

Olfactory Derived Stem Cells Delivered in a Biphasic Conduit Promote Peripheral Nerve Repair In Vivo.

AUTHOR(S)

Phoebe E. Roche Ms, Tijna Alekseeva, Amro Widaa, Alan J. Ryan, Amos Matsiko, Michael Walsh, Garry P. Duffy, Fergal J. O'Brien

CITATION

Ms, Phoebe E. Roche; Alekseeva, Tijna; Widaa, Amro; Ryan, Alan J.; Matsiko, Amos; Walsh, Michael; et al. (2017): Olfactory Derived Stem Cells Delivered in a Biphasic Conduit Promote Peripheral Nerve Repair In Vivo.. figshare. Journal contribution. <https://hdl.handle.net/10779/rcsi.10764572.v1>

HANDLE

[10779/rcsi.10764572.v1](https://hdl.handle.net/10779/rcsi.10764572.v1)

LICENCE

CC BY-NC-SA 4.0

This work is made available under the above open licence by RCSI and has been printed from <https://repository.rcsi.com>. For more information please contact repository@rcsi.com

URL

https://repository.rcsi.com/articles/Olfactory_Derived_Stem_Cells_Delivered_in_a_Biphasic_Conduit_Promote_Peripheral_Nerve_Repair_In_Vivo_/10764572/1



Olfactory Derived Stem Cells Delivered in a Biphasic Conduit Promote Peripheral Nerve Repair In Vivo

PHOEBE ROCHE ¹,^a TIJNA ALEKSEEVA,^a AMRO WIDAA,^a ALAN RYAN,^a AMOS MATSIKO,^a MICHAEL WALSH,^a GARRY P. DUFFY,^{a,b} FERGAL J. O'BRIEN^{a,c,d}

Key Words. Peripheral nerve injury • Nerve guidance conduit • Olfactory derived stem cells • Biomaterials

^aTissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, Dublin, Ireland; ^bNational University of Ireland Galway, University Road, Galway, Ireland; ^cTrinity Centre for Bioengineering, Trinity College Dublin (TCD), College Green, Dublin 2, Ireland; ^dAdvanced Materials and Bioengineering Research (AMBER) Centre, RCSI & TCD, Ireland

Correspondence: Fergal J. O'Brien, Ph.D., Tissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, 123 St. Stephens Green, Dublin 2, Ireland. Telephone: 35318034345; Fax: +35314022355; e-mail: fjobrien@rcsi.ie

Received October 3, 2016; accepted for publication July 5, 2017; first published Month 00, 2017.

<http://dx.doi.org/10.1002/sctm.16-0420>

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

ABSTRACT

Peripheral nerve injury presents significant therapeutic challenges for recovery of motor and sensory function in patients. Different clinical approaches exist but to date there has been no consensus on the most effective method of treatment. Here, we investigate a novel approach to peripheral nerve repair using olfactory derived stem (ONS) cells delivered in a biphasic collagen and laminin functionalized hyaluronic acid based nerve guidance conduit (NGC). Nerve regeneration was studied across a 10-mm sciatic nerve gap in Sprague Dawley rats. The effect of ONS cell loading of NGCs with or without nerve growth factor (NGF) supplementation on nerve repair was compared to a cell-free NGC across a variety of clinical, functional, electrophysiological, and morphologic parameters. Animals implanted with ONS cell loaded NGCs demonstrated improved clinical and electrophysiological outcomes compared to cell free NGC controls. The nerves regenerated across ONS cell loaded NGCs contained significantly more axons than cell-free NGCs. A return of the nociceptive withdrawal reflex in ONS cell treated animals indicated an advanced repair stage at a relatively early time point of 8 weeks post implantation. The addition of NGF further improved the outcomes of the repair indicating the potential beneficial effect of a combined stem cell/growth factor treatment strategy delivered on NGCs. *STEM CELLS TRANSLATIONAL MEDICINE* 2017;6:1894–1904

SIGNIFICANCE STATEMENT

Peripheral nerve injury affects millions of patients each year resulting in reduced quality of life, and billions of dollars in health care costs and lost work capacity. Surgical intervention with autografts work reasonably well for small lesions but for larger injuries, the functional outcomes are often unfavorable. Combining a surgical approach with contemporary developments in tissue engineering offers a potentially rewarding way forward. This study indicates that olfactory derived stem cells can have a regenerative effect on peripheral nerve repair in vivo when delivered on a novel biomaterial consisting of a collagen and laminin functionalized hyaluronic acid nerve guidance conduit.

INTRODUCTION

Millions of individuals suffer some degree of nerve injury leading to reduced motor or sensory function each year [1]. Injuries are predominantly caused by laceration events, crush injuries or burns; with almost 3% of critical trauma patients presenting with some form of peripheral nerve deficit [2].

In traumatic nerve injury, spontaneous functional recovery occurs only if the injury is incomplete or if the injury gap is small [3]. In practice, if the injury size is large, or direct suturing not possible, cable graft autografts are the current reference standard of care [4]. Autografting has a restricted role, first because donor tissue is finite, and second because despite advances in

techniques of microsurgical repair, the prognosis for functional recovery at both the donor and recipient sites remains poor [5]. The frequency, with which patients require treatment for the poor long-term outcomes typically associated with peripheral nerve injury, indicates that there remains a significant need for the identification of new approaches in peripheral nerve repair.

Tissue engineering offers an alternative approach through the development of biological substitutes, which promote regeneration of damaged tissues. Biomaterial scaffolds, cells, and growth factors are the key components of tissue-engineered approaches to peripheral neurogenesis [6]. Scaffold based artificial nerve grafts, which mimic the properties of autologous grafts, are now being extensively investigated although none

have yet superseded the results achieved with autografts.

Increasingly, there is evidence in the literature supporting cell based therapeutic approaches to peripheral nerve repair [7]. Denervated Schwann cells are key to the intrinsic process of regeneration but prolonged or extensive loss of axonal contact can render their effects obsolete through the loss of extracellular matrix (ECM)-integrin interactions and oxidative stresses. Supporting the denervated environment with progenitor cells in a cell-based therapeutic approach is, therefore, a logical progression and a number of biological scaffolds been successfully demonstrated to function synergistically with neural progenitor cells to enable functional neural tissue growth [7–11]. One important aspect of stem cell use in therapeutic applications is the means of acquisition of the source cells. In practical terms, the location of many progenitor cells within the human body can render them both challenging to access surgically, and unacceptable to patients. Olfactory neuroepithelial derived stem (ONS) cells reside in the nasal mucosa. They represent a desirable source of progenitor cell for peripheral nerve repair because the harvesting process has a low morbidity profile and they have been shown to demonstrate better clonogenicity, higher proliferation rates with a more natural inclination for differentiation toward neural and glial lineage than other neural derived stem cells [12]. ONS cells and their progeny—olfactory ensheathing cells—have previously been demonstrated to express ECM receptors such as CD44 and β 1 integrin, and NGF receptors [13, 14]. Although they have been studied extensively in CNS models where they have been shown to promote axonal regeneration [15], the role of ONS cells in peripheral nerve regeneration has been less well reported—partly because the optimum means of delivery within a large gap peripheral nerve injury has yet to be reported.

