Bridging the injured spinal cord with neural stem cells

Spinal cord injury (SCI) damages not only the gray matter neurons, but also the white matter axonal tracts that carry signals to and from the brain, resulting in permanent loss of function below injury. Neural stem cells (NSCs) have high therapeutic potential for reconstruction of the injured spinal cord since they can potentially form neuronal relays to bridge functional connectivity between separated spinal cord segments. This requires host axonal regeneration into and connectivity with donor neurons, and axonal growth and connectivity of donor neurons to host central nervous system (CNS) circuitry. In this mini-review, we will discuss key studies that explore novel neuronal relay formation by grafting NSCs in models of SCI, with emphasis on long-distance axonal growth and connectivity of NSCs grafted into injured spinal cord.

Traumatic spinal cord injury results in permanent loss of motor, sensory and autonomic function below injury. Unlike neurons of the peripheral nervous system, injured neurons of the CNS do not undergo spontaneous regeneration (Ramón y Cajal et al., 1991). One strategy to repair the injured spinal cord is transplantation of cells that can replace lost neural tissue in sites of SCI for potential functional recovery. This is especially true following severe SCI, in which there is little sparing of tissue and thus little chance to restore functional connectivity between severed spinal cord segments.

Neural stem cells, which can proliferate and differentiate into neurons and glia, have high therapeutic potential for reconstruction of the injured spinal cord. This includes host axonal regeneration and connection into permissive transplanted NSCs, and growth and connectivity of graft-derived axons with host neurons (Jakeman and Reier, 1991; Bregman et al., 1993; Bamber et al., 1999). This reciprocal growth of host axons into the grafted neurons and from grafted neurons into host constitutes novel relay circuits that can bridge the injured spinal cord (Figure 1). In addition, transplanted NSCs may reduce secondary injury (Cummings et al., 2005) and have potential to re-myelinate spared, demyelinated axons (Keirstead et al., 2005).

**Grafted NSCs as a permissive substrate for host axonal regeneration**

One important objective for NSC transplantation is to promote regeneration of injured host axons, since differentiation of NSCs into neural tissue mimics a state of CNS development that can provide a permissive environment for host axonal growth and regeneration. In addition, NSCs are unique among other cell types since they can generate new neurons that can serve as targets for regenerating host axons. Indeed, early studies report that host axons could penetrate fetal spinal cord grafts, including some long tract systems, such as corticospinal, serotoninergic, noradrenergic, local propriospinal axons, and sensory axons (Jakeman and Reier, 1991; Bregman et al., 1993; Bamber et al., 1999). However, the number of regenerated axons is not known and the distance into the grafts remains modest and the distance into the graft is relatively short (within 0.5 mm). This is in contrast to robust regeneration or regrowth into and beyond fetal spinal cord grafts by host axons in the neonatal stage where CNS neurons still have great regenerative capacity (Bregman et al., 1993; Ishii et al., 1994). Recent studies, including ours, confirmed regeneration of both ascending and descending long tract axons into NSC grafts (Bonner et al., 2003; Coumans et al., 2012b). Furthermore, these regenerated host axons formed synaptic connections with graft-derived neurons.

To enhance the intrinsic growth capacity of injured adult CNS axons, additional therapeutic manipulations are needed (Seiffers and Benowitz, 2008). One strategy is to deliver neurotrophins to the injured spinal cord for promotion of host axonal regeneration into NSC grafts and potentially beyond (Bregman et al., 1997; Coumans et al., 2001; Lu et al., 2003). Neurotrophins delivered via protein infusion can increase the number and extent of supraspinal axons penetrating the fetal graft (Bregman et al., 1997; Coumans et al., 2001). We have successfully delivered neurotrophins into NSC grafts or beyond to promote host axonal regeneration using gene therapy for precise local delivery (Lu et al., 2003; Lu et al., 2012a). However, prolonged delivery of neurotrophins may modulate activity in host axonal circuitry and affect overall behavioral outcomes (Lu et al., 2012a). Another strategy is to enhance intrinsic growth ability of injured adult neurons by genetic manipulation of growth associated genes, such as deletion of phosphatase and tensin homolog (PTEN) (Liu et al., 2010) or overexpression of aGrp’s like Factor 7 (the graft is too short (within 0.5 mm). The combination of these strategies with NSC grafts may greatly enhance host axonal regeneration and connectivity with graft-derived neurons.

