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Report

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

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SUMMARY

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 CNS. 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).

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 2 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 grafts and also formed synapses.

RESULTS

Human 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 fluorescence-activated cell sorting (FACS)-purified using $CD184^{+}$, $CD15^{+}$, $CD44^{-}$, and $CD271^{-}$. Purified neural stem cells were then cultured on polyornithine/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 *GFP* (Taylor et al., 2006). FACS analysis reviewed that more than 99% of NSCs expressed GFP (Figure S1A available online). In addition, NSCs, whether transduced with GFP or not, dramatically downregulated expression of the conventional pluripotency markers Tra1-81 and SSEA4 (Figures S1B and S1C) and maintained expression of NSC-associated markers, including nestin and Sox2 (Figures S1D and S1E) (Israel et al., 2012). GFP-expressing NSCs were harvested and embedded in fibrin matrices containing a growth factor cocktail (see Experimental Procedures; 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), 2 weeks after the original spinal cord injury. Control

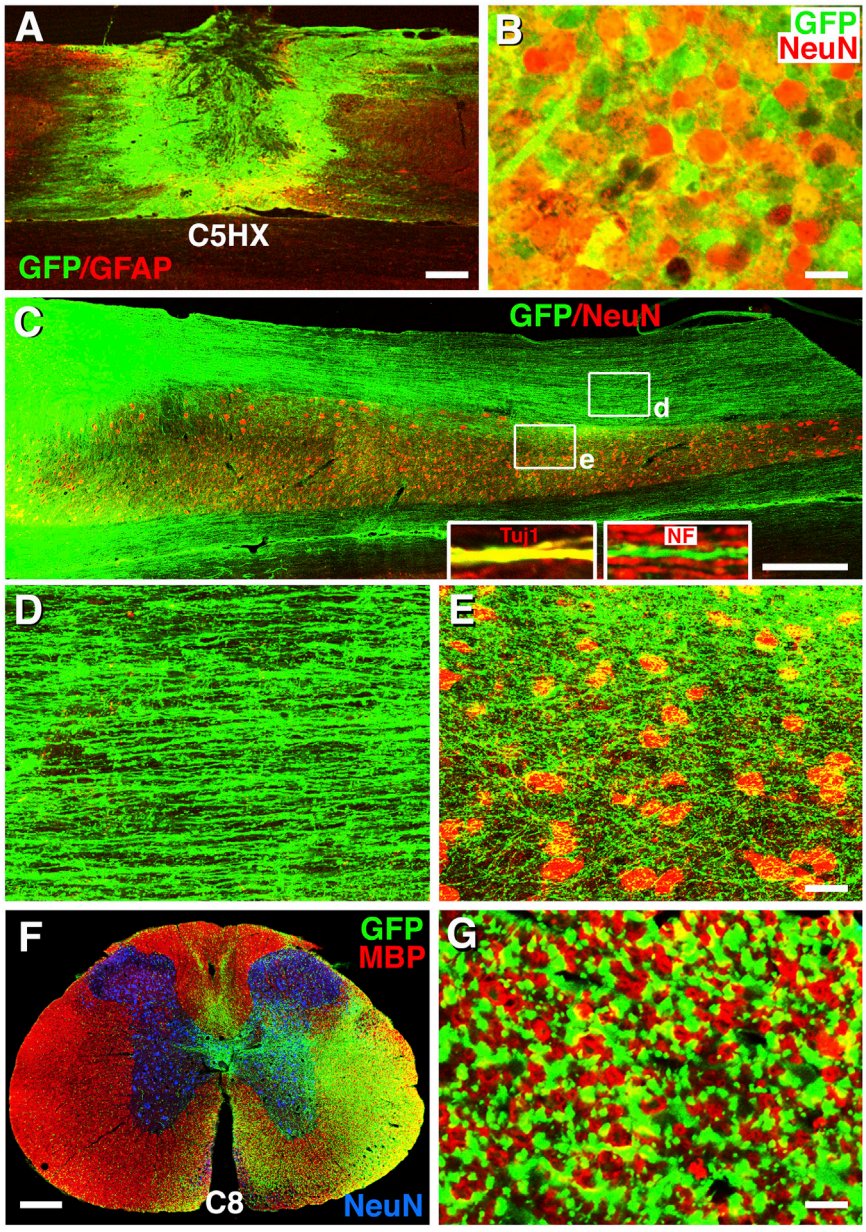

Figure 1. Survival, Differentiation, and Growth of Human iPSC-Derived Neural Stem Cells in Sites of Spinal Cord Injury

(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, and 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 (C) indicate that axons colocalize with Tuj1 but not neurofilament (NF). (F and G) GFP, MBP, and NeuN triple labeling of a coronal section three 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 (F) from lateral white matter demonstrates remarkably high number of human axons interspersed in white matter. Scale bar indicates 350 μm in (A), 10 μm in (B), 600 μm in (C), 32 μm in (D) and (E), 250 μm in (F), and 20 μm in (G).

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 postgrafting.

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

