SPINE Volume XX, Number XX, pp 000–000
©2010, Lippincott Williams & Wilkins

Cervical Multilevel Intraspinal Stem Cell Therapy

Assessment of Surgical Risks in Gottingen Minipigs

Bethwel Raore, MD,* Thais Federici, PhD,* Jason Taub, MD,* Michael C. Wu, PhD,†
Jonathan Riley, MD,* Colin K. Franz, PhD,* Michele A. Kliem, PhD,* Brooke Snyder, PhD,*
Eva L. Feldman, MD,‡ Karl Johe, PhD,§ and Nicholas M. Boulis, MD*

Study Design. Assessment of long-term surgical risks from multiple intraspinal cell injections.

Objective. To prove that multilevel-targeted cell injection to the spinal cord can be a feasible and safe procedure.

Summary of Background Data. Neural cell transplantation has been proposed as a treatment for a variety of neurologic disorders, including degenerative, ischemic, autoimmune, and traumatic etiologies. Among these diseases, the lack of effective treatment for amyotrophic lateral sclerosis has prompted the search for cell-based neuroprotection or motor neuron-replacement therapies.

Methods. Fifteen female minipigs, divided into 3 experimental groups, underwent either 5 or 10 unilateral injections of neural stem cells or 10 vehicle injections into the C3–C5 segments of the spinal cord, using a device and technique developed for safe and accurate injection into the human spinal cord. All animals received intravenous Tacrolimus (0.025 mg/kg) BID during the course of the study. Sensory and motor functions as well as general morbidity were assessed for 28 days. Full necropsy was performed and spinal cords were analyzed for graft survival. This study was performed under Good Laboratory Practice conditions.

Results. Neither mortality nor permanent surgical complications were observed within the 28-day study period. All animals returned to preoperative baseline showing full motor function recovery. Graft survival was demonstrated by immunohistochemistry.

Conclusion. Clinically acceptable neural progenitor survival, distribution, and density were achieved using the number of injections and surgical techniques specifically developed for this purpose.

Key words: microinjection, neural stem cells, spinal cord, cell therapy, pigs. **Spine 2010;XX:000–000**

From the *Department of Neurosurgery, Emory University, Atlanta, GA; †Neurodigitech, LLC, San Diego, CA; ‡Department of Neurology, University of Michigan, Ann Arbor, GA; and §Neuralstem Inc, Rockville, MD.

Acknowledgment date: September 21, 2009. Revision date: December 22, 2009. Acceptance date: January 25, 2010.
The device(s)/drug(s) that is/are the subject of this manuscript is/are being evaluated as a part of an ongoing FDA-approved investigational protocol (IDE) or corresponding national protocol.
Corporate/Industry funds were received in support of this work. One or more of the author(s) has/have received or will receive benefits for personal or professional use from a commercial party related directly or indirectly to the subject of this manuscript: e.g., royalties, stocks, stock options, decision making position.
B. R. and T. F. contributed equally to this work.
Supported by Neuralstem Inc.
The recipient of these funds (T.F.) has directed them to a research fund that has been used solely for the purpose of research. In addition, Neuralstem Inc. has purchased a nonexclusive license for the surgical device—Spinal Platform and Delivery Method—developed by N.M.B. from the Cleveland Clinic. N.M.B. received a percentage of this licensing fee. The Contract Research Organization (CRO) has no conflict of interest and is held responsible for the quality of the data.

Address correspondence and reprint requests to Nicholas M. Boulis, MD, Department of Neurosurgery, Emory University, 1365B Clifton Rd, NE, Suite B6200, Atlanta, GA 30322; E-mail: nboulis@emory.edu

Cell transplantation has been proposed as a treatment for a variety of neurologic disorders, including Parkinson, Alzheimer, and Huntington diseases, stroke, epilepsy, multiple sclerosis (MS), spinal cord injury (SCI), and amyotrophic lateral sclerosis (ALS).[1–3]

Cell therapy trials for SCI and ALS have been launched in several centers worldwide (Table 1). As the number of potential cellular, molecular, and small molecule spinal cord therapeutics expands, regulatory bodies are becoming increasingly sophisticated about the risks of spinal cord surgery. When a biologic product is used in combination with a surgical device, the Food and Drug Administration (FDA) requires that preclinical large animal studies mimic the route and method of administration to be used in subsequent clinical studies to assess the risk to human subjects. To date, free-hand injection of therapeutics has been permitted below the segmental level of complete spinal cord injuries. The risks of exacerbating deficits in vulnerable spinal cord parenchyma above the level of injury or in cords affected by partial injuries, degenerative and autoimmune diseases require rigorous means to minimize secondary injury.