**Growth and connectivity of grafted NSCs**

One of the hallmark features of a functional neuron is its connectivity to target cells by an axon and axonal terminals. Therefore, successful NSC graft for SCI will display integration and connectivity of donor neurons to host CNS circuitry, Jakeman and Reier (1991) were among the first to perform extensive anatomical assessments of such projection patterns between graft and host tissue at chronic time points after grafting fetal spinal cord tissue. They demonstrated that graft-derived axons extended up to 5 mm into host spinal cord tissue, though the majority of axons associated with the graft-host border as determined by anterograde and retrograde tracer labeling. Fischer’s group (Lepore and Fischer, 2005) was the first to directly demonstrate axonal growth of grafted embryonic spinal cord cells into host spinal cord as far as 5 mm using the reporter gene alkaline phosphatase. However, grafts of cultured neuronal-restricted precursor (NRP) cells-gial-restricted precursor (GRP) cells into injured spinal cord failed to extend axons into host unless neurotrophins were delivered near the graft (Lepore and Fischer, 2005; Bonner et al., 2011). Graft-derived axon terminals formed synaptic connections with host dorsal column neurons where brain-derived neurotrophic factor (BDNF) was delivered (Bonner et al., 2011). Study of human NSCs from normal human NSC donors implanted into SCI tissue have been reported to extend axons into host CNS tissue when transplanted into the injured spinal cord. Wictorin and Björklund (1992) grafted human fetal spinal cord cell suspension into adult rat spinal cord one segment above or below a partial lesion site, and observed human axonal extension into host white matter for up to 10 mm at 3-4 months post-graft using a human specific neurofilament marker. This finding along with a previous study of human axonal growth in adult lesioned rat brain (Wictorin and Björklund, 1992). Whether human NSCs implanted into sites of SCI can survive or differentiate into neurons that extend their axons into host spinal cord to establish functional connections is unknown.

Giovanini and colleagues (Giovanini et al., 1997) characterized human fetal spinal cord transplantation into sites of adult rat SCI under different lesion and grafting conditions. Although they demonstrated extensive human neurite growth into host, these neurites grew only into host gray matter for a short distance, but not into host white matter. The short distance growth of human axons into host may relate to poor survival and integration of human fetal spinal cord cells into lesion center of host since immunosuppression was described in the methodology of this xenograft study (Giovanini et al., 1997).

Recently, our group focused on characterization of axonal growth and...
connectivity of grafted NSCs in models of severe SCI. We developed new methodology to improve NSC tracking, survival, and differentiation/matura-
ration in the injured spinal cord (Lu et al., 2012b). We began with iso-
lated of NSCs/progenitor cells from a stable transgenic Fischer 344 rat line expressing a green fluorescent protein (GFP) reporter gene to unequivocally label grafted cells in order to follow their axonal growth and connectivity. The Fischer 344 rats are inbred and isogenic, thereby avoiding immunological rejection when cells are grafted between rats of the same strain. Next, we used committed NSCs and progenitor cells from freshly dissociated embryonic dorsal spinal cord tissue to avoid glial differentiation or lack of axonal growth from cultured rat NSCs (Cao et al., 2001; Lepore and Fischer, 2005). In addition, we embedded isolated NSCs/progenitor cells into a fibrin matrix containing growth factor cocktails to retain these cells and support their survival and differentiation in severe lesioned spinal cord. Finally, we delayed grafting of NSCs/neural progenitor cells for two weeks after SCI, a clinically relevant time point, to avoid peak inflammation for further enhancement of their survival (Lu et al., 2012b). We observed that adult NSCs/progenitor cells survived well, completely filled the upper thoracic complete transection site and differentiated into both neurons and glia (Lu et al., 2012b). Most remarkably, the grafted neu-ons extended their axons in remarkable densities in both rostral and caudal directions (29,000 axons emerging from the graft in the caudal direction) and over very long distances (7–9 spinal cord segments and over 20 mm) in the host spinal cord. Such numbers and distance of growth of graft-derived axons are unprecedented to the best of our knowledge. Furthermore, many grafted axons were myelinated and exhibited extensive synapse formation with host neurons (Lu et al., 2012b). Similarly, E14 brainstem derived NSCs grafted into T1 transection site also extended large number of axons into host for long distances, including graft-derived catecholaminergic and serotonergic neurons and their innervations of caudal sympathetic pregan-
glionic neurons (Hou et al., 2013). These results indicate that, despite the initial non-growth-supporting environment of the lesioned adult CNS, our protocols transformed the lesion site into one that is highly permissive for ax-
onal growth from graft-derived neurons within the lesion site.

