Title

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Journal

Neuron, 83(4)

ISSN

0896-6273

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Publication Date

2014-08-01

DOI

10.1016/j.neuron.2014.07.014

Peer reviewed

Published in final edited form as:
Neuron. 2014 August 20; 83(4): 789–796. doi:10.1016/j.neuron.2014.07.014.

Long-Distance Axonal Growth from Human Induced Pluripotent Stem Cells After Spinal Cord Injury

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Abstract

Human induced pluripotent stem cells (iPSCs) from a healthy 86 year-old male were differentiated into neural stem cells and grafted into adult immunodeficient rats after spinal cord injury. Three months after C5 lateral hemisections, iPSCs survived and differentiated into neurons and glia, and extended tens of thousands of axons from the lesion site over virtually the entire length of the rat central nervous system. These iPSC-derived axons extended through adult white matter of the injured spinal cord, frequently penetrating gray matter and forming synapses with rat neurons. In turn, host supraspinal motor axons penetrated human iPSC grafts and formed synapses. These findings indicate that intrinsic neuronal mechanisms readily overcome the inhibitory milieu of the adult injured spinal cord to extend many axons over very long distances; these capabilities persist even in neurons reprogrammed from very aged human cells.

INTRODUCTION

Recent findings indicate that multipotent neural progenitor cells derived from either embryonic spinal cord or embryonic stem (ES) cells extend abundant numbers of axons over long distances when implanted into sites of spinal cord injury (SCI) (Lu et al., 2012b). These cells form functional relays across sites of complete spinal cord transection to restore conductivity across a lesion site and improve functional outcomes (Lu et al., 2012b). Grafts of neural progenitor cells can also remyelinate spared axons surrounding sites of partial spinal cord injury, and may have neuroprotective properties (Cummings et al., 2005; Keirstead et al., 2005; Plemel et al., 2011).

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SUPPLEMENTAL INFORMATION
Supplemental Information includes 4 Supplemental Figures and Supplemental Experimental Procedures.

These properties of neural stem cells raise the possibility of developing experimental treatments for spinal cord injury. However, immunosuppression to allow neural stem cell grafting in humans with spinal cord injury is potentially hazardous, given compromised health and high risk of infection in traumatized patients. Alternative cell sources that may not require immunosuppression could constitute a major advance. Accordingly, we explored whether human induced pluripotent stem cells (iPSCs) exhibit similar properties of engraftment, differentiation and axonal outgrowth when driven to a neural stem cell fate. Human iPSCs were differentiated into neural stem cells and implanted into immunodeficient rats two weeks after C5 lateral hemisection lesions, a clinically relevant treatment time period. Three months later, we found an unprecedented degree of axonal outgrowth: indeed, axons extended nearly the entire rostral-to-caudal extent of the adult rat nervous system and formed synapses with rodent neurons, while host axons penetrated human iPSC cell grafts and also formed synapses.

RESULTS

Human induced pluripotent stem cells (iPSCs) were derived from a healthy 86 year-old male and driven toward a neural stem cell (NSC) lineage, as previously described (Yuan et al., 2011; Israel et al., 2012). Briefly, primary dermal fibroblasts were transduced with retroviral vectors expressing OCT4, SOX2, KLF4, and c-MYC, cultured on PA6 cells in the presence of SMAD inhibitors for induction of neural stem cells (Yuan et al., 2011), and FACS-purified using $CD184^{+}$, $CD15^{+}$, $CD44^{-}$, and $CD271^{-}$. Purified neural stem cells were then cultured on poly-ornithine/laminin-coated plates with DMEM/F12 supplemented with N2, B27 and basic fibroblast growth factor. This iPSC derived NSC line was extensively characterized in vitro, including immunocytochemistry, genome wide mRNA expression profiles and electrophysiology (Israel et al., 2012). Proliferating NSCs were transduced with lentiviral vectors expressing green fluorescent protein (GFP) (Taylor et al., 2006). FACS analysis reviewed that more than 99% of NSCs expressed GFP (Fig. S1A). In addition, NSCs, whether transduced with GFP or not, dramatically down-regulated expression of the conventional pluripotency markers Tra1-81 and SSEA4 (Fig. S1B-C) and maintained expression of NSC-associated markers, including nestin and Sox2 (Fig. S1D-E) (Isreal et al., 2012). GFP-expressing NSCs were harvested and embedded in fibrin matrices containing a growth factor cocktail (see Methods; Lu et al., 2012b) to promote graft survival and retention in the lesion site. Cells were grafted into C5 spinal cord hemisection lesion sites (N=7), two weeks after the original spinal cord injury. Control subjects (N=5) underwent the same lesions and injections of the fibrin matrix containing the growth factor cocktail lacking neural stem cells. Subjects underwent weekly functional assessment and were perfused 3 months post-grafting.