A variety of safety, efficacy, and ethical concerns have been raised regarding the biologic products, including appropriate cell types (neural progenitor cells, olfactory ensheathing cells, mesenchymal stem cells, *etc.*), the source (embryonic or fetal), the therapeutic effect (neuroprotection or neurorestoration), and the possibility of rejection and tumor formation.[11–14] In contrast, little attention has been paid to the surgical procedures and devices proposed for cell transplantation into the spinal cord. Surgical penetration of the functional human spinal cord has an acceptable risk profile with a long history of application in a variety of neurosurgical disorders. There is significant precedent in the field of surgical treatment of pain.[15] Percutaneous cordotomy and dorsal root entry zone (DREZ) procedures require spinal cord penetration and accurate lesion formation. Similarly, the treatment of syringomyelia requires the insertion of small chronically indwelling cannulas into the spinal cord to drain the cyst cavity. Finally, the resection of vascular malformations and tumors often requires surgery in the highly eloquent tissue of the functioning spinal cord tissue.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Table 1. Clinical Protocols for Intraspinal Cell Transplantation

Year	Country/ Sponsor	Cells	Indication	Delivery	Inclusion Criteria	Status	Reference
2000–2003	Israel and Belgium (Proneuron biotech.)	Adult autologous macrophages	SCI	Syringe with a 30-gauge fixed needle Hand-held injections at the caudal border of the lesion	Acute complete SCI (ASIA Grade A) between C5 and T11 n = 8	Phase I. Completed	Knoller *et al*[4]
2001–2002	China	Cultured fetal OECs	SCI	Free-hand injections (personal communication)	Chronic SCI n = 16	38-mo follow-up report	Huang *et al*[5]
2001–2002	Italy	Adult autologous MSCs	ALS	18-gauge cannula Pump injector supported by a table-fixed arm	Definite ALS n = 9	Completed	Mazzini *et al*[6]
2003	China	Cultured fetal OECs	SCI	Free-hand injections (personal communication)	Chronic SCI (cervical, thoracic, or thoracolumbar) n = 171	2–8 wk post-operative follow-up report	Huang *et al*[7]
2004	Australia	Cultured autologous OECs	SCI	Hamilton syringe with a 28-gauge beveled needle Stabilized system mounted to the operating table	Chronic complete SCI (ASIA Grade A) between T4 and T10 n = 6	Phase I clinical trial. 1 year follow-up. Ongoing.	Feron *et al*[8]
2003–2006	China	Cultured fetal OECs	ALS	Free-hand injections (personal communication)	Probable or definite ALS n = 327	Short-term outcome (4 wk postoperative)	Chen *et al*[9]
2007	Spain	Adult autologous BMCs	ALS	Intraspinal Not described.	ALS n = 10	Phase I/II clinical trial. Ongoing	Not published
2008	Turkey	Adult autologous BMCs	ALS	Intraspinal Not described.	ALS n = 13	1-year follow-up. Ongoing.	Deda *et al*[10]
2009	United States (Geron Corp.)	Human ESC-derived oligodendrocytes	SCI	Syringe positioning device that attaches to the frame of the operating room table	Subacute complete thoracic SCI (ASIA Grade A)	Phase I. Approved.	(www.geron.com)

SCI indicates spinal cord injury; MSCs, mesenchymal stem cells; ALS, amyotrophic lateral sclerosis; ASIA, American spinal injury association; OEC, olfactory ensheathing cells; BMCs, bone marrow cells; ESC, embryonic stem cells.

Cord injections in humans have been performed in a free-hand injection fashion[4,5,7,9] or using table-mounted operating devices.[6,8,16] Free-hand intraparenchymal injection represents an ill-advised delivery strategy in humans for a variety of reasons: (1) It cannot reliably reproduce anatomic targeting of the ventral horn; (2) It has an increased potential for spinal cord mass effect from uncontrolled pressure; (3) Movement of the unsteady needle can sheer white matter tracts; (4) Finally, it provides an imprecise rate of infusion predisposing the injection to reflux up the catheter. Table-mounted devices are usually designed in combination with micromanipulators and microinjectors, offering better stability, better control of volume and speed of injection, and anatomic precision through 3-dimensional positioning. Despite these advantages, these systems allow for movement of the patient with respect to the injection needle both during respiration in the prone position, and as a result of inadvertent jostling of the patient. Each of these issues carries significant potential to promote suboptimal efficacy and the generation of significant neurologic morbidity.

Clinical translation of spinal cord approaches still requires optimized means for intraspinal delivery. Limited literature exists addressing intraspinal surgical techniques in large animals.[17–19] We have previously demonstrated that single injections of cell suspensions[20,21] and viral vectors[22] can be safely and accurately performed into the healthy porcine cervical spinal cord, using a microinjection platform specifically designed for this purpose. Our injection platform fixes to the patients' spine rather than the operating table. The platform allows for rostrocaudal displacement to accommodate multilevel/multiple injections. In our studies, surgical risks and morbidity have been assessed in healthy large animals. The size and morphologic similarity of the swine and human spines and spinal cords renders the pig optimal for reliable safety and feasibility studies of grafting approaches in the spinal cord. In the present study, we validate the safety and accuracy of multiple unilateral cervical intraspinal injections and demonstrate postoperative graft survival in Göttingen minipigs.

