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Biomedical Materials

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RECEIVED
31 July 2017

REVISED
18 October 2017

ACCEPTED FOR PUBLICATION
30 October 2017

PUBLISHED
1 February 2018

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Keywords: spinal cord injury, cell transplantation, affinity release, hydrogel, stem cells

Supplementary material for this article is available online

Abstract

The lack of tissue regeneration after traumatic spinal cord injury in animal models is largely attributed to the local inhibitory microenvironment. To overcome this inhibitory environment while promoting tissue regeneration, we investigated the combined delivery of chondroitinase ABC (chABC) with human induced pluripotent stem cell-derived neuroepithelial stem cells (NESCs). ChABC was delivered to the injured spinal cord at the site of injury by affinity release from a crosslinked methylcellulose (MC) hydrogel by injection into the intrathecal space. NESCs were distributed in a hydrogel comprised of hyaluronan and MC and injected into the spinal cord tissue both rostral and caudal to the site of injury. Cell transplantation led to reduced cavity formation, but did not improve motor function. While few surviving cells were found 2 weeks post injury, the majority of live cells were neurons, with only few astrocytes, oligodendrocytes, and progenitor cells. At 9 weeks post injury, there were more progenitor cells and a more even distribution of cell types compared to those at 2 weeks post injury, suggesting preferential survival and differentiation. Interestingly, animals that received cells and chABC had more neurons than animals that received cells alone, suggesting that chABC influenced the injury environment such that neuronal differentiation or survival was favoured.

Introduction

In pre-clinical animal models of spinal cord injury (SCI), robust recovery with a single treatment has been sparse [1], reflecting clinical outcomes. Combination strategies can target different mechanisms of repair and hence greater opportunity for recovery. For example, the co-delivery of cells with the enzyme chondroitinase ABC (chABC) has shown promise, where cells promote tissue repair and chABC degrades the glycosaminoglycan component of the inhibitory chondroitin sulphate proteoglycans (CSPGs) [2, 3]. Greater recovery was observed following the co-delivery of chABC with Schwann cells or neural stem cells than either on its own [4–7].

CSPGs are mostly known for their inhibitory effect on axonal outgrowth [8, 9]; however, they also affect stem cell maintenance, proliferation, survival, and differentiation [10, 11]. CSPGs promote the proliferation of neural stem/progenitor cells (NSCs), and their differentiation into astrocytes [11–13]. One study demonstrated an increase in oligodendrogenesis at the expense of astrocytic differentiation of mouse NSCs following CSPG receptor knockout [13]. Unfortunately, this study did not include an analysis of neuronal differentiation. The effect of CSPGs is partly

mediated by the EGF-receptor (EGFR): blocking EGFR led to increased neuronal differentiation of endogenous or co-transplanted rat NSCs [14–16]. However, it is unclear how chABC affects human stem cell survival and differentiation.

Many different cell types have been transplanted following injury, including NSCs and their progeny, as well as peripheral glia cells, with some studies demonstrating promising results; however, it remains unclear which cell type promotes the greatest recovery [1]. NSC and oligodendrocyte progenitor cell transplantations are the focus of clinical trials in SCI [17, 18]. Neuronal cell transplantation has shown promise, especially since many motor- and interneurons are lost following injury; however, it is still unclear which neuronal subtype elicits the greatest benefit [19–21]. We have previously demonstrated that pre-differentiation has an impact on cell survival following transplantation into the injured brain and spinal cord [22, 23]. Pre-differentiation of human induced pluripotent stem cell (iPSC)-derived cortically-specified neuroepithelial stem cells (NESCs) demonstrated good survival and significantly more β-III tubulin-positive cells than immature or more mature populations one week post-transplantation into the stroke-injured rat brain [22]. The NESCs generate mainly glutamanergic excitatory neurons, which usually extend long projecting distance axons towards lower motor neurons within the spinal cord [24]. Therefore, we chose to deliver pre-differentiated NESCs in this study.

The delivery of both chABC and cells are challenging because chABC is a fragile enzyme that degrades rapidly while transplanted cells, especially neurons, show limited survival [25–27]. Since the outcome can worsen when stem cells differentiate uncontrollably or die, it is imperative to maintain cell survival and guide their differentiation following transplantation [28–30]. To this end, we developed a minimally invasive hydrogel for cell delivery [27, 29, 31]. The physical blend of hyaluronan and MC (HAMC) promotes the survival of transplanted cells [29, 31]. To achieve local release of bioactive chABC, we synthesized a crosslinked MC (XMC) hydrogel for affinity-based chABC delivery, which reduces CSPG levels for at least 2 weeks *in vivo* and promotes functional repair [32].

Here, we deliver chABC intrathecally using the XMC hydrogel (figure 1(a)) in combination with intraspinal injections of pre-differentiated NESCs in a HAMC hydrogel (figure 1(b)) to investigate the effect of chABC on the fate of transplanted cells and their combined effect on spinal cord regeneration. Figure 1(b) outlines the study design. We demonstrate a reduction in cavity formation following cell and cell +chABC delivery compared to injury only controls. Most transplanted NESCs express neuronal markers at 2 weeks, yet at 9 weeks post injury the distribution is more evenly split between progenitor cells, astrocytes, oligodendrocytes, and neurons. Interestingly, we find a higher percentage of neurons when the cells are delivered in combination with chABC, indicating that CSPGs influence the fate of grafted cells.

Materials and methods

chABC-SH3 expression and purification

Chemically competent BL21(DE3) *Escherichia coli* cells were transformed with pet28b plasmids containing the His-SH3-chABC-FLAG DNA vector and cultured as previously described [26].

chABC kinetic activity assay

The kinetic chABC-SH3 activity was determined as previously described [26]. Briefly, the activity of chABC was estimated by measuring the rate at which chABC (0.1 mg ml^{-1}) degraded chondroitin sulphate A (CS-A, 10 mg ml^{-1}, Sigma-Aldrich) at a ratio of 1:10 v/v.

Synthesis of MC for affinity release

MC (300 kDa, Shin-Etsu, Tokyo, Japan, 4% w/v) was modified as previously described to enable affinity release [27, 33]. Briefly, MC was first modified to introduce carboxylic acid groups (MC-COOH) using bromoacetic acid under basic conditions. Then MC was thiolated to MC-SH with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride, 3,3′-dithiobis(propionic dihydrazide) and dithiothreitol. MC-SH was reacted with a maleimide-modified peptide, 3-maleimidopropionic-GGGKPPVVKKPHYLS, by Michael-type addition resulting in peptide-modified MC (MC-peptide). The peptide sequence was synthesized by solid phase synthesis and is known to bind weakly to Src homology 3 (SH3), which was expressed as a fusion protein with chABC to allow affinity release.