Survival, Differentiation and Axonal Outgrowth from Grafted Human iPSC-Derived NSCs

When examined three months post-transplantation, grafted human iPSC-derived NSCs survived and were distributed through most of the lesion (Fig. 1A, S2A-G). Grafts in 4 of 7 animals exhibited a rift near the center of the graft (Fig. S2D-G) that contained collagen that effectively segregated the graft into rostral and caudal components (Fig. S2H-I), while grafts in the remaining three animals were free of rifts but exhibited some attenuation of cell

density near the central region of the graft (Fig. S2A-C, J). The majority (71.2 ± 3.1%) of grafted human iPSC-derived NSCs expressed the mature neuronal marker NeuN (Fig. 1B; Fig. S2K). Grafted cells also expressed the neuronal markers MAP2 and Tuj1 (Fig. S2L-M), whereas expression of the immature neuronal marker doublecortin (DCX) was rarely detected by 3 months post-grafting (not shown). 17.7 ± 2.8% of grafted cells expressed the mature astrocyte marker GFAP (Fig. S2N). We could not detect expression of the mature oligodendrocyte marker APC in NSC grafts: while NG2-labeled oligodendrocyte progenitor cells were present in the graft, they did not co-localize with GFP or human nuclear antigen (Fig. S2O-P), suggesting origin from the host; our iPSC selection conditions favored neuronal and astrocyte enrichment (see methods). In addition to general neuronal markers, 4.4 ± 0.9% of grafted cells expressed choline acetyltransferase (ChAT), characteristic of spinal motor neurons (Fig. S2Q-R). No cells within grafts detectably expressed the serotonergic neuronal marker, 5HT. Some grafted cells migrated into the host spinal cord adjoining the lesion site (Fig. 1A) and expressed the mature astrocytic marker GFAP (Fig. S2S-T). 7% of grafted cells expressed Ki67 (Fig. S3A-D), suggesting continued proliferation of a proportion of grafted human cells three months post-implantation. No teratomas were observed.

Grafted human iPSC-derived NSCs extended very large numbers of GFP-expressing immunoreactive axons directly out of the lesion site and into the host spinal cord (Fig. 1C-G). The axonal nature of these GFP-expressing projections from the lesion site was confirmed by GFP co-localization with Tuj1 (ßIII-tubulin, Fig. 1C inset). Interestingly, despite expression of mature neuronal markers by cell somata in the graft, few GFP-expressing graft-derived axons that had grown out of the graft expressed the mature axonal marker neurofilament (NF), three months post-grafting (Fig. 1C inset). The number of GFP-labeled human axons emerging caudally from the hemi-lesion site was quantified in a series of horizontal sections at a point 0.5mm caudal to the lesion site in the entire right hemicord of a representative subject: 20,500 axons were identified, a conservative estimate, because bundles of axons that could not be independently resolved were counted as single axons. This number is 41% higher than the number of axons emerging from grafts of rat-derived neural progenitor cells per hemicord in our previous report (Lu et al, 2012b). Indeed, within three spinal segments of implantation, the density of graft-derived human axons appeared to qualitatively equal that of rodent host axons in some regions of white matter (Fig. 1G). Notably, human axons grew in organized, rostro-caudal linear trajectories in host white matter (Fig. 1D, F-G). Throughout the course of their white matter projections, human axons gave off branches that dove into host gray matter (Figs. 1E and S3E-H). Human axons in host gray matter were highly ramified (Figs. 1E and S3E-H), similar to patterns of endogenous axonal branching and termination in host gray matter. Many human axons terminated in lamina IV to VII (Fig. 1F). The density of human axons extending in rostral and caudal directions from the implant site qualitatively appeared equal (Fig. S3I-P).

Long-Distance Growth and Connectivity of Grafted Human iPSC-Derived NSCs

Human NSC graft-derived axons extended over very long-distances in the host spinal cord, continuing to extend into the brain and even reaching the olfactory bulb (Fig. 2). Indeed, from the C5 lesion site axons extended through adult host white matter as far caudally as the

distal lumbar spinal cord (Fig. 2A-D), and as far rostrally as the frontal cortex and olfactory bulb (Fig. 2E-K); human axons essentially extended the entire length of the adult rat neuraxis. Most rostrally extending axons grew as a bundle through the ventrolateral spinal cord and ventral portion of the brainstem (Fig. 2E), continuing to extend rostrally through the region of the hypothalamus (Fig. 2E). The total distance traversed corresponded to more than 26 spinal segments, greater than 9 cm in length; this exceeds by two-fold the distance over which axons emerged from grafts of rat multipotent neural precursor cells in our previous study (Lu et al, 2012b). Axon density progressively diminished as a function of distance from the graft.

