Complete rat spinal cord transection as a faithful model of spinal cord injury for translational cell transplantation

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Spinal cord injury (SCI) results in neural loss and consequently motor and sensory impairment below the injury. There are currently no effective therapies for the treatment of traumatic SCI in humans. Various animal models have been developed to mimic human SCI. Widely used animal models of SCI are complete or partial transection or experimental contusion and compression, with both bearing controversy as to which one more appropriately reproduces the human SCI functional consequences. Here we present in details the widely used procedure of complete spinal cord transection as a faithful animal model to investigate neural and functional repair of the damaged tissue by exogenous human transplanted cells. This injury model offers the advantage of complete damage to a spinal cord at a defined place and time, is relatively simple to standardize and is highly reproducible.

Successful clinical trials in treatment of SCI cannot be initiated without previous preclinical studies using adequate animal model that closely mimics the loss of function that occurs in humans. In the last decade diverse cell-based therapies have shown certain potential incorporating new neural cells into the milieu of a traumatic spinal cord injury. These cell-based treatments are designed to regenerate or remyelinate axons providing new oligodendrocytes or simply reconnecting injured tissue with newly generated neurons1–4. However, a proper and standard animal model of injury will allow better understanding of the biological and molecular changes along the injury and easily set up a platform to test potential therapeutic strategies. Widely used animal models of SCI include complete or partial transection or experimental contusion and compression, with both bearing controversy as to which one is more faithful to the human SCI functional and anatomical consequences. Human traumatic SCI is classified into five categories by the American Spinal Injury Association (ASIA) and the International Spinal Cord Injury Classification System, from incomplete to complete (E to A, respectively). The complete transection animal model reproduces the complete spinal cord injury in humans defined by ASIA as “no preservation of motor and/or sensory function exists more than 3 segments below the neurological level of injury”9. Nowadays, the human SCIs are likely to be much more complex than those experimentally provoked in rat models. Rats result to be a convenient model for spinal cord study, due to a low cost, easiness to care for, low incidence of surgical infections, and well established functional analysis techniques, although anatomical differences of the axonal tracts should be taken into account. Here we present a detailed surgical procedure of complete spinal cord transection and oligodendrocyte (OPC)-directed differentiated human embryonic stem cells (hESC) transplantation. Functional locomotion rescue of OPC transplanted group indeed support the use of this model for functional repair after severe SCI by exogenous human transplanted cells. Complete spinal cord transaction model was widely used to investigate the regenerative potential of different types of human cells: umbilical mesenchymal stem cells10, embryonic stem cells derived oligodendrocyte progenitors4, dental pulp-derived stem cells4,11 motoneuron progenitors4, olfactory ensheathing cells12,13, neural stem cells8. However, a surgery and cell transplantation protocol has not been so far sufficiently detailed to efficiently generate a reproducible and standardized model.
Results

Presurgical Procedure. All small surgical instruments were sterilized in stainless Steel Sterilization Container by autoclave. Large instruments and equipment were sterilized with 70% ethanol. The heating pad, stereotaxic instrument and Rat Spinal Cord Unit were mounted as shown on Figure 1 A–C. The small surgical instruments were additionally sterilized maintaining them in Hot bead Sterilizer. Small cuts of GORETEX (1×2 cm) were sterilized under the UV light and kept in flow laminar hood until use. The Anesthesia Workstation and the instruments on the surgical table are set up as shown in Figure 1 C and 1 D. For analgesia purpose, half an hour before the surgery the morphine is injected subcutaneously (2.5 mg/kg b.w.). Enrofloxacine is also administrated subcutaneously according to the animal body weight (12 mg/kg b.w.) for antibiotic prophylaxis purposes and later every 24 hours during 7 days of postoperative period. For cell transplantation from different species of the

Figure 1 | Instruments and equipment used in the protocol. (A). DKI 900 Small Animal Stereotaxic instrument mounted on DKI 980 Rat Spinal Cord Unit. (B). DKI 5000 Microinjection Unit with 5001 Holder. (C). Anesthesia Workstation (FABIUS, Draeger) connected to Plexiglas chamber and Gas absorber (CA-AG1000, Cibertec, Spain). (D). The list of surgical instruments used in the study.