Because of the therapeutic implications for human cell transplantation, we tested whether cultured human fetal spinal cord derived NSCs grafted into severe injured rodent spinal cord exhibit similar axonal growth and connectivity as rodent NSCs. We chose an FDA approved human cell line (566BSC cells) that is approved for clinical trials for amyotrophic lateral sclerosis (ALS) and is well characterized both in vivo and in vitro for different cell lines (Cao et al., 2005) and grafted these cells into sites of T1 complete transection in adult immunodeficient adult hybryg rats (Lu et al., 2012b). Again, we observed long-distance growth and connectivity of GFP-labeled human axons from the graft even at the short survival time of 7 weeks. In a follow-up study, the same human line was grafted into a compression injury site of adult rats under immunosuppressive conditions (van Gorp et al., 2013). Numerous human neurites extended from the graft into the host in both rostral and caudal directions, especially along the lateral white matter. In addition, human neurites expressed the pre-synaptic mark

er synaptophysin, suggesting connection with host neurons. These findings indicate that human NSCs have similar intrinsic growth capacity to provide sufficient information for extensive, long-distance axonal growth even in an inhibitory adult CNS environment.

The generation of embryonic stem (ES) cells and induced pluripotent stem (IPS) cells opens new doors for SCI repair since these pluripotent stem cells are able to proliferate indefinitely and can differentiate into various types of cells, including neurons and glia, therefore providing endless re-
source for cell transplantation. In addition, IPS cells are adult derived and can be transplanted autologously to avoid both ethical and immunorejec-
tion concerns. Several studies reported grafting of either ES cell-derived NSCs (Kimura et al., 2005; Erceg et al., 2010; Rossi et al., 2010; Cui et al., 2011; Niapour et al., 2012) or IPS cell-derived NSCs (Tsuji et al., 2010; Nori et al., 2011; Fujimoto et al., 2012) into injured rodent spinal cord. These studies, however, did not explore the growth and connectivity of grafted neurons with host. Our group first showed extensive outgrowth of human axons over long distance into host from human ES cells (HUES7) derived NSC graft placed in C5 hemisection site for 3 months. Human ES cell graft-derived axons reached the brainstem in the rostral direction and the lumbar spinal cord in caudal direction, with axonal growth spanning over 17 spinal segments and more than 50 mm (Lu et al., 2012b). Graft of another human ES cell line (H9) derived NSCs into C5 hemisection site for 3 months also exhibited similar extensive axonal growth (Figure 2, unpublished result). Notably, human axons grew in highly organized, rostro-cau-
dal linear trajectories in host white matter (Figure 2A). Throughout the course of their white matter projections, human axons sent their collateral branches into host gray matter for innervation of target neurons (Figure 2B). Immunohistochemistry demonstrates that human axons expressing the pre-synaptic marker synaptophysin surrounded host gray matter neurons, including choline acetyltransferase expressing host spinal motor neurons, indicating synaptic connections with host neurons (Lu et al., 2012b). Sim-
ilarly, grafted primitive neural stem cells (pNSCs) derived from human ES cells (HUES9) into sites of SCI also extended long axonal projections into host spinal cord, including human serotonergic axons from the graft (Zhao et al., 2013). Recently, we also transplanted human IPS cells derived NSCs into C5 hemisection site of adult immunodeficient rats for 3 months and found, once again, extensive axonal outgrowth in a preliminary study (Lu et al., unpublished results). Indeed, human axons traversed virtually the en-
tire rostral-caudal extent of the rat CNS. The robust growth of human IPS
cell-derived NSCs may relate to the integration of four genes that induce steness of adult somatic cells (Takashiki and Yamanaka, 2006). These findings indicate that NSCs derived from human ES or IPS cells have simi-
lar properties of axonal growth and connectivity as CNS derived NSCs.