Recently it was reported that grafts of multipotent neural progenitor cells can spread from an implantation site in approximately half of cases, although teratomas are not observed (Steward et al., 2014 and Tuszynski et al., 2014). After grafting human iPSCs in the present study, we observed cell spread into the central canal for up to three segments from the implantation site in four of seven grafted animals (Fig. S3Q-T). There were not, however, ectopic collections or nodules of cells at more remote segments from the lesion, including the distal spinal cord, brainstem or brain after examining every 6^{th} section labeled for GFP throughout the neuraxis. Graft cells that spread into the central canal extended axons into the immediately adjoining spinal cord, but these did not extend farther than 0.5 – 1mm into host parenchyma. Notably, the extraordinarily high number of axons extending into host white matter surrounding the lesion site emerged directly from grafts in the lesion site (Figs. 1A, C), and not from the modest numbers of neurons located in ectopic cells nests in the central canal (which extended axons into the host spinal cord for more limited distances). Similarly, axons extending into the brainstem and brain were direct extensions of axons in ventromedial white matter emerging from grafts placed in the lesion site, as observed in Fig. 2E; these axons did not arise from the modest numbers of neurons located in ectopic cells nests in the central canal. Tracing of individual axons from the olfactory bulb and lumbar spinal cord to the mid-cervical spinal cord graft would be required to state with certainty that axons visualized as far rostrally as the olfactory bulb and as far caudally as the lumbar spinal cord arose from the graft, rather than these isolated cell nests. However, the preponderance of evidence suggests that these long-growing axons originated from the graft in the lesion site: axons emerging from the graft numbered in the tens of thousands and could be visualized extending directly into the brainstem, whereas: 1) the number of cells in ectopic nests was far lower, 2) cell nests were present only up to three segments from the lesion site, and 3) axons arising form these nests extended few axons in comparison to grafts in the lesion site.

Previously, we reported that grafted rat NSC-derived axons were frequently myelinated by host rat oligodendrocytes in spinal cord white matter, seven weeks post grafting (Lu et al., 2012b). Three months post-grafting an extremely high density of human iPSC-derived axons extended through adult white matter (Figs. 1C,D,F,G; 2A,B,E,H; 3A,B; S3E-H, S3I-P). These extending human axons frequently directly contacted host myelin membranes (Fig. 3A-C), suggesting that graft-derived axons are not inhibited by adult myelin (Schwab et al., 2005). However, graft-derived human axons were not detectably myelinated by rat host oligodendrocytes (Fig. 3A-C).

GFP-labeled human axons formed bouton-like terminals in host rat gray matter (Fig. 3D). These structures co-localized with synaptophysin and were present at all levels of the rat spinal cord in close apposition to host neurons and dendrites, including choline acetyltransferase-expressing host spinal motor neurons (Fig. 3E). Human-specific synaptophysin labeling was detected at human axon terminals in host spinal cord gray matter, and was distributed predominantly in laminae VI-VII (Fig. 3F; Fig. S4A-B, D-E). Human axon terminals expressing human-specific synaptophysin were frequently in direct contact with host neuronal dendrites labeled with MAP-2 (Fig. 3F). Given the availability of a human-specific synaptophysin label (hSyn, Chemicon), we were able to quantify the relative contribution of human synaptophysin to total synaptophysin at a location three spinal segments caudal to the graft, at the C8 level: human synaptophysin accounted for 9% of total synaptophysin at this level (Fig. S4A-F). In addition, a modest number of human axon terminals expressed vesicular glutamate transporter 1 (vGlut1), suggesting the presence of glutamatergic terminals (Fig. 3G), whereas expression of GABAergic markers was not detected. However, the overall expression of transmitter-associated markers was relatively modest, possibly reflecting the still-developing nature of many human axons at this stage. Immunoelectron microscopy confirmed the presence of synaptic structures forming between GFP-labeled, graft-derived human axons and host dendrites (Fig. 3H).

Long-Distance Growth and Connectivity of Human Axons Is Not a Result of Cell Fusion

A priori it is extremely unlikely that the human axons observed in this study were a result of fusion with host rat spinal cord neurons, given: 1) the very high density of GFP-expressing axons, 2) their presence in axonal tracts originating from the brain (where fusion could not have occurred), and 3) their projection to ectopic locations (such as the olfactory bulb). To confirm the absence of fusion, we performed an additional study. Human iPSC-derived NSCs were transduced with lentiviral vectors to express red fluorescent protein (RFP) and were grafted into C5 hemisection lesion sites in transgenic GFP-expressing mice (N=4). The recipient mice were immunodeficient (Niclou et al., 2008). When examined one month after grafting, numerous graft-derived axons emerged from the lesion site; cells or axons double labeled for RFP (human) and GFP (rats) were never detected (Fig. 4A-B).