Synthesis of XMC for chABC-SH3 delivery

XMC was prepared as described previously [27]. Briefly, thiolated MC (MC-SH) [33] and unmodified MC (300 kDa, Shin-Etsu Corp.) were dissolved in phosphate buffer (PB) to obtain a final concentration of 5% w/v total MC and 0.1 μmol thiol/100 μl gel. The gel was crosslinked using poly(ethylene glycol)-bismaleimide (PEG-MI$_2$, 3000 Da, Rapp Polymere, Tuebingen, Germany) for a final molar ratio of 0.75:1 maleimide to thiol. MC-peptide [33] and recombinant chABC-SH3 fusion protein were added to XMC prior to crosslinking for a final chABC-SH3 concentration of 0.3 U/5 μl and a final molar ratio of 1:100 chABC-SH3:SH3 binding peptide.

Affinity release of chABC

The release of chABC-SH3 from XMC-peptide was measured as previously described [26]. Briefly, 100 μl of the XMC-peptide containing chABC-SH3 was injected into a 2 ml Eppendorf tube at 37 °C. After

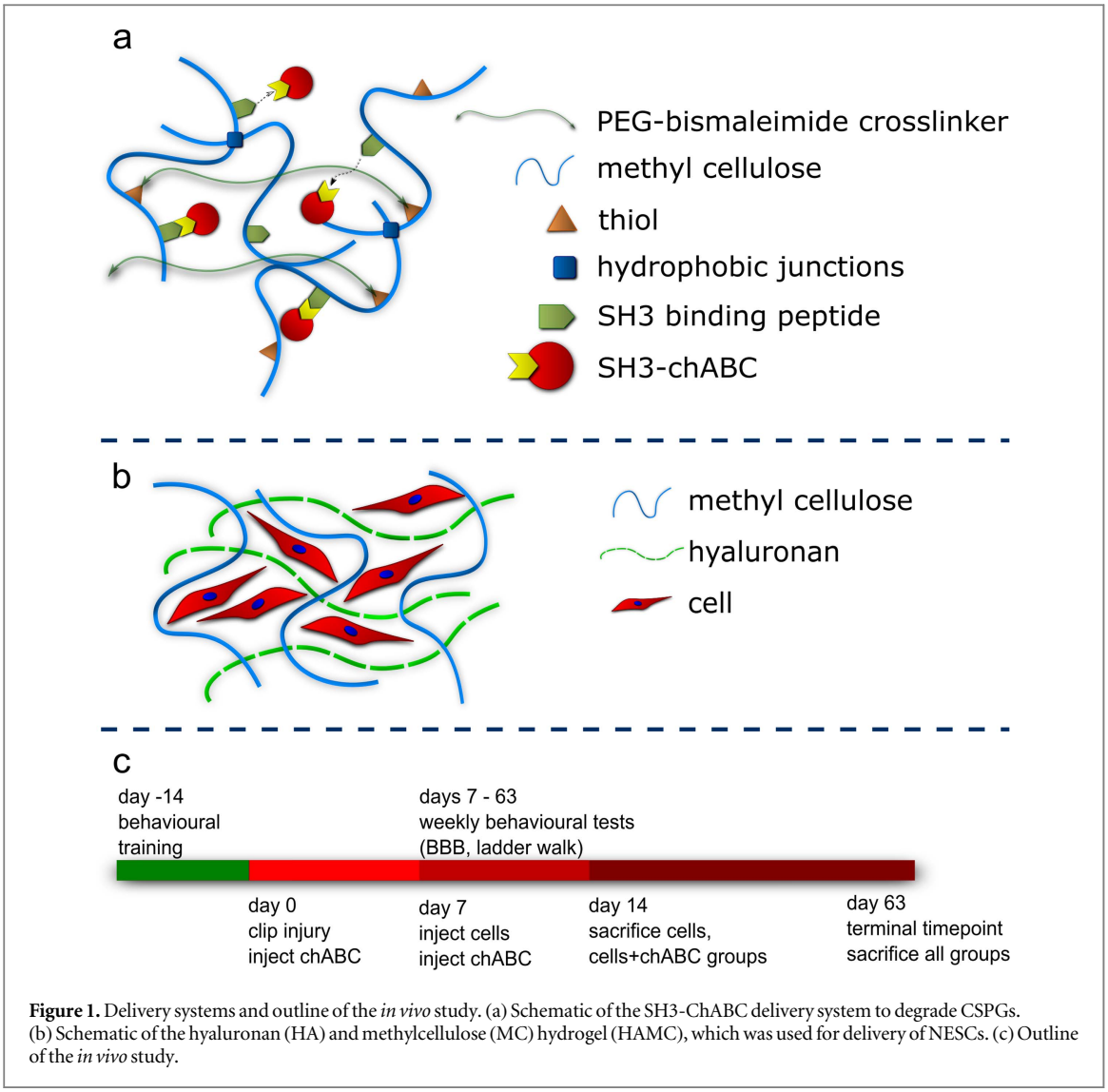

Figure 1. Delivery systems and outline of the *in vivo* study. (a) Schematic of the SH3-ChABC delivery system to degrade CSPGs. (b) Schematic of the hyaluronan (HA) and methylcellulose (MC) hydrogel (HAMC), which was used for delivery of NESCs. (c) Outline of the *in vivo* study.

gelation, 400 μl of warm artificial cerebrospinal fluid (aCSF: 350 mM NaCl, 3 mM KCl, 0.8 mM $MgCl_2$, 1.4 mM $CaCl_2$, 1.5 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, pH 7.4) was carefully added on top of the gel. At 0, 1, 2, 5, and 7 d the supernatant was completely removed and replaced with fresh aCSF. MC-peptide gels without chABC-SH3 were used as blanks for each time point. Release samples were analysed by ELISA using a 96-well Ni-NTA plate (Qiagen, Toronto, ON) and an anti-FLAG antibody coupled with HRP (1:5000) for detection. The absorbance levels were read on a TECAN plate reader at 405 nm with a wavelength correction at 650 nm. Protein concentration was calculated based on the linear range of a standard curve from the same plate.

HAMC preparation

A physical blend of hyaluronan (HA, 1400–1800 kDa, Novamatrix, Drammen, Norway) and MC (300 kDa, Shin-Etsu, Tokyo, Japan) was used to prepare HAMC as previously described [29]. Briefly, sterile filtered HA and MC were dissolved in aCSF at a concentration of 1% HA (w/v) and 1% MC (w/v), mixed in a SpeedMixer (DAC 150 FVZ, Siemens) and allowed to fully dissolve on a shaker at 4 °C overnight prior to use.