To facilitate delivery of therapeutic ONS cells within a peripheral nerve model, a nerve guidance conduit (NGC) was used. The NGC consisted of an outer tubular collagen conduit with hyaluronic acid (HA) hydrogel luminal filler. These two base components have shown promising results in peripheral nerve regeneration, and studies have shown that ONS cells have an affinity to these ECM molecules [14, 16]. A limiting factor in the use of HA for tissue engineering is its lack of cell binding sites. Functionalizing biomaterials with molecules such as laminin, which promotes attachment of cells and enhances myelination of axons through β 1 integrin dependent processes [17], can enhance their ability to promote axonal growth and integration within damaged nerve tissue. Therefore, in this study, we evaluated laminin as a potential agent for facilitating ONS cell binding to the HA hydrogel component of the NGC.

Additionally, we investigated the use of the neurotrophin nerve growth factor (NGF), to complement the regenerative capacity of the ONS cells within the NGC. Improved functional and morphologic outcomes have been demonstrated when tissue engineered scaffolds are supplemented with nerve growth factor (NGF) [18]. Studies have also shown improved outcomes in peripheral nerve injuries using NGF in the absence of scaffolds or stem cells [19] suggesting that NGF serves two distinct functions in peripheral nerve repair; supporting implanted stem cell differentiation, and promoting out-sprouting of native axons from the proximal stump *in vivo*. To the best of our knowledge, this is a first report on the use of ONS cells for peripheral nerve repair delivered in a collagen and laminin-functionalized HA based system.

The objective of this study was to investigate the potential of ONS cells delivered in a biphasic collagen and laminin

functionalized HA NGC to promote peripheral nerve repair *in vivo*. The specific aims were to:

- Isolate ONS cells from Sprague Dawley Rats and evaluate the effect of laminin and nerve growth factor (NGF) on metabolic activity and differentiation.
- Evaluate the effects of functionalizing the HA component of a collagen-HA NGC with laminin.
- Evaluate the modulating effects of NGF on ONS cells within a collagen-hyaluronic acid NGC.
- Evaluate the degree of peripheral nerve recovery following implantation of ONS cells within the biphasic NGC, with and without NGF, across a panel of clinical, functional, electrophysiological and morphologic parameters.

MATERIALS AND METHODS

Isolation of ONS Cells from Sprague Dawley Rats and Evaluation the Effect of Laminin and Nerve Growth Factor on Metabolic Activity and Differentiation

Prior to commencement, full ethics committee approval was obtained from the Royal College of Surgeons in Ireland Research Ethics Committee (REC000-754) in addition to appropriate project licenses from the Department of Health, Ireland with permits for the import of animals from Harlan U.K. from the Department of Agriculture. All chemicals were purchased from Sigma Ireland (Arklow, Co. Wicklow, Ireland, <http://www.sigmaaldrich.com>) or BD bioscience (Dun Laoghaire, Co. Dublin, Ireland, <http://www.bd.com/uk>) and used per the manufacturer's instructions unless otherwise indicated. Multipotent ONS cells were harvested and cultured from explants taken from the nasal cavities of 6-week-old Sprague Dawley rats (Harlan U.K., Blackthorn, U.K., <http://www.envigo.com>), using protocols developed by Féron et al. (Supporting Information 1: Methods) [20].

To confirm the capacity of the ONS cells to differentiate, brightfield microscopy images were taken and immunofluorescence for stem cell and neural markers, and nerve growth factor (NGF) receptor p75 was performed (Supporting Information 2: Methods).

To evaluate the effect of both laminin and NGF on ONS cell activity, assays comparing laminin (5 μ g/ml) coated culture surface conditions against uncoated tissue culture surface conditions (with and without NGF enhanced media at 100 ng/ml) were performed (Supporting Information 2: methods). Metabolic activity of ONS cells following 7 days in culture in each condition was assessed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) assay with photometric assessment (average absorbance at 490 nm).

Qualitative analysis for the expression of Nestin, glial fibrillary acid protein (GFAP) and β -tubulin by ONS cells in two-dimensional (2D) culture was performed using immunostaining with the following primary antibodies: anti-nestin (raised in rabbit, diluted at 1:100), anti β -tubulin (raised in mouse, diluted at 1:350; anti Glial Fibrillary Acid Protein (GFAP raised in mouse, diluted at 1:40). Secondary antibodies (AlexaFluor, goat anti-mouse 488, and goat anti-rabbit 546 in 1:1,000) and hoechst nuclear stain were applied, prior to imaging. Once expression of the key neural and glial markers in standard conditions was demonstrated, quantitative assessment using flow cytometry was performed. The antibody solutions here were made using anti Nestin, anti β -tubulin,

and anti GFAP in dilution buffer [0.3% Triton X in PBS or 0.5% Tween 10 in phosphate buffered saline (PBS)].

Evaluation of the Effects of Functionalizing the HA Component of the NGC with Laminin and the Further Modulating Effects of NGF on ONS Cells Within the NGC

The external tubular component of the NGC was fabricated using a suspension of 2.5% bovine fibrillar collagen type I in 0.05 M acetic acid which was lyophilized in custom made tubular stainless-steel molds (2 mm lumen diameter, 840 μm thick walls) using an in house refined lyophilization cycle (cooling rate 0.9°C from 20°C to a final freeze temperature of -40°C) which has been shown to generate scaffolds with large porosity and a homogenously interconnected pore architecture [21, 22]. Once the tubular constructs were fabricated, they were subjected to dehydrothermal cross-linking treatment (DHT) which resulted in NGCs with a porosity of 95.7%, tensile modulus of 417 kPa, and an estimated degradation half-life of 2–3 weeks in vivo [23].

A previously described procedure was used to produce the HA hydrogel filler [24]. Prior to use in vivo, the physical properties of the hydrogels were assessed and characterized in house followed by cell viability analysis (Supporting Information 3: methods). Two variants (nonfunctionalized and laminin functionalized) were produced for potential inclusion in the end product NGC. The functionalized variant was produced by adding laminin (5 $\mu\text{g}/\text{ml}$) to the HA solution before freeze-drying. In order to evaluate the effect of laminin and NGF inclusion in the hydrogel luminal filler on ONS cell differentiation, ONS cells were cultured for 7 days on the hydrogels in media (DMEM/F12/FBS/P/S) or media containing 50 ng/ml recombinant rat NGF and examined using immunostaining.