Host Axonal Growth into Grafted Human iPSC-Derived NSCs

We examined whether host axons also grew into human iPSC-derived NSC grafts. Host serotonergic axons, which exert important roles in modulating motor function (Ribotta et al., 2000; Musienko et al., 2011), penetrated human iPSC-derived NSC grafts and expressed the presynaptic terminal marker synaptophysin (Fig. 4C-F). Their growth into iPSC grafts significantly exceeded growth into the lesion cavity in control animals by 6-fold ($P<0.001$, Fig 4G). The reticulospinal motor projection to the spinal cord was anterogradely labeled by injecting biotinylated dextran amine (BDA) into the pontine paragigantocellular reticular nucleus (Lu et al., 2012a). Reticulospinal motor axons also penetrated human iPSC-derived NSC grafts (Fig. S4G-I). Thus, reciprocal connections from host-to-graft (Fig. 4; Fig. S4G-I) and graft-to-host (Fig. 3) were present.

The presence of both host-to-graft and graft-to-host synaptic structures generated a potential mechanism for creating relays across lesions that could support functional recovery (Lu et

al., 2012b). However, as noted earlier, collagenous rifts were present within the centers of most grafts, and axons did not cross these rifts (Fig. S2D-I); this could lead to disconnection of proximal and distal graft segments. In addition, among grafts lacking clear midline rifts, there were regions of cell density attenuation in the graft core (Fig. 1A and S2A-C, J) that also reduced potential substrates for neural relay formation. When behavioral outcomes were assessed on three tasks sensitive to forelimb function (grid-walking, grooming and vertical exploration), functional recovery was not observed (Fig. S4J-L).

DISCUSSION

We recently reported that neural stem cells extend axons over very long distances when grafted to the injured spinal cord (Lu et al., 2012b), despite the inhibitory milieu of the adult central nervous system. We now report yet more extensive growth of neural stem cells derived from human iPSCs: human axons extend in both greater numbers and over virtually the entire length of the rat central nervous system. Indeed, in some regions of the injured spinal cord, the number of human axons appears nearly equal to rat axons (Fig. 3A-B). Human axons form synapses onto rodent neurons, and rodent axons penetrate human grafts and express pre-synaptic proteins. Notably, this degree of axonal extension occurs from cells of an 86 year-old human, indicating that age does not appear to be a barrier to the expression of highly plastic properties of neural stem cells grafted into models of central injury. These findings provide further support that intrinsic neuronal growth mechanisms overcome the inhibitory milieu of the injured adult CNS, permitting extensive and very long-distance axonal growth.

While previous studies grafted iPSC-derived neural stem cells in models of spinal cord injury, the extensive degree of axonal growth that we detect was not previously recognized, possibly because reporter genes or their promoters were weaker (Tsuji et al., 2010; Nori et al., 2011; Fujimoto et al., 2012; Nutt et al., 2013). Our vectors robustly express enhanced GFP using a hybrid chick ß-actin promoter (Taylor et al., 2006). Moreover, our grafting method appears to result in superior graft survival and filling of the lesion site, which may support emergence of greater numbers of axons.

Human axons in this experiment were in direct physical contact with rat myelin (Fig. 3C), yet their growth was not inhibited. It is possible that the lengthened developmental period of human compared to rat neurons allowed human axons to grow for longer distances prior to the expression of cell surface receptors that mediate axon-glial interactions and myelin-associated inhibition. Indeed, human axons emerging from grafts commonly expressed the early axonal marker Tuj1 but not the mature axonal marker neurofilament, suggesting that as axons extend outward from the lesion site they are still at a developmental stage. The expression of neurofilament in the developing human CNS starts as late as gestational week 25 (>6 months) (Pundir et al., 2012), and it is possible that axons had not yet reached this stage of relative maturity. A prolonged developmental period of human neurons could also account for the lack of detectable myelination of human axons in the rat spinal cord in this experiment: human axon myelination might require neuronal maturation, or contact/stabilization with a host target neuron. Previous reports demonstrate that human oligodendrocytes are capable of myelinating rodent axons (Keirstead et al., 2005; Wang et

al., 2013), suggesting that axon-glial signals governing myelination are conserved across these species.

Host axons grew into human iPSC-derived neural stem cell grafts placed in the C5 hemisection lesion site and formed synaptic structures, and human axons grew in both directions from the lesion site and formed synaptic structures with host neurons and dendrites. Yet persistent functional improvement was not detected, in contrast to our previous study grafting rat or human neural progenitors to sites of spinal cord injury (Lu et al., 2012b). Numerous mechanisms may account for a lack of functional benefit in this study, including: 1) separation of the graft into rostral and caudal compartments by a central collagenous fissure, across which relays did not extend, 2) continuing maturation of grafted cells, such that too few neurons were *fully* mature and functional at the time of behavioral assessment to support recovery, 3) an absence of myelination of grafted axons (Alto et al., 2009), 4) formation of ectopic projections that were functionally detrimental, 5) insufficient expression of neurotransmitters that are compatible with normal circuit function, or 6) a type I error in which too few animals were studied to yield significant differences.