Figure 2 | Presurgery procedure. (A). Rat in the chamber during the anesthesia. (B). Surgery unit connected with rat anesthesia mask. (C, D, E and F). Rat positioned in surgery unit with rat anesthesia mask. (G). Rat ready for introducing intravenous cannula in caudal tale venous. (H and I). Procedure of introducing intravenous cannula in caudal tale venous of the animal.
The Anesthesia Workstation is first set up at 2% of isofluorane. Dorsal area 2F) and the Anesthesia Workstation is set up at 2% of isofluorane. This flow is maintained until surgery is completed. Dorsal area different from the surgery zone). The intravenous catheter is introduced in caudal venous tale for fluidotherapy (Figure 2G–I). The syringe holder with the 20 ml syringe filled with physiologic saline solution 0.9%, connected to a perfusion device (Figure 2G–I), is turned on and the intravenous catheter connected to the syringe. The flow is set up at 2 ml/h and maintained throughout all surgical procedure. Eye dehydrratation is prevented by applying carbomer ophthalmic gel 2% (1 drop to each eye). A longitudinal incision of approximately 2.5 cm is performed with scalpel blade (Figure 3A). After removing the fat tissue (Figure 3B and 3C) the moisture is kept with physiologic saline solution 0.9%. The muscles overlying the vertebral column are reflected exposing the vertebral column T7–T10 (Figure 3D) and the alm retractor positioned to keep the incision widely open (Figure 3D). The spinotrapezium muscle is detached from bone on the spinal laminae using the scalpel blades or raspatory-peek handle (Figure 3E and 3F) and the connective tissue and remaining muscles are removed by iris scissors to be able to see clearly the bone structures (Figure 3G and 3H). The thoraco-dorsal arteria usually is visualized crossing throught T6 and is important to maintain it intact to avoid any hemorrhage complication. Figure 3S shows a draw of the thoracic segments T5–T11. Under the headband magnifier the T9 spine segment backwards is carefully lifted while introducing slowly a Rougeur of a very fine-pointed side-cutting. First remove T9 spine and then partially remove the lateral apohasis at T9 and T8 level (Figure 3I and 3J)). Dura is cut using Von Graefe Knife (Figure 3J) and 1 drop of Lidocaine solution (2%) is added directly on the lesion. The spinal cord is lifted using Spinal Cord Hook (Figure 3K, 3L and 3M) and the cross-sectional transection of the spinal cord is performed using thin scissors (Vanna Spring Scissors) (Figure 3N and 3O). This procedure frequently causes meninges to bleed, being handled with compression of the affected portion with wet surgical gauze. In order to remove the tissue between two cuts Vessel Dilating Probe needs to be passed through meninges tissue (Figure 3P). It is very important to cut any residual fibers and to verify complete transection. It has been reported that 5–10% of spared white matter in the ventrolateral funiculi is sufficient for sustained rat locomotion14,15. The spinous processes are immobilized using vertebral clamps fixed to the Spinal Cord Unit securing the T10 vertebral segment (Figure 3Q and 3R). Cells, prepared as described in Methods are positioned into the glass pipette fixed to Hamilton syringe which is adapted to a microinjector at the stereotaxic unit over the spinal cord surface; 3 mm (caudally) bellow the lesion as is shown on Figure 4E. The glass pipette is lowered 1 mm and a total of 5 μl of cell suspension is delivered at 2 μl/min. An automatic microinjector is also recommended like a Nanomite, Infuse/Withdraw (70-3601, Harvard Apparatus, USA). Excessive rate of injection leads to the grafting decrease. The glass pipette is left in the injection site for 2 minutes without injection to avoid leaking of the injected cells. Last steps are repeated by injecting the glass pipette in the second and third caudal position as well as in the equivalent three positions rostrally, 3 mm above the lesion. The injections in host spinal cord cranial and caudal to the lesion epicenter were performed in order to avoid the cavitations epicenter, hemorrhagic necrosis, and inflammation, which might decrease cell survival and integration. The glass pipette is

Figure 3 | Surgery procedure. (A), A longitudinal incision of skin with scalpel blade. (B and C). Removal of fat tissue. (D). Alm retractors positioned to keep the incision widely open. (E). Exposed vertebral column T7–T10 after muscle reflection. (F). Exposed vertebral column T7–T10 after detachment of the spinotrapezium muscle from bone on the spinal laminae. (G and H). Removal of residual connective tissue and remaining muscles by iris scissors. (I). Position of wet surgical gauze to stop eventual bleeding. J. Spinal cord at T7–T9 level after laminectomy. (K and L). Positioning of Spinal Cord Hof. (M). Lifting the spinal cord. (N). Cross-sectional transection of the spinal cord using scissors. (O). Lifting the meninges to verify the complete transection. (P). Spinal cord cut. (Q and R). Immobilization of the spinous processes using vertebral clamps vertebral column T10. (S). Rat spine vertebrae from T5–T11. To recognize T9 it is very important to localize T10 which have different spinous processes.

recipient immunosuppression is required and Cyclosporine A (20 mg/kg b.w.) should be injected subcutaneously daily starting two days before the transplantation until the end of experiment.

