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Harnessing the power of cell transplantation to target respiratory dysfunction following spinal cord injury

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Abstract

The therapeutic benefit of cell transplantation has been assessed in a host of central nervous system (CNS) diseases, including disorders of the spinal cord such as traumatic spinal cord injury (SCI). The promise of cell transplantation to preserve and/or restore normal function can be aimed at a variety of therapeutic mechanisms, including replacement of lost or damaged CNS cell types, promotion of axonal regeneration or sprouting, neuroprotection, immune response modulation, and delivery of gene products such as neurotrophic factors, amongst other possibilities. Despite significant work in the field of transplantation in models of SCI, limited attention has been directed at harnessing the therapeutic potential of cell grafting for preserving respiratory function after SCI, despite the critical role pulmonary compromise plays in patient outcome in this devastating disease. Here, we will review the limited number of studies that have demonstrated the therapeutic potential of intraspinal transplantation of a variety of cell types for addressing respiratory dysfunction in SCI.

Keywords

transplant; graft; stem cell; progenitor; cell replacement; breathing; diaphragm; regeneration; SCI

Spinal cord injury

Spinal cord injury (SCI) represents a heterogeneous set of conditions resulting from trauma to the spinal cord. The specific collection of functional deficits after SCI depends on factors such as location, type, and severity of the traumatic event (McDonald and Becker, 2003). Greater than half of all SCI cases occur in the cervical region. Damage in this location can result in respiratory compromise that is physically and psychologically debilitating, because injury frequently disrupts the neural circuitry that controls critical muscles such as the

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diaphragm (Lane et al., 2008a). Even injuries at more caudal levels, such as those in the thoracic spinal cord, can result in significant and persistent pulmonary dysfunction, including compromised breathing and effects on critical non-ventilatory functions, like coughing (Warren and Alilain, 2014).

The pathophysiology of SCI is complex and multi-factorial due to both the variety of cell types and the complexity of the neuronal connections affected. However, this also provides a number of potential therapeutic mechanisms to target with transplantation (Goulao and Lepore, 2016) specifically in the context of respiratory dysfunction post-SCI. These mechanisms include the obvious but challenging replacement of injured long-distance projecting neurons of the circuits controlling breathing. To date, transplantation-based interventions have been used to promote plasticity of axonal connections that are part of these respiratory circuits (Alilain et al., 2011; Decherchi and Gauthier, 2002; Decherchi et al., 1996; Li et al., 2003; Polentes et al., 2004; Stamegna et al., 2011), replacement of glial cell types (Li et al., 2015a; Li et al., 2015b) and local respiratory interneurons (Lee et al., 2014; White et al., 2010) of the cervical spinal cord, delivery of neurotrophic factor support (Gransee et al., 2015), and restoration of neurotransmitter signaling (Dougherty et al., 2016; Li et al., 2015a; Li et al., 2015b). While this list only represents a few of the possible benefits that transplantation can provide, the fact that only a limited number of studies have to date been conducted in this field means that many of the potential mechanistic benefits of transplanted cells have yet to be explored for targeting respiratory dysfunction in SCI.

Promoting axonal regrowth

Regeneration of damaged axons and reconnection with appropriate post-synaptic structures is a major therapeutic goal for SCI treatment. In addition, promoting sprouting of damaged and/or spared fibers is another important and possibly more easily achievable goal, which would involve generation of novel connections that could underlie meaningful recovery of function (Bareyre et al., 2004). Unfortunately, a host of neuronal-intrinsic (Luo and Park, 2012) and environmental (Bradbury et al., 2002; Giger et al., 2010) factors limit or prevent robust axonal regrowth in CNS diseases such as SCI. A number of studies have used transplants to induce plasticity of axonal populations that are involved in controlling breathing (Alilain et al., 2011; Decherchi and Gauthier, 2002; Li et al., 2003; Polentes et al., 2004; Stamegna et al., 2011). The premise behind these approaches is that a variety of cell types, some of which are not derived from the CNS, appear to have properties that can promote robust axonal growth, even in the injured spinal cord.

Many of these studies focused on axonal regrowth targeting a specific neural circuit that is central to inspiration via diaphragmatic control (Figure 1A). Each hemi-diaphragm is separately innervated by a pool of phrenic motor neurons (PhMNs) located at mid-cervical levels (Lane et al., 2009). These PhMNs are not spontaneously active; instead, they receive primarily monosynaptic drive from bulbospinal axonal input from neurons whose cell bodies are located ipsilaterally in a medullary nucleus called the rostral Ventral Respiratory Group (rVRG), in addition to some contralateral input from the crossed phrenic pathway (Boulenguez et al., 2007; Goshgarian et al., 1991; Lane et al., 2009). Cervical SCI can result in damage both to PhMNs and to descending rVRG connections to spared PhMNs (Zimmer

et al., 2007). There is also growing appreciation for the important role that intraspinal respiratory interneuron populations play in diaphragm function (Lane et al., 2008b).