Can one conclude from the present study that human iPSC-derived neurons are inferior to ES-derived or neural progenitor cells in supporting functional recovery, since we observed significant functional improvement in our previous study after grafting multipotent neural precursor cells to sites of T3 complete transection (Lu et al., 2012b), but no functional improvement in the present study? No. The comparison of behavioral outcomes between studies is complex. Our previous study (Lu et al., 2012b) assessed outcomes on hindlimb locomotion which reflects whether supraspinal inputs from above the lesion are activating pattern generators for locomotion that are located entirely below the lesion; reconstruction of the (preserved) pattern generator is not necessary, only the activation of this circuitry. In contrast, the motor endpoints in the current study consist of more complex use and placement of the forepaw, features that are not driven by local pattern generators and that likely require more accurate innervation and integration of several supraspinal inputs to local segmental motor output neurons. Moreover, this lesion was a hemisection rather than a complete transection, thus host axon sparing and sprouting may support, or interfere with, functional improvement (Tuszynski and Steward, 2012). A determination of relative functional benefits of iPSC-derived neural stem cells, embryonic-derived neural stem cells and rodent-derived neural stem cells requires concurrent study in the same model system to draw clear conclusions. Moreover, in the present iPSC grafts contained collagenous rifts that divided grafts into rostral and caudal compartments across which axons did not travel, precluding formation of neuronal relays across the lesion to restore function. Grafts in our previous study did not contain large rifts, although smaller rifts were present in some cases (Lu et al., 2012b). We also observe collagenous rifts when grafting neural stem cells into animals with chronic injuries (unpublished observations). We are currently attempting to attenuate or eliminate rifts by degrading collagen in the lesion cavity at the time of neural stem cell grafting. Interestingly, grafts into closed contusion cavities do not consistently form rifts (unpublished observations), suggesting that cells from the leptomeninges in open lesion cavities form these scars. Spinal cord contusion is the most common mechanism of

human injury, and rift formation may therefore not be a limiting factor in considering future human translation.

iPSC-derived cells offer the possibility of avoiding immunosuppressant drugs that are problematic in an already severely injured and vulnerable human population. One human iPSC line was the subject of the present study, and additional lines need to be tested to determine the extent to which heterogeneity in donor source and methods of cell preparation (Toivonen et al., 2013) may influence graft survival, axon outgrowth and functional connectivity. In addition, specific iPSC lines have differing teratogenic properties (Tsuji et al., 2010; Nori et al., 2011; Fujimoto et al., 2012). Yet the present demonstration that human neural stem cells exhibit the most extensive outgrowth of axons from a spinal cord lesion site yet reported, that these properties are observed even when using donor cells of old age, and that grafting can be performed after clinically relevant treatment delays, motivates the performance of additional studies that more fully identify and utilize optimal cell types for non-human studies of neural injury and for potential future translational trials. Prior to considering clinical trials, several additional points should be addressed: can extending axons be guided toward appropriate targets and away from inappropriate targets? Might axons that project to ectopic targets result in adverse behavioral consequences? How will grafts affect sensory and autonomic outcomes? Will projections arising from grafted neural stem cells, like the naturally developing nervous system, undergo pruning, as some projections are strengthened and others eliminated? These and related studies will determine whether the extensive axonal growth observed from iPSC-derived neural stem cells can be harnessed for therapeutic benefit.

EXPERIMENTAL PROCEDURES

Human iPS cells and NSCs

The generation of human iPS cells and NSCs was described previously (Yuan et al., 2011; Israel et al., 2012). NSCs were transduced with lentiviral vectors expressing green fluorescent protein (GFP) (Taylor et al., 2006).

In Vivo Studies

Adult female athymic nude rats (N=14) and adult SCID mice (N=4) underwent C5 lateral spinal cord lesions; 7 rats received human iPSC-derived NSC grafts, and 5 controls received no grafts; all underwent weekly behavioral analysis for 3 months post-grafting. Two additional subjects were grafted and dedicated to ultrastructural analysis. Four SCID-GFP mice received RFP-expressing iPSC grafts for study of cell fusion over a 1 month survival period.

Anatomical Analysis

Spinal cord blocks containing iPSC grafts, the rest of spinal cord and brain were sectioned and immunolabeled for GFP and additional cell markers. We quantified neural cell differentiation, the density of human axons and synaptophysin. Host axonal growth was identified by 5-HT immunolabeling and BDA anterograde tracing.

Statistical Methods are noted in Supplementary information.

Additional details are in Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Veterans Administration, NIH (NS09881 and EB014986), the Craig H. Neilsen Foundation, the California Institute for Regenerative Medicine, and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation.