To produce the fully assembled NGC, the external tubular collagen conduits (95% porosity) were rehydrated in 0.025 M acetic acid. HA hydrogels were resuspended in H_2O and injected into the lumen of the tubular conduit using 30-gauge needles. The filled biphasic NGCs were then lyophilized using directional freeze-drying to produce unidirectional aligned pores within the luminal filler. The final constructs were then cross-linked and sterilized by DHT.

To evaluate the effect of cell seeding, ONS cells were injected into the core of the NGC at a concentration of 50×10^4 cells using 24-gauge hypodermic needles. Cell retention and attachment was evaluated after 72 hours by flash freezing and embedding specimens in OCT medium. Twenty micrometer longitudinal sections were cut and the cytoskeleton was stained using phalloidin conjugated with tetramethylrhodamine and nuclear counterstaining.

Animal Model

Animals were obtained from Harlan Laboratories U.K. The in vivo study incorporated one negative control group and three experimental treatment groups (a) cell-free NGC, (b) ONS cell-loaded NGC (NGC and ONS), and (c) ONS cell-loaded NGC with recombinant rat NGF (NGC, ONS, and NGF). In order to confirm the lack of spontaneous regeneration through the defect, negative control animals were maintained on study for an extended period of time (16 weeks) whilst experimental treatment groups were maintained until euthanasia at 8 weeks.

Animals were anesthetized with 5% isoflurane in oxygen within a sealed induction chamber. Anesthesia was maintained with 2%–3% isoflurane administered via a sealed dual lumen facemask. Animals were prepped with chlorhexidine and placed prone in the operative field. The surgical site was shaved and

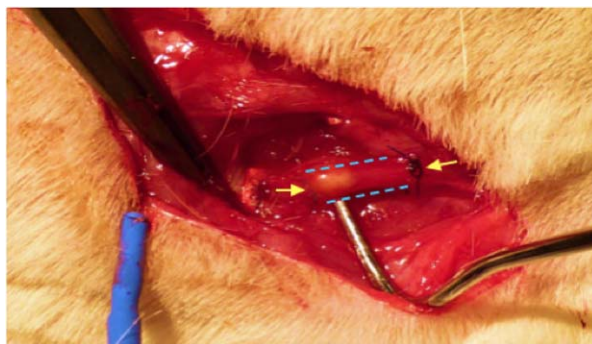


Figure 1. Nerve guidance conduit (NGC) in situ. Cuff sutured to perineurium. Treatment groups were implanted with either NGC alone, NGC and ONS cells, or NGC, ONS, and nerve growth factor.

procedures were performed on a heated mat to maintain core body temperature.

The right sciatic nerve was approached via a horizontal skin incision over the sciatic notch, using a posterior compartment muscle splitting technique. The sciatic nerve was identified using a Zeiss optical microscope and confirmed with a 2-mA disposable nerve stimulator set at 0.5 mA. A 10-mm interstump defect was resected. Muscle opposition was achieved with two loosely placed interrupted vicryl 4.0 sutures and the skin was closed with three to four interrupted 4.0 vicryl rapide sutures with derma bond tissue glue to reinforce the wound. In the negative control group, this marked the end of the procedure and the wound was closed, animals were recovered and monitored as previously described.

In experimental treatment group animals (Fig. 1), the gap in the sciatic nerve was bridged with the NGC with or without ONS cells and NGF. A 1-mm cuff of the external collagen conduit component of the NGC was fixed to the perineurium of the nerve stumps with 6–0-prolene sutures before muscle opposition and skin closure. 9 animals were treated with the NGC alone, 9 animals were treated with NGC and ONS (5×10^4 ONS cells at passage 3), and 9 animals were treated with the ONS cell seeded NGC with 50 ng recombinant rat NGF incorporated in the cell solute (NGC, ONS, and NGF).

Following closure, the wound was cleaned and dried. A local anesthetic block using 0.25% lignocaine was given before reversing the anesthetic. Once the operative procedure was complete, animals were monitored for signs of stress, pain, or wound infection. Additional analgesia was given as required. Where ulceration or marked self-mutilation by autophagy occurred, a protocol of daily washing with chlorhexidine, application of local anesthetic cream and maintenance of dry bedding was followed to prevent the development of secondary soft tissue infections.

Evaluation of the Degree of Peripheral Nerve Recovery Following Implantation of ONS Cells Within the Biphasic NGC

Animals were compared in terms of clinical and functional recovery in groups classified by intervention. Clinical signs of sciatic nerve injury including: muscle atrophy, contracture formation, presence of ulceration and self-mutilation were recorded at week 8 post intervention. The presence of sensation was evaluated by means of the nociceptive withdrawal reflex. The reflex was evaluated by stimulating the lateral plantar surface of the footpad using a sterile 24-gauge hypodermic needle.