Several neuroanatomical mechanisms associated with restoration of this circuit have been targeted using cell transplantation to achieve recovery of diaphragm function in rodent models of unilateral cervical SCI. Though technically challenging due to intrinsic (Luo and Park, 2012) and extrinsic (Bartus et al., 2012) inhibitors of axonal growth, transplants could be used to promote regeneration of injured rVRG axons through and/or around the lesion and back toward PhMNs and/or local respiratory interneuron populations. Spared contralateral rVRG input has also been shown to be a potential substrate for diaphragm recovery - even spontaneously - through mechanisms that promote plasticity such as activation of latent contralateral rVRG synaptic input to denervated PhMNs located ipsilateral to the lesion (Warren et al., 2014). Futhermore, cell transplants could be used to activate latent connections from the crossed phrenic pathway by modulating synaptic transmission between contralateral rVRG fibers and PhMNs and/or by promoting actual sprouting of these contralateral rVRG axons toward the ipsilateral PhMN pool. Transplant cells may also have the capacity to promote growth of other descending axonal populations that are important to respiratory function, such as serotonergic fibers that can affect PhMN excitability by modulating, for example, synaptic input from rVRG axons (Dale-Nagle et al., 2010).

Researchers in the field have primarily employed two major models of cervical SCI to study the value of transplantation-based therapies on respiratory recovery. The C2 hemisection model involves surgical cutting of the entire hemi-cord in order to completely interrupt bulbospinal input from the ipsilateral rVRG, while the ipsilateral PhMN pool is completely spared as these neurons reside at levels caudal to the lesion, i.e. C3-C5 (Figure 1B) (Lee et al., 2013). Mid-cervical hemi-contusion models (conducted mostly at C3 or C4) have been more recently employed as they provide the advantage of closely modeling many aspects of the human condition (Li et al., 2014; Nicaise et al., 2013; Nicaise et al., 2012a; Nicaise et al., 2012b). Cervical contusion results in damage to both PhMN cell bodies/dendrites and rVRG axons (Figure 1C), which can make it challenging to determine the mechanistic basis of any observed improvement, particularly with studies aimed at promoting axonal growth. Furthermore, the contusion results in significant sparing of ipsilateral tissue at the level of the lesion, including rVRG fibers running in the ventrolateral white matter, while the hemisection provides the advantage of being able to completely remove all ipsilateral rVRG input and spare all PhMNs. Additionally, a model introduced by Awad and colleagues uses a C3/C4 hemi-contusion injury followed by C2 contralateral hemi-section (Awad et al., 2013). In this manner, it is possible to determine the contribution of descending ipsilateral pathways to any observed recovery (in regards to the hemi-contusion). One model is not superior to another for studying respiratory dysfunction; instead, all should be used by investigators in the field as valuable tools to test cell-based therapies and to understand the mechanism(s) by which these cells can exert their effects.

Peripheral nerve graft

Compared to the CNS, the adult peripheral nervous system (PNS) is relatively permissive for axon regrowth after injury (Wright et al., 2014). Decherchi and Gauthier attempted to harness the regenerative capacity of the PNS for promoting growth of descending respiratory axon populations. In a first attempt, Decherchi and colleagues inserted a peripheral nerve graft (PNG) into level C2 of the uninjured spinal cord at the location of bulbospinal projections within the ventrolateral white matter (Decherchi et al., 1996). By conducting electrophysiological recording of individually-stimulated nerve strands, the authors reported that axons originating from respiratory neurons in the medulla grew into the PNG. As the PNG was transplanted in a blind-end fashion, they did not assess regrowth back into the host spinal cord. Next, Decherchi and Gauthier tested the feasibility of a PNG as a conduit or bridge for regenerating axons around a site of injury, thereby transferring the permissive PNS environment to the CNS after SCI (Decherchi and Gauthier, 2002). They found that respiratory-associated projections assessed electrophysiologically could grow into a PNG when transplanted acutely or at 3 weeks or 3 months after C3 hemisection. However, greater ingrowth was noted when the PNG was delivered acutely or at 3 weeks after injury, rather than with a long delay of 3 months. About 37.5% of regenerated axons within the PNG transplanted acutely, 47% of axons regenerated within the PNG transplanted at 3 weeks, and 30% regenerated axons within the PNG transplanted at 3 months exhibited neuronal firing patterns. Of those, approximately 13% of the axons from the acute transplantation, 11% of axons from the transplantation at 3 weeks, and 8% of axons from the transplantation at 3 months had activity consistent with respiratory neurons. While the authors conducted histological analysis of the graft, the level of synapse formation between PNG fibers and PhMNs was not analyzed, perhaps underestimating the degree of ingrowth and/or incorrectly determining the cell body source of axons in the PNG. Though these studies did not assess functional diaphragm recovery, the results indicate that PNG transplantation can elicit regrowth of respiratory-associated axons and, therefore, PNG transplantation is a clinically-relevant method in which respiratory recovery might be achieved, even when delivered with a delay post-injury. Furthermore, these studies reveal that the timing of PNG transplantation post-injury has a significant effect on the amount of regrowth of injured respiratory-associated axons. Another study by Gauthier and colleagues fully integrated the PNG from the medulla to the spinal cord just caudal to a C3 hemi-section (Gauthier et al., 2002). Here, they found that descending PNG fibers had electrophysiological patterns similar to respiratory neurons and were able to reconnect within the ventral horn caudal to the injury. This reconnection coincided with phrenic nerve bursting, which suggests that the PNG was able to bridge medullary respiratory axons with PhMNs.