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Highlights

- Human iPSC-derived neural stem cells are grafted to sites of rat spinal cord injury
- Human neurons extend numerous axons into host spinal cord and form synaptic contacts
- Graft-derived human axons extend virtually the entire length of the rat CNS
- Host axons penetrate human iPSC grafts and form synapses

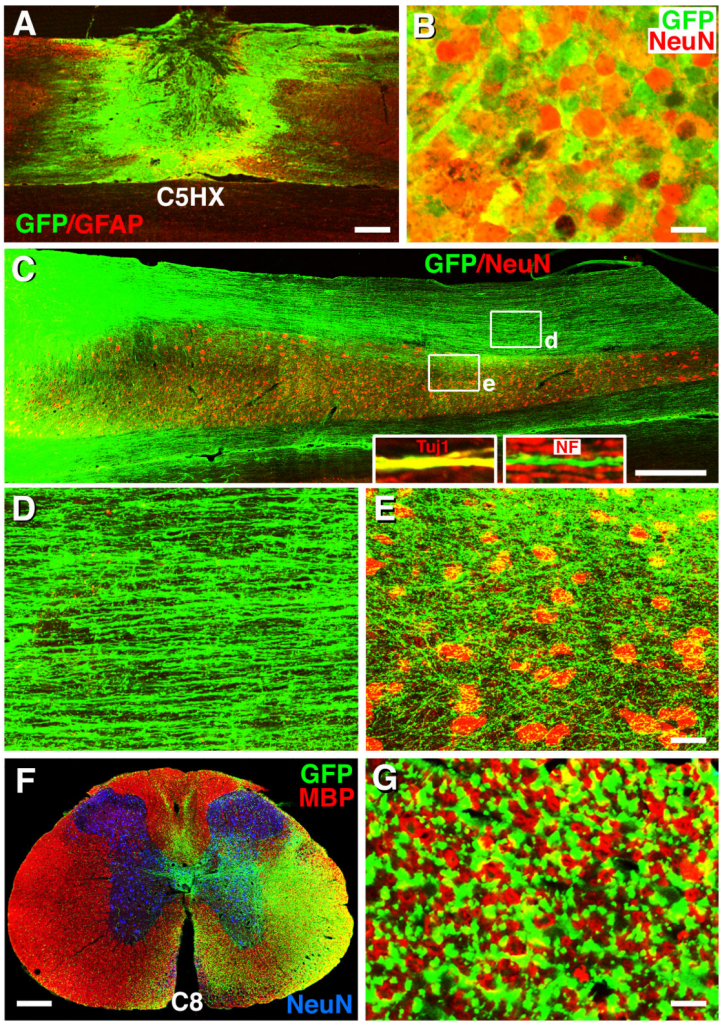

Figure 1. Survival, Differentiation and Growth of Human iPSC-Derived Neural Stem Cells in Sites of Spinal Cord Injury

(**A**) **GFP**-labeled human iPSC-derived neural stem cells were grafted into sites of C5 hemisection spinal cord injury. Horizontal section immunolabeled for **GFP** and **GFAP** indicates that implants survive well and distribute through the lesion cavity. Rostral is to left, caudal is to right. **(B)** The majority of cells within the graft immunolabel for mature neuronal markers **NeuN**, indicating neuronal differentiation. (**C-E**) Very large numbers of **GFP**-labeled axons extend caudally into the host spinal cord **(D)** white matter and **(E)** gray matter (region of **NeuN** labeling). Insets in panel C indicate that axons co-localize with **Tuj1**, but not neurofilament (**NF**)**. (F-G) GFP**, **MBP,** and **NeuN** triple labeling of a coronal section 3 segments (C8) caudal to the graft shows dense distribution of human axons predominantly on right, lesioned side of the spinal cord. (**G**) Higher magnification of panel **F** from lateral white matter demonstrates remarkably high number of human axons interspersed in white matter. Scale bar: A, 350 μm; B, 10 μm; C, 600 μm; D-E, 32 μm; F, 250 μm; G, 20 μm.