■ Materials and Methods

This study was conducted under Good Laboratory Practices conditions. A Contract Research Organization assured the quality of the data. Surgical procedures, animal care, and collection of tissue were performed at the Saint Joseph's Translational Research Institute, in Norcross, GA following protocol approved by the Institutional Animal Care and Use Committee at the same institution.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Table 2. Experimental Design

Group	Treatment (Graft Site)	Cohort Size (n)	No. Injections	Dose (No. cells/volume)
A	C3–C5, unilateral	5	5	$2 \times 10^4/\mu L$; 6 μL/inj
B	C3–C5, unilateral	5	10	$2 \times 10^4/\mu L$; 6 μL/inj
C	C3–C5, unilateral	5	10	None; 6 μL/inj

inj indicates injection.

Study Design

The study design is summarized in Table 2. Briefly, 15 to 20 kg female Göttingen minipigs (Marshall BioResources, North Rose, NY), divided into 3 groups (n = 5/group), underwent multiple unilateral injections of cells or vehicle (hibernation buffer) into the spinal cord. Groups 1 and 2 received 5 and 10 unilateral injections of cells, respectively. Group 3 received 10 unilateral injections of vehicle.

Cells

A human neural stem cell line, NSI-566RSC, derived from the cervical-thoracic cord of a single 8-week human fetus[23] was supplied by Neuralstem Inc. (Rockville, MD). Cells from passage 12 were used in this study.

At no more than 36 hours before the scheduled surgeries, live cell suspensions comprising the different densities (Table 2) were prepared in a current Good Manufacturing Practices facility and shipped overnight directly to Saint Joseph's Translational Research Institute in an insulated shipping container held at 2 to 8°C. After arrival, cell density and viability were confirmed by the Trypan Blue exclusion method (0.4% solution, Sigma, St Louis, MO) and only cell suspensions with >85% viability were used.

Immunosuppression

Starting on the day of the surgery, Tacrolimus (Prograf—Astellas Pharma US Inc, Deerfield, IL) at a dose of 0.025 mg/kg, BID, was administered intravenously until euthanasia. A jugular vein 10 French chronic silicone catheter (Access Technologies—Norfolk Medical Inc, Skokie, IL) was placed under general anesthesia for drug administration.

Additional Medication

Cefazolin (500 mg/day, IM) was administered 1 day before the surgery and maintained until euthanasia. Buprenorphine (0.01 mg/kg, BID, IM) was given before the surgical procedure and maintained for 3 days. Methylprednisolone was administered as a bolus (125 mg, intravenous) just before the first cell injection and then daily (1 mg/kg, intravenous) during the first 7 postoperative days.

Surgical Procedure

Anesthesia. Animals were fasted 12 to 16 hours before surgery. Animal sedation and anesthesia induction consisted of a cocktail of IM ketamine 35 mg/kg, xylazine 1 mg/kg, and atropine 0.04 mg/kg. The animals were then intubated and maintained on oxygen and 1% to 3% isoflurane general anesthesia.

Laminectomy. Each animal underwent a C3–C5 laminectomy. Briefly, the animal was placed in prone position on a frame custom designed to mimic the patient positioning on a Jackson spinal surgical table which maximize exposure to the spine and minimize pressure on the abdomen and chest, therefore, minimizing epidural venous bleeding. The table uses adjustable slings that are placed under the chest and pelvis of the animal, allowing the abdomen to hang free. The design of the table was generously provided by Martin Marsala.[24] The operative field was shaved, washed, and draped. The cervical multilevel C3–C5 laminectomy was then performed as previously described.[21,22] Before dural opening, each animal received an intraoperative dose of intravenous methylprednisolone (125 mg). Following dural opening, the microinjection platform was fixed rostrally to the occiput and caudally to the C7 spinous process. Side rails were adjusted to fit the appropriate length between these 2 points. A gondola device bearing a Narishige microdrive model MO-97 (Narishige Scientific Instrument Lab, Tokyo, Japan) was placed on the rails (Figure 1). This gondola can be positioned at the appropriate position for the initial injection by sliding along the rails. It is then locked into position. A rigid cannula was placed onto the microdrive and secured approximately 5-mm above the dorsal surface of the spinal cord. The gondola is equipped with a turntable and screw adjustment to allow for positioning at the desired point on the radius of the circle. Thus, any point within the circle created by the rotation of the turntable with the screw set at the maximum radius can be reached. This device allows for microadjustment in the x (mediolateral) and y (rostrocaudal) directions. Finally, the gondola is equipped with a universal joint that allows for correction of the sagittal and coronal angles.[10]

Cell Injection. The right DREZ was identified under 3.5× surgical loupe magnification and penetrated on an orthogonal trajectory to the cord surface at a point <1 mm medial to the DREZ. The injection rate was programmed to 6 μL/min, using