A common theme in SCI therapeutics is the need for combinatorial strategies to achieve robust recovery, particularly for achieving axonal regeneration given the large number of sources of growth inhibition. Toward this end, Alilain and colleagues used a PNG while simultaneously removing the growth-inhibitory environment of the CNS by injecting chondroitinase ABC (ChABC), which degrades the glycosaminoglycan (GAG) side chains of chondroitin sulfate proteoglycans (CSPGs) that limit axon extension in the CNS (Bradbury et al., 2002). The authors combined ChABC injection with the PNG immediately following C2 hemisection and then reconnected the graft one week later caudal to the injury

at C4 along with another injection of ChABC. Functional recovery in the ipsilateral hemi-diaphragm was achieved and was greater than the effects of ChABC or PNG treatments alone (Alilain et al., 2011). Electromyography (EMG) recordings demonstrated that the peak inspiratory amplitude of the ipsilateral hemi-diaphragm reached and often surpassed recordings from the uninjured contralateral hemi-diaphragm of the same animal and of uninjured control animals; however, on average, the bursting duration was less on the ipsilateral side. By tracing axons from the medulla, the authors were able to show bulbospinal axonal regrowth into the PNG and surrounding cervical spinal cord. Co-localization of these axons with synapsin puncta suggests that regenerating axons could form synaptic connections. Axonal tracing from the PNG also revealed a subset of axons in the PNG originating from respiratory locations in the medulla, including the rVRG (but also other nuclei such as serotonergic neurons of the raphe). Ipsilateral hemi-diaphragm activity ceased only when the PNG was transected, suggesting that diaphragm recovery was mediated by input from respiratory centers in the brainstem exclusively via regrowth through the PNG. Interestingly, an increase in tonic EMG recordings occurred after PNG transection, suggesting that local interneurons and/or intersegmental propriospinal neurons also played a role in rearranging respiratory circuitry controlling PhMN activation. The authors found that the recovery observed with ChABC treatment alone was associated with increased serotonergic (5-HT) fiber input. However, the densities of 5-HT fibers between ChABC treatment alone and PNG+ChABC injection were similar (despite greater EMG responses with the combination group), suggesting that the PNG contributed distinctly to functional recovery possibly through regrowth of respiratory axons originating in the brainstem.

Olfactory ensheathing cells

The evidence described thus far supports the ability of a PNG to promote rewiring of damaged respiratory neural circuitry after SCI. The use of PNG therapy is technically challenging in clinical practice, particularly inserting these relatively large pieces into spinal cord parenchyma; however, the use of isolated Schwann cell transplants may be a powerful approach for harnessing the benefits of the PNG without the practical difficulties (Guest et al., 2013). A significant number of defined cell types that provide practical advantages over PNG have been characterized *in vitro* and tested *in vivo* in SCI models (Li and Lepski, 2013). Advantages include greater ease of derivation, extensive capacity for *in vitro* expansion without losing phenotypic potential or therapeutic properties, and ability for long-term storage, amongst other benefits. These include multipotent stem cells (Lepore et al., 2004) and lineage-restricted progenitors (Lepore et al., 2005; Lepore and Fischer, 2005; Lepore et al., 2006) obtained from the CNS, PNS and even non-nervous system sources, as well as more mature cell classes such as differentiated neurons.

One such class of cells that has received extensive attention in the SCI field is the olfactory ensheathing cell (OEC), a type of glia that ushers axons of olfactory receptor neurons across the PNS-to-CNS transition even in the adult (Richter and Roskams, 2008). This normal function of the OEC combined with its ease of derivation, including in an autologous fashion, makes it a potentially useful candidate for promoting axonal growth in SCI. Despite the potential of OECs, only a small number of studies have analyzed the effects of OEC

transplantation on recovery of respiratory function following SCI (Li et al., 2003; Polentes et al., 2004; Stamegna et al., 2011).

Li and colleagues transplanted an equal ratio of rat OECs and olfactory nerve fibroblasts to serve as a supportive matrix for the OECs into the lesion site at the time of C2 hemisection (Li et al., 2003). Two months later, the majority of transplanted rats exhibited respiratory bursting in the phrenic nerve during spontaneous breathing. The authors showed that this effect was not due to activation of the CPP. This study was the first to demonstrate the therapeutic power of OEC transplantation for promoting plasticity of neural connections involved in respiratory control after SCI; however, these experiments did not conduct any assessment of whether improvement occurred because severed ipsilateral supraspinal connections had regenerated and/or spared fibers had sprouted.

