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Engraftment, Migration and Differentiation of Neural Stem Cells in Contused Rat Spinal Cord

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Keywords

Regeneration, spinal cord injury, transplantation, stereology, glial scar.

Introduction

Spinal cord contusion injury can be severe and rapidly progresses over time (1, 2). The mechanisms of spinal cord injury can be considered to have two phases of injury, a primary or acute phase and the secondary phase leading to chronic injury (3-5).

Expansion of NS cells in vitro may provide a potential source for graft material needed to attempt regeneration of injured spinal cord. Currently two methods are used: neurosphere culture (6) and adherent culture (7-9). Transplantation of NS cells has been evaluated and anticipated as a possible treatment for spinal cord injury. Multipotent NS cell transplantation is a promising way to provide treatment for spinal cord injury including replacement of lost cells, reestablishment of functional connections and remyelination of spared nerve fibers (9, 10).

NS cells are able to differentiate into multiple neural cell types, including neurons, oligodendrocytes and astrocytes. They also secrete neurotrophic factors and cytokines (11-13). After spinal cord injury it is thought NS cells could boost neural repair by encouraging host cell regeneration and by replacing host cells which have died at the site of injury (8). Grafting NS cells to areas of injury has been shown to promote axonal growth (14). NS cells can also influence the mobilisation of endogenous stem cells (15). NS cells have shown considerable migratory ability within the nervous system. Intravenously delivered NS cells migrate into lesion sites, differentiate into neurons and glia and improve functional recovery (16). Transplanted NS cells have been shown to form cellular bridges capable of spanning the lesion and to protect against secondary damage (17).

The NS cells used in this study express markers of neurogenic radial glia. Radial glia have been proposed to act as a guide or scaffold to induce cell migration in injured spinal cord (18, 19). The ability of mouse NS cells to integrate, migrate and

differentiate in injured rat spinal cord tissue was analysed. Cyclosporin-A (CsA) immunosuppressant was used to prevent any rejection of mouse cells by rat tissue. Lentiviral vector expressing GFP was used to transduce NS cells so that they could be traced following implantation into spinal cord tissue (20, 21). We expanded adherent mouse NS cells in vitro and delivered them to contused rat spinal cord. NS cells were shown to migrate extensively within the injured spinal cord tissue. Migration appeared to be directed toward the lesion centre. The cellular environment within NS cell treated animals showed many differences in cellular composition compared to control animals. NS cells were observed to differentiate into oligodendrocytes and astrocytes. Some limited evidence of neuronal differentiation was also observed. Although functional recovery was not observed, these cells may create important scaffold for regenerating axons to reach their target destinations following injury.

Materials and methods

NS cell culture and characterisation

NS cells were isolated from E16.5 mouse foetal forebrain and transduced with lenti-GFP (15). NS cells were cultured in RHB-A medium (available from Stem Cell Sciences Ltd.) supplemented with 2mM L-glutamine (Sigma-Aldrich), 1% B27 (Invitrogen), N2 (1:200) and 10ng/ml of both EGF and FGF-2 (Peprotech, USA). Cells were plated at $1x10^5$ cells/well in 8 well chamber slides and characterised by immunocytochemistry. Cells were fixed with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS) and incubated for 30 minutes in blocking solution containing 1% Bovine serum albumin (BSA), 3% normal goat serum (NGS) and 0.05% tween-20 in 0.1M PBS. Cells were incubated with the primary antibodies

Nestin (Chemicon), Pax6 (Stem Cell Sciences) and GFAP (Dakocytomation) for 1 hour. After three PBS washes, cells were incubated in 1:100 Rhodamine (Sigma-Aldrich) secondary antibody for 30 minutes. A negative control was carried out for each antibody staining by substituting PBS for the primary antibody. Images were captured at 20X on an Olympus IX71 fluorescent microscope.

NS cell viability

Cell viability in RHB-A medium was determined. Cells were suspended in RHB-A media at density of $1x10^5$ cells/µl and then left for up to 4 hours at room temperature. Cells were incubated in Via-count stain for 5 minutes, fixed in 4% paraformaldehyde and resuspended in 0.1M PBS. Percentage viability was determined using a Guava Cytosoft instrument (Guava Technologies, Philadelphia, USA).

Spinal cord injury

Twenty eight female Sprague Dawley rats weighing between 200-220g were used in this study. The rats were anaesthetised by intraperitoneal (I.P.) injection of ketamine and xylazine (100mg/kg and 10mg/kg respectively) following which a laminectomy was performed at T8-T10. All animals received a 200 Kilodynes moderate contusion injury at T9 using an Infinite Horizon Impactor Device (Precision Systems and Instrumentation, Lexington, KY, USA). The muscle and skin was sutured with absorbable suture material and animals kept warm on a heated blanket until fully recovered from intervention.

NS cell transplantation

Animals were randomly divided into a NS cell treated group (n = 11) and a control (vehicle) group (n = 17). Administration of a subcutaneous injection of 5mg/kg Cyclosporin-A (CsA; Sandimmun®, Sandoz) started 3 days post injury and continued on a daily basis throughout the study in all animals. NS cells were suspended in RHB-A media and transplanted into the NS cell treated group one week post injury. On the day of cell transplantation animals were reanaesthetised and spinal cords reexposed. A 2µl injection of NS cells (containing 50,000 cells/µl) or vehicle (RHB-A medium) was injected into the injured spinal cords 2mm rostral and 2mm caudal to the site of injury. The muscle and skin was again sutured. Both the control and the NS cell-treated groups were subdivided into two groups and were sacrificed 2 and 6 weeks post injection of NS cells/vehicle.

Postoperative care

Animal care was in accordance with institutional guidelines. Each animal received a subcutaneous injection 5-10mg/Kg Enrofloxacin (Baytril® 5%, Bayer) antibiotic once daily for a minimum period of a week. Pain relief was provided by administering Buprenorphine (Torbugesic® Injection, FortDodge Animal Health Ltd) at a dose rate of 0.1-0.25mg/Kg twice daily for 7 days after surgery. Saline solution (3.5ml) was administered subcutaneously for 3 days following surgery. Bladders were manually expressed twice daily from day of injury.

Behavioural analysis

All animals were assessed using BBB locomotor rating scale to determine their motor function. Only animals with a score of 15 or less on the day following injury were included in the study. All BBB scoring was performed by three trained blinded

examiners. BBB assessment was carried out 2 weeks, 4 weeks and 6 weeks post injection of NS cells/vehicle. Mean BBB ± standard error (SE) was calculated. GraphPad Prism statistical program was used to estimate significance of the difference between the change in BBB score for each animal over the entire treatment period.

Tissue Processing

Animals were deeply anaesthetised by I.P. injection of Sodium pentabarbitone and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1M PBS. Spinal cords were dissected out, postfixed overnight with 4% paraformaldehyde, immersed in 30% sucrose cryoprotectant overnight and frozen in liquid nitrogen chilled isopentane. Spinal cords were cryosectioned transversely at 20μm thickness in a rostral to caudal direction. Sampling regions were separated by a distance of 200μm. Eight spinal cord sections were collected at each sampling region.