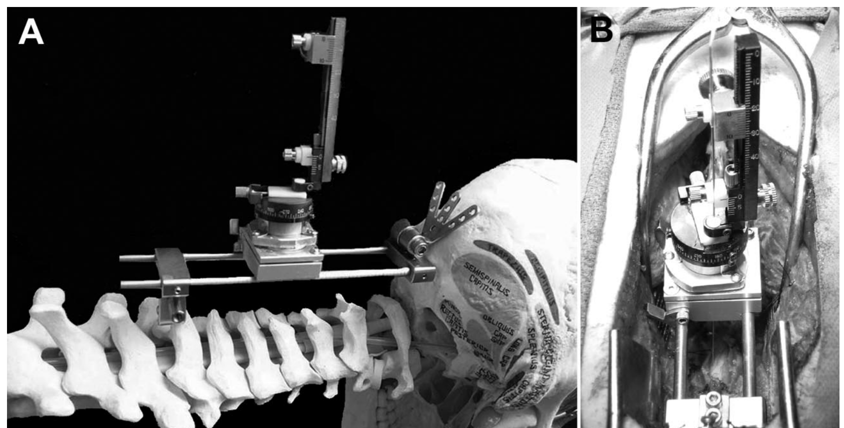

Figure 1. Cervical intraspinal microinjection platform. **A,** The microinjection platform is demonstrated while attached rostrally to the occiput and caudally to the spinous process of C7. **B,** The platform and microdrive are demonstrated following a C3/4 laminectomy and dural opening before unilateral microinjection into the porcine cervical spinal cord. Adapted from Clinical and Translational Science 2009;2:2. Copyright John Wiley And Sons.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

a precalibrated MINJ-PD microINJECTOR pump (Tritech Research, Inc, Los Angeles, CA). Doses were administered through the pump connected to a 250 μL Hamilton syringe (Hamilton Co, Reno, NV) and a cannula assembly consisting of a 30-gauge beveled needle attached to 1 meter of silicone tubing. The cell suspension was infused at a depth of 4 mm from pial contact following initial cannula introduction to a depth of 5 mm. By passing to a depth of 5 mm and then pulling back 1 mm, a small reservoir is created to reduce reflux. This maneuver also serves to correct the position of the surface of the cord which is depressed by approximately 1 mm as the cannula passes through the pia in a downward direction. Unilateral microinjections were performed at 2-mm intervals along the rostrocaudal axis at C3–C5 in the 10 injection group, and at 4-mm intervals in the 5 injection group. Following infusion, the cannula was left in place for 1 extra minute to minimize the potential for cell suspension reflux along the cannula track. Following watertight dural closure, muscular and fascial layers were closed using 2–0 and 0 Vicryl sutures, respectively. Skin closure was completed using 3–0 nylon suture.

Postoperative Care

Animals were extubated and monitored for 2 hours following anesthesia recovery. Next, they were transferred to individual cages and monitored at least once daily for food consumption, defecation, and micturition.

Behavioral Assessment

Clinical and behavioral observations were performed and recorded on days 1 through 7, 14, 21, and 28. Behavioral data were collected to assess neurologic morbidity. Sensory function was assessed by presence or absence of a withdrawal response to mechanical stimulus to the toes of front and hind limbs. Motor examination followed the Tarlov score system:

0 = Paralysis; no movement

1 = Perceptible tonus in the hind limbs; slight movement

2 = Movement in the hind limbs, but unable to sit or stand

3 = Ability to stand and walk but ataxic and for short periods

4 = Complete recovery; normal motor function

Euthanasia, Perfusion, and Tissue Collection

Animals were euthanized 28 days after surgery. Following euthanasia, transcardiac perfusion was performed with heparinized saline solution followed by buffered 4% paraformaldehyde solution. The appropriate spinal cord sections were collected for histologic analysis.

Histology

Spinal cords were embedded into gelatin matrix, postfixed, cryoprotected, and sectioned on a freezing-microtome at 40 μm. The transplantation area of the cervical segments and the 2 flanking areas were processed. Sections at 0.24 mm intervals (*i.e.*, every sixth section) were stained with a monoclonal human-nuclei antibody (HuNu, Chemicon, MA) or a polyclonal antibody against a neuronal-specifc marker, Doublecortin (DCX; Santa Cruz, CA). HuNu recognizes only human cell grafts and was used for graft identification. Doublecortin (DCX) is a marker of young neurons and was used to further characterize neuronal differentiation of the grafts. DCX is not expressed in adult porcine spinal cord. Intervening slides were stained with antiglial fibrillary acidic protein (Thermo-Fisher, IL), a marker of astrocytes, anti-Iba-1 (WAKO, VA), a marker of microglia, and hematoxylin and eosin to reveal the general condition of the tissues.

Statistical Analysis

Each experimental group contained 5 animals. Behavior scores of each animal were documented every day and expressed as mean± standard deviation (SD) for each experimental group. Experimental conditions were compared with a one-way analysis of variance (ANOVA).

Results

Behavior Outcome

All animals returned to their preoperative neurologic baseline (Figure 2) by postoperative day (POD) 7 except for 1 animal in group B. Neither mortalities nor surgical complications were reported in the study.

Sensory Function

Sensory function was assessed on days 1 through 7 and then on POD 14, 21, and 28. The toes of the front and hind limbs were progressively compressed with a surgical forceps and the response was scored as present (+) or absent (−). All the animals had adequate withdrawal response before surgery and in the first postsurgical evaluation except for 1 animal in group A which showed an absent withdrawal response in the right hind limb. That animal recovered sensory function by POD 1 and did not show further deficits in sensory function over the 28-day period.