When examined 3 months posttransplantation, grafted human iPSC-derived NSCs survived and were distributed through most of the lesion (Figure 1A; Figures S2A–S2G). Grafts in four of seven animals exhibited a rift near the center of the graft (Figures S2D–S2G) that contained collagen that effectively segregated the graft into rostral and caudal components (Figures S2H and S2I), 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 (Figures S2A–S2C and S2J). The majority (71.2% ± 3.1%) of grafted human iPSC-derived NSCs expressed the mature neuronal marker NeuN (Figure 1B; Figure S2K). Grafted cells also expressed the neuronal markers MAP2 and Tuj1 (Figures S2L and S2M), whereas expression of the immature neuronal marker doublecortin (DCX) was rarely detected by 3 months postgrafting (data not shown); 17.7% ± 2.8% of grafted cells expressed the mature astrocyte marker GFAP (Figure 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 colocalize with GFP or human nuclear antigen (Figures S2O and S2P), suggesting origin from the host; our iPSC selection conditions favored neuronal and astrocyte enrichment (see Experimental Procedures). In addition to general neuronal markers, 4.4% ± 0.9% of grafted cells expressed choline acetyltransferase (ChAT), characteristic of spinal motor neurons (Figures S2Q and S2R). No cells within grafts detectably expressed the serotonergic neuronal marker, 5-HT. Some grafted cells migrated into the host spinal cord adjoining the lesion site (Figure 1A) and expressed the mature astrocytic marker GFAP (Figures S2S and S2T). Seven percent of grafted cells expressed Ki67 (Figures S3A–S3D), suggesting continued proliferation of a proportion of grafted human cells 3 months postimplantation. 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 (Figures 1C–1G). The axonal nature of these GFP-expressing projections from the lesion site was confirmed by GFP colocalization with Tuj1 (βIII-tubulin, Figure 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) 3 months postgrafting (Figure 1C, inset). The number of GFP-labeled human axons emerging caudally from the hemilesion site was quantified in a series of horizontal sections at a point 0.5 mm 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 (Figure 1G). Notably, human axons grew in organized, rostrocaudal linear trajectories in host white matter (Figures 1D, 1F, and 1G). Throughout the course of their white matter projections, human axons gave off branches that dove into host gray matter (Figures 1E; Figures S3E–S3H). Human axons in host gray matter were highly ramified (Figures 1E; Figures S3E–S3H), similar to patterns of endogenous axonal branching and termination in host gray matter. Many human axons terminated in lamina IV to VII (Figure 1F). The density of human axons extending in rostral and caudal directions from the implant site qualitatively appeared equal (Figures S3I–S3P).