Cell culture

Human iPSC-derived cortically-specified NESCs were characterized and generated as previously described [22]. Prior to use, cells were differentiated for 16 d on poly-D-lysine (20 μl ml^{-1})/laminin (5 $\mu g\,ml^{-1}$) coated plates at a concentration of 37 500 cells cm^{-2} in media comprised of 25 ml 10X DMEM/F12 supplemented with glutamine and HEPES, 25 ml Neurobasal (Gibco), 1 ml penicillin/streptomycin, 1 ml B27, and 50 μl BSA fraction V (Thermo Fischer), with media changes every 2–3 d.

Cell preparation

Pre-differentiated NESCs (16 d) were magnetically sorted for the cell surface marker PSA-neural cell adhesion molecule (NCAM) using MicroBeads (Miltenyi Biotec). The cells were centrifuged at 1400 rpm for 4 min, re-suspended in 60 μl of buffer (PBS pH 7.2, 0.5% BSA, 2 mM EDTA), and stored at 4 °C for 10 min 20 μl of the MicroBeads were then mixed into

the cell suspension, and the mixture incubated at 4 °C for 15 min. The cells were transferred to pre-rinsed columns (LS, Miltenyi Biotec), and washed three times with 3 ml of buffer. To remove labelled cells, the column was removed from the magnet and washed out with 5 ml of buffer using a plunger. Cells were re-suspended in the hydrogel for a final concentration of 0.75 w/v HA and 0.75 w/v MC (HAMC) and 20 000 cells μl^{-1} (figure 1(B)). Cell-seeded hydrogels were stored on ice until transplantation.

Animal surgeries

All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care) and protocols were approved by the Animal Care Committee of the Research Institute of the University Health Network. An outline of the *in vivo* study is depicted in figure 1(C). 60 female Sprague Dawley rats (300 g, Charles River, Montreal, QC) were used to assess the effects of cell- and chABC-delivery in terms of long term CSPG levels, cell survival, integration and fate, and animal functional behaviour. Five groups were compared: (1) cells+chABC, (2) cells alone, (3) chABC alone, (4) vehicle control, (5) buffer control (injury). See supplemental table 1, available online at stacks.iop.org/BMM/13/024103/mmedia, for a summary of the animal treatment groups. Three animals were lost due to surgical complications or dehydration, one each from the groups (1), (3), and (4). Clip compression injury was performed as described previously [29]. Briefly, animals were anaesthetized with isoflurane and subjected to a laminectomy at thoracic level 1 and 2 (T1-2). The spinal cord was injured by cord compression with a 21 g modified aneurysm clip for 1 min, resulting in a moderate SCI [34]. Immediately after the injury, 5 μl of chABC-XMC (or XMC in vehicle control group or PB in buffer control group) was injected into the intrathecal space through a 30 G angled blunt-tipped needle. The needle was kept in place for 1 min to prevent backflow of the hydrogel. After closing the overlying muscles, fascia and skin, rats were placed under a heating lamp and allowed to recover.

7 d following clip compression injury and chABC-XMC (or XMC or buffer control) delivery, all rats underwent a second operative procedure. Rats were anaesthetized, and the previous operative site re-exposed. Cell injections were made stereotactically through the intact dura with the aid of an operating microscope using a motorized microinjector at a rate of 1 $\mu l\,min^{-1}$. A total of four injections were made using a 10 μl Hamilton syringe with a customized 32 gauge needle: two injections, 1 mm rostral and two injections, 1 mm caudal from the lesion site, each 1 mm lateral from the midline, containing 2 μl of control solutions (aCSF or HAMC) or cells in HAMC (2×10^4 cells μl^{-1}). The needle was left in place for an additional 2 min after injection to prevent backflow. To aid transplant survival, all animals (including the non-cell control) were given cyclosporine A (10 $mg\,kg^{-1}\,d^{-1}$, Sandimmune, Novartis Pharma, Canada Inc., Dorval, Quebec, Canada) via subcutaneous implanted osmotic pumps (Alzet) starting 1 d prior to transplantation until sacrifice, as previously described [29]. Immediately after the intraspinal injections, an additional 5 μl of chABC-XMC (or XMC or buffer) was intrathecally injected through a 30 gauge angled blunt-tipped needle. Upon injection, the needle was held in place for 1 min to prevent backflow. Lastly, the skin was closed and the rats were placed under a heating lamp and allowed to recover.

Animals were sacrificed and transcardially perfused with 4% PFA in 0.1 M (PB, pH 7.4) at 2 and 9 weeks post injury, the spinal cords removed, cut into blocks of 1.5 cm encompassing the site of injury/injection and processed for serial longitudinal cryosectioning (20 μm).

Housing and post-operative care

Buprenorphine (0.05 $mg\,kg^{-1}$) was administered twice daily every 12 h for 48 h after surgery. Animals were housed individually in a temperature-controlled room with a 12 h light/dark cycle. Clavamox was added to the water for 2 d prior to surgery, and for 5 d post-surgery to prevent infections. Bladders were manually expressed three times per day until bladder function returned. Water and food were provided ad libitum. Skin clips were removed 10–14 d post-operatively.

Immunofluorescence

After fixation, samples were processed for immunohistochemical staining as described previously [35]. Primary antibodies were diluted in PBS and incubated with the sections overnight at RT. The following primary antibodies were used: anti-glial fibrillary acidic protein (GFAP, 1:2000, DAKO Z0334), anti-human nuclei (hNUC, 1:500, Millipore MAB1281), anti-human cytoplasm (STEM121, 1:2000, Takara Bio Y40410), anti-ß-III-tubulin (TUBB3, 1:2000, Abcam ab41489), anti SOX10 (1:250, Abcam, ab155279), anti-nestin (1:200, Abcam ab105389), anti-CSPG (clone CS-56, 1:100, Sigma C8035), and anti-Ki67 (1:500, Abcam ab15580). Primary antibodies were detected by a combination of highly cross-absorbed secondary antibodies incubated for 3 h at RT: Alexa Fluor© 488 or 633 goat anti-mouse IgG (1:500), Alexa Fluor© 546 goat anti-rabbit IgG (1:500), and Alexa Fluor© 568 or 633 goat anti-chicken IgG (1:500, all Thermo Fisher Scientific) diluted in PBS. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1 $\mu g\,ml^{-1}$, Thermo Fisher Scientific). Samples were mounted using ProLong Gold antifade reagent (ThermoFisher Scientific) and visualized using an inverted confocal

microscope with a motorized stage (Olympus FV1000) at 20× (NA 0.75) magnification to create overview images or at 40× (NA 0.6) magnification for demonstration of cell fate. Secondary antibody only staining was performed to ensure specificity of the secondary antibodies.

Morphological analysis

At least four longitudinal sections, evenly distributed throughout the entire thickness of each rat spinal cord ($n \geqslant 3$ animals per group) were analysed by a user blinded to the treatment. The slide numbers of the serial sections were matched to analyse similar locations between groups. Image overviews encompassing the lesion site/transplanted cells were taken using a motorized stage with the same settings for all groups.