Motor Function

The Tarlov score system was used to evaluate motor function. The majority of animals (10 of 15) recovered

Pre-operative

Post-operative Day 3

Figure 2. Behavioral outcome. All the animals showed full recovery returning to the preoperative baseline. Note the cervical surgical incision and the bandage protecting the intravenous catheter in 1 animal of group B at POD3.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

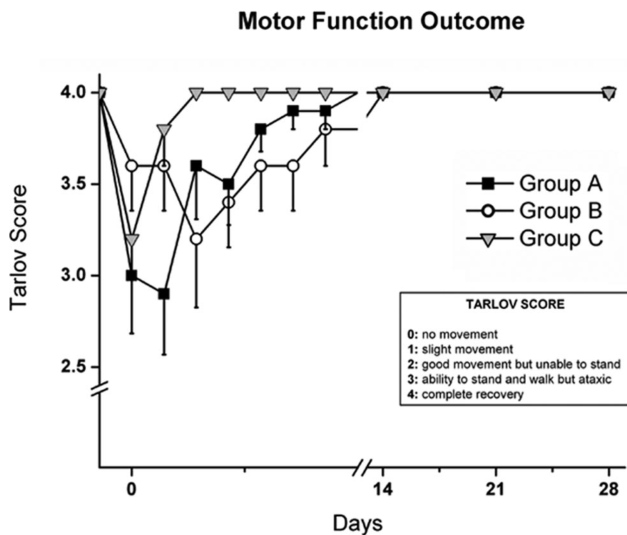

Figure 3. Motor function outcome. Regardless of the differences in the number of injections (5 *vs.* 10) or the treatment (cells *vs.* vehicle), all the animals showed complete motor function recovery which was maintained over the 28 day period.

full motor ability (score 4) by POD 2. The rest of the animals progressively recovered to preoperative baseline by POD 6. Only 1 animal (the same that experienced transient absent sensory response) had difficulty moving the right hind limb after surgery, but recovered to score 3 by POD 7 and completely recovered by POD 14. Despite some variations in the duration of transient neurologic deficits among the 3 groups, all the animals showed full motor function recovery which was maintained over the 28-day period (Figure 3). No statistically significant differences were detected in the behavior outcome between the experimental groups (one-way ANOVA: POD 1 $P = 0.096$; POD 4 $P = 0.27$; POD 7 $P = 0.368$).

No deficits in micturition or defecation were noted.

Histopathology

General Observations. There were a total of 125 needle injections into spinal cords of the 15 pigs from this study. Needle tracks and associated changes occurred primarily in the injected side of the spinal cords. There were no apparent differences among the groups. Responses in all but 2 pigs were generally relatively minor and consisted of changes that would be expected following multiple needle injections. Accordingly, needle tracks with associated hypercellularity were observed in all but 1 control pig, which consisted of microglial cells. Axonal dystrophy (axonal swelling) of minimal or mild severity was present in 3/5, 2/5, and 2/5 pigs in control, low, and high dose groups, respectively, in the transplant area; in 1/5 low dose pigs in the cervical cord cranial to the transplant area; and in 1/5 and 1/5 pigs in the low and high dose groups, respectively, in the cervical cord caudal to the transplant area. More profound lesions occurred at 1 site in each of 2 pigs. These changes consisted of degeneration, necrosis, and malacia which were present in the lateral and ventral funiculi from the "region of interest" (ROI) of 1 control pig and in the dorsal funiculi from the cervical region caudal to the ROI in 1 high dose pig. They were present in the white matter of both pigs and were associated with gliosis and loss of neurons in the adjacent gray matter of the control pig apparently due to retrograde degeneration. Other changes included mild nerve fiber degeneration in the cervical cord cranial to the cervical ROI in 1 low dose pig and minimal gliosis in 1 low dose pig in the ROI. There were no changes in the thoracolumbar segments. Glial cells (GFAP-positive) in and surrounding needle tracks were detected in all control and treated spinal cords (data not shown). Reactive microglial cells (Iba-1-positive) were also identified in these sites. The microglial response was apparently a reaction to injury from needle punctures, since positive cells were present in the needle tracks from control cords as well (data not shown).

Graft Survival and Differentiation. HuNu positive cells were present in the transplant areas in 0/5 controls, 5/5 low dose, and 3/5 high dose pigs where they occurred primarily along needle tracks with some migration to the immediately surrounding areas. The grafts were generally concentrated in the intermediate zone and a medial portion of ventral horn (Figure 4A). The grafted cells appeared to be migrating away from the injection site (Figure 4B). Graft survival and its neuronal differentiation were also confirmed by staining the cord sections with anti-DCX antibody since adult pig spinal cords do not express DCX. The HuNu and DCX localization in the cord were identical (Figure 4A, C). In the 2/5 high dose pigs where HuNu staining was absent (most likely due to epitope masking from overfixation), presence of human graft was confirmed by staining with anti-DCX antibody. Dense DCX stain was detected adjacent to motor neurons in the ipsilateral ventral horn of the spinal cord of treated animals (Figure 4D).