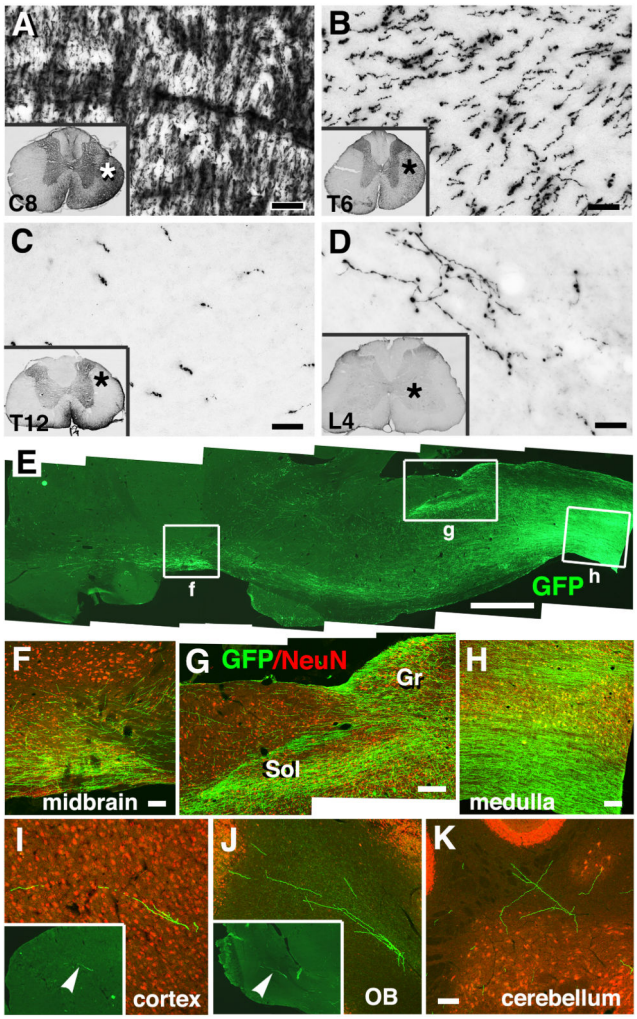

Figure 2. Long-Distance Growth of iPSC-Derived NSCs

(A-D) Light-level **GFP** immunolabeling of human iPSC-derived axons in coronal sections shows very large numbers of axons extending into *caudal* host spinal cord. Insets in each panel show the sampled region from which higher magnification views were obtained: **(A)** C8, **(B)** T6, **(C)** T12 and **(D)** L4. **(E)** Fluorescent **GFP** labeled human iPSC-derived axons extend *rostrally* into brain in a sagittal section at a low magnification. (**F-H)** Higher magnification views from the boxed areas in panel **E** show that **GFP** labeled human axons extend into **(F)** midbrain and **(G)** gracile (Gr) and solitary (Sol) nuclei; **(H)** very large numbers of axons enter the medulla. **NeuN** labels host brain neurons. **(I-K)** Individual **GFP**-labeled human axons are present in **(I)** cortex, **(J)** olfactory bulb, and **(K)** cerebellum. Insets indicate region of sampling. Scale bar: A-C, 20 μm; D, 60 μm; E, 1.7 mm; F, H, 110 μm, G, 180 μm, I-K, 100 μm.

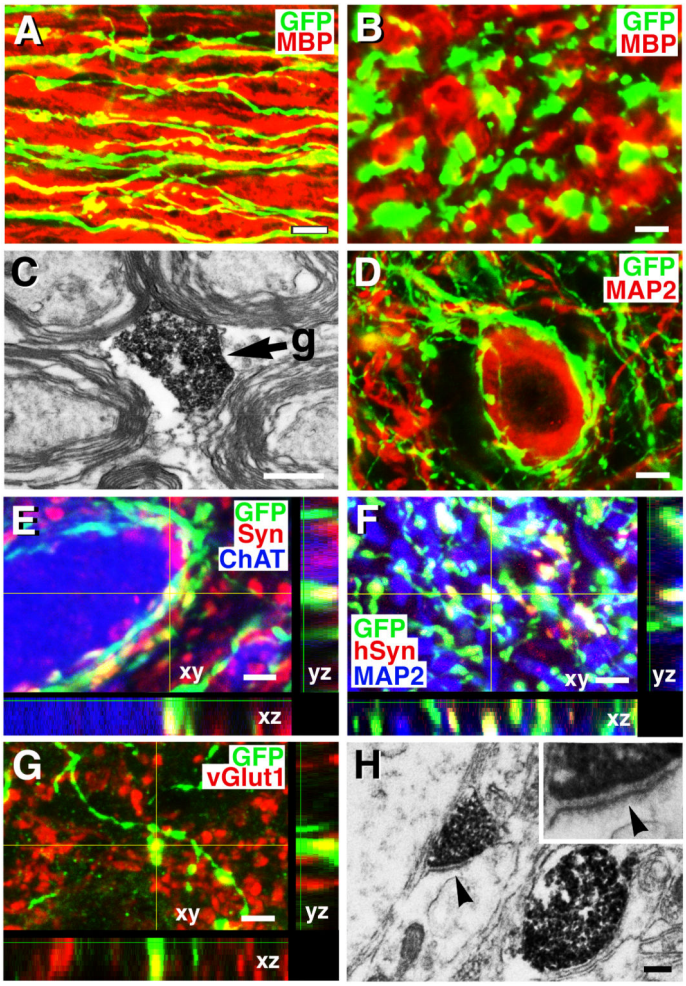

Figure 3. Association of Human Axons with Host Myelin, and Connectivity with Host