Immunohistochemistry

Immunohistochemistry was carried out on five animals from each of the four experimental groups. Frozen sections were rehydrated and incubated with 20% NGS block solution in PBS containing 0.2% Triton X for 20 minutes. The primary antibodies (NeuN: Ms IgG, Chemicon, 1:100; Growth associated protein-43 (GAP-43): Ms IgG, Sigma-Aldrich, 1:100; Myelin basic protein (MBP): Rabbit polyclonal, Chemicon, 1:100; Brain lipid binding protein (BLBP): Rabbit polyclonal, Chemicon, 1:100; Vimentin: Ms IgG, Sigma-Aldrich, 1:100; GFAP: Rabbit Polyclonal, DakoCytomation, 1:300; NG2: Ms IgG, Invitrogen, 1:100; CD11B: Ms IgG, Chemicon, 1:100); CHOP: Rabbit polyclonal, Santa Cruz Biotechnology, 1:200;

Fractin: Rabbit polyclonal, Chemicon, 1:200; Heat Shock Protein 70 (HSP 70): Rabbit polyclonal, Stressgen, 1:200) were diluted in PBS containing 2% NGS + 0.02% Triton X. Sections were incubated in primary antibody for 2 hours at room temperature. Sections were washed 3 times in PBS. The corresponding secondary antibody, Rhodamine (Sigma-Aldrich) or Cy5 (Chemicon), was diluted 1:100 in PBS and incubated for 1 hour at room temperature. Sections were again washed and counter-stained with 1μg/ml DAPI (Sigma) for 5 minutes. A negative control was carried out for each antibody stain by substituting PBS for the primary antibody. Images were captured on an Olympus IX81 fluorescent microscope at 20X magnification using a Volocity grid confocal acquisition system.

SEM

One slide from each animal within the NS cell treated and untreated group was processed for SEM to visualize surface features of injured spinal cord sections. The cryosections were fixed in 1% Osmium for 1 hour, dehydrated in alcohol and hexamethyl disilazine for 20 minutes and air dried. The sections were mounted on carbon-painted stubs. Sections were gold-coated with a sputter-coater at 30mA for 4 minutes and visualized with a Hitachi 2600N SEM using an accelerating voltage of 20kV.

Stereology

Lesion size

Images of the first section of each sampling region of all four animals in both control and NS cell treated group were captured at 4X using a Nikon Eclipse light microscope. Estimation of lesion volume was carried out using the Cavalieri method

(22). A point grid was randomly placed onto images of spinal cord sections. All points hitting spinal cord tissue, lesion and artefact were counted and recorded. Volume was calculated for each group using the following formula,

$$\mathbf{V= \sum P \cdot A \cdot T}$$

That is, V=Volume, P= Number of points hitting region of interest, A=Area associated with each point and T=Distance between each sampled section,

Where,

Area associated with each point = (Distance between adjacent points/Linear Magnification) ^{2}.

The proportion of spinal cord occupied by the lesion, i.e., volume fraction (V_v), was calculated by dividing the volume of the lesion by the volume of the spinal cord less the volume of any artefact present. Mean lesion size ± SE was calculated for each experimental group.

Cellular environment of lesion

Stereological analysis was also carried out on immunostained spinal cord sections. For each antibody, 4 images were captured per section at 20X. The images were captured from random fields of view around the edge of the lesion in each animal. A point grid was again placed randomly on the images captured and the number of points hitting immunoreactive structures and the entire field of view were counted and recorded in Microsoft Excel. V_v of the immunoreactive tissue was calculated by dividing the number of points overlying immunoreactive cells by the total number of

points hitting tissue. Any points hitting immunoreactive GFP positive NS cells were eliminated. Mean Vv immunoreactivity ± SE was calculated for experimental group.

NS cell quantification

Stereological analysis was carried out to estimate the volume of NS cells and the number per unit volume of NS cells in each spinal cord. Volume of NS cells was calculated using 20X images, by counting the number of points hitting cells and entering this number into the Cavalieri method formula above. The volume of lesion was plotted on the same graph with volume of NS cells to determine the migration pattern of NS cells toward or away from lesioned region. Number of NS cells per unit volume was also calculated. This was carried out by dividing the volume of NS cells per spinal cord by the average volume of a NS cell in each spinal cord.

Statistics

Statistical calculations were performed using Minitab software. A two-way analysis of variance was performed to examine effect of time post injury, treatment and interaction. Post-hoc comparisons were undertaken by Fisher's test. Differences were considered to be statistically significant at a probability value (P) < 0.05.

Results

NS cell characterisation and viability

NS cells were positive for expression of Nestin (Figure 1A-D) and Pax6 (Figure 1E-H) but negative for the astrocytic marker GFAP (Figure 1I-L). No difference in viability over time was found even up to 4 hours in RHB-A medium at room temperature (Figure 1M). To be certain that viability of cells before transplantation

was not an issue, it was decided that NS cells would not be left more than 30 minutes at room temperature prior to transplantation.

Analysis of size of spinal cord lesion

Spinal cords showed thinning at contused region in both 2 (Figures 2A, B) and 6 week groups (Figures 2C, D). Thinning was more obvious at the 6 week time point, where a discolored region was present at the lesion site. Estimation of size of lesion using stereological parameters showed that that although there was a trend toward bigger lesion in the NS cell treated groups compared to control groups at 2 and 6 weeks, no significant difference in size of lesion was detected. This was true for both volume (Figure 2E) and Vv of lesion (Figure 2F). There was however a significant increase in lesion size between the 2 and 6 week time points.

Analysis of cellular environment of lesion

The Vv of immunoreactive cells in the sampled images was calculated for both 2 (Figure 3 and 4) and 6 week (Figure 5 and 6) time points. This reveals any changes in cellular environment of lesion due to the presence of engrafted NS cells.

<u>Astrocytes</u>

Vv of GFAP positive astrocytes was significantly higher in the control compared to NS cell treated animals at both time points (Figures 4 and 6A, B). This indicates that the presence of NS cells does not cause increase in expression of normal astrocytes within injured spinal cord and in fact may indicate reduction in glial scarring following NS cell administration. Vimentin and BLBP are normally only seen in radial glia during CNS development; however following injury astrocytes that have

become reactive begin to express these markers as well as GFAP. Vimentin positive reactive astrocytes were significantly higher in NS cell treated animals at both time points (Figures 4 and 6C, D). There was also a significant increase observed in Vimentin staining between the 2 and 6 week time points within both animal groups. However, BLBP positive reactive astrocytes only showed increase in expression in NS cell treated animals at the 6 week time point (Figure 6E, F).

Neurons

No difference in expression of NeuN immunoreactive neurons was observed between both animal groups at the 2 and 6 week time points (Figures 3 and 5). An increase in GAP43 staining was observed between 2 and 6 weeks in the control group. At 6 weeks there was also an increase in GAP43 staining in the control group compared to NS cell treated animals (Figure 6G, H).

Oligodendrocytes

Presence of MBP immunoreactive oligodendrocytes increased in the NS cell treated group at 2 weeks post transplantation (Figure 4E, F). A decrease in MBP positive oligodendrocytes was observed in the lesion environment of NS cell treated animals by the 6 week time point.

NG2 chondroitin sulphate proteoglycan

NS cell treated animals showed significantly higher amounts of NG2 immunoreactive cells (Figure 4G, H and Figure 6I, J). Increased NG2 has been shown to be associated with radial glia, this may explain the increased amounts of inhibitory NG2 in the lesion site of NS cell treated animals.

Microglia/macrophages

At the 2 week time point a higher proportion of CD11B positive macrophages/microglia was observed in the control group (Figure 4I, J). This indicates less inflammation associated with NS cell treated animals.