■ Discussion

In this study, we have compared safety and feasibility of multiple intraspinal injections in the cervical spinal cord using a stereotaxic device newly designed specifically for this purpose. Stable human neural stem cells originally derived from a fetal spinal cord tissue were injected 10 times at 2 mm or 5 times at 4 mm intervals along 1 side of C3–C5 segments. There were no functional or pathologic differences between animals receiving either 10 injections or 5 injections or between animals receiving 10 cell injections or 10 buffer injections. Most of the injections were within 1 mm of the intended target, at or near the ventral horn. Our data demonstrated that we can safely and accurately target the ventral horn of the spinal cord. Despite some transient motor dysfunction, all the animals returned to normal function, demonstrating the feasibility of multiple unilateral injections into the cervical spinal cord. Histopathology evaluation revealed that, as expected, there were minor tissue injuries associated with the needle penetration into the healthy cords. There

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

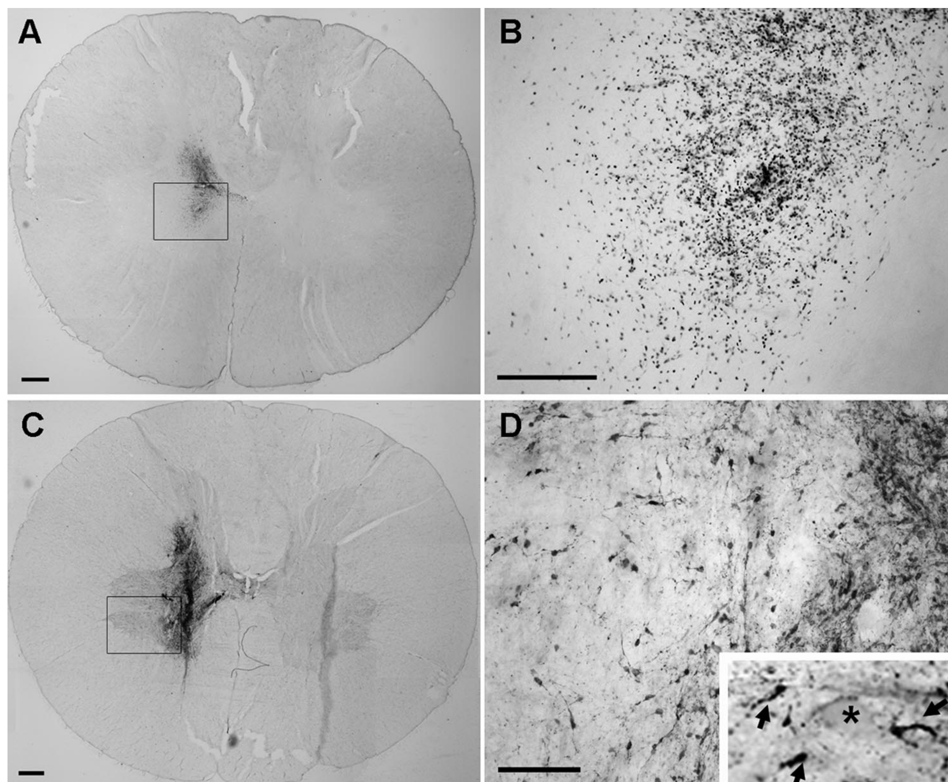

Figure 4. Graft identification and migration/differentiation. Two adjacent coronal sections of a different animal from Group A, stained with HuNu (**A, B**) or DCX antibodies (**C, D**) are shown. **B,** HuNu staining—Migrating edge of the grafted cells in the gray matter; **D,** DCX staining—Note neurites from the graft (arrows) extending out and surrounding motor neurons (*) in the ventral horn. Scale bars = 1 mm (**A, C**); 250 μm (**B, D**).

were no pathologies detected from the cell grafts themselves. This study demonstrates that multiple intraspinal cell placements serially along the cervical segments are safe and feasible for human testing. The development of safe and accurate means for spinal cord stem cell transplantation will pave the way for a wide array of therapies for spinal cord conditions, such as SCI, ALS, and MS.

With the emerging promise of stem cell therapies, several trials for SCI and ALS have been launched around the world.[4–10,16] Clinical trials have mainly focused on thoracic injections based on the sole premise that Phase 1 safety trials can demonstrate safety and tolerability of the biologic product. In SCI trials to date, human spinal cord injection has been targeted below the level of complete injury. This approach has the advantage of minimizing the potential for exacerbating the neurologic deficit of the patient. It has the disadvantage, however, of making it impossible to detect any injury associated with injection. Thus, these “safety” trials are only capable of detecting formation of tumors, but not deterioration of white or gray matter function at the segmental level of injection. Because SCI is not a terminal disease, histologic evaluation of the graft sites will occur sporadically at best. Thus, these phase I trials are not designed to detect segmental damage. In contrast, in the vulnerable spinal cords of ALS, partial spinal cord injury, and MS patients, segmental damage will be immediately apparent.

Use of large animal models is critical for evaluating safety of combined surgical procedure, cell injection device, and the candidate cell products before clinical trials. Large animal models are also crucial for the understanding of graft rejection. For evaluating safety of intraspinal cell transplantation, minipigs were quite useful due to anatomic similarity and size compared to the adult human spines and spinal cords. While much preclinical proof-of-principle work has been done in rodents, the tolerance of the rodent spinal cord for trauma, and its native capacity for recovery vastly exceeds that of humans. The porcine spinal cord mimicked closely to the minimal tolerance of the human spinal cord to injuries. The healthy minipigs also provided the means to: (1) evaluate sensory, motor, and urinary function after surgery; (2) assess the impact of spinal cord pulsation observed *in vivo*; (3) address the impact of epidural and intraparenchymal vasculature on the risk of hemorrhage; and (4) evaluate the risk of infection and tolerance of immunosuppressive regimens.