Polentes and colleagues transplanted a more purified population of cells (approximately 75% OECs) immediately after C2 hemisection (Polentes et al., 2004). The authors showed partial recovery with OEC transplantation via EMG and phrenic nerve recordings ipsilateral to the lesion. They also showed some degree of spontaneous recovery post-injury; however, OEC transplantation resulted in an even greater effect. Using a variety of targeted lesions of the ipsilateral, contralateral or midline spinal cord after recovery, the authors demonstrated that the observed beneficial effect of OECs was primarily due to the contribution of descending ipsilateral respiratory pathways and not to spared contralateral input (though some contralateral contribution was likely), whereas contralateral pathways were responsible for the observed spontaneous recovery. Detailed histological analyses such as axonal tract tracing from the ipsilateral and contralateral brainstem were not conducted; therefore, the specific neuroanatomical effects of OECs on axonal growth are unknown.

Stamegna and colleagues followed up these previous studies by investigating the extent of recovery of descending respiratory pathways when transplants, purified to contain more than 90% OECs, were delayed to two weeks after C2 hemi-contusion (Stamegna et al., 2011). The authors found that, compared to non-transplanted injured controls, OEC transplantation resulted in increased recovery in phrenic nerve and diaphragm EMG recordings, as well as improvements in overall ventilatory behavior as assessed by whole-body plethysmgraphy. Similar to the study by Polentes and colleagues, the therapeutic effects of OECs appeared to be due to contribution of descending ipsilateral pathways. This study was the first to demonstrate the therapeutic effects of OECs on respiratory function in the clinically-relevant cervical contusion model.

Collectively, the studies published to date in models of SCI-induced respiratory dysfunction demonstrate that OECs hold potential to enhance axonal regrowth and sprouting following SCI, including from critical respiratory neuronal populations. However, more work is necessary to understand the neuroanatomical basis of this plasticity in the cervical hemisection and contusion paradigms, as well as to investigate whether combinatorial approaches with other interventions can enhance the benefit of OECs on axonal regrowth and functional recovery.

Neuronal replacement

Replacement of mature CNS cell types, particularly neurons, is a promising yet challenging endeavor for achieving recovery of damaged neural circuitry. Replacement of PhMNs using transplanted multipotent neural stem cells (NSCs) or lineage-restricted neural progenitor cells (NPCs) has not yet been attempted and faces many challenges, including integration of MNs in the ventral horn, long-distance extension of axonal projections to the periphery, and correct innervation of target muscle. Cell replacement of bulbospinal neurons in areas responsible for respiratory drive faces similar challenges of axonal growth through the brainstem and spinal cord and correct innervation of neurons at various spinal cord regions where MNs that control respiratory muscles are located.

Researchers in this field have also considered relay formation via delivery of transplant-derived interneurons to establish novel connections to bridge and restore damaged respiratory circuits in SCI (Lee et al., 2014; White et al., 2010). These cells have the potential to integrate into the recipient CNS and mature into neurons with the capacity to fire action potentials and make synaptic connections with host neurons and other transplanted cells. However, integration of these neuronal transplants into host tissue raises questions, including how afferent and efferent connections to and from these cells, respectively, would form to complete appropriate relays. With the complex network of connections present in the CNS, it will be challenging to guide desired connections between host and graft neurons; nevertheless, strategies toward this end are being developed (Bonner et al., 2011). In addition to the biological issue of achieving appropriate host-graft connectivity, a challenge involves actually being able to experimentally determine whether the transplanted cells are forming meaningful connections and whether these connections are even contributing to any observed recovery. For example, the benefit elicited by transplanted cells might not be resulting from neuronal integration, but instead from the fulfillment of a different role entirely (such as neuroprotection) that is not necessarily even associated specifically with a neuronal identity.

Fetal spinal cord

To date, neuronal transplantation in the context of SCI-induced respiratory compromise has primarily been tested using grafts of rodent fetal spinal cord (FSC) tissue. Although the use of human fetal-derived cells for clinical translation is associated with ethical issues, investigating FSC grafts provides valuable information about how various classes of NSCs and NPCs (the predominant cell types that make up these grafts) can aid in the restoration of function (Anderson et al., 1995). It also represents a powerful tool both to define the candidate populations of cells most useful in restoring damaged neural circuitry and to understand whether a particular combination of cell types (e.g. specific neuronal subtypes, glia, NSCs, NPCs) is necessary (Lepore and Fischer, 2005).