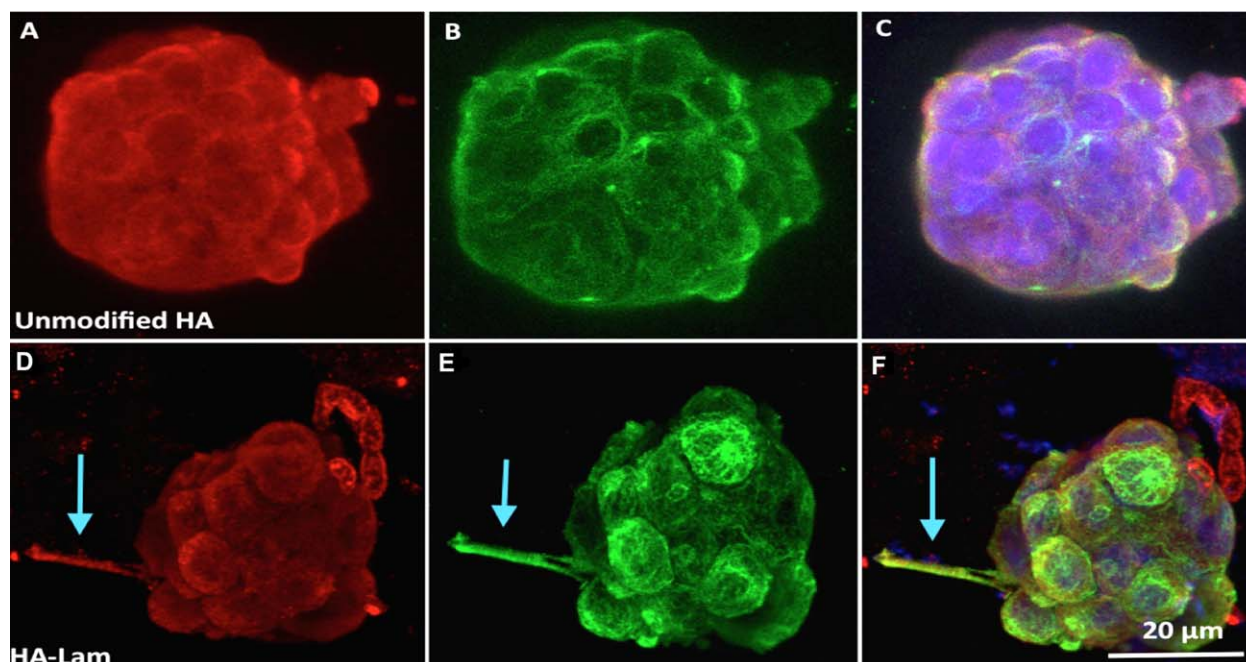


Figure 2. Olfactory neuroepithelial derived stem (ONS) cells in culture on HA hydrogels. **(A–C):** Spheroidal configuration of ONS cells on unmodified HA hydrogel. **(D–F):** ONS cells on HA-Lam hydrogel. In comparison with unmodified HA hydrogels, ONS cells demonstrated more phenotypic extension (blue arrows). **(A, D)** single staining for nestin, **(B, E)** single staining for β -tubulin **(C, F)** composite images with nestin (red) nuclear stain (blue) and β -tubulin (green). The scale bar for all images is 20 μ m. Abbreviations: HA, hyaluronic acid; HA-Lam, HA-Laminin.

Animals without the presence of autophagy ($N = 7$) were compared in terms of static variant sciatic function index (SFI) as described by Baptista et al. [25]. Each animal was placed on a clear plexi glass surface and distances between the points of maximum contact were measured. Static SFI was calculated using the following formula: $SFI = 118.9(\text{Toe spread factor [TSF]}) - 51.2(\text{paw length factor [PLF]}) - 7.5$. TSF and PLF of the operated limb was calculated with the formula $(\text{injured} - \text{uninjured})/\text{uninjured}$. Once obtained, the TSF and PLF were used to estimate the static SFI.

Electrophysiological testing was performed by means of compound muscle action potential (CMAP) measurement and gross peak tensile and compressive force analysis in response to the application of electrical stimulation to the treated nerve.

At 8 weeks for experimental treatment groups, animals were placed under a second general anesthetic. At second surgery, the sciatic nerve was exposed in both the operated limb and the contralateral nonoperated limb. CMAP evaluation was performed using a Neurosign 100 two-channel surgical nerve monitor.

A 0.5 mA electrical stimulus was passed from the stimulator probe first through the contralateral nonoperated sciatic nerve to establish a baseline CMAP value for the nonoperated sciatic nerve. The same current was passed through the interventional sciatic nerve from the proximal stump, through the device to the muscle innervated by the distal segment. For each test, both the nonoperated and the treated nerve were stimulated three times and the best measurement documented in mA. Because all animals' weights, size, nerve diameter and best nonoperated nerve conductivity differed, the CMAP results of the treated nerve were reported as a percentage of the value obtained from the animal's contralateral nonoperated nerve.

Assessment of the gross force generated in response to the applied electrical stimulus was performed using a 10 N digital push pull force gauge (accuracy ± 0.1 N) attached to the pad of

each foot with a 2.0 silk-braided suture. The maximum peak tensile and peak compressive force generated by the hind limb in response to a 0.5mA stimulus along a standardized anterior-posterior vector was measured. A percentage of the value of the nonoperated limb was calculated.

Whilst under second anesthesia, both the nonoperated and treated sciatic nerve (including the implanted NGC) were removed and placed directly into formalin for morphologic evaluation. Animals were then euthanized by cervical dislocation and decapitation under deep anesthesia.

Following sacrifice, an evaluation of gastrocnemius muscle weight was performed. The upper insertion of both bellies of the gastrocnemius muscle was carefully dissected on both the operated and nonoperated limb and the lower limbs were amputated at the tibiofemoral joint to allow assessment of the gastrocnemius muscle weight of each limb. Mean weight loss values as a percentage of the weight of the nonoperated limb were calculated.

To evaluate the degree of morphologic recovery, excised nerve tissue was processed for histological analysis. To examine the extent of morphologic recovery achieved following treatment, qualitative analysis by means of immunofluorescence staining and quantitative analysis by means of a semiautomated axon counting image analysis software program was performed. Nerve regeneration in the three treatment groups was assessed in terms of marker expression, axonal number, axonal calibre and axonal alignment.

Specimens from experimental treatment group animals and specimens from the contralateral nonoperated positive control nerve sections were fixed in 10% normal buffered formalin cryopreserved in 30% sucrose, flash-frozen in liquid nitrogen and embedded in OCT medium. The implant midpoint was isolated and transverse sections at 20 μ m thickness were mounted onto PDL precoated slides.

The following antibodies were used to determine neural and glial cell presence: anti-neurofilament, anti β -tubulin III (axons)