The percentage of lesion volume was calculated as the area of the cavitated region divided by the total area of the spinal cord cross section according to the GFAP-staining. CSPG, Iba1, GFAP, and TUBB3 expression was estimated by quantifying the number of positive pixels for each antibody. Images were converted to black and white using the same threshold between groups for each antibody and using ImageJ software. Data was standardized to the area of tissue analysed, as previously described [35]. To analyse the host cell response, a 100 μm thick band around the lesion was analysed for GFAP and TUBB3 at the 9 weeks survival time point. GFAP and TUBB3 staining co-localized with stem121 were excluded in the cell groups. Analysis for Iba1 and CSPG at 2 weeks post injury included the lesion core.

Cell survival was estimated by counting the hNUC positive cells in each section, as described previously [29]. Ki67 was used to identify proliferating cells and double positive cells (Ki67 and hNUC positive cells) were counted to estimate the percentage of proliferating human cells.

Cell fate was estimated by outlining the cells positive for either the human cytoplasmic (STEM121) or nuclear (hNUC) stains and measuring the overlap of this region with markers for progenitor cells (nestin), oligodendrocytes (SOX10), astrocytes (GFAP), or neurons (TUBB3).

Behavioural test

For animals surviving 9 weeks, motor behaviour was evaluated weekly by two blinded observers using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale [36] and a Ladder walk test [37]. Animals were trained to cross a 1 m long ladder without interruption to reach their food pellet rewards daily for 14 d prior to the injury. Baseline values were obtained 2 d prior to injury and testing resumed 2 weeks after injury for animals with a BBB score of ⩾11. The number of gaps was kept consistent; however, their location was changed. The number of foot faults made by either hindlimb were counted and averaged over three recorded runs per animal. Animals that had a BBB score less than 11 were given a maximum score of 10, the average number of steps taken by the animals to cross the ladder.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 6. Data are plotted as mean ± standard error of the mean (SEM). Data was subjected to an analysis of variance followed by a Bonferroni's post-hoc test for comparisons between means of multiple groups, a t-test was performed to assess differences between the cell groups (1) and (2) at 2 weeks post injury. A p value < 0.05 was regarded as statistically significant (i.e. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Results

NESCs are enriched for *β*-III-tubulin *in vitro*

The NESCs used comprise of a population of self-renewing cells, which were derived from a human iPSC line. Their *in vitro* differentiation profile was described in detail previously [22]. Briefly, during *in vitro* differentiation, NESCs downregulate the stem/progenitor markers, nestin and SOX2, and upregulate the neuronal marker *ß-III-tubulin* (TUBB3). In addition to differentiating the cells for 16 d *in vitro*, we used magnetic-activated cell sorting for the NCAM to further enrich the population of neuronal cells. This led to an increase of TUBB3-positive cells from 36% ± 3% to 81% ± 5% prior to *in vivo* injection.

chABC release and effect on CSPG expression

The XMC-peptide slowly released bioactive chABC-SH3 over 7 d *in vitro*, as described previously [27]. CSPG positive staining was found in all animals at the injury site (figures 2(a), (b)), with some cells, including astrocytes, expressing CSPGs within the lesion at 9 weeks post injury. Animals that received cells and chABC had reduced CSPG levels compared to animals that received cells only at 2 weeks post injury (figure 2(C)). No significant differences in CSPG levels between the groups were found at the later time point ($p > 0.05$, data not shown).

Cell transplantation reduces cavity formation

The large cystic cavitations at the lesion site, which are characteristic of contusion/compression-type injuries (figure 3(a)), were reduced with the delivery of NESCs with and without chABC (figure 3(b)) at 9 weeks post-injury. Quantification of the lesion volumes demonstrated that animals receiving NESCs developed statistically significant smaller cystic cavitations than control animals (figure 3(c), $p < 0.05$). Quantification of GFAP levels around the lesion site did not reveal

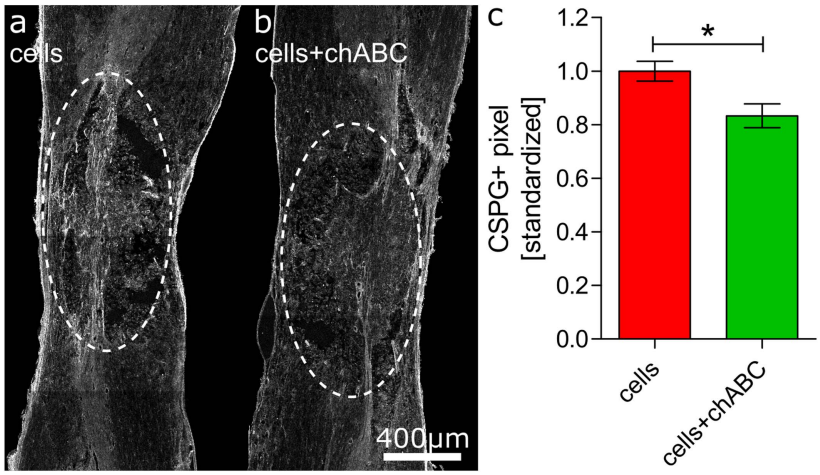

Figure 2. Delivery of chABC reduces CSPG levels at 2 weeks post injury (a), (b) CSPGs expression at the lesion site in animals receiving either (a) cells or cells with (b) chABC, where dashed line denotes lesion boundary. (c) Quantitative analysis of the amount of CSPG-positive pixels demonstrated that chABC-delivery significantly reduces the amount of CSPGs. Data are plotted as mean ± standard error, $n \geqslant 4$ animals, $^{*}p < 0.05$.

any significant differences between the groups (figure 3(d)). Since local delivery of chABC-SH3 alone did not reduce the lesion volume, yet that of NESCs alone and in combination with chABC did, the reduced volume observed with the co-delivery of NESCs and chABC was attributed to the NESCs.