To investigate whether cells derived from particular anatomical regions of the developing spinal cord differentially affect respiratory recovery after SCI, White and colleagues transplanted either dorsal (FSC_D) or ventral (FSC_V) subregions of rat spinal cord obtained from embryonic day 13.5-14 directly into the lesion cavity immediately post C2 hemisection (White et al., 2010). SCI-only and FSC_V transplanted animals showed recovery in ipsilateral

phrenic nerve bursting under conditions of eupnea, while this response was absent in a subset of FSC_D transplanted rats. Furthermore, enhanced phrenic nerve bursting was observed in response to hypercapnic and hypoxic challenge in the FSC_V groups, but not in the FSC_D or injury-only animals. These findings demonstrate that the anatomical source of FSC tissue (and presumably the makeup of NSCs and NPCs) can exert significant effects on the therapeutic properties of these transplants. Histological observation of FSC_D and FSC_V revealed relatively dramatic differences in the neuronal populations comprising these grafts and their anatomical organization; however, the authors did not examine the contribution of the diverse NSC/NPC types present in each graft type to differences in transplant fate *in vivo* or functional effect. It will be critical going forward to systematically determine the most appropriate class(es) of NSCs/NPCs needed for transplantation, which will likely differ depending on factors such as the type and location of SCI and the therapeutic mechanism being targeted. Using the trans-synaptic tracer pseudo-rabies virus (PRV), the authors also reported that transplant-derived neurons made synaptic connections with host neurons, including those in the phrenic nucleus (PhMNs and/or interneurons) and brainstem. While these initial studies were able to show integration of transplanted cells into host neural circuitry, the mechanistic contribution of this integration (if it is even relevant to functional recovery – as discussed above) was not examined.

Lee and colleagues continued to explore the potential of neuronal integration – and potentially host-graft relay formation – with FSC transplants in the C2 hemisection model (Lee et al., 2014). Transplant-derived cells displayed firing activity in a non-random burst pattern, suggesting possible connectivity with host neuronal circuitry, though this bursting was not synchronous with respiratory behavior. Interestingly, grafted cells increased their burst frequency upon exposure to hypoxic challenge but not significantly during hypercapnia, suggesting the existence of some respiratory-associated afferent input to the transplanted cells. This bursting was also associated with increased tidal volume and decreased respiratory frequency in the FSC transplanted animals in response to hypoxia. These findings suggest that transplant-derived neurons can synaptically integrate with host neuronal populations and can significantly modulate functional respiratory output after severe SCI. The response of the transplanted cells to respiratory-associated stimuli, for instance hypoxia, also suggests that regimens such as intermittent hypoxia could be used to strengthen graft-host connectivity and possibly even recruit and “train” exogenous cells into host respiratory circuitry. Similar to the previous study by White and colleagues (White et al., 2010), these results represent proof-of-principle for the potential of neuronal transplantation and point toward some mechanisms by which these graft-derived cells can exert functional benefit. However, it will be important to follow up with experiments aimed at determining critical details, including the appropriate NSC/NPC types to employ, whether these graft-host connections are actually relevant to recovery, and, if so, the exact neuroanatomical basis of this novel circuitry.

Glial replacement

Replacement of various glial lineages also holds great promise (Falnikar et al., 2015). Astrocytes play a host of key roles in the CNS, including regulation of extracellular ionic and neurotransmitter homeostasis, active participation in synaptic transmission, delivery of

energy substrates to neurons, regulation of blood vessel dynamics and blood brain barrier, generation of extracellular matrix molecules, and expression of neurotrophic factors, amongst a long list of other critical functions (Pekny and Nilsson, 2005). Oligodendrocyte replacement can also be used to remyelinate denuded hosts axons, as well as potentially the projections of transplant-derived neurons (Faulkner and Keirstead, 2005).

Astrocyte replacement with glial progenitors

A number of previous studies employed astrocyte and glial progenitor transplantation in SCI models mostly for harnessing the axonal growth promoting properties of astrocytes (Davies et al., 2006; Davies et al., 2008; Davies et al., 2011; Haas et al., 2012; Shih et al., 2014; Smith et al., 1986; Smith et al., 1990; Smith and Silver, 1988). However, these studies did not target the normal homeostatic functions of astrocytes in the CNS. To begin to utilize astrocyte replacement in a mechanistically-targeted fashion based on their crucial functions in the intact nervous system, we have used glial-restricted precursor (GRP) transplantation to restore extracellular glutamate uptake in the injured spinal cord. Astrocytes are responsible for the vast majority of glutamate uptake throughout the CNS via expression of the plasma membrane transporter, glutamate transporter 1 (GLT1), thereby playing a central role in maintaining normal synaptic communication and preventing glutamate-mediated excitotoxicity (Maragakis and Rothstein, 2004; Maragakis and Rothstein, 2006). Following SCI, astrocyte GLT1 expression and function are severely compromised, which contributes to excitotoxicity-induced cell death and consequent functional loss during the delayed secondary injury phase (Lepore et al., 2011a; Lepore et al., 2011b; Li et al., 2014). In unilateral C4 contusion, we injected rodent GRP-derived astrocytes engineered to overexpress GLT1 into the cervical ventral horn as a therapeutic strategy for achieving transplantation-based delivery of astrocytes, reconstituting GLT1 function, preventing excitotoxicity, and consequently protecting PhMNs and preserving diaphragm function (Li et al., 2015a). Unmodified transplants robustly survived but expressed relatively low levels of GLT1 in the injured cervical spinal cord, suggesting that the injured host spinal cord exerts similar effects on GLT1 expression by these cells as on endogenous astrocytes. On the contrary, GLT1 overexpressing cells expressed persistently high levels of GLT1 protein (which was accompanied by an increase in functional glutamate uptake capacity) following transplantation into the injured spinal cord, including at early time points post-injection during the time frame of PhMN loss. In addition, these overexpressing astrocyte transplants significantly promoted survival of PhMNs, preservation of diaphragm neuromuscular junction (NMJ) innervation, and protection of diaphragmatic respiratory function as assessed by EMG and compound muscle action potential (CMAP) recordings. GLT1 overexpression did not change the *in vivo* properties of the cells (compared to control transplants), such as proliferation and efficient astrocyte differentiation, suggesting that the therapeutic benefit was a product of increased GLT1 levels and not some other changes in transplanted cell function. Our findings demonstrate the therapeutic value of targeting specific mature astrocyte properties using cell transplantation for preserving respiratory function after SCI. Given the long list of important astrocyte functions, our approach represents only one example of this powerful method for treatment in SCI.