Apoptosis

Significantly less Fractin immunoreactive apoptotic cells were found in NS cell treated animals at both time points (Figures 4 and 6K, L). Quantification of markers of ER stress (CHOP) and HSP 70 did not change between treatment groups.

Engraftment of NS cells in injured spinal cord

Examination of spinal cord sections in both 2 and 6 week vehicle groups confirmed absence of green fluorescent NS cells. Examples of NS cells and the region of spinal cord where they are found at 2 and 6 week are shown in Figure 7A, B and C, D respectively. Some transplanted cells adhered to cavity wall and did not migrate into host spinal cord (Figure 7A, C). NS cells that successfully engrafted extended cell processes. Toward peripheral regions of the spinal cord, the NS cells displayed a radial morphology similar to radial glia (Figure 7B). These cells may act as a guide or scaffold to induce cell migration in injured spinal cord. Many macrophages are observed which display background autofluoescence (Figure 7B and 4D).

SEM analysis of NS cells at the edge of the lesion shows long cell processes belonging to NS cells at 2 week (Figure 7E, F) and 6 week (Figure 7G, H) time points. Some cells appear to be using the NS cell processes as a migratory scaffold (Figure 7F).

The number of NS cells found within the NS cell treated groups varied somewhat between individual animals, this may be a reflection of the injection of the NS cells. Although $2x10^5$ cells were injected into each spinal cord (half at each injection site), only $1x10^5$ cells on average were found within animals at each time point (Figure 8). Although there appears to be more cells present at the 6 week time point compared to 2 weeks, there was no significant difference in numbers of NS cells found at the two survival time points.

Migration of NS cells in injured spinal cord

Volume of NS cells was calculated in spinal cords of each animal. This value was plotted on a graph with lesion volume to determine the migratory pattern of NS cells in injured spinal cord tissue at 2 weeks (Figure 9A) and 6 weeks post injury (Figure 9B). NS cells appear to migrate toward the lesion centre, from their injection sites rostral and caudal to the lesion. This is particularly evident at the 6 week time point.

Differentiation of NS cells

Immunohistochemical analysis of NS cells at 2 and 6 weeks revealed some very limited immunoreactivity within NS cells for the neuronal marker NeuN at 2 and 6 weeks (Figure 10A, G). GAP43 immunoreactivity was only detected in association with NS cells at the 2 week time point (Figure 10B). Co-labelled NS cells and oligodendrocytes were detected at the 2 week time point only (Figure 10C). GFAP immunoreactive NS cells were abundant at both time points (Figure 10D, H) particularly at the edge of the lesion and cavities. Some vimentin and BLBP immunoreactive NS cells were found at 2 weeks (Figure 10E, F respectively). Very few BLBP immunoreactive NS cells were detected by 6 weeks (Figure 10I).

Analysis of motor function

BBB scores of left (Figure 11A) and right (Figure 11B) hindlimbs show a definite trend to more improvement in the NS cell-treated group at 6 weeks. This was consistent across all three blinded observers; however the effect is overall not statistically significant. The mean BBB from the three blinded observers was calculated for each animal over the entire treatment period. Mean change in BBB at 6 weeks was 8.1 for cell treatment group and 6.2 for vehicle group.

Discussion

We investigated the influence of NS cells as multipotent cells, capable of delivery of therapeutic factors to provide trophic support, mobilisation of endogenous stem cells and replacement of lost cells are all crucial factors in relation to their use in treatment of spinal cord injury.

NS cells did not affect the lesion size at either time point analysed. The effect of NS cell treatment on cellular environment of the lesion site showed that less GFAP immunoreactive cells were present within the glial scar, indicating NS cells prevented some of the astrocytic cells from forming the physical glial barrier at the lesion site. Animals that were treated with NS cells showed less influx of microglia at the lesion site at the 2 week time point, perhaps indicating a role for NS cells in preventing inflammation. In addition, more oligodendrocytes were present in NS cell treated animals, these are an important factor in enabling regeneration at the lesion site and are likely host oligodendrocytes that differentiated from endogenous precursor cells. The NS cell treated group displayed increased levels of the inhibitory chondroitin sulphate proteoglycan (CSPG) NG2 at the lesion site. NG2 has been shown in

association with radial glia (23) and this may explain the increase in NG2 associated with animals treated with the NS cells, which express markers of neurogenic radial glia. CSPG deposition may have diminished the effect of NS cells on promoting regeneration of pathfinding axons of spared neurons, as shown by decreased expression of GAP43 at the lesion site in the NS cell treated group (24, 25). NS cell treated animals showed reduced apoptosis at the lesion site. This effect of NS cells has been documented previously in studies where the stem cells zoned into the injured areas and suppressed apoptosis (26; 27).

NS cells displayed a morphological phenotype similar to radial glia, in particular within white matter regions. Presence of endogenous cells expressing markers of radial glia has also been shown in white matter of injured spinal cord (23). SEM analysis of these NS cells indicates they act as a contact guidance cellular scaffold upon which cells can migrate. This latter role may be exploited following spinal cord injury to encourage neuronal regrowth across the glial scar (18). Transplanted NS cells have also been shown to form cellular bridges capable of spanning the lesion and protecting against secondary damage (17, 28).

We show that NS cells migrate extensively in the direction of the spinal cord lesion itself. This migratory capacity of NS cells has already been documented and shown to be influenced by the secretion of chemoattractants by the lesion (16, 29).

Differentiation of NS cells transplanted into adult spinal cord has previously been described as mainly restricted to glial lineage (30, 31). In this study, we found that at the 2 week time point NS cells differentiated into astrocytes, oligodendrocytes and neurons. However, at 6 weeks only astrocytes and neurons were detected. NS cells have been shown to differentiate into neurons and oligodendrocytes in the hostile environment of the spinal cord (32, 33) and neurogenic ability of NS cells has been

implied to be related to timing of grafting (34). Inflammatory cytokines may be involved in restricted differentiation of NS cells after grafting into injured spinal cord (35). In this study the transplantation of NS cells 7 days post injury may have provided the opportune window for differentiation into oligodendrocytes, astrocytes and neurons.

This study shows promising results for the potential use of NS cells in treatment of spinal cord injury. NS cells contributed to a lesion environment composed of less microglia and more oligodendrocytes. These are important factors for consideration of endogenous cellular activity following transplantation of therapeutic cells. NS cells engrafted into the host tissue and appeared to provide a cellular scaffold, which could act as a potential guide across the lesion site. Migration of NS cells toward the lesion site is important in consideration of genetic manipulation of stem cells to act as delivery vehicles of therapeutic genes to a site of injury. We have shown that these NS cells are capable of replacing lost and damaged oligodendrocytes and neurons by differentiating into these two cell types.

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Author disclosure statement

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Figure legends

Figure 1

Staining of GFP NS cells with DAPI and antibodies for Nestin (A-D), Pax6 (E-H) and GFAP (I-L). Scalebar = 20μm. Viacount staining shows GFP NS cells after 4 hours in RHB-A medium (M). No change in viability is noted

Figure 2

Digital images showing spinal cords immediately after dissection in 2 week vehicle (A), 2 week NS cell treated (B), 6 week vehicle (C) and 6 week NS cell treated (D) animals. Boxes highlight injured region. Scalebar = 5mm. Graphs show (E) volume of lesion and (F) Vv of lesion in NS cell treated and control groups of animals at 2 and 6 week time points. Error bars represent standard error of the mean. * = $P < 0.05$

Figure 3

Graph shows Vv of immunoreactive cells in the lesion area at 2 weeks post transplantation of NS cells. This illustrates the proportion of each contributing cell type to the lesion environment in NS cell treated and control groups. Error bars represent standard error of the mean. * = $P < 0.05$

Figure 4

Immunohistochemically stained transverse sections of injured spinal cord tissue taken at 2 weeks post injury in control (A, C, E, G, I, K) and NS cell treated (B, D, F, H, J, L) groups showing cellular environment of lesion stained with A, B: GFAP; C, D: vimentin; E, F: MBP; G, H: NG2; I, J: CD11B; K, L: fractin. Scalebars = 100μm.