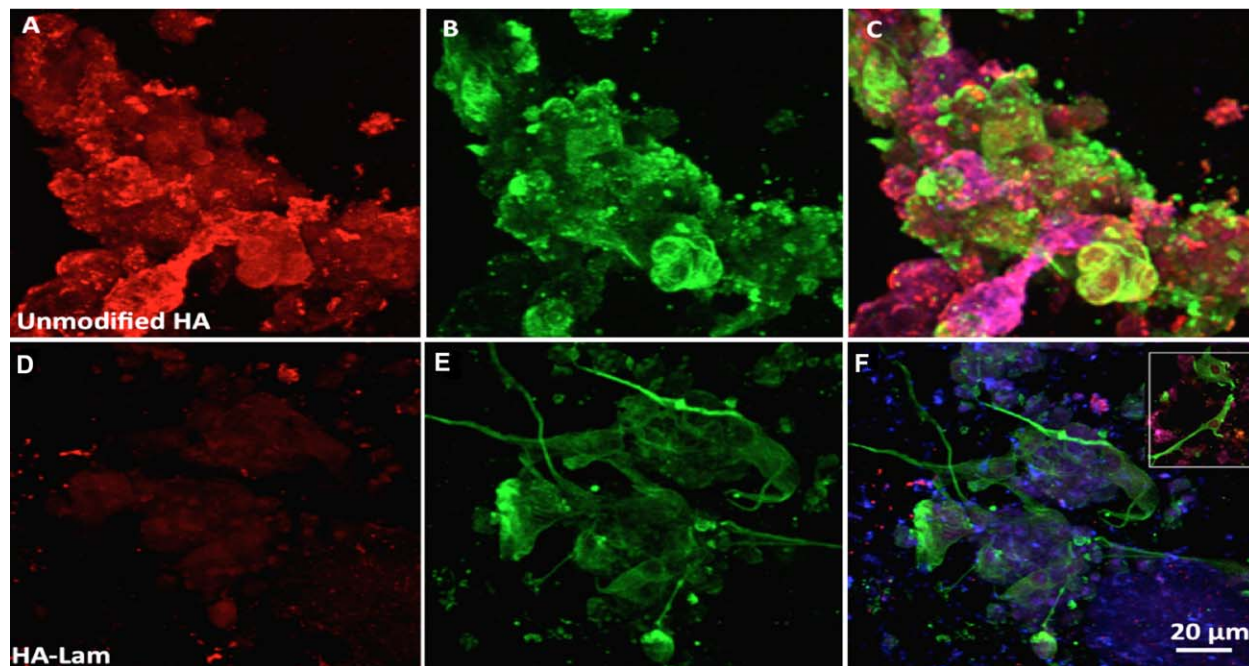


Figure 3. Olfactory neuroepithelial derived stem (ONS) cells in culture on HA hydrogels with media supplemented with nerve growth factor (NGF). **(A–C):** Spheroidal configuration of ONS cells on unmodified HA hydrogel supplemented with NGF. Matrix invasion and cell extension were not observed. **(D–F):** ONS cells on HA-Lam hydrogel supplemented with NGF. In comparison with unmodified HA hydrogels, ONS cells cultured on HA-Lam with NGF demonstrated marked phenotypic extension (F Insert) and matrix invasion. **(A, D)** single staining for nestin, **(B, E)** single staining for β -tubulin **(C, F)** composite images with nestin (red) nuclear stain (blue) and β -tubulin (green). The scale bar for all images is 20 μ m. Abbreviations: HA, hyaluronic acid; HA-Lam, HA-Laminin.

anti-S100 β and anti-myelin basic protein (MBP) (Schwann cells) anti-laminin and anti-fibronectin (basal membrane proteins). Required antibody dilutions were determined using intact peripheral nerve. After staining, the sections were covered with visualized using a fluorescent Nikon Eclipse 90i Microscope. Images were taken using Nikon DS-Ri1 camera.

Following imaging, the number, calibre, and alignment of axons at the mid-section of the explanted tissue was assessed. Images stained for axonal markers were processed for semiautomated quantitative analysis using java based image processing and analysis software. Images of β -tubulin and neurofilament stained sections of the same scale and magnification were converted into an 8-bit gray scale image. Manual adjustment of contrast, brightness and threshold values, allowed a representative image of the number of axons present per field of view to be generated and quantified. Areas of extra axonal debris or clumping were identified by visual inspection and eliminated from the analysis.

Statistical Methods

Statistical analysis was performed using one-way analysis of variance (ANOVA). Where statistically significant results were obtained, Dunnett's post hoc test was performed to compare groups versus the control group, NGC alone.

RESULTS

ONS cells with the potential to differentiate along neural and glial lineages in vitro were successfully isolated from 6-week-old Sprague Dawley rats (Supporting Information 4: results).

Laminin and Nerve Growth Factor Influenced Metabolic Activity and Differentiation of ONS Cells

2D immunofluorescence studies demonstrated that rat derived ONS cells have the potential to differentiate toward neural and glial lineage. Metabolic activity was improved in the presence of laminin (data not shown). Expression of β -tubulin, nestin and GFAP changed in response to the presence of laminin, indicating tunable differentiation. NGF addition to culture media had a pro-differentiation effect similar to that of laminin, but when added to laminin cultured cells, its effects were not synergistic (Supporting Information 4: results).

The Biphasic Collagen and Laminin Functionalized NGC Supported ONS Cells While the Addition of NGF Promoted Cell Elongation

Improved metabolic activity (98% viable cells) was achieved through functionalization of the luminal filler with laminin when compared to nonfunctionalized HA (64% viable cells). Furthermore, confocal images of ONS cells cultured in hydrogels functionalized with laminin demonstrated that NGF promoted cell extension in laminin functionalized conditions only (Figs. 2, 3).

Following demonstration that laminin enhanced ONS activity, it was incorporated into the biphasic NGC and ONS cells were injected. Nuclear staining demonstrated that migration of the injected ONS cells occurred through the center of the biphasic NGC at the 72-hour time point. This demonstrated first, that the laminin functionalized hydrogel was porous enough to allow infiltration and retention of cells; and second, that while the cells readily diffused through the hydrogel, they did not migrate into the tubular collagen conduit outer wall of the device, which was desirable to ensure that cells were retained in the center of the

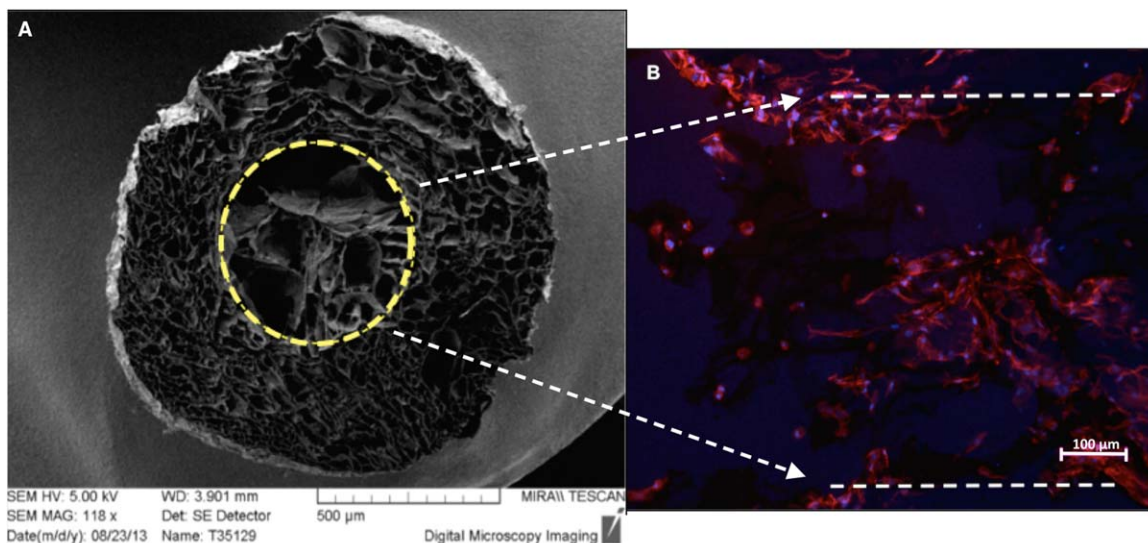


Figure 4. Biphasic NGC. **(A):** SEM photomicrograph demonstrating the cross-sectional appearance of the biphasic nerve guidance conduit (NGC). The dotted yellow line demonstrates the tubular collagen conduit—HA-Laminin luminal filler interface (Image Courtesy of Austyn Matheson and Alan Ryan TERG). **(B):** High magnification image of longitudinal section of the insert is shown with phalloidin stained cytoskeleton by cells distributed through the bi-layered tissue engineered NGC. Cell nuclei were counter stained with Hoechst nuclear stain.

NGC. ONS cells stained for F-actin, demonstrating that cells migrating through the gel were also able to extend processes (Fig. 4).