Surgical procedure. The Anesthesia Workstation is first set up at 3% of isofluorane within 0.5% oxygen flow connected to the Plexiglas inducer chamber as it is shown in Figure 2A. Rats go into deep anesthesia plane about 1 minute after, when the muscles are relaxed. The anesthesia stage is confirmed checking the pedal, palpebral and corneal reflexes. The rat’s head is positioned on heating pad over the Spinal Cord Unit with stretched anterior and posterior legs (Figure 2D and 2C). The rat’s head is positioned in rat anesthesia mask (Figure 2D, 2E and 2F) and the Anesthesia Workstation is set up at 2% of isofluorane. This flow is maintained until surgery is completed. Dorsal area between the neck and hindlimbs is shaved extending ~2 cm bilaterally from the spine (Figure 3A) and then disinfected with Chlorhexidine digluconate 1% (It is recommended to do this procedure in the area different from the surgery zone). The intravenous catheter is introduced in caudal venous tale for fluidotherapy (Figure 2G–I). The syringe holder with the 20 ml syringe filled with physiologic saline solution 0.9%, connected to a perfusion device (Figure 2G–I), is turned on and the intravenous catheter connected to the syringe. The flow is set up at 2 ml/h and maintained throughout all surgical procedure. Eye dehydrratation is prevented by applying carbomer ophthalmic gel 2% (1 drop to each eye). A longitudinal incision of approximately 2.5 cm is performed with scalpel blade (Figure 3A). After removing the fat tissue (Figure 3B and 3C) the moisture is kept with physiologic saline solution 0.9%. The muscles overlying the vertebral column are reflected exposing the vertebral column T7–T10 (Figure 3D) and the alm retractor positioned to keep the incision widely open (Figure 3D). The spinotrapezium muscle is detached from bone on the spinal laminae using the scalpel blades or raspatory-peek handle (Figure 3E and 3F) and the connective tissue and remaining muscles are removed by iris scissors to be able to see clearly the bone structures (Figure 3G and 3H). The thoraco-dorsal arteria usually is visualized crossing throught T6 and is important to maintain it intact to avoid any hemorrhage complication. Figure 3S shows a draw of the thoracic segments T5–T11. Under the headband magnifier the T9 spine segment backwards is carefully lifted while introducing slowly a Rougeur of a very fine-pointed side-cutting. First remove T9 spine and then partially remove the lateral apohasis at T9 and T8 level (Figure 3I and 3J)). Dura is cut using Von Graefe Knife (Figure 3J) and 1 drop of Lidocaine solution (2%) is added directly on the lesion. The spinal cord is lifted using Spinal Cord Hook (Figure 3K, 3L and 3M) and the cross-sectional transection of the spinal cord is performed using thin scissors (Vanna Spring Scissors) (Figure 3N and 3O). This procedure frequently causes meninges to bleed, being handled with compression of the affected portion with wet surgical gauze. In order to remove the tissue between two cuts Vessel Dilating Probe needs to be passed through meninges tissue (Figure 3P). It is very important to cut any residual fibers and to verify complete transection. It has been reported that 5–10% of spared white matter in the ventrolateral funiculi is sufficient for sustained rat locomotion14,15. The spinous processes are immobilized using vertebral clamps fixed to the Spinal Cord Unit securing the T10 vertebral segment (Figure 3Q and 3R). Cells, prepared as described in Methods are positioned into the glass pipette fixed to Hamilton syringe which is adapted to a microinjector at the stereotaxic unit over the spinal cord surface; 3 mm (caudally) bellow the lesion as is shown on Figure 4E. The glass pipette is lowered 1 mm and a total of 5 μl of cell suspension is delivered at 2 μl/min. An automatic microinjector is also recommended like a Nanomite, Infuse/Withdraw (70-3601, Harvard Apparatus, USA). Excessive rate of injection leads to the grafting decrease. The glass pipette is left in the injection site for 2 minutes without injection to avoid leaking of the injected cells. Last steps are repeated by injecting the glass pipette in the second and third caudal position as well as in the equivalent three positions rostrally, 3 mm above the lesion. The injections in host spinal cord cranial and caudal to the lesion epicenter were performed in order to avoid the cavitations epicenter, hemorrhagic necrosis, and inflammation, which might decrease cell survival and integration. The glass pipette is
removed carefully from the injection site. Vertebral clamps and retractors are removed. The laminectomy was covered with GORETEX synthetic dura (Figure 4E). The deep and superficial muscle layers and the skin are carefully sutured with Monosyn violet 4/0 (Figure 4F, 4G and 4H). The animal is then recovered from anesthesia on a heating pad until alert and mobile (Figure 4I and 4J) and the bladder is manually pressed until completely empty.