Functional neuronal relay formation

The reciprocal growth and connection of grafted neurons into host and

host neurons into graft could result in potential functional neuronal relay

formation. Early studies partially documented the ingrowth of host axons
to graft and outgrowth of grafted neurons into host, supporting this hy-

pothesis (Jakeman and Reier, 1991; Bregman et al., 1993; Bregman et al., 1997; Bamber et al., 1999). Recent studies confirmed functional connec-
tivity between grafted neurons and host target neurons by injection of the transneuronal tracer pseudorabies virus and electrophysiological recordings (White et al., 2010; Lee et al., 2014). The formation of a functional neuronal relay by grafted neurons was first documented by Bonner et al. (2011) who demonstrated that host ascending sensory axons formed active synapses with graft neurons at the dorsal column injury site with the signal propagating by graft derived axons to the dorsal column nuclei using stimulus-evoked c-Fos expression and electrophysiological recording. Our group first documented partial restoration of descending neuronal transmission signals crossing com-
plete transected spinal cord that was allowed NSC graft using electrophysiological recording (Lu et al., 2012b). In addition, we observed locomotor behavioral improvement in NSC transplanted subjects compared to controls (Lu et al., 2012b). Re-transsection above the graft abolished this recovery, indicating novel relay circuit formation by grafted neurons that connected host neurons above and below the lesion. Other studies have reported various degrees of functional improvement with NSC graft, probably partially due to this novel neuronal relay formation (Coumanis et al., 2001; Cummings et al., 2005; Low-
ry et al., 2008; Erceg et al., 2010; Rossi et al., 2010; Nori et al., 2011; Fujimoto et al., 2012; Hou et al., 2013). Together, these findings demonstrate growth and connectivity between NSC graft and host tissue, lending strong support to the concept that donor neurons can serve as a novel functional relay be-
tween intact host neurons separated by a spinal cord lesion.

Future perspectives and challenges

Despite these recent advances, there remain several challenges facing future research toward therapeutic repair for SCI. Our recent work reveals the incredible capacity for intrinsic growth of transplanted NSCs within the injured adult CNS (Lu et al., 2012b); however, fine-tuning graft-derived projections by rehabilitation training and promoting their growth toward specific targets by axon guiding molecules and neurotrophins will require further investigation. Additional future studies are also needed to charac-
terize graft-derived neuronal and glial populations, in order to understand and shape the nature of host-graft relay circuitry. An ongoing goal remains to identify new methods to enhance regeneration of host axons into NSC
grafts, especially for functionally important pathways like the corticospinal tract, which is particularly refractory to regeneration. The potential for formation of novel neuronal relay circuits by NSC transplantation opens up new possibilities for combinatorial therapies for the treatment of SCI.

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Figure 2 Growth and innervation of human axons after spinal cord injury in rats. (A) Growth of green fluorescent protein (GFP) expressing human axons (H9 derived) in the host white matter 3 mm caudal to a neural stem cell (NSC) graft placed in C, hemisection site for 3 months. Inset shows individual axons at higher magnification in a horizontal section. (B) Innervation of human axons (green) from host white matter (WM) into gray matter (GM) is indicated by arrows. Host neurons are labeled with NeuN (red). Dashed lines indicate white matter and gray matter interface. Scale bar: 32 µm.

References