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 (Figure 2). Indeed, from the C5 lesion site axons extended through adult host white matter as far caudally as the distal lumbar spinal cord (Figures 2A–2D) and as far rostrally as the frontal cortex and olfactory bulb (Figures 2E–2K); 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 (Figure 2E), continuing to extend rostrally through the region of the hypothalamus (Figure 2E). The total distance traversed corresponded to more than 26 spinal segments, greater than 9 cm in length; this exceeds by 2-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; 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 (Figures S3Q–S3T). 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 sixth 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 0.5–1 mm 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 (Figures 1A and 1C) and not from the modest numbers of neurons located in ectopic cell 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 Figure 2E; these axons did not arise from the modest numbers of neurons located in ectopic cell nests in the central canal. Tracing of individual axons from the olfactory bulb and lumbar spinal cord to the midcervical 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 from 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 7 weeks postgrafting (Lu et al., 2012b). Three months postgrafting an extremely high density of human iPSC-derived axons extended through adult white matter (Figures 1C, 1D, 1F, 1G, 2A, 2B, 2E, 2H, 3A, and 3B; Figures S3E–S3P). These extending human axons frequently directly contacted host myelin membranes (Figures 3A–3C), 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 (Figures 3A–3C).

GFP-labeled human axons formed bouton-like terminals in host rat gray matter (Figure 3D). These structures colocalized 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 (Figure 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 (Figure 3F; Figures S4A, S4B, S4D, and S4E). Human axon terminals expressing human-specific synaptophysin were frequently in direct contact with host neuronal dendrites labeled with MAP-2 (Figure 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