Transplanted cells survive, migrate, and proliferate

In order to understand why the lesion volume was reduced when animals were transplanted NESCs, we investigated NESC migration to the lesion site. As shown in figure 4(a), NESCs migrated from the four injection sites around the lesion towards the lesion epicentre where they partly filled out the cavity at 9 weeks post injury. We observed this behaviour in both cell groups. Figure 4(b) shows that initial survival was low, with only approximately 3%–5% of the grafted cells found at 2 weeks post-injury (or 1 week after transplantation). However, the number of human cells increased over the next 8 weeks, indicating that the transplanted cells proliferated. Interestingly, proliferating human cells were indeed found after transplantation, mainly within the lesion site (figures 4(c)–(e)). Quantification of human nuclei and Ki67 double positive cells demonstrated that approximately 13%–14% of cells were proliferative 9 weeks post-injury (figure 4(f)). We observed no difference in the numbers of hNUC+ and Ki67+ cells with or without co-delivery of chABC, indicating that chABC neither affected survival nor proliferation of transplanted NESCs ($p > 0.05$). Furthermore, chondroitinase treatment did not influence the general inflammatory response 2 weeks post injury, as assessed by Iba1 staining (supplemental figure S1). This indicates that chABC did not influence the inflammatory response.

chABC co-delivery results in an increase of neuronal cells

Although the transplanted cells were biased towards cortical glutamanergic neurons and the majority of cells expressed neuronal markers prior to transplantation, they still represent a heterogeneous population and any surviving progenitor cells can differentiate into the three main cell types of the central nervous system: astrocytes, oligodendrocytes, and neurons. Therefore, we investigated the cell fate at 2 and 9 weeks post-injury and detected progenitors, astrocytes, oligodendrocytes and neurons (figures 5(a)–(d)). Their ratio changed significantly between the two time points (figure 5(e)). At 2 weeks following injury, 65%–75% of the human cells expressed the neuronal marker TUBB3, with few nestin-positive progenitors, even less SOX10+ oligodendrocytes and 15%–25% GFAP+ astrocytes. At 9 weeks post-injury, the fate of the transplanted NESCs changed to having more nestin+ progenitors, more SOX10+ oligodendrocytes, similar GFAP+ astrocytes and fewer TUBB3+ neurons. While the percentages of NESCs positive for nestin, GFAP, and SOX10 were similar between animals transplanted with cells alone and cells co-delivered with chABC at 9 weeks post-injury, the percentage of TUBB3+ neurons was significantly different: 32% ± 3.02% of the transplanted NESCs were positive for TUBB3 when transplanted with chABC and only 16% ± 2.5% were positive when NESCs were transplanted alone ($p < 0.05$). The neurons extended short axons (figure 5(d)). While few mature NeuN+ neurons were found (data not shown), the difference in the number of immature TUBB3+ neurons between NESCs transplanted with or without chABC, suggested a role for CSPGs in terms of cell fate. No differences between animals were found in terms of

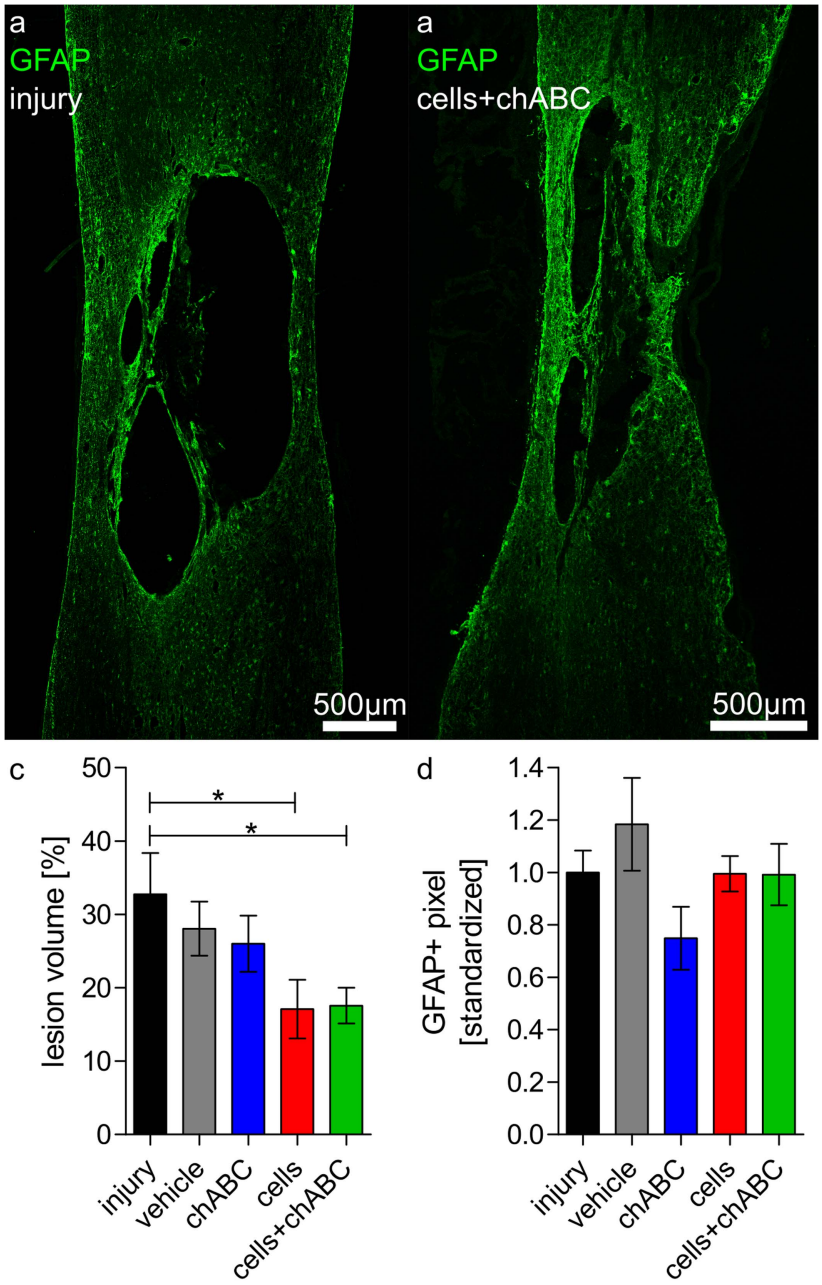

Figure 3. Cell transplants reduce the lesion size at 9 weeks post-injury. (a) Animals without cell grafts had large cavities with clearly delineated GFAP-positive astrocytes lining the glial scar. (b) Animals with transplanted cells had smaller cavities. (c) Quantitative analysis of the lesion volume demonstrated that the cavities of animals receiving cells were significantly smaller than those in control animals. (d) No statistically significant differences in GFAP-reactivity around the lesion site were observed between groups. Data are plotted as mean ± standard error, $n \geqslant 4$ animals, $^{*}p < 0.05$.

axonal density in the tissue adjacent to the lesion (supplemental figure 2).

Treatments do not influence the behavioural outcome at 9 weeks post injury

All animals demonstrated normal motor function prior to the injury (BBB score of 21), with a significant drop after clip compression injury, where animals only showed some movement in all joints, but no stepping, with an average BBB score of 8 (figure 6(a)). Animals slowly recovered to an average BBB score of 12, with approximately half of the animals demonstrating coordinated stepping. No differences between the groups were observed ($p > 0.05$).