Improved Clinical, Functional, Electrophysiological, and Morphologic Outcomes Indicated Peripheral Nerve Recovery Following Implantation of ONS Cells Within the Biphasic NGC

There was one peri operative death due to respiratory arrest. The remaining 35 animals completed the experimental period. At 8 weeks post-surgery, all nine animals in the negative control group demonstrated muscle atrophy and contracture formation. Seven animals had developed ulceration and seven demonstrated moderate to severe autophagy. Failure of recovery in the negative control group was confirmed by electrophysiological testing at 16-weeks. No recordable electrical conductivity was measured in any animal. Inspection of the defect site demonstrated prolific scar tissue formation between the proximal and distal nerve stumps.

Improved clinical outcomes were noted in all experimental treatment groups. Wasting, contractures and autophagy were less prevalent in the animals, which were treated with ONS cells compared to the cell free NGC. Ulceration was found in similar numbers across groups (Fig. 5A–5C). A statistically significant improvement in sensory re-innervation (Fig. 5) was detected in animals treated with ONS cells compared to untreated animals or animals treated with NGC alone (p value $< .05$). The nociceptive withdrawal reflex was observed in three animals within the NGC and ONS treatment group and four animals within the NGC, ONS, and NGF treatment group. No reflex was detected in animals treated with the NGC alone.

Animals without evidence of autophagy ($N = 7$) were evaluated in terms of static variant SFI. The average static variant SFI for animals treated with the NGC alone was -28.5 the NGC and ONS was -26.8 and the NGC, ONS, and NGF was -55.09 . The differences in SFI between treatment groups were not statistically significant.

Statistically significant improvement in electrophysiological recovery (Fig. 5) was detected in all treatment groups compared to the negative control (p value $< .05$). No readable CMAP

response to the stimulus applied was detected negative control animals. Animals treated with the NGC and ONS or the NGC, ONS, and NGF showed a statistically significant improvement in CMAP values (as a percentage of the contralateral nonoperated nerve) when compared with animals treated with the NGC alone (p value $< .05$ for both). The mean percentage CMAP detected in both groups of ONS cell treated animals was 60% compared with 21% in the NGC alone group.

Muscle responses to electrical stimulation of the implant were present in all experimental treatment groups. Treatment with ONS cells improved electrophysiological outcomes in terms of peak tensile and compressive force generated compared with the cell-free control. Measured peak tension responses of the hind limb demonstrated significantly ($p > .05$) higher magnitude tensile force generation in animals treated with the NGC and ONS (mean = 47%) or the NGC, ONS, and NGF (mean = 52%) compared with animals treated with the NGC alone (mean = 18%). Similarly, peak compressive responses were improved (although this was nonsignificant— $p = .092$). In animals treated with either the NGC and ONS (mean = 52%) or the NGC, ONS, and NGF (mean = 64%) compared to animals treated with the NGC alone (mean = 24%). The dispersion of the results indicated a trend toward a higher concentration of relative peak compression in the NGC, ONS, and NGF treated animals when compared to the NGC alone. Further pair wise evaluation of the differences across treatment groups showed that there was a statistically significant improvement in peak compressive force generated when NGF with ONS cells were added to the NGC compared to the NGC alone (p value $< .05$) suggesting that the addition of NGF improved peak compression force values.

The mean gastrocnemius muscle weight loss was 2.5 g for NGC, ONS, and NGF treated animals compared to 3 g for NGC and ONS treated animals and 4.78 g for animals treated with the NGC alone. There was a statistically significant reduction in gastrocnemius muscle depletion in the NGC, ONS, and NGF treated group indicating that the addition of NGF with ONS cells significantly modulated the effect of the NGC alone (p value $< .05$).

Parameter tested	NGC	NGC & ONS	NGC, ONS & NGF
Muscle atrophy	9	7	7
Contracture	7	3	3
Ulceration	3	2	2
Autophagy	6	3	3
Nocioceptive withdrawal reflex	0	3	4
Sciatic function inde	-28.5	-26.79	-55.09

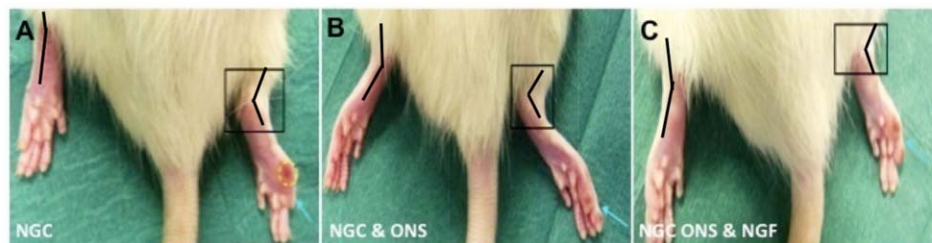


Figure 5. Improved clinical outcomes were noted in all experimental treatment groups. Table (top) demonstrating clinical outcomes per group. Images (bottom) showing recovery of the nerve morphology following treatment (A–C) compared to the nonoperated positive control (Left hind-limb). Contractures (reduced angle shown within the black squares), ulcers (yellow circles) and autophagy of the lateral toes (blue arrows) are highlighted. (A): Animal treated with the NGC alone. (B): Animal treated with NGC and ONS. (C): Animal treated with NGC, ONS, and NGF. Abbreviations: NGC, nerve guidance conduit; NGF, nerve growth factor; ONS, olfactory neuroepithelial derived stem.