Figure 5

Graph shows Vv of immunoreactive cells in the lesion area at 6 weeks post transplantation of NS cells. Error bars represent standard error of the mean. * = $P < 0.05$

Figure 6

Immunohistochemically stained transverse sections of injured spinal cord tissue taken at 6 weeks post injury in control (A, C, E, G, I, K) and NS cell treated (B, D, F, H, J, L) groups showing cellular environment of lesion stained with A, B: GFAP; C, D: vimentin; E, F: BLBP; G, H: GAP43; I, J: NG2; K, L: fractin. Scalebars = 100µm.

Figure 7

Photomicrographs showing merged brightfield and fluorescent images of NS cells at the 2 week (A) and 6 week (C) time points. Higher magnification images show engrafted NS cells at 2 weeks (B) and 6 weeks (D). Arrows indicate macrophages which may have engulfed dead GFP-expressing NS cells. Arrowhead indicates linear arranged NS cell resembling radial glial morphology. Scalebars A, C = 1000µm, B, D = 200µm. SEM images show surface features of NS cells at 2 weeks (E, F) and 6 weeks (G, H) post transplantation. Arrow in F shows cell nucleus located along cell process. Scalebars E, G = 40µm, F, H = 20µm.

Figure 8

Graph shows number of GFP NS cells per unit volume at 2 and 6 weeks survival time post injury. Error bars represent standard error of the mean.

Figure 9

Graphs show volume of NS cells and volume of lesion in injured spinal cords 2 weeks (A) and 6 weeks (B) post transplantation.

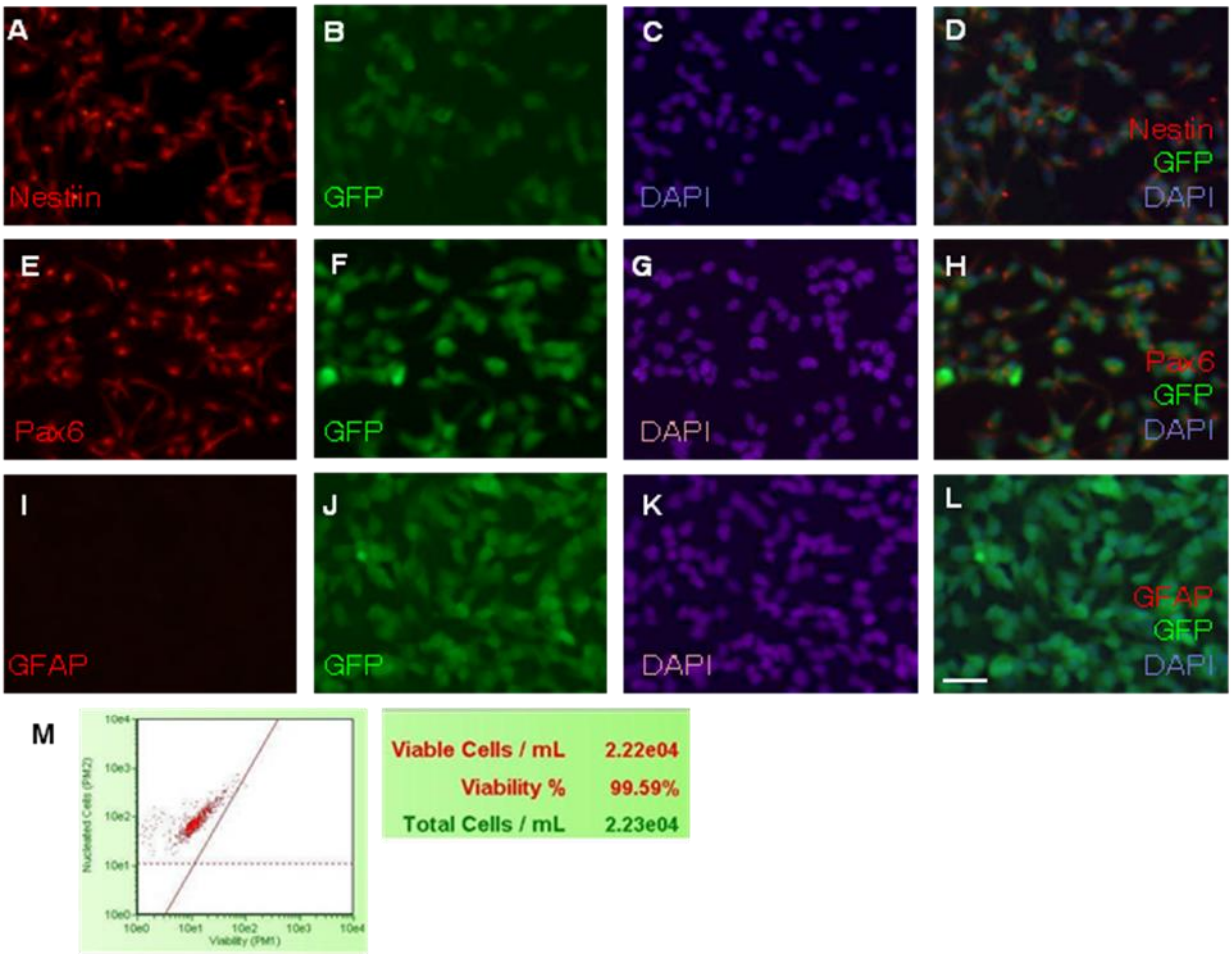
Figure 10

Merged images of immunohistochemically stained transverse sections of injured spinal cord tissue taken from NS cell treated groups at 2 week and 6 week time points. Immunoreactivity is shown at 2 weeks for (A) NeuN, (B) GAP43, (C) MBP, (D) GFAP, (E) vimentin, (F) BLBP, and at 6 weeks for (G) NeuN, (H) GFAP and (I) BLBP. Arrows highlight some of the immunoreactive NS cells. Scale bars = 50μm.