Cell preparation for transplantation. For transplantation the cells are disaggregated mechanically with a glass pipette and centrifuged for 2 min at 50 g, room temperature. The cells are disaggregated by pipetting to single cell suspension in culture medium. Immediately before transplantation, the cell viability is checked by trypan blue and cell populations with >95% viability are used for transplantation. The cell solution is prepared at 100,000 cells/μl and 1.6 million cells injected per animal. The silicon-coated 100 μm glass tip with silicon tube connected to the 50 μl Hamilton syringe needle and microinjector are mounted on stereotaxic frame (Figure 4A). Using a stereotaxic manipulator arm and injection unit the glass tip are immersed in vegetal oil (Figure 4A). The role of the oil is to push uniformly the cell sample. Approx. 20 μl of vegetal oil is aspirated in the glass pipette using microinjection unit. Using a stereotaxic manipulator arm and injection unit (Figure 4B) 16 μl of cell suspension is aspirated in the glass pipette using microinjection unit (Figure 4C and 4D).

Results Supporting the Procedure

Nuclear magnetic resonance (NMR) imaging provides a noninvasive method for studying the integrity of spinal cord and in the case of spinal cord injury faithful tool to follow the spinal cord damage after spinal cord transection in vivo. NMR images showed a clear and persistent lesion with no spared axons in the lesion site as shown in Figure 5B and 5C. Regenerative effects of OPC cells in rat’s transected spinal cord are already described with more details1. Locomotor tests such as open field locomotor scale, described by Basso, Beattie and Bresnahan (BBB) are used to test locomotor recovery after complete transection injuries in rat spinal cord with and without transplanted cells. In this locomotor assay rats are trained weekly to move in an open field which is a molded-plastic circular enclosure with a smooth, nonslip floor. Rats were allowed to move freely and are scored during 4 minutes for their ability to use their hindlimbs. Joint movements, paw placement, weight support, and fore/hindlimb coordination are judged according to the 21-point BBB locomotion scale. Before the injury, all animals showed normal locomotor activity, scored as 21 on the BBB scale, although all injured rats manifested complete hind limb paralysis 7 days after injury, resulting in a score of 0. The BBB scores were in the range of 0–1 or 2 in the control animals during the 4 months after SCI (Figure 5D). In contrast animal group transplanted with OPC showed hind limb functional locomotor recovery which increased gradually after 3 weeks of transplantation. Four months after transplantation OPC transplanted animals displayed BBB scores significantly (P < 0.001) higher than that achieved by the control group reaching a final average BBB score of 6 (Figure 5D).

The degree and functional significance of complete transection in the host tissue is evaluated by immunohistological techniques. Immunohistochemistry analysis using the antibody against Neurofilament 200 fibers has been shown that area of surviving white matter in cross-section through the center of lesion site has disappeared or has significantly reduced compared with intact animals, indicating the absence of spontaneous axonal regeneration in non-transplanted rats after complete transection (Figure 5E). Immunohistochemistry analysis confirmed previous findings that regenerative effect after transplantation is due to transplanted cells differentiated to neurons which coincides with locomotor activity1. The presence of neurons of human origin in the lesion site is confirmed by immunoreactivity against NF70, human specific marker (Figure 5F).