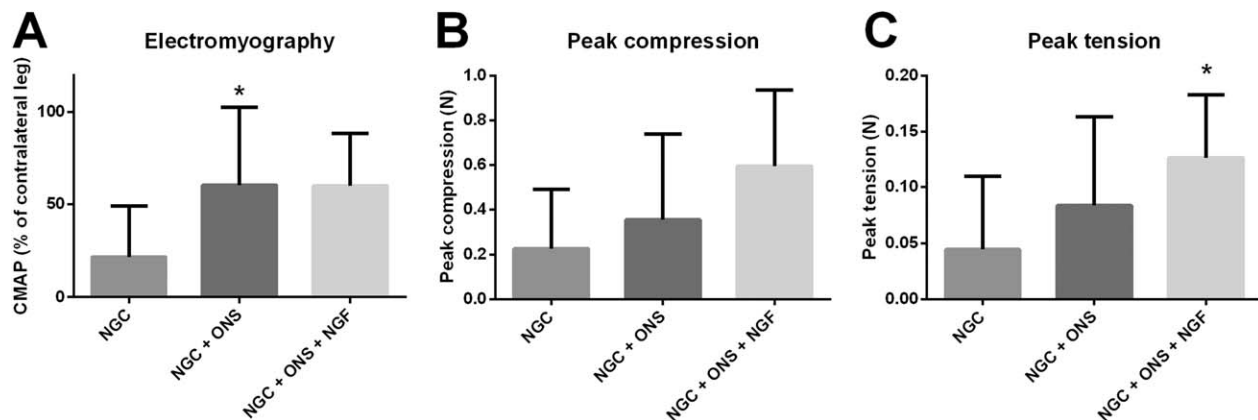


Figure 6. Electrophysiological recovery was detected in all treatment groups. Electromyographical testing (A) demonstrated that the addition of ONS cells resulted in 2.79-fold increase in compound muscle action potential versus the NGC alone ($p < .05$), while the further addition of NGF resulted in a similar increase of 2.76-fold ($p > .05$). (B) Peak compression force was increased by 2.6-fold with the addition of ONS cells + NGF, although this was nonsignificant ($p = .092$). (C) Peak tension was improved by 1.9-fold with the addition of ONS cells ($p > .05$) and by 2.9-fold with the further addition of NGF ($p < .05$). Asterisk indicates statistically significant difference ($<0.05\%$) versus NGC control group. Abbreviations: NGC, nerve guidance conduit; NGF, nerve growth factor; ONS, olfactory neuroepithelial derived stem.

In addition to ECM proteins laminin and fibronectin, infiltrating cells, axonal ingrowth and Schwann cells were identified in all treatment groups (Fig. 7A–7D). Statistically significant increases in axonal number were observed between ONS cell treated animals compared to cell free NGC treated animals. NGF enhancement of ONS cells also had a significant impact on the same parameter; there was a stepwise improvement in average axonal count across the treatment groups. NGC treated animals yielded an average

axonal count per field of view of 4,671 compared to the NGC and ONS or NGC ONS and NGF treated animals, which yielded average axonal counts of 6,751 and 9,925 respectively. Overall, the addition of ONS cells resulted in a 44.5% increase in axon count ($p > .05$), while the combination of ONS cells + NGF resulted in a 212.5% increase in axon count versus the NGC alone ($p < .05$).

The average diameter of axons generated in the NGC treatment group was 3.6 μm . By comparison, the average diameter of

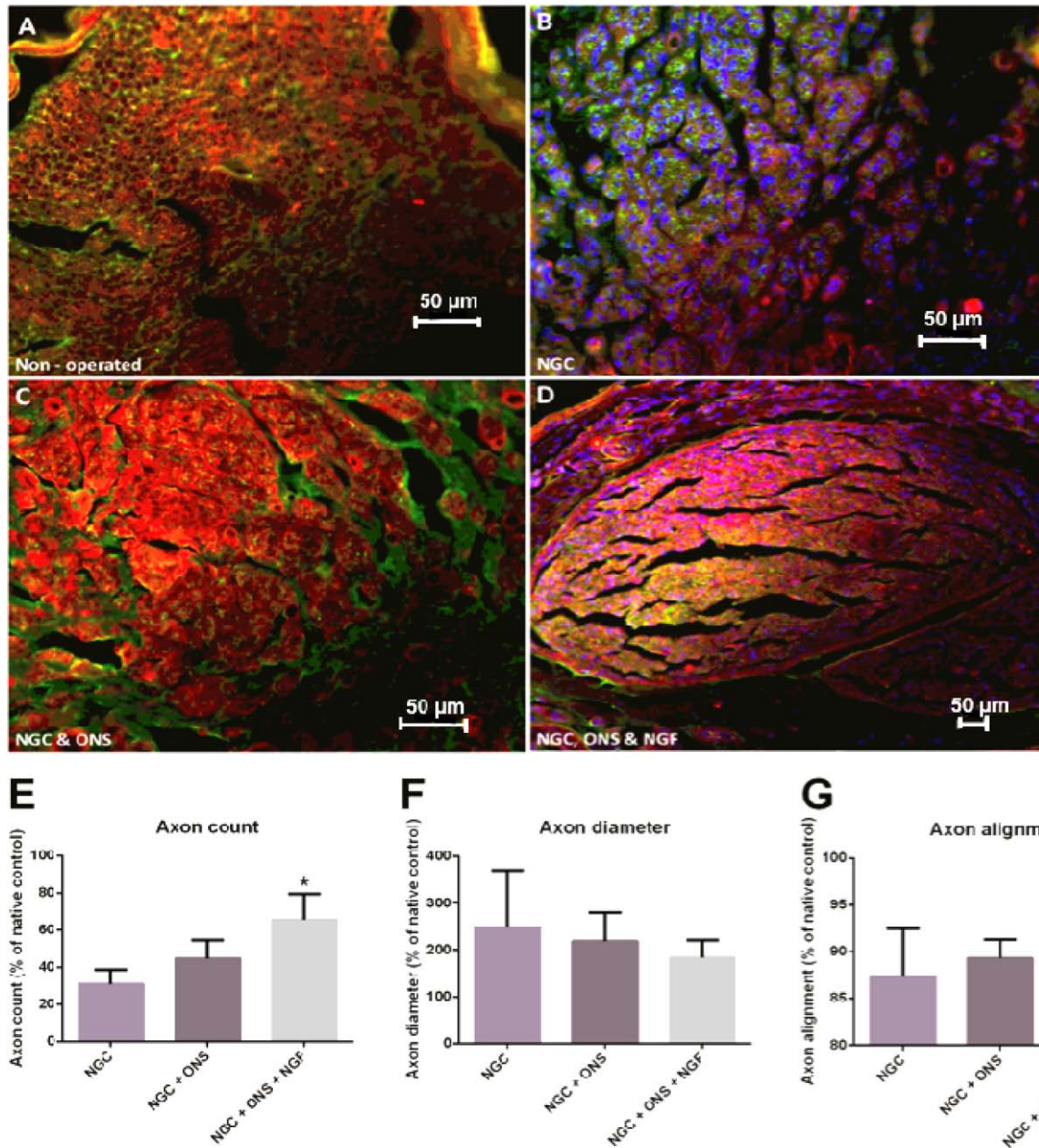


Figure 7. Recovery of nerve morphology was detected in all treatment groups. Representative immunofluorescent images showing recovery of the nerve morphology versus the control (A) following treatment with NGC (B), NGF + ONS (C), and NGF + ONS + NGF (D). Mid-conduit sections in all treatment showed signs of morphological recovery comparable to the positive control. Nuclei = blue. Extracellular matrix laminin = red and fibronectin = green. Scale bar in all images – 50 μm. The addition of ONS cells resulted in (E) a 44.5% increase in axon count ($p > .05$), while the combination of ONS cells + NGF resulted in a 212.5% increase in axon count versus the NGC alone ($p < .05$). Axon diameter (F) was improved by 11.7% ($p > .05$) with ONS cell addition and 26% by the combination of ONS cells + NGF ($p > .05$). Axon alignment (G) was not significantly altered with the addition of either ONS cells alone or in combination with NGF (2.2% and 4.4% increases, respectively, $p > .05$). Asterisk indicates statistically significant difference ($<0.05\%$) versus NGC control group. Abbreviations: NGC, nerve guidance conduit; NGF, nerve growth factor; ONS, olfactory neuroepithelial derived stem.

axons generated in the NGC and ONS treatment group was 4 μm and the average diameter of axons generated in the NGC, ONS, and NGF treatment group was: 3.61 μm, equating to an 11.7% improvement in diameter with ONS cell addition and 26% improvement with the combination of ONS cells + NGF. Axonal diameter in all treated animals was not significantly different from axonal diameter in the normal contralateral nonoperated nerve (p value $> .05$). Anisotropy was also evident in all treatment groups (ranging from 79% to 93%) but the addition of ONS cells and NGF

modulated the effect of the NGC. Axonal alignment in NGC, ONS, and NGF treated animals was not significantly different from axonal alignment in the normal contralateral nonoperated nerve (p value $> .05$).