Uninjured animals crossed the gridwalk with few, if any, errors (<1 foot fault, figure 6(b)). By week 2 post-injury, approximately 20% of animals were able to cross the gridwalk, which increased to more than 90% of animals by 9 weeks. All animals demonstrated a significant increase in the number of footfalls following the injury. By 9 weeks the average number of errors per crossing was reduced by 3–4 foot faults in all groups. Values for all groups at 9 weeks post injury were below the initial baseline values, yet no

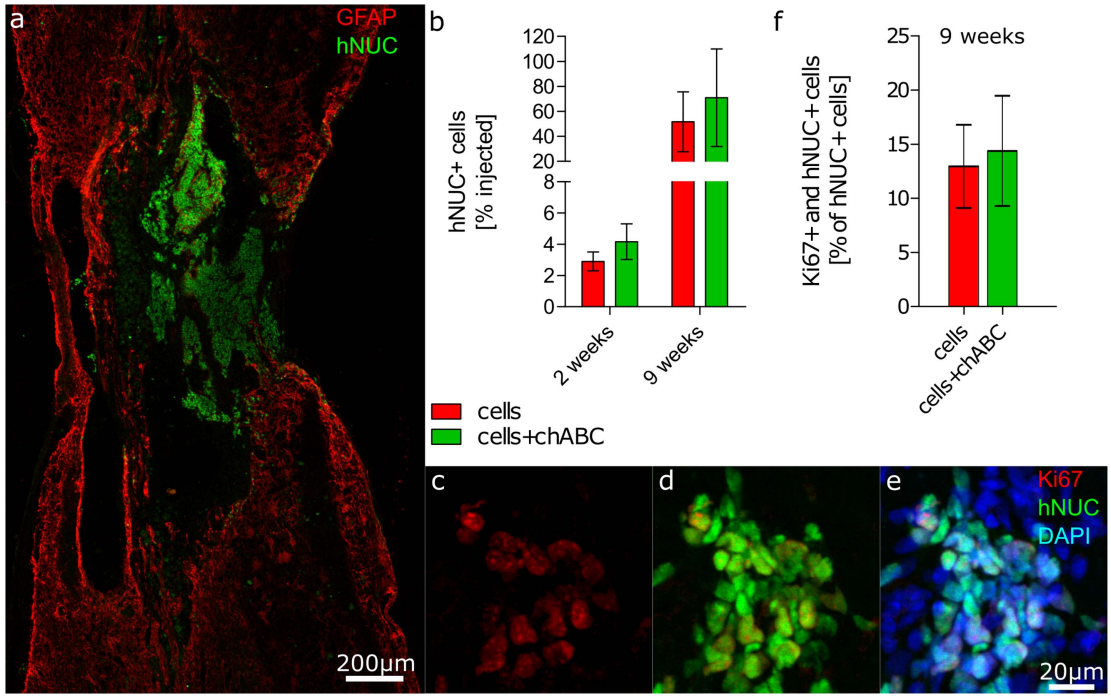

Figure 4. Transplanted cells survive, migrate, and proliferate. (a) Low magnification image demonstrating human nuclei positive cells (hNUC, green) within the lesion site, which is outlined by GFAP positive astrocytes (red, 9 weeks post-injury). (b) Initial survival of transplanted NESCs was low at 2 weeks post-injury, but increased at 9 weeks post-injury, indicating cell proliferation. (c)–(e) Ki67 (red), human nuclei (green) double positive cells were found at 9 weeks post-injury, indicating proliferative cells were still present. (f) Quantification of the number of proliferating human cells by double staining for Ki67 and hNUC. Observations between the two cell groups were similar and no statistical differences were found between them regarding cell survival and cell proliferation. Data are plotted as mean ± standard error, $n \geqslant 4$ animals.

significant differences could be detected between individual groups ($p > 0.05$).

Discussion

The complex pathophysiology of spinal cord injuries requires a combination therapy for tissue and functional repair. Biomaterials have been shown to improve cell- and drug-delivery for neural tissue repair [29, 32]. The present study investigated the effect of hydrogel-based co-delivery of the CSPG degrading enzyme chABC with human cortically-specified NESCs on transplanted cell survival, integration, and differentiation, and their combined effect on spinal cord regeneration.

Many proteins, and especially enzymes, are unstable within the body or are cleared rapidly when injected systemically. A delivery system that can be tuned to achieve controlled and sustained release can promote tissue repair. To this end, treatment of the injured spinal cord with chABC to degrade CSPGs has been shown to be beneficial, but the sustained delivery of chABC has been limited by its thermal instability [38]. Previously, we demonstrated that an affinity based release system to deliver chABC is able to reduce CSPG levels at the injury site for 2, but not 8, weeks *in vivo* following a single injection at time of injury [26, 32]. To further enhance the benefit of local chABC release, we delivered it twice—at the time of injury and one week later with cell transplantation; however, we avoided more injections as it can be harmful to the animals to undergo multiple surgeries. We saw reduced CSPG expression at 2 weeks in animals treated with chABC compared to untreated, spinal cord injured animals, but not at 9 weeks post injury. Based on prior experience, we estimate a reduction in CSPG levels for up to three weeks [32]. To extend the time of bioactive delivery, either the concentration of SH3 binding peptide within the gel could be increased or a stronger binding affinity peptide could be used [39]. To improve chABC stability, it may be modified by site-directed mutagenesis to prevent protein unfolding and denaturation [40]. The lack of chABC affect on CSPGs levels at the later time point might also be due to a general down regulation of CSPGs at the injury site. Jones *et al* demonstrated that the peak for most CSPGs occurs around 2 weeks post injury and that their levels decrease afterwards following a dorsal column lesion [41]. However, CSPG levels may be more consistently upregulated following contusion type injuries [32, 42]. In addition, the glial scar was thought to form a mechanical barrier to neuronal regeneration; however, a recent study demonstrated that the scar tissue is softer than its surroundings [43]. It is unclear whether the delivery of chABC changes the stiffness of the glial scar or only affects its chemical composition.