Pluripotent stem cells

Pluripotent embryonic stem (ES) cells have been used extensively as a source for deriving large numbers of homogenous CNS cell types for transplantation into the injured spinal cord (Tetzlaff et al., 2011). The relatively recent discovery of induced pluripotent stem (iPS) cells (Takahashi et al., 2007) has generated great excitement as they represent a clinically-relevant source of pluripotent cells generated from adult somatic cell types, avoiding ethical issues of ES cell derivation. This technology also allows for homogeneous derivation of mature cell types in large quantities, potentially in an autologous fashion from the eventual transplant recipient (Das and Pal, 2010; Kiskinis and Eggan, 2010). Despite the promise of this approach, the iPS cell field is in its infancy with respect to evaluating *in vivo* graft integration and therapeutic usefulness in relevant SCI models (Goulao and Lepore, 2016; Salewski et al., 2010), particularly in the context of respiratory dysfunction (Li et al., 2015b).

As an extension of our work targeting astrocyte GLT1 using rodent GRPs (Li et al., 2015a), we took a similar approach with human iPS cells. We derived pluripotent iPS cells from non-diseased human donors, generated glial progenitors and then differentiated these cells into astrocytes prior to transplantation into the unilateral C4 contusion model (Li et al., 2015b). Similar to rodent GRPs, human iPS cell-derived transplants survived for long periods of time in the injured cervical spinal, did not form tumors or show uncontrolled proliferation, differentiated into only GFAP-positive astrocytes, and did not localize to ectopic locations or differentiate into unexpected lineages. In addition, these cells could be engineered to overexpress GLT1, resulting in robust and persistent transporter expression in the injured spinal cord. Importantly, GLT1-overexpressing human iPS cell-derived astrocytes decreased lesion size within the injured cervical spinal cord, morphological denervation by PhMNs at the diaphragm NMJ, and functional diaphragm denervation as measured by EMGs and CMAPs. These findings show that clinically-relevant populations of human iPS cells can be used as a safe and therapeutically efficacious source of transplants for targeted astrocyte replacement in SCI. It will now be important to test transplantation of additional classes of NSCs, NPCs and more mature lineages derived from iPS cells in models of SCI-induced respiratory dysfunction, including studies of long-term integration and safety and potential variability in therapeutic outcomes associated with various donor cell lines.

Gene delivery

Cell transplantation can be used as a vehicle to deliver therapeutic molecules to the injured – or even surrounding intact – spinal cord. A number of candidate cell types, including NSCs, NPCs and mesenchymal stem cells (MSCs), naturally express therapeutically-relevant molecules such as trophic (Neuhuber et al., 2005), immunomodulatory (Pluchino et al., 2003) and axonal growth-inducing (Shih et al., 2014) factors. In addition, cells can be engineered *in vitro* prior to transplantation to modify or enhance their expression profile. As described earlier, we employed such an approach to boost glutamate transporter expression in transplant-derived astrocytes and achieved robust protection of diaphragm function following cervical contusion SCI (Li et al., 2015a; Li et al., 2015b).

Neurotrophic factor delivery using mesenchymal stem cells

Stable cell integration into host tissue is often an important requirement for transplantation-based gene delivery. Cells native to the CNS would presumably be the optimal candidates for such an approach. However, long-term survival may not always be necessary, such as when only transient delivery of a given factor is desired. In such cases, cells derived from sources outside the nervous system may also be useful.