Feasibility of intraspinal cell transplantation is being investigated for treatment of SCI and ALS.[11,25,26] Macrophage cell therapy has been suggested to modulate the immune response at the site of the spinal cord injury.[5] Olfactory ensheathing cells and oligodendrocyte precursors have been postulated to support myelination of regrowing axons in SCI.[5,7,27] Mesenchymal stem cells, which can differentiate into neural cells, as well as glial-restricted precursors have been demonstrated to secrete growth factors and modulate gliosis in ALS.[7,28,29] Finally, it has been demonstrated that human embryonic stem cell-derived motor neurons[30] and human neural stem cells[31] are able to differentiate into neurons and form axons and synapses in rodent models of SCI and

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

ALS. In the current study, we used stable neural stem cells (NSI-566RSC) that have been proven to induce growth factor expression and provide segmental neuroprotection as well as functional benefits in animal models of SCI[32,33] and ALS.[23,31,34] In these previous studies, grafted cells formed synaptic contacts on host MN and expressed multiple growth factors, including glial-derived neurotrophic factor, brain-derived neurotrophic factor, and insulin-like growth factor-1. Detailed characterization of survival and neuronal differentiation of NSI-566RSC after intraspinal transplantation in minipigs has been undertaken in previous studies[24,35,36] and was not the primary aim of this study. However, after 28 days, there were quantitative survival and robust neuronal differentiation in all grafts. Consistent with the previous studies,[24,35,36] each graft occupied approximately 1-mm sphere in the tissue. Thus, 2-mm graft intervals used in this study represented the maximum frequency reasonable from therapeutic and safety perspectives.

This study has shown the safety of multiple unilateral cervical spinal cord injections. Ideally cell therapy for ALS would require the treatment of the whole motor neuron columns, however, given that death in ALS occurs secondary to respiratory failure and the loss of upper airway control, our rationale is aimed at prolonging life by preserving the phrenic motor neurons and strengthening the proximal upper extremities. We were able to establish clinically acceptable levels of cell density and number of injections using the surgical techniques and devices previously described.[21,22] Overall, up to 10 unilateral injections of NSI-566RSC at 2 mm intervals into the C3–C5 cervical spinal cord proved to be feasible and safe. Additional refinements of the injection device are ongoing to accommodate lumbar spinal cord injections and perform the injections in a minimally invasive fashion.

■ Key Points

- Free-hand injections and table-attached devices are currently being used for spinal cord injections but carry significant potential for inconsistent graft targeting leading to suboptimal efficacy in addition to causing neurologic morbidity.
- We have developed a spinal cord microinjection platform designed to facilitate safe and accurate administration of cellular and molecular therapeutics to humans.
- Validation in large animal models is a required step in progressing towards a clinical trial.
- The size and morphologic similarity in spines of the swine and of human beings renders the pig optimal for safety studies in the spinal cord.
- We can accurately target the ventral horn of the spinal cord. Multiple unilateral injections of cells into the cervical spinal cord of minipigs proved to be feasible and safe.

Acknowledgments

The authors thank Neuralstem, Inc. and ALS Association NINDS 5K02NS059416.