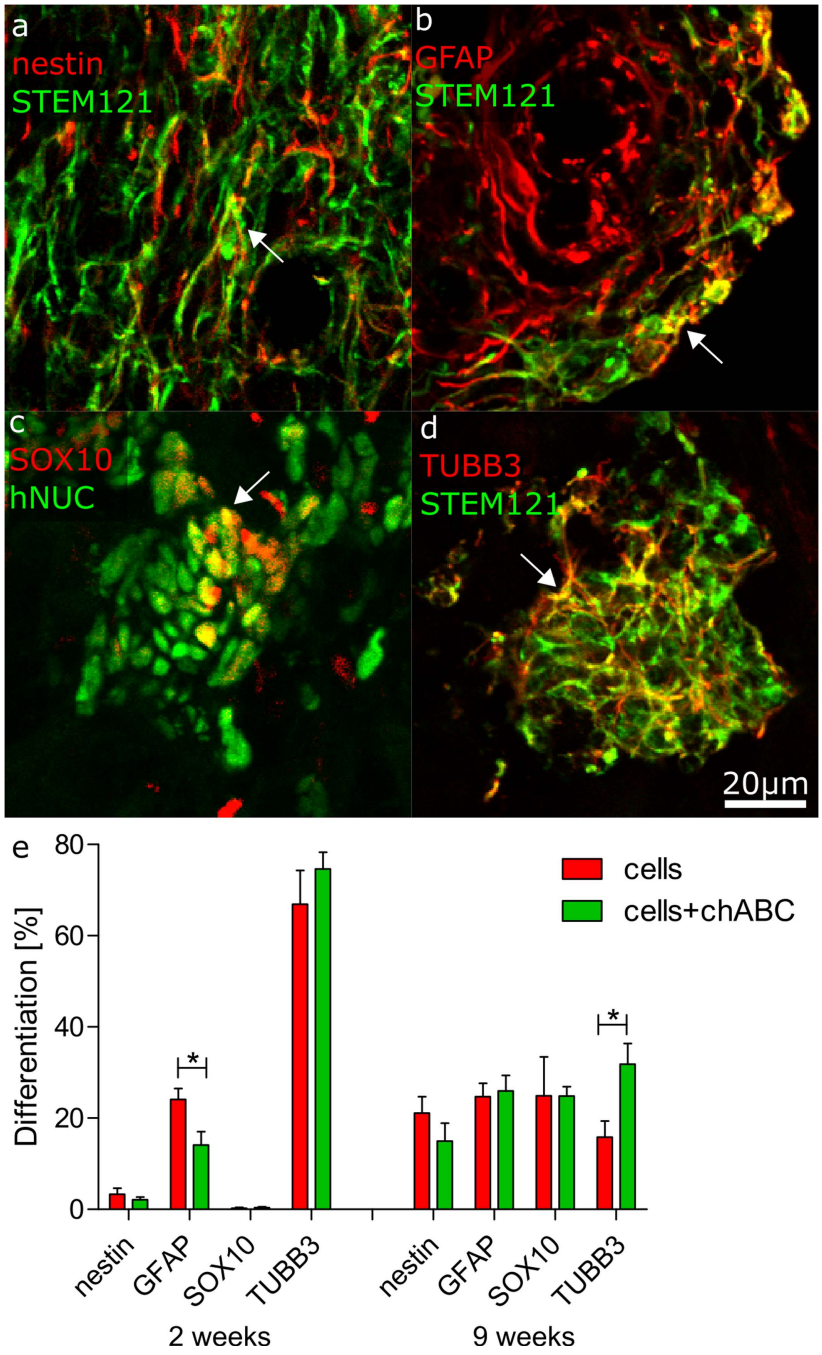

Figure 5. chABC promotes neuronal differentiation of transplanted cells. (a)–(d) Transplanted human cells were either identified with an antibody against human cytoplasm (STEM121, green, (a), (b), (d)) or human nuclei (hNUC, green, (c)). Cells were double stained to identify: (a) progenitor cells (nestin, red), (b) astrocytes (GFAP, red), (c) oligodendrocytes (SOX10, red), (d) neurons (TUBB3, red). Arrows indicate examples of double labelled NESCs. Images are from the 9 week post-injury time point. (e) Histogram showing the percentage of NESCs positive for the markers identifying progenitor cells, astrocytes, oligodendrocytes, and neurons at 2 and 9 weeks following injury. Data are plotted as mean ± standard error, $n \geqslant 3$ animals, $^{*}p < 0.05$.

Cell transplantation promises to replace lost cells and restore function; however, cell survival typically is low following transplantation. Biomaterials can provide a scaffold for cell adhesion, thereby reducing cell death due anoikis, and promoting cell survival and differentiation with the presence of other factors [44, 45]. The physical blend of hyaluronan and MC (HAMC) promotes cell survival alone and when modified with adhesive peptide sequences and growth factors, e.g. GRGDS and PDGF-A, it enhances differentiation [29, 31, 46].

The NESCs used here were derived from human iPSCs. They demonstrate self-renewal and multipotency when cultured in the presence of inhibitory factors [22]; however, the majority are committed to a neuronal lineage, express markers associated with cortical layers, and are either glutamatergic or GABAergic. Glutamanergic excitatory neurons in the motor cortex generally extend long axons towards lower motor neurons within the spinal cord that are damaged after injury [24]. Cells were transplanted in

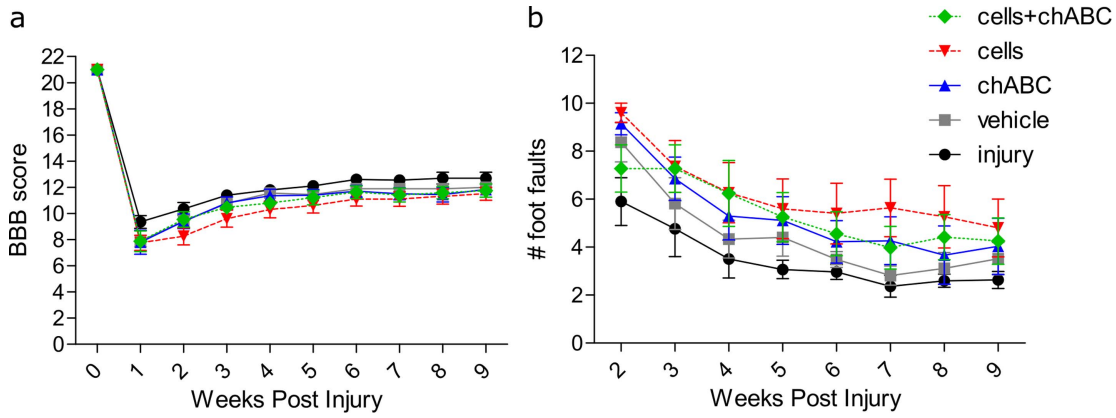

Figure 6. Functional recovery over time. (a) Animals demonstrated normal locomotor function prior to the injury, but were only able to partly move their hindlimbs 1 week post-injury due to the clip compression injury. Animals slowly recovered to an average BBB score of 12, indicating some coordinated stepping. (b) Animals made few, if any, mistakes crossing the ladder prior to injury. Following injury, animals had problems crossing the ladder and the majority were not able to cross at all, but they recovered slightly to an average of four foot faults by week 9. No statistical differences between groups were found for either behavioural test ($p > 0.05$). Data are plotted as mean $\pm$ standard error, $n \geqslant 9$ animals.