Figure 11

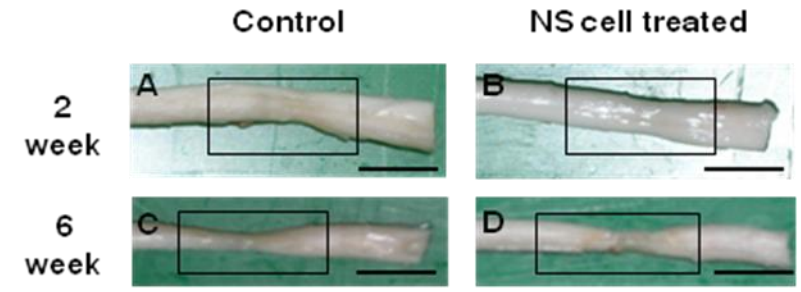
Graphs show percentage improvement of BBB scores in left (A) and right (B) hindlimbs of animals in control and NS cell treated groups at 2, 4 and 6 weeks post injury. Error bars represent standard error of the mean

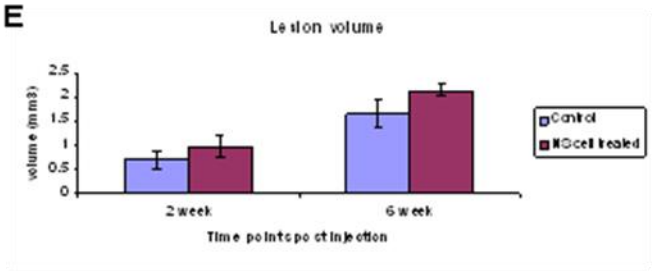
Figure 1


A
Nestin
B
GFP
C
DAPI
D
Nestin
GFP
DAPI
E
Pax6
F
GFP
G
DAPI
H
Pax6
GFP
DAPI
I
GFAP
J
GFP
K
DAPI
L
GFAP
GFP
DAPI

M
Viable Cells / mL 2.22e04
Viability % 99.59%
Total Cells / mL 2.23e04

Figure 2


Control
NS cell treated
2 week
6 week
A
B
C
D


E
Lesion volume
volume (mm3)
2.5
2
1.5
1
0.5
0
2week
6week
Time points post injection
Control
NScell treated

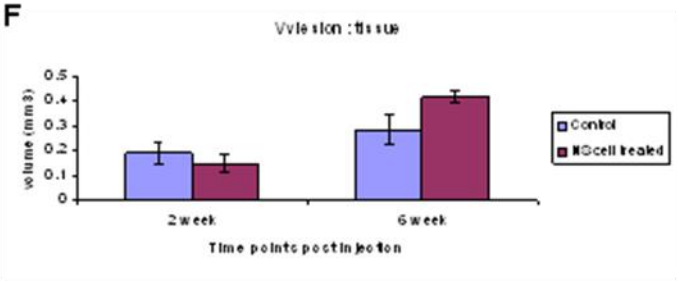
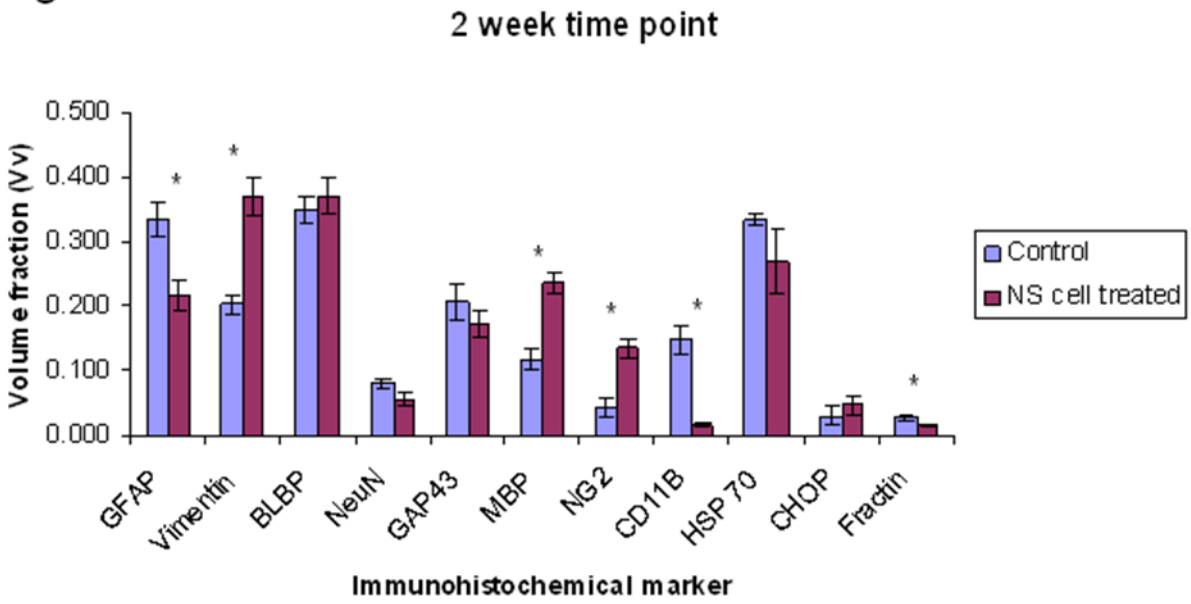

F
Lesion tissue
volume (mm3)
0.5
0.4
0.3
0.2
0.1
0
2week
6week
Time points post injection
Control
NScell treated

Figure 3


2 week time point
Volume fraction (Vv)
0.500
0.400
0.300
0.200
0.100
0.000
GFAP
Vimentin
BLBP
NeuN
GAP43
MBP
NG2
CD11B
HSP 70
CHOP
Fractin
Immunohistochemical marker
Control
NS cell treated

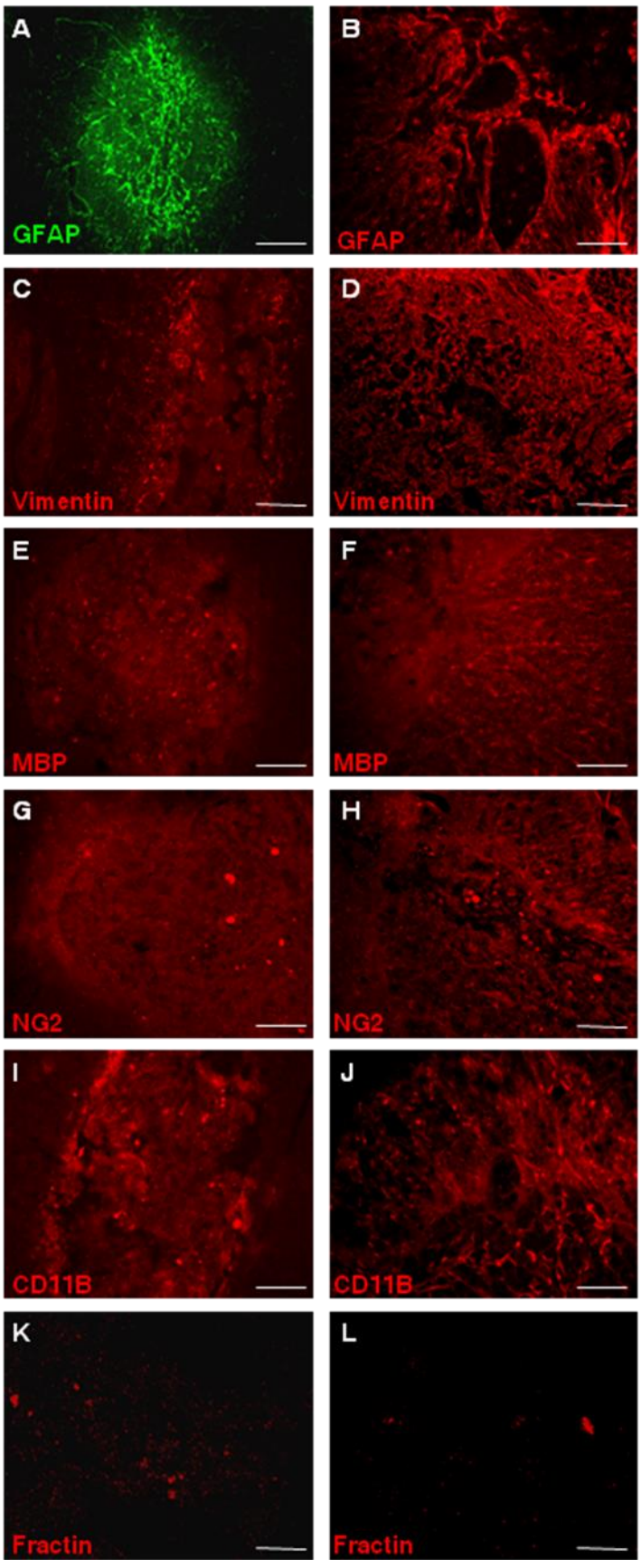
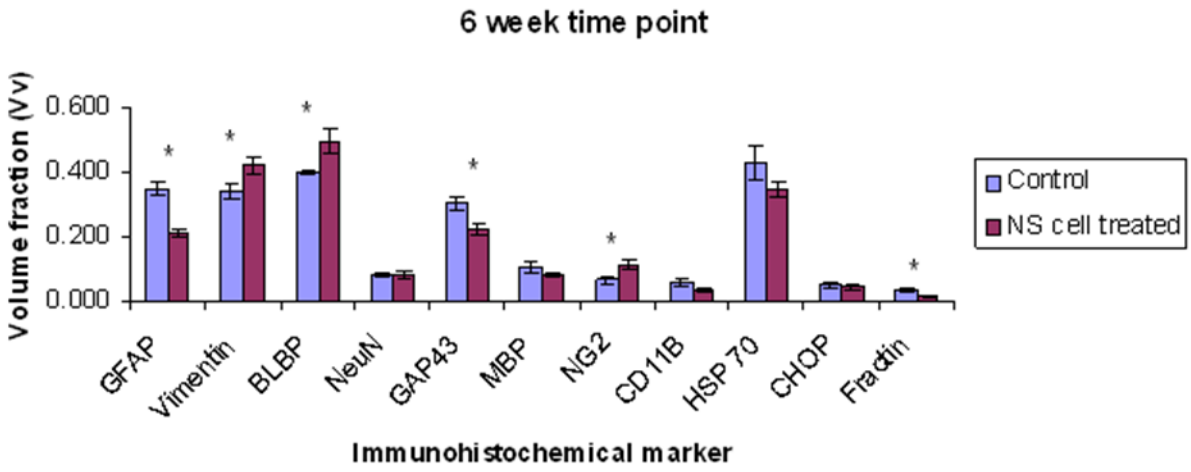

