted us to use adult NSCs without exogenous gene induction as sources of insulin-producing cells from the patient's own organs to treat diabetes. HPC NSCs are well established and have been studied extensively, whereas, OB NSCs are of potential interest because of their easily accessible location (Curtis et al, 2007; Liu and Martin, 2003; Pagano et al, 2000; Zhang et al, 2004).

HPC NSCs were round and retained this shape when expanded as a monolayer (Fig 3A, left). OB NSCs grew as heterogeneous forms with adherent property and neurosphere morphologies (Fig 3A, right). Under neuron differentiation conditions, the cell morphologies changed markedly: both HPC and OB NSCs extended prolonged neurites (Fig 3B). Gene expression profiles were measured using Agilent rat genome microarrays (Table 1 of Supporting information). HPC NSC, OB NSC and HPC and OB neuron expression profiles were grouped by hierarchical clustering, and correlation coefficients were computed for all pair-wise comparisons (Fig S5 of Supporting information). Both HPC NSCs and OB NSCs expressed the NSC marker *Sox2*, the radial glial cell marker *nestin* and the neural progenitor markers *Olig2* and *Sox1* (NSC lineage; Fig 3C). NSC marker gene expression was down-regulated in HPC and OB neurons (HPC Neu and OB Neu in Fig 3C), and neuronal marker gene expression was up-regulated (mature neuron marker; Fig 3C), indicating their successful commitment into neuronal lineages.

Importantly, *insulin-1* and *insulin-2* expression increased extensively in HPC and OB neurons (pancreatic genes; Fig 3C). IHC revealed that rat OB neurons were insulin+ natively (Fig S3D of Supporting information), consistent with the microarray data. *Pax3*, an early developmental stage neural crest marker was not detectable. Another neural crest marker, *Sox10*, was expressed in these cells, but the levels were unchanged upon neuronal differentiation. These data suggested that insulin was up-regulated in neurons derived from adult NSCs in both the HPC and OB and that its regulation in adult neuronal lineages could be distinguished from the commitment event in embryonic neural crest lineages.

In the pancreatic lineage, insulin gene expression is regulated by several transcription factors abundant in islets such as NeuroD1, neurogenin 3 Pdx1 and MafA (Melloul et al, 2002; Sander and German, 1997; Servitja and Ferrer, 2004). MafA and MafB are co-expressed in insulin+ β cells during embryogenesis, whereas, in the adult pancreas, only MafA is produced in β cells and MafB in glucagon+ α cells (Nishimura et al, 2006). Our microarray analysis revealed that neither HPC nor OB neurons expressed MafA, Pdx1 or neurogenin 3, which are necessary to activate the insulin gene in pancreatic β cell lineages. Quantitative real-time RT-PCR analysis confirmed that *insulin-1* mRNA induction was correlated with *NeuroD1* mRNA up-regulation in both the HPC and OB (Fig 3D). The *insulin-1* mRNA induction resulting from NeuroD1 siRNA transfection led to its down-regulation in neurons, whereas, *MafA*, *Pdx1* or *neurogenin 3* siRNAs had no influence (Fig 3E). These data suggested that *NeuroD1* expression is required for insulin expression in adult NSCs *in vitro*.