HAMC, with and without co-delivery of chABC. Few surviving cells were present one week following transplantation; however, due to proliferation, the number increased more than tenfold by week nine. The cells migrated away from the four injection sites surrounding the lesion towards the lesion cavity, which they partially filled, thereby reducing the cavity volume. Similar cell behaviour was reported with other cell types, such as human iPSC-derived oligodendrocyte progenitor cells and mouse (iPSC)-derived NSCs [29, 47], whereas others reported a more widespread distribution around the lesion site when human foetal brain tissue-derived NSCs were transplanted [48, 49]. While we observed no difference in NESC survival with chABC delivery, mouse NSC survival was enhanced when they were co-transplanted with chABC in a chronic model of SCI [5]. This difference may be attributed to the different cell type, injury model, or method of chABC delivery. Typically, reduced cell proliferation and increased cell differentiation is desired, as a greater number of proliferating cells may lead to tumour formation [50]. Cell proliferation can decrease naturally with time, as was suggested previously for human NSCs [48], or can be induced by pre-treatment with a γ-secretase inhibitor [50].

Most of the grafted NESCs expressed neuronal markers initially, but a more even distribution between immature progenitor cells, astrocytes, oligodendrocytes, and neurons was found at the 9 week endpoint. This is likely due to differentiation of the proliferating progenitor cells that were present at low numbers following transplantation. The high number of neurons at the earlier time point matches our observation in the stroke-injured rat brain [22]. Interestingly, greater neuronal differentiation was observed when NESCs were delivered with chABC, suggesting that CSPGs play a role in NESC differentiation or survival.

CSPGs have been shown to influence stem cell maintenance [10, 11], and CSPG receptor knockout increases the differentiation of mouse NSCs to oligodendrocytes [13]. We did not observe an effect on oligodendroglial differentiation, which might be due to the difference in cell types investigated. Others have demonstrated that the effect of CSPGs is at least partly mediated by EGFR [16], and that blocking the receptor increases neuronal differentiation [14, 15]. Although chABC did not influence overall cell survival or proliferation, it might have selectively promoted neuronal survival. The delivery of chABC has been shown to attenuate the inflammatory response, which could have a beneficial effect on neuronal survival and host tissue regeneration [51, 52]. Cytokine signalling has further been implicated in neuronal differentiation, indicating that modulating the inflammatory response could influence cell differentiation [53, 54]. However, chABC did not affect Iba1 expression at two weeks post injury between animals that received cell grafts plus chABC and those that received cell grafts only. This is consistent with our previous observation where affinity-released chABC, as delivered here, had no effect on ED1 or M2 macrophages [32]. The difference in macrophage response between our observations and previous ones is most likely attributed to the difference in chABC delivery. Here, chABC was delivered intrathecally using small volumes of our hydrogel whereas Bradbury *et al* used either large volume lumbar injections or intraspinal injections of lentiviral vectors [51, 52]. While neurons, the main cell type transplanted and present up to at least 2 weeks, have been demonstrated to secrete cytokines and chemokines, their influence on the immune response is not well understood [55, 56]. In contrast to mesenchymal stromal cells, which have been demonstrated to elevate

levels of anti-inflammatory cytokines and reduce levels of proinflammatory cytokines [57, 58], they are thought to provide a neuronal relay across the lesion site rather than influencing the host environment. Thus we attribute the influence of chABC on graft cell differentiation on the early decrease in CSPG levels.

Recently, it was demonstrated that myelin basic protein is inhibitory to NSC proliferation [59], further demonstrating that the injury environment influences stem cell fate. Cell density of grafted cells also influences differentiation, with higher density reducing oligodendrocytic differentiation and promoting neuronal differentiation [49]. While chABC delivery correlated with an increased number neurons, a more pronounced increase might lead to better functional benefits. To improve neuronal cell survival or differentiation of progenitor cells into neurons, the HAMC hydrogel can be modified with factors that increase neuronal populations similar to a previous study where GRGDS and PDGF-A modified HAMC improved oligodendrocyte progenitor cell survival and differentiation [29].

Neither the individual treatments (cells or chABC alone) nor their combination had a clear effect on endogenous regeneration. Although there was a trend of reduced astrocyte reactivity around the lesion site in the chABC group, this was not significant. This is consistent with our earlier observations using the same delivery system and those from others [5, 32, 60]. Similarly, the number of axons around the lesion was comparable between the groups. Although chABC has been demonstrated to increase axonal regeneration, this effect seems to be more pronounced in animals with transection type injuries [42, 61]. The lack of a robust affect on host axonal regeneration may be attributed to the limited time frame for which chABC is available, the amount delivered and the inherent limitations of the enzyme [62, 63]. More specifically, chABC removes much, but not all, of the sugar chains from CSPGs, leaving the protein core and carbohydrate stubs behind, which may impair axonal outgrowth.

Despite the increase in neuronal differentiation, we did not see any behavioural recovery with the combined treatment. This may be partly due to the poor survival of human cells in immune competent animals, even in the presence of immunosuppression [28]. However, human cells have the ability to integrate functionally into the central nervous system of rodents and elicit positive effects, as was demonstrated in animal models of Parkinson's disease and SCI [64, 65]. Recently, it was also suggested that regional specificity is important for axonal regeneration of, for example, the cortical spinal tract (CST). Transplantation of spinal cord NSCs induced greater axonal outgrowth of the CST than forebrain NSCs [65], whereas forebrain instead of spinal stem cell-derived GABA neurons were needed to correct locomotor deficits in an animal model of Huntington's disease [66]. Although forebrain neurons have extensive connections to the spinal cord, regional differences are likely important for cell transplantation studies and grafted cells may need to be matched to the transplant region. However, a previous comparison between foetal spinal cord- and forebrain-derived NSCs failed to demonstrate a difference [67]. In addition, few cells demonstrated mature neuronal markers in this study, indicating that longer survival times may be needed to have a greater effect on motor recovery.

In conclusion, delivery of NESCs resulted in reduced lesion volume by graft cell migration into the lesion site, thereby promoting tissue repair. Moreover, NESCs transplanted with chABC maintained a greater percentage of neurons at 9 weeks post-injury than NESCs transplanted alone, suggesting that CSPGs are involved in stem cell differentiation. Despite the significant numbers of neurons and increased tissue repair, no functional repair was observed. This is likely due, in part, to the injury not being severe enough to capture differences and that the NESCs are forebrain rather than spinal cord cells. In on-going studies, we are investigating hiPSC-derived spinal neuronal cells for transplantation and their co-delivery in hydrogels modified with pro-survival and pro-differentiation factors.

Acknowledgments

We thank Mr Peter Poon for performing spinal cord injuries and helping with behavioural testing. We thank Dr Andrea Mothe for helpful advice and discussions, Dr Malgosia Ip for help with material synthesis and chondroitinase ABC expression, and Mr Bartlomiej Maracewicz for help with image analysis. We are grateful to funding from the Craig H Neilsen Foundation (Grant to MSS and AN), the Canadian Institutes of Health Research (Foundation grant to MSS), and the Natural Sciences and Engineering Research Council of Canada (Discovery grant to MSS and CGSM to PA).

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