A
GFAP
B
GFAP
C
Vimentin
D
Vimentin
E
MBP
F
MBP
G
NG2
H
NG2
I
CD11B
J
CD11B
K
Fractin
L
Fractin

Figure 4

Figure 5


6 week time point
Volume fraction (Vv)
0.600
0.400
0.200
0.000
GFAP
Vimentin
BLBP
NeuN
GAP43
MBP
NG2
CD11B
HSP 70
CHOP
Fractin
Immunohistochemical marker
Control
NS cell treated

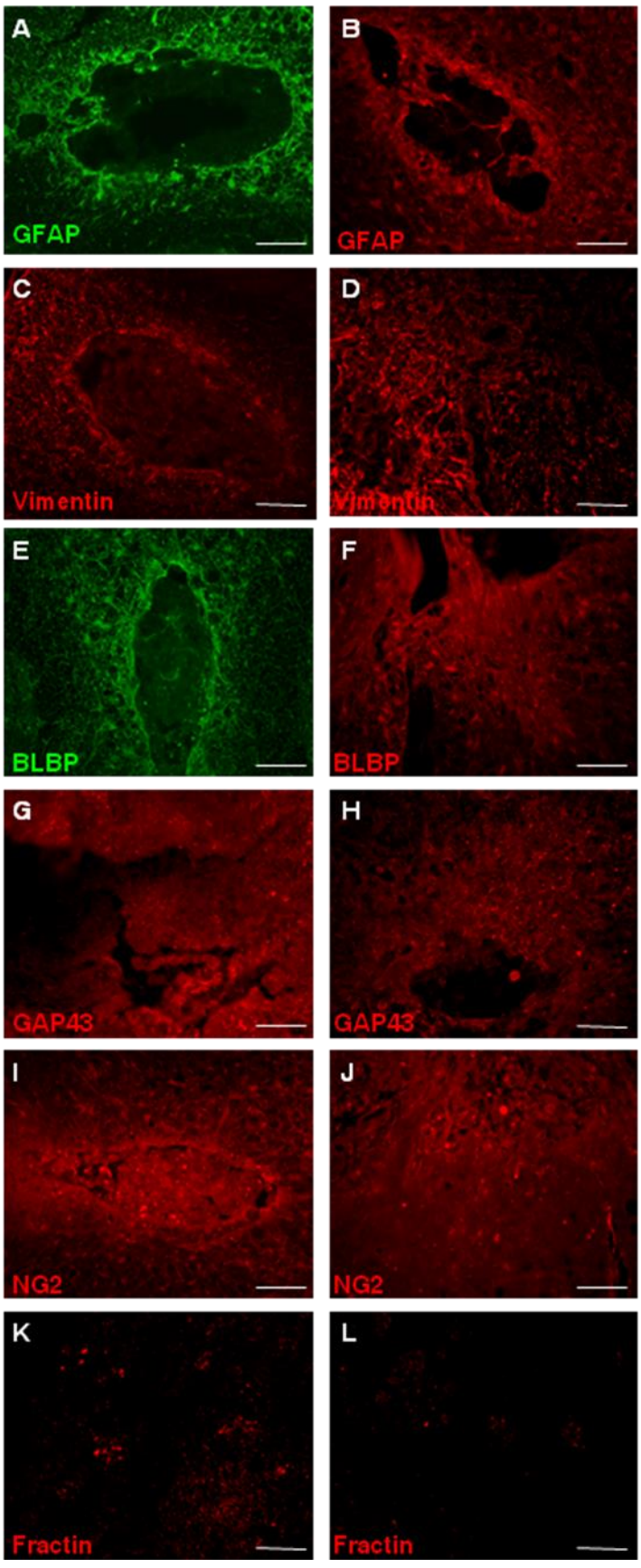

A
GFAP
B
GFAP
C
Vimentin
D
Vimentin
E
BLBP
F
BLBP
G
GAP43
H
GAP43
I
NG2
J
NG2
K
Fractin
L
Fractin