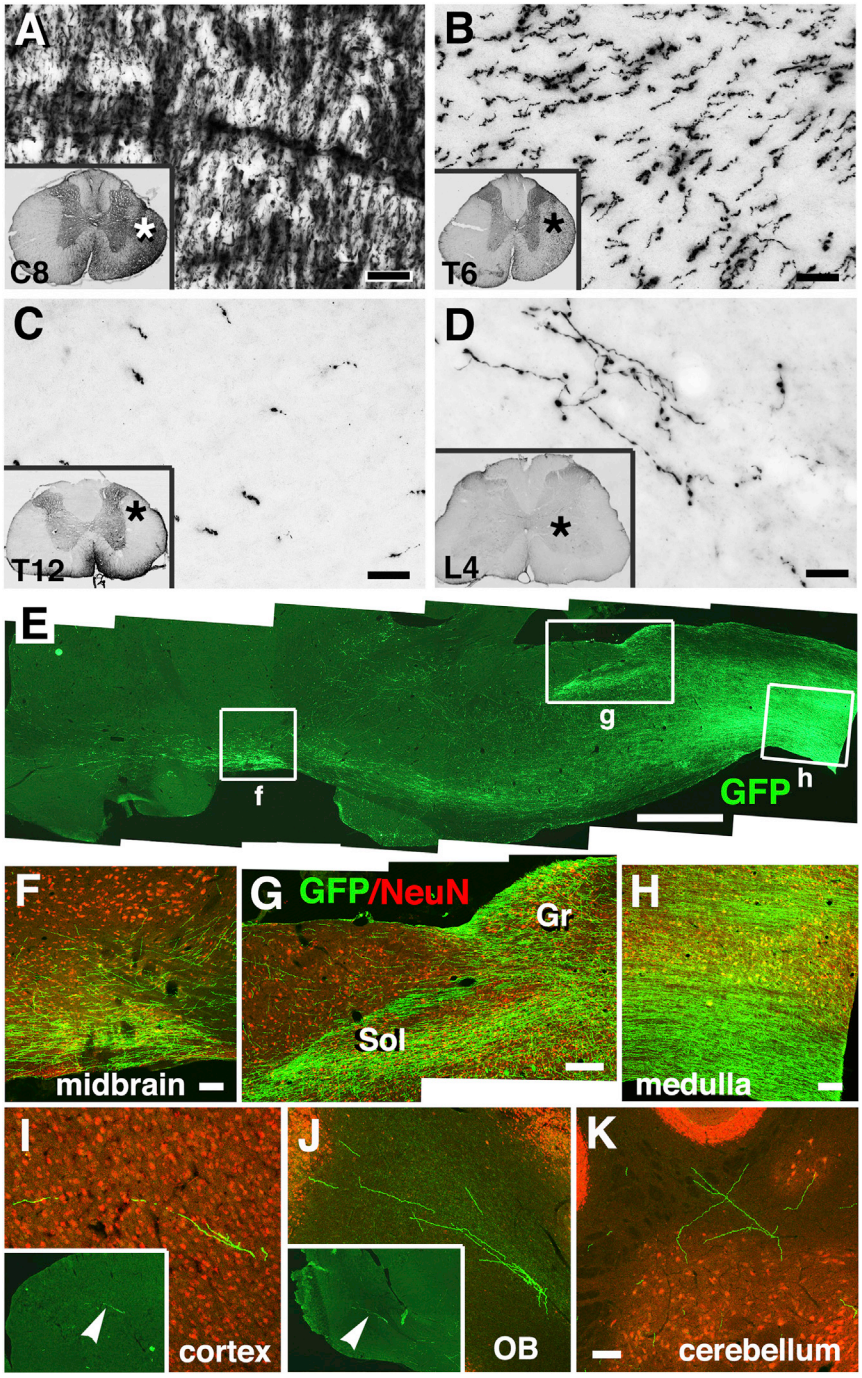

Figure 2. Long-Distance Growth of iPSC-Derived NSCs

(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 (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 indicates 20 μm in (A)–(C), 60 μm in (D), 1.7 mm in (E), 110 μm in (F) and (H), 180 μm in (G), and 100 μm in (I)–(K).

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 (Figures S4A–S4F). In addition, a modest number of human axon terminals expressed vesicular glutamate transporter 1 (vGlut1), suggesting the presence of glutamatergic terminals (Figure 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 (Figure 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 1 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 (Figures 4A and 4B).