(A-B) GFP and **MBP** double labeling indicate close association of human axons with host myelin in white matter. **(A)** Horizontal and **(B)** coronal section (C8). Myelination of human axons is not evident. **(C)** Electron microscopy confirms that extending iPSC-derived graft (**g**) axons contact host myelin sheaths. **(D) GFP**-expressing human axon terminals are closely associated with **MAP-2**-expressing host neurons and dendrites caudal to the lesion site. **(E)** A z-stack image triple labeled for **GFP**, synaptophysin (**Syn**), and **ChAT**, indicating co-association of graft-derived human axon terminals with a synaptic marker in direct association with host motor neurons. **(F)** A z-stack image triple labeled for **GFP**, human-specific synaptophysin (**hSyn**), and **MAP2**, showing graft-derived human axon terminals with a synaptic marker in direct association with host dendrites. **(G)** Double labeling for **GFP**, vesicular glutamate transporter 1 (**vGlut1**) showing a graft-derived human axon terminal co-expression of **vGlut1**. **(H)** Electron microscopy confirms that DAB-labeled, **GFP**-expressing human axon terminals form synapses (arrowhead) with host dendrites (see inset). Scale bar: A, D, 4 μm; B, E, G, 2 μm; C, 500 nm; F, 1.8 μm; H 200 nm.

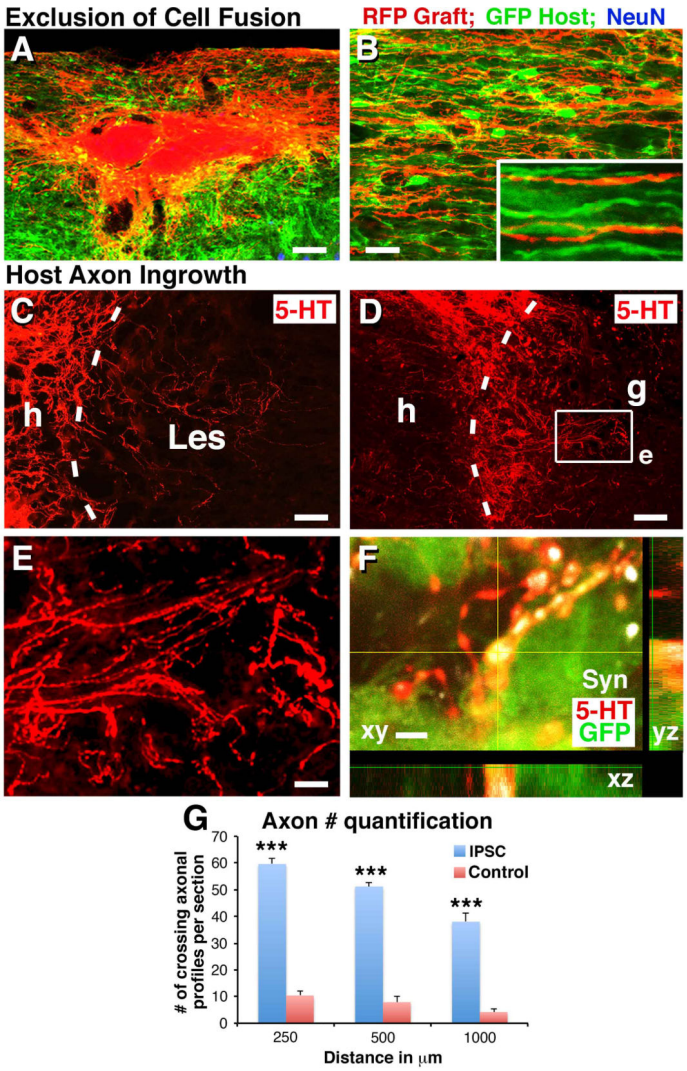

Figure 4. Lack of Cell Fusion; Host Axonal Ingrowth into Grafts

(A) Triple labeling for **RFP**, **GFP**, and **NeuN** reveals survival and neurite extension from **RFP**-expressing human iPSC-derived NSCs into **GFP** transgenic adult mice with C5 spinal cord lesions. **(B)** Higher magnification view from host white matter 2 mm caudal to the graft shows that **RFP**-labeled human axons do not co-localize with **GFP**-expressing host white matter processes. Individual **RFP** axons are distinct from host **GFP** axons in inset. **(C)** Host (**h**) raphespinal axons penetrate a control lesion (**Les**) site (injected at the time of grafting with a fibrin/thrombin matrix, but no cells); dashed lines indicate host/lesion interface. **(D)** Host raphespinal axons penetrate an iPSC graft (**g**) in the lesion site; the boxed region is shown in panel **E. (F)** Serotonergic axons penetrating grafts in the lesion site express the pre-synaptic protein synaptophysin. **(G)** Quantification shows a 6-fold increase in penetration of host serotonergic axons into the lesion site compared to controls, $P<0.001$. Scale bar: A, 100 μm; B, 22 μm; C-D, 120μm; E, 10μm; G, 5 μm.