References

1. Miller RH. The promise of stem cells for neural repair. *Brain Res* 2006;1091:258–64.
2. Webber DJ, Minger SL. Therapeutic potential of stem cells in central nervous system regeneration. *Curr Opin Investig Drugs* 2004;5:714–19.
3. Yu D, Silva GA. Stem cell sources and therapeutic approaches for central nervous system and neural retinal disorders. *Neurosurg Focus* 2008;24:E11.
4. Knoller N, Auerbach G, Fulga V, et al. Clinical experience using incubated autologous macrophages as a treatment for complete spinal cord injury: phase I study results. *J Neurosurg Spine* 2005;3:173–81.
5. Huang H, Chen L, Wang H, et al. Safety of fetal olfactory ensheathing cell transplantation in patients with chronic spinal cord injury. A 38-month follow-up with MRI. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 2006;20:439–43.
6. Mazzini L, Mareschi K, Ferrero I, et al. Stem cell treatment in amyotrophic lateral sclerosis. *J Neurol Sci* 2008;265:78–83.
7. Huang H, Chen L, Wang H, et al. Influence of patients' age on functional recovery after transplantation of olfactory ensheathing cells into injured spinal cord injury. *Chin Med J (Engl)* 2003;116:1488–91.
8. Feron F, Perry C, Cochrane J, et al. Autologous olfactory ensheathing cell transplantation in human spinal cord injury. *Brain* 2005;128:2951–60.
9. Chen L, Huang H, Zhang J, et al. Short-term outcome of olfactory ensheathing cells transplantation for treatment of amyotrophic lateral sclerosis. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 2007;21:961–6.
10. Deda H, Inci MC, Kurekci AE, et al. Treatment of amyotrophic lateral sclerosis patients by autologous bone marrow-derived hematopoietic stem cell transplantation: a 1-year follow-up. *Cytotherapy* 2009;11:18–25.
11. Bradley JA. Repairing spinal cord injury by cell transplantation. *Transplantation* 2008;86:769–71.
12. Fehlings MG. Complete cord injury. *J Neurosurg Spine* 2005;3:171–2; discussion 172.
13. Blight A, Curt A, Ditunno JF, et al. Position statement on the sale of unproven cellular therapies for spinal cord injury: the international campaign for cures of spinal cord injury paralysis. *Spinal Cord* 2009;47:713–14.
14. Owens J. Stem-cell treatments for spinal-cord injury may be worth the risk. *Nature* 2009;458:1101.
15. Gabriel EM, Nashold BS Jr. History of spinal cord stereotaxy. *J Neurosurg* 1996;85:725–31.
16. Geron Corporation. World's first clinical trial of human embryonic stem cell therapy cleared. *Regen Med* 2009;4:161.
17. Lonser RR, Gogate N, Morrison PF, et al. Direct convective delivery of macromolecules to the spinal cord. *J Neurosurg* 1998;89:616–22.
18. Jeffery ND, Smith PM, Lakatos A, et al. Clinical canine spinal cord injury provides an opportunity to examine the issues in translating laboratory techniques into practical therapy. *Spinal Cord* 2006;44:584–93.
19. Zurita M, Vaquero J, Bonilla C, et al. Functional recovery of chronic paraplegic pigs after autologous transplantation of bone marrow stromal cells. *Transplantation* 2008;86:845–53.
20. Riley J, Butler J, Park J, et al. Targeted spinal cord therapeutics delivery: stabilized platform and MER guidance validation. *Stereotact Funct Neurosurg* 2007;86:67–74.
21. Riley J, Federici T, Park J, et al. Cervical spinal cord therapeutic delivery: preclinical safety validation of a stabilized microinjection platform. *Neurosurgery* 2009;65:754–61.
22. Federici T, Riley J, Park J, et al. Preclinical safety validation of a stabilized viral vector direct injection approach to the cervical spinal cord. *Clin Transl Sci* 2009;2:165–7.
23. Xu L, Yan J, Chen D, et al. Human neural stem cell grafts ameliorate motor neuron disease in SOD-1 transgenic rats. *Transplantation* 2006;82:865–75.
24. Usvald D, Motlick J, Marsala M. Analysis of dosing regimen and reproducibility of intraspinal grafting of human spinal stem cells in immunosuppressed minipigs. *Cell Transplant.* In press.
25. Nayak MS, Kim YS, Goldman M, et al. Cellular therapies in motor neuron diseases. *Biochim Biophys Acta* 2006;1762:1128–38.
26. Lunn JS, Hefferan MP, Marsala M, et al. Stem cells: comprehensive treatments for amyotrophic lateral sclerosis in conjunction with growth factor delivery. *Growth Factors* 2009;27:133–40.
27. Sharp J, Keirstead HS. Therapeutic applications of oligodendrocyte precur-

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

sors derived from human embryonic stem cells. *Curr Opin Biotechnol* 2007; 18:434–40.

28. Lepore AC, Rauck B, Dejea C, et al. Focal transplantation-based astrocyte replacement is neuroprotective in a model of motor neuron disease. *Nat Neurosci* 2008;11:1294–301.
29. Mazzini L, Fagioli F, Boccaletti R, et al. Stem cell therapy in amyotrophic lateral sclerosis: a methodological approach in humans. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2003;4:158–61.
30. Deshpande DM, Kim YS, Martinez T, et al. Recovery from paralysis in adult rats using embryonic stem cells. *Ann Neurol* 2006;60:32–44.
31. Xu L, Ryugo DK, Pongstaporn T, et al. Human neural stem cell grafts in the spinal cord of SOD1 transgenic rats: differentiation and structural integration into the segmental motor circuitry. *J Comp Neurol* 2009;514:297–309.
32. Cizkova D, Kakinohana O, Kucharova K, et al. Functional recovery in rats with ischemic paraplegia after spinal grafting of human spinal stem cells. *Neuroscience* 2007;147:546–60.
33. Yan J, Xu L, Welsh AM, et al. Extensive neuronal differentiation of human neural stem cell grafts in adult rat spinal cord. *PLoS Med* 2007;4:e39.
34. Yan J, Xu L, Welsh AM, et al. Combined immunosuppressive agents or CD4 antibodies prolong survival of human neural stem cell grafts and improve disease outcomes in amyotrophic lateral sclerosis transgenic mice. *Stem Cells* 2006;24:1976–85.
35. Hefferan MP, Kakinohana O, Santucci C, et al. Lumbar transplantation of human spinal stem cells for the treatment of amyotrophic lateral sclerosis. Chicago, IL: Society for Neuroscience; 2009.
36. Marsala M, Usvald D, Vodicka P, et al. Spinal grafting of human spinal neural precursors: a preclinical safety and dosing study in immunosupressed minipig. San Diego, CA: Society for Neuroscience; 2009.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.