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.,

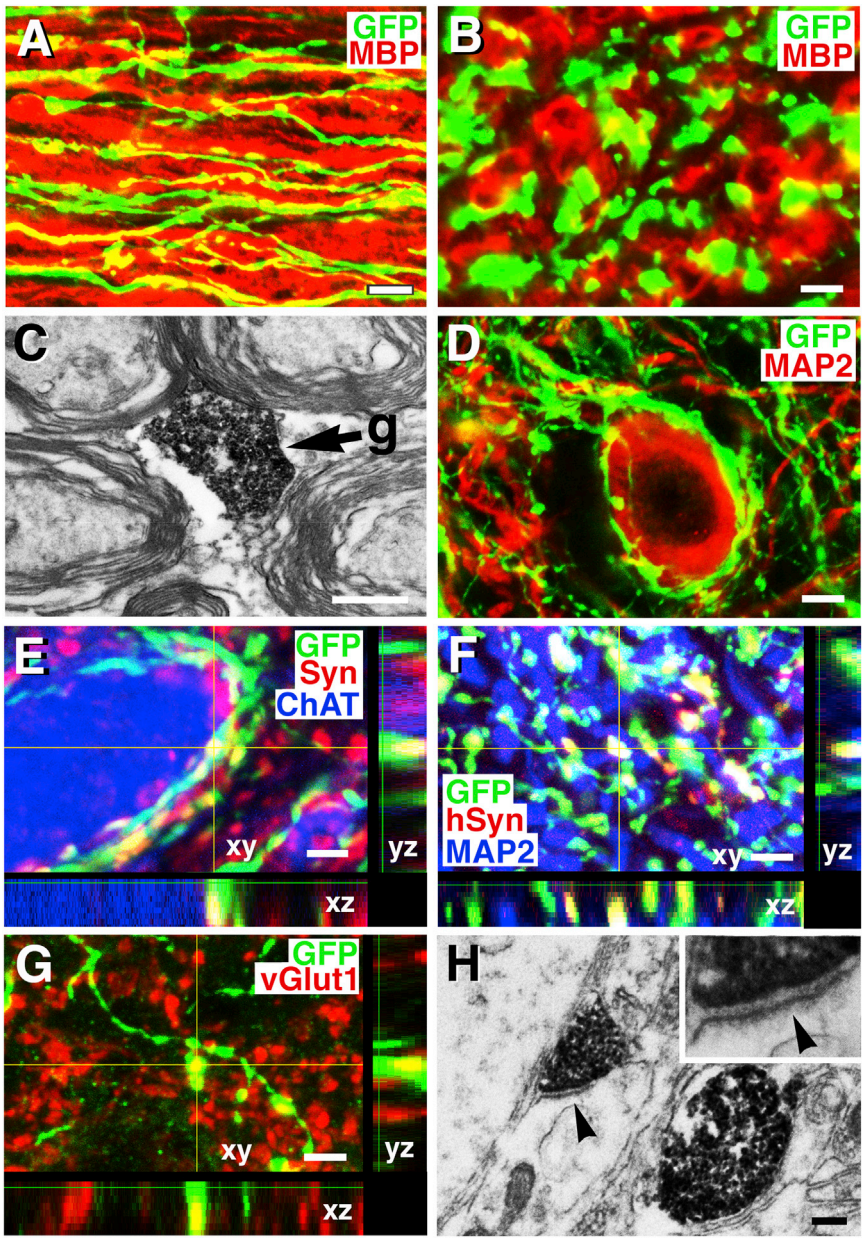

Figure 3. Association of Human Axons with Host Myelin and Connectivity with Host

(A and 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 coassociation 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 coexpression of vGlut1. (H) Electron microscopy confirms that DAB-labeled, GFP-expressing human axon terminals form synapses (arrowhead) with host dendrites (see inset). Scale bar indicates 4 μm in (A) and (D), 2 μm in (B), (E), and (G), 500 nm in (C), 1.8 μm in (F), and 200 nm in (H).

2000; Musienko et al., 2011), penetrated human iPSC-derived NSC grafts and expressed the presynaptic terminal marker synaptophysin (Figures 4C–4F). Their growth into iPSC grafts significantly exceeded growth into the lesion cavity in control animals by 6-fold ($p < 0.001$; Figure 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 (Figures S4G–S4I). Thus, reciprocal connections from host-to-graft (Figure 4; Figures S4G–S4I) and graft-to-host (Figure 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 (Figures S2D–S2I); 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 (Figures 1A; Figures S2A–S2C and S2J) 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 (Figures S4J and S4L).

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 CNS. 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 CNS. Indeed, in some regions of the injured spinal cord, the number of human axons appears nearly equal to rat axons (Figures 3A and 3B). Human axons form synapses onto rodent neurons,