Another method of reconnection of damaged tissue is assessing motor pathways is the simultaneous stimulation of the motor cortex using transcranial magnetic stimulation (TMS) and voluntarily contraction in a target muscle as a non-invasive, painless and safe method in assessment of human central and peripheral motor pathways17–19. The lower limb motor evoked potentials (MEP) determine the severity of spinal motor damage. Complete transection of spinal cord produces flat MEP after surgery, without recovery after 4 months (Figure 5G). Our results have shown that OPC cells transplantation immediately after surgery induces MEP after 1 month which is maintained for another 4 months, clearly indicating the regenerative effects of these cells2 (Figure 5G).

Discussion

Our results confirm that rat model of complete transection is reproducible and simple to standardize model for SCI, faithfully mimicking the most severe clinical cases of SCI in humans. The most important advantage of this model is completeness of the injury that can be performed at defined time and place. As there are no spared...
Figure 5 | (A–C) NMR of the rat with and without complete transaction. (A). Sham. (B). Injured rats (laminaectomy with complete transaction of spinal cord) 1 week post surgery. (C). Injured rats (laminaectomy with complete transaction of spinal cord) after 2 weeks post surgery. (D) BBB score during 4 month postsurgery. Starting 4 weeks after the transplantation, a significant increase (P < 0.001) in locomotor recovery, determined by the BBB locomotor rating scale, was observed in OPC transplanted animals compared to controls. The values are presented as mean ± s.e.m. (E, F) Immunohistological analysis of completely transected spinal cord with and without cell treatment. (E). Spinal cord (Control) 4 months after complete transaction. No signs of axonal regrowth of existing neurons (green-NF200). GFAP (red), DAPI-blue. (F). Spinal cord 4 months after complete transaction. No signs of axonal regrowth of existing neurons (green-NF200). (G) CMAP- compound motor action potential.

In conclusion, complete transection animal model of SCI causes severe behavioral (locomotor) and histological (axonal damage) changes, and has proved both useful and reliable for evaluation of rodents using different cell or pharmacological strategies.

Methods

All material and reagents as well as detailed procedure of animal care after the lesion and transplantation were listed and described in Supplementary Methods.

Experimental individuals. Adult female rats, 2 months old, 200 g of body weight. Food and water provided ad libitum during the entire experiment. All surgical procedure steps have to be performed according to ethical procedures for the use of animals in laboratory experiments. The experimental protocol used here was approved by the Animal Care Committee of the Research Institute Principe Felipe (Valencia, Spain) in accordance with the National Guide to the Care and Use of Experimental Animals (Real Decreto 1201/2005).

Post-surgery procedure. Post surgery procedure are provided in Supplementary Methods.

Cell culture and differentiation. Primary hESC colonies (H9-GFP, WiCell Inc., Madison, WI) are cultured on mitomycin C inactivated commercially available human foreskin fibroblasts (American Type Culture Collection, Manassas, VA, USA), in ES medium containing Knockout-DMEM (Invitrogen), 100 μM β-mercaptoethanol (Sigma), 1 mM L-glutamine (Invitrogen), 100 mM nonessential amino acids, 20% serum replacement (SR; Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 8 ng/ml basic fibroblast growth factor (bFGF; Invitrogen). ESC medium is changed every other day. Human ESC are passaged by incubation in

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1 mg/ml collagenase IV (animal-free, Invitrogen) for 5 hours to mechanically dissect and move to freshly prepared feeder cells. Cells are differentiated toward OPC according to previously published protocols. Briefly, cells are plated for 2 days in 50% HESC growth media and 50% glutaraldehyde media (GRM) in 24-well plates (Corning). This medium is then replaced with 100% GRM supplemented with 20 ng/ml EGF (Sigma-Aldrich) for 48 h. After permeabilization with 0.5% Triton X-100 (X100, Sigma) tissue was included in 30% sucrose (S84097, Sigma) during two days before inclusion paraformaldehyde (PFA; 158127, Sigma) in phosphate buffered saline (PBS). The number of averages are ready for transplantation.

Behavioral testing (open field locomotor scale). Functional recovery is assessed by evaluators blinded to treatment groups. Open field locomotor test using the Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale is performed in a plastic tray (50 x 80 x 20 cm) 2 weeks after spinal cord injury. Each animal was adapted to the environment for 1 h at room temperature. All cells were counterstained by incubation with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) from Molecular Probes (Invitrogen, USA) for 3 min at room temperature followed by washing steps. Signals were visualized by Confocal Microscopy (Leica, Germany).

Statistical methods. BBB scores are analyzed by repeated measures 2way ANOVA with Bonferroni multiple comparison test at each time point. The differences were significant when P < 0.05.

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Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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