Figure 6

Figure 7

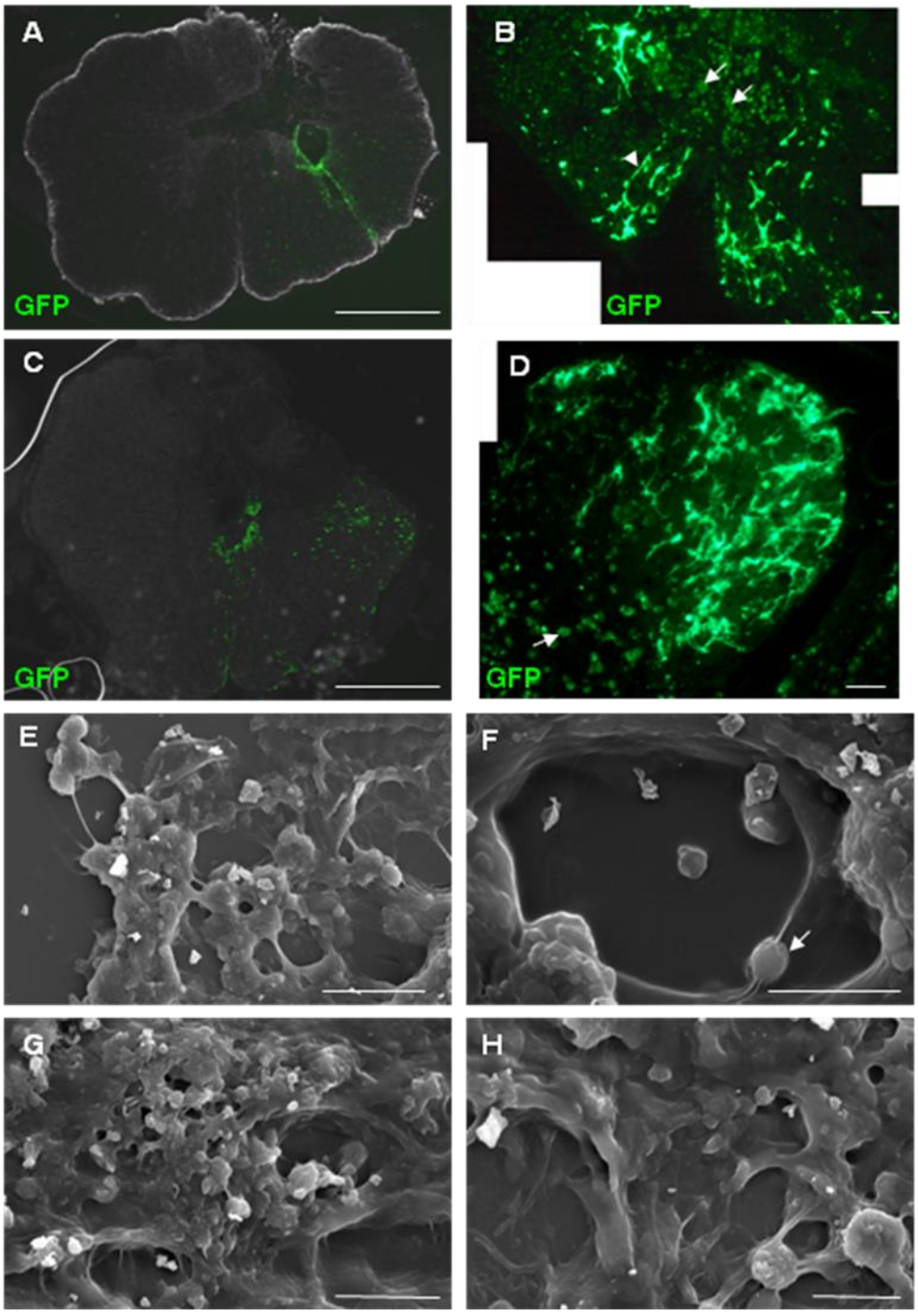


Figure 8

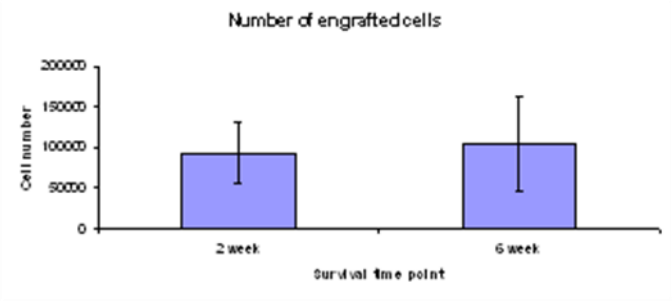
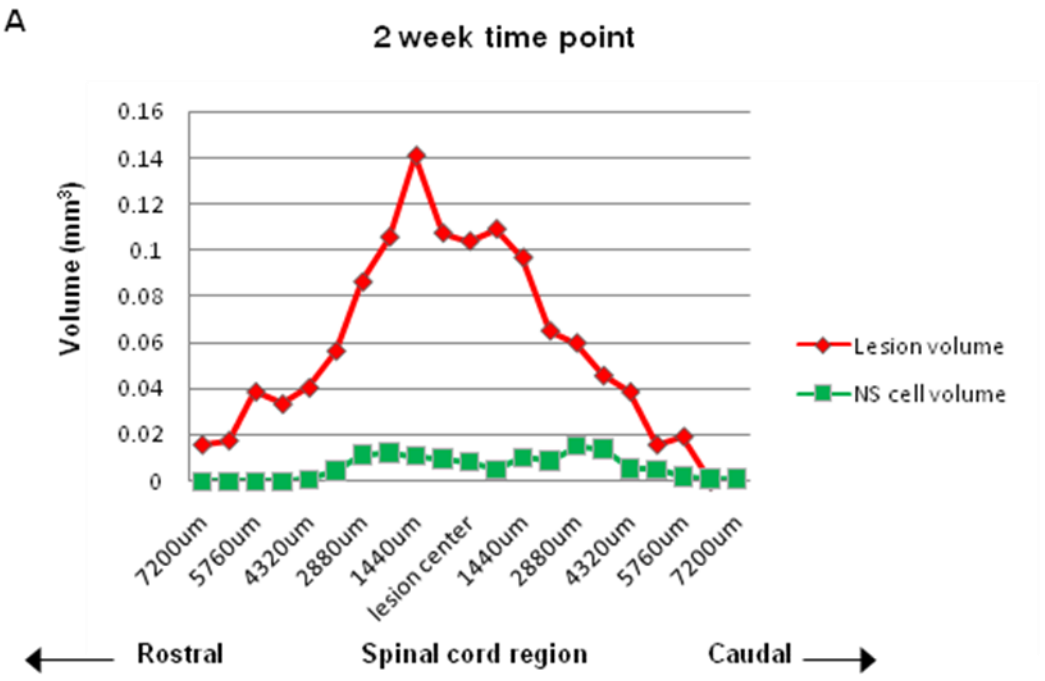

Number of engrafted cells
200000
150000
100000
50000
0
Cell number
2 week
6 week
Survival time point

Figure 9


A
2 week time point
Volume (mm3)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
7200um
5760um
4320um
2880um
1440um
lesion center
1440um
2880um
4320um
5760um
7200um
Lesion volume
NS cell volume
Rostral
Spinal cord region
Caudal