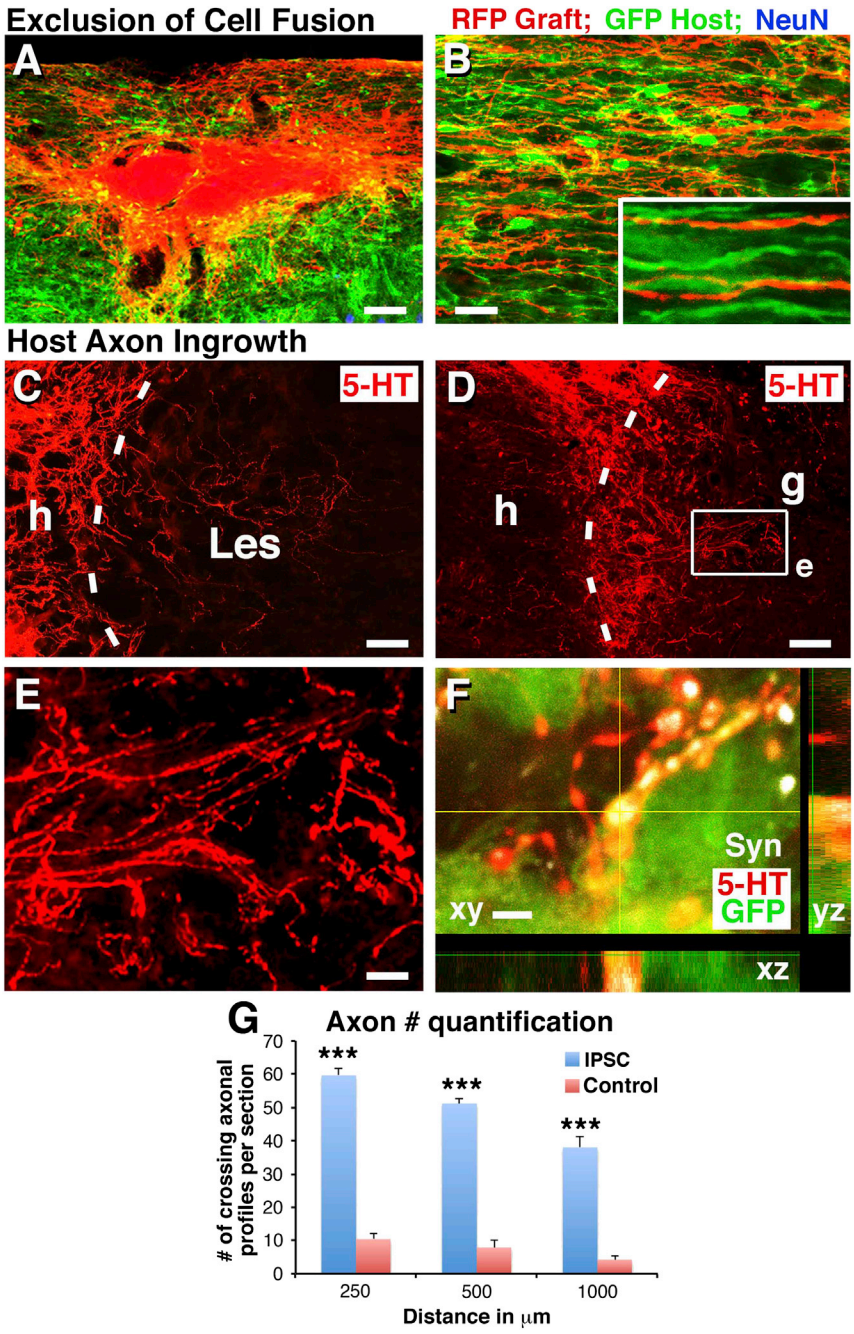

Figure 4. Lack of Cell Fusion, Host Axonal Ingrowth into Grafts

(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 colocalize 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 (E). (F) Serotonergic axons penetrating grafts in the lesion site express the presynaptic protein synaptophysin. (G) Quantification shows a 6-fold increase in penetration of host serotonergic axons into the lesion site compared to controls, $p < 0.001$. Data are represented as mean ± SEM. Scale bar indicates 100 μm in (A), 22 μm in (B), 120 μm in (C) and (D), 10 μm in (E), and 5 μm in (F).

and rodent axons penetrate human grafts and express presynaptic 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 β-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 (Figure 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 probably 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 (P.L. et al., unpublished data). 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 (P.L. et al., unpublished data), 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 nonhuman 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 iPSCs and NSCs

The generation of human iPSCs and NSCs was described previously (Yuan et al., 2011; Israel et al., 2012). NSCs were transduced with lentiviral vectors expressing *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; seven rats received human iPSC-derived NSC grafts, and five controls received no grafts; all underwent weekly behavioral analysis for 3 months postgrafting. 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 Supplemental Experimental Procedures.

Additional details are in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.07.014.

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