Along these lines, Gransee and colleagues engineered bone marrow-derived rat MSCs to express brain-derived neurotrophic factor (BDNF) at concentrations significantly above normal spinal cord levels (Gransee et al., 2015). A large body of work in the SCI field has illustrated the therapeutic potential of MSC transplantation (Oliveri et al., 2014). MSCs provide advantages that include expression of a number of trophic factors, the potential for autologous derivation, and limited proliferation after engraftment (thereby avoiding tumor formation issues) (Dasari et al., 2014). Transdifferentiating into mature CNS lineages is likely not occurring with MSCs; therefore, MSCs are not a candidate for cell replacement (Wright et al., 2011). The authors intraspinally transplanted BDNF-MSCs at the time of C2 hemisection, resulting in diaphragm EMG recovery starting several weeks after injury (Gransee et al., 2015). An interesting aspect of this study was that functional improvement occurred despite most MSCs not surviving beyond the initial week post-transplantation, suggesting that BDNF delivery was only necessary at very early time points. However, the mechanism by which the BDNF-MSCs exerted their benefit was not examined. BDNF is associated with neuronal survival, axonal regrowth and sprouting, effects on neuronal excitability, and synaptic plasticity (Weishaupt et al., 2012). It is possible that BDNF exerted a number of potential effects on PhMN properties such as cell body size, dendritic morphology or even survival, which would have possible effects on excitability, synaptic input from rVRG axons and diaphragm innervation, respectively (Boyce and Mendell, 2014). The BDNF-MSCs may have modified synaptic transmission at the diaphragm NMJ, as this same group has also shown BDNF effects on NMJ transmission (Mantilla et al., 2004) and on diaphragm activation in the C2 hemisection using intrapleural AAV-BDNF injection (Gransee et al., 2013). Mitchell and colleagues have systematically demonstrated that BDNF plays a key role in plasticity of synaptic input to PhMNs from spared rVRG axons after hemisection (Dale-Nagle et al., 2010). Therefore, it is possible that the BDNF-MSCs strengthened input from the latent contralateral rVRG input that is mostly inactive prior to injury, though the study by Gransee and colleagues did not test this. As BDNF has neurotropic properties (Weishaupt et al., 2012), the MSC transplants may have induced axonal plasticity, such as a collateral sprouting response from spared contralateral rVRG axons and innervation of ipsilateral PhMNs and/or respiratory interneuron populations (Lane et al., 2008b).

5-HT expressing neurons

Descending serotonergic input is important in PhMN activation, including in synaptic plasticity mechanisms that mediate diaphragm recovery due to strengthening of spared rVRG input to PhMNs after cervical SCI (Dale-Nagle et al., 2010). Dougherty and colleagues transplanted embryonic midline brainstem (MB) cells that contain significant numbers of serotonin-producing raphé neurons one week after C2 hemisection (Dougherty

et al., 2016). In response to respiratory challenge, MD-transplanted rats exhibited an increased ventilatory response as assessed with plethysmography, as well as increased phrenic nerve activity. Importantly, recovery was only noted in animals in which the MB transplants survived out to six weeks post-engraftment, suggesting the need for persistent integration and 5-HT production. This study demonstrates the potential of restoring neurotransmission in order to modulate the activation of neurons within the respiratory circuit. Going forward, it may be important to develop ways to regulate neurotransmitter release as these transplanted neurons were injected into the spinal cord and not into their endogenous location, a place where they normally receive afferent input to appropriately control their activity and 5-HT release.

Clinical trial assessing effect of transplantation on respiratory function

A number of clinical trials have been conducted or are currently in progress with SCI patients using a variety of cell types for transplantation, including MSCs, macrophages, NSCs/NPCs, OECs, Schwann cells, and ES cell-derived oligodendrocyte progenitors (Zhu et al., 2014). While the pre-clinical studies detailed in this review have demonstrated the utility of transplantation for targeting breathing compromise, almost no data on respiratory outcome are available in these patient studies.

In a very preliminary assessment, Jarocha and colleagues described a case in which stem cell transplantation may have aided in respiratory recovery (Jarocha et al., 2014). Patients received a combination of intraspinal (at the site of injury), intravenous and lumbar puncture injections of autologous bone marrow nucleated cells (BMNCs). Of the five pediatric patients who had undergone transplantation, one showed promising effects. A 2.5-year-old female patient sustained a SCI at C2-C4 that caused loss of the swallowing reflex and respiratory drive, resulting in the need for continuous mechanical ventilation. This patient showed no recovery for more than a year before receiving a total of six cell transplantations over four years. After the fifth transplantation (50 months from the first injection), the authors stated that active tongue movements reappeared and the swallowing and cough reflexes were restored. The authors also described beneficial effects on respiratory function, as assessed by the Spinal Cord Independence Measure scale that takes into account respiratory and sphincter management. Although the authors stated that no negative consequences were associated with transplantation, evidence was not provided to demonstrate that the recovery in respiratory function in this one patient was actually due to the BMNC transplants, as there was no control group. Although there was a year of no recovery prior to transplantation, the pediatric population, which was the focus of this study, is likely more prone to spontaneous improvement than adults. Therefore, any observed recovery may have been due to spontaneous recovery mechanisms. Alternatively, the developing nervous system may be more therapeutically responsive to the BMNC transplants. This trial was focused on safety rather than efficacy, leaving a number of questions for further evaluation. Though preliminary, this study does support the potential feasibility of cell transplantation in human SCI for targeting respiratory dysfunction and also stresses the critical need to test pulmonary functions in SCI clinical trials going forward, particularly in transplantation studies targeting spinal cord regions involved in respiratory control.