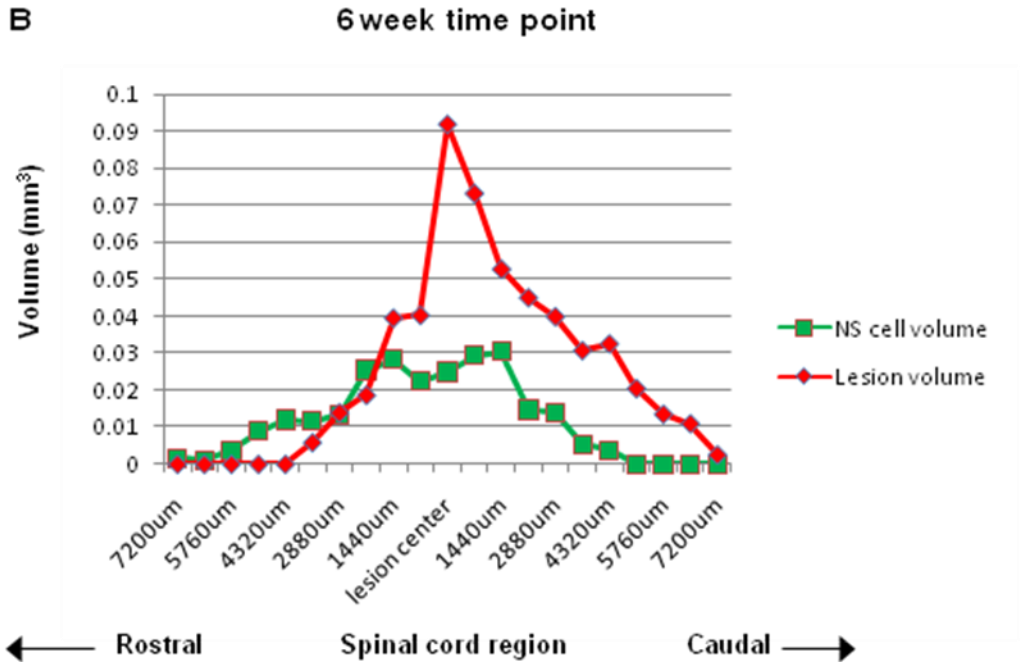

B
6 week time point
Volume (mm3)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
7200um
5760um
4320um
2880um
1440um
lesion center
1440um
2880um
4320um
5760um
7200um
NS cell volume
Lesion volume
Rostral
Spinal cord region
Caudal

Figure 10

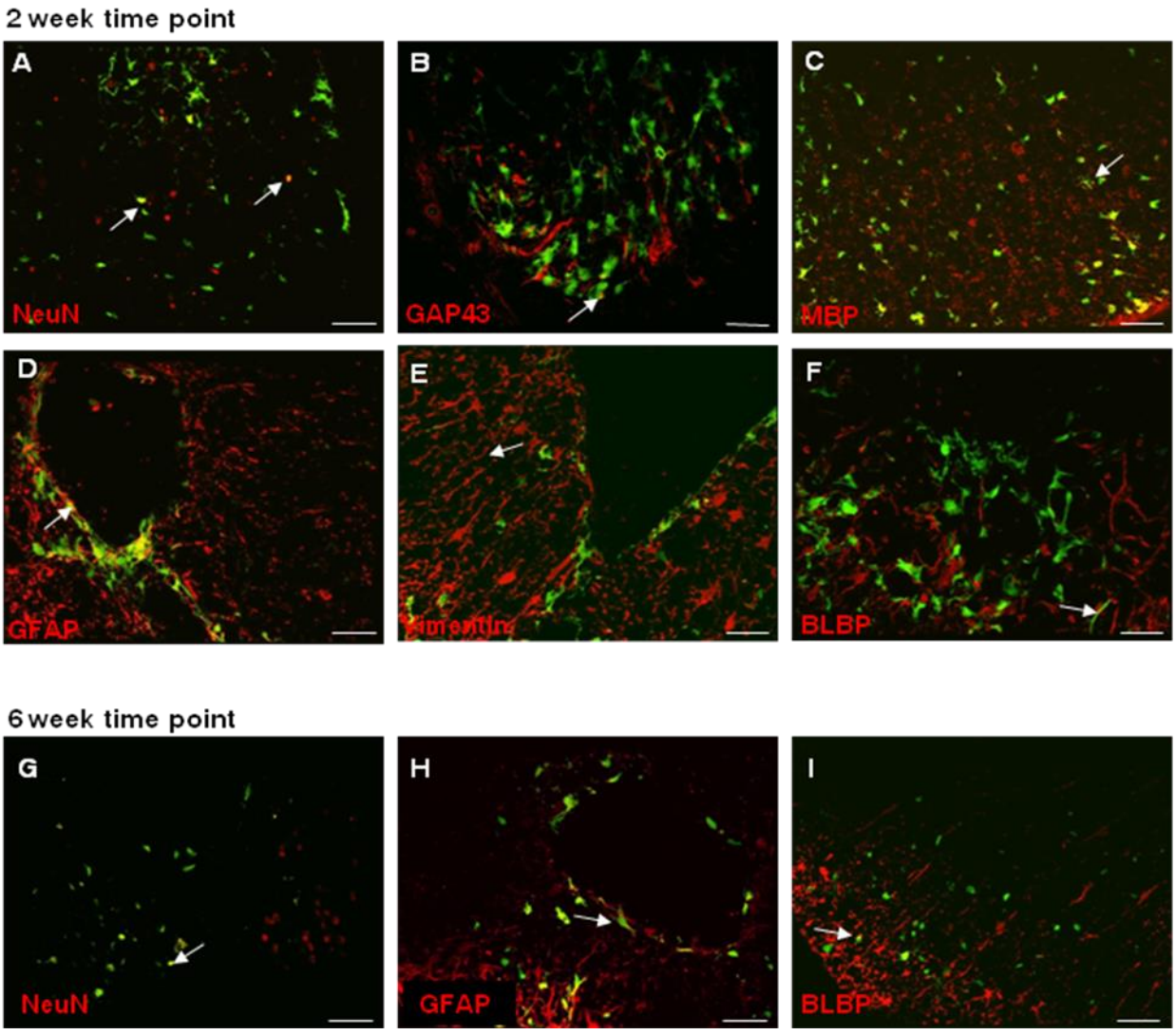
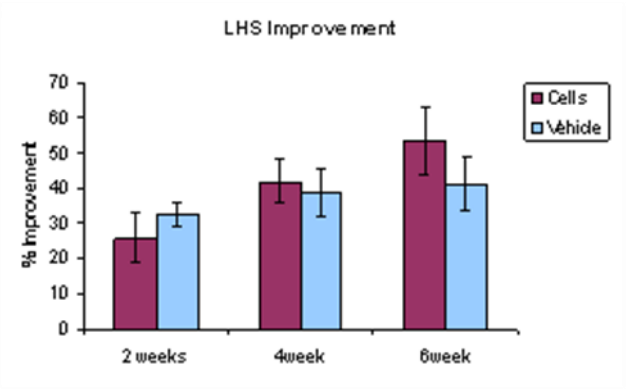
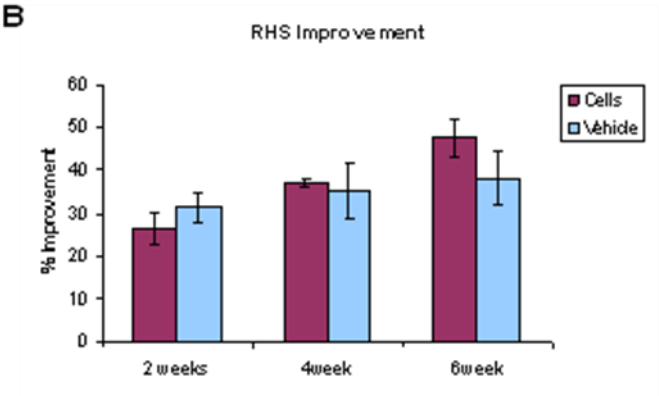

2 week time point
A
NeuN
B
GAP43
C
MBP
D
GFAP
E
Vimentin
F
BLBP
6 week time point
G
NeuN
H
GFAP
I
BLBP

Figure 11

A


LHS Improvement
% Improvement
70
60
50
40
30
20
10
0
2 weeks
4week
6week
Cells
Vehicle

B


RHS Improvement
% Improvement
60
50
40
30
20
10
0
2 weeks
4week
6week
Cells
Vehicle