Conclusions

The studies to date that have investigated the therapeutic potential of transplant-based strategies for addressing respiratory compromise represent only a small fraction of the major body of work that has tested transplantation in models of SCI, despite the critical relevance of breathing dysfunction to patients. Nevertheless, this relatively small number of studies has demonstrated both the power of such an approach and the success of transplantation to target multiple cellular mechanisms for achieving recovery of respiratory function. These mechanisms include promotion of axonal regrowth and sprouting, replacement of neurons and glia, relay formation, generation of novel neural circuitry, neuroprotection, and delivery of trophic factors and neurotransmitters. Furthermore, the studies discussed in this review evaluated only a small subset of the various classes of cell types that could potentially be used for transplantation. Future work needs to assess additional classes of stem and progenitor cells and mature cell types derived from nervous system and non-neural tissues, as well as derived from clinically-relevant sources such as iPS cells. Given the growing appreciation for studying respiratory dysfunction in models of SCI, we expect that this important body of work will rapidly expand in the coming years, which we anticipate will translate to much-needed therapies for patients suffering from this debilitating outcome of SCI.

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Abbreviations

BDNF	brain-derived neurotrophic factor
BMNC	bone marrow nucleated cell
C2	3, 4, etc., cervical level 2, 3, 4, etc
ChABC	chondroitinase ABC
CMAP	compound muscle action potential
CNS	central nervous system
CPP	crossed phrenic phenomenon
CSPG	chondroitin sulfate proteoglycan
EMG	electromyography
ES cells	embryonic stem cells
FSC	fetal spinal cord

GAG	glycosaminoglycan
GLT1	glutamate transporter 1
GRP	glial-restricted precursor
iPS cell	induced Pluripotent Stem cell
MB cell	embryonic midline brainstem cell
MSC	mesenchymal stem cell
NMJ	neuromuscular junction
NPC	lineage-restricted neural progenitor cell
NSC	multipotent neural stem cell
OEC	olfactory ensheathing cell
PhMN	phrenic motor neuron
PNG	peripheral nerve graft
PNS	peripheral nervous system
PRV	pseudo-rabies virus
rVRG	rostral Ventral Respiratory Group
SCI	spinal cord injury
5-HT	5-hydroxytryptamine (serotonin)

Highlights

- Cell transplantation is a promising therapeutic strategy for SCI.
- Respiratory dysfunction plays a critical role in patient outcome following SCI.
- Transplant-based targeting of respiratory compromise has not been extensively explored.
- A small number of studies to date have shown the potential power of such an approach.

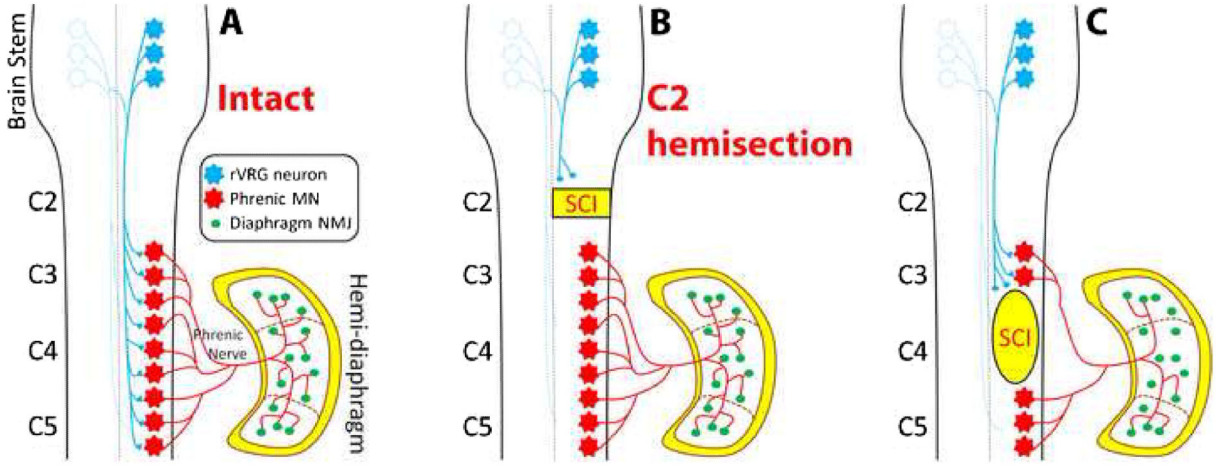

Figure 1. Animal models of cervical SCI used to study diaphragm dysfunction

Intact rVRG-PhMN-diaphragm circuitry (**A**). C2 hemisection SCI (**B**). Unilateral C4 contusion SCI (**C**). Contralateral bulbospinal input is illustrated in light blue dashed lines in all panels. rVRG: rostral Ventral Respiratory Group. PhMN: phrenic motor neuron. NMJ: neuromuscular junction. SCI: spinal cord injury site. C2, C3, C4, C5: cervical spinal cord levels 2, 3, 4 and 5.