DISCUSSION

The objective of this study was to investigate the potential of ONS cells delivered in a biphasic NGC to promote peripheral nerve

repair. Our data indicates that ONS cells can have a pro-regenerative effect on peripheral nerve repair *in vivo*. ONS cell treated animals performed better than those treated with a cell free NGC across a comprehensive panel of clinical, functional, electrophysiological, and morphological parameters. The results presented in this study have implications for future therapeutic applications using autologous stem cells to enhance peripheral nerve repair.

We targeted ONS cells as a potential stem cell source for a number of reasons. Whilst previously, Schwann cells were considered the reference standard cells for peripheral nerve repair models, in large gap injury zones they have been found to be inadequate and the extraction processes required to harvest them have for many an unacceptable morbidity profile. Stem cells are becoming a preferable option, and less invasive sources that retain clonogenicity whilst demonstrating preferential differentiation toward neural and glial lineages are especially desirable [26, 27]. In this regard, ONS cells represent a very good option, they are multipotent, easy to access and the harvesting process in humans has an acceptable safety profile. In addition to possessing an excellent capacity for regeneration, ONS cells have also been shown to express nerve growth factor receptors, successfully survive transplantation into different environments and produce extracellular matrix receptors, which are not present in other neural stem cells [28–30].

In this study, ONS cells demonstrated multipotency through the expression of stem cell markers β -tubulin, nestin, and GFAP, which validated our choice of cells. However, in order to maximize the potential of cell based therapeutic approaches to peripheral nerve repair, a biomaterial-based carrier for the cells is ideally required. We specifically aimed to use a carrier with a composition and structure optimized to harness the potency of the ONS cells. Although collagen and HA have both been used extensively in nerve regeneration, functionalizing agents are often required to promote cell attachment and enhance myelination. ONS cells are known to have an excellent affinity to ECM molecules. In this study, laminin enhanced the metabolic activity of the cells. Furthermore, enhanced cell outgrowth was seen when NGF was added as a secondary stimulus. These findings helped to optimize the composition and culture regime used in the NGC, which was then taken forward in order to attempt to facilitate functional peripheral nerve regeneration in a critical nerve defect *in vivo*.

In the *in vivo* study, evidence of functional and morphological regeneration (most notably the nociceptive withdrawal reflex CMAP measurement and axonal count) of the injured nerve was seen across all experimental treatment groups in comparison to the nontreated control. Histomorphological analysis indicated statistically significant differences in axon count between the treatment groups; with improved counts achieved in ONS and NGC, and ONS and NGF treated animals versus the cell free NGC. The data indicated that the NGC promoted regeneration and that its effects were enhanced with the addition of ONS cells and NGF across almost all parameters tested. Moreover, a return of the nociceptive withdrawal reflex in ONS cell treated animals (indicating regeneration of both efferent and afferent axons), which was not detected in animals treated with the NGC alone, suggested an advanced repair stage at a relatively early time point of 8 weeks post implantation. Improvement in terms of CMAP and morphological repair (axon count, axonal calibre, and anisotropy) clearly demonstrated that the addition of ONS cells with and

without NGF modulated the functional regenerative effects of the developed NGC.

In terms of the current state of the art, there are typically two approaches to peripheral nerve regeneration: cell-free tissue-engineered nerve grafts and cell based therapeutic approaches. Taking the carrier in isolation, morphologically, the cell free NGC performed well in comparison to recent studies over similar defect distances on other biomaterials [31, 32]. The axons generated by the NGC in this study gave better results in terms of total axon count (NGC gave a mean axon count of 4,671 compared to Costas [30] reported 4,225.2 or de Ruiters [32] reported 2,925 for a PGLA multichannel tube) and critically, axonal calibre (3.6 μm compared to de Ruiters 4.01). Interestingly, a significant difference in axonal caliber between the NGC treatment group and the calibre of the axons of nonoperated nerve was not observed. However, variances in axon diameter in the NGC treatment group suggested that regenerated axons were wider than the mature axons found in the nonoperated limb, reflecting recent studies demonstrating variable calibres in axonal regeneration in rats [32]. There are a number of possible explanations for why this may have occurred (i.e., target specific signals; cell extensions effects on growth cones; configurational alterations in axonal microtubules; differences in the biophysical environment) [33, 34]. From this analysis, it was not possible to ascertain the precise reason behind the variation but given the importance of axonal diameter on the speed of neuronal impulse transmission, it is a finding, which warrants closer investigation.

Although morphologically, the NGC alone performed well, the addition of ONS cells and NGF enhanced its biological effect resulting in improved clinical, functional and electrophysiological outcomes when compared to the NGC alone. In comparison to cell based therapeutic approaches in the recent literature, using alternative cell sources (Schwann cells, bone marrow derived mesenchymal stem cells (MSCs) and adult adipose derived stem cells (ADCs) [35]) the ONS cell treated groups in this study performed exceptionally well. MSCs have been shown to improve functional outcomes and morphological appearances of regenerated nerve tissue in rats [36], while a 3.5-fold increase in axon numbers has been demonstrated to occur when ADCs were added to engineered neural tubes (mean axon count 3,400) [37]. The ONS treated groups in our study generated more axons (6,751 for NGC and ONS treated animals, and 9,925 in animals treated with NGC, ONS, and NGF) than our cell free treatment groups. Further to increasing axon counts, improved alignment was demonstrated with the addition of ONS cells with and without NGF (91% and 89%, respectively). This was an important finding because achieving functional peripheral nerve regeneration requires alignment of neurons to bridge the nerve gap. It supported the theory that cell and growth factor inclusion in a tissue-engineered scaffold approach to peripheral nerve regeneration can enhance the directional growth of new axons and neurons toward the target organ [38].

In addition to the morphological findings in both cell free and ONS treated groups, the manner by which the regenerated axons affected functionality provided further indication of the benefit of ONS cell inclusion in the system. The assessment of nociceptive responses clearly indicated that regrowth of sensory fibers was supported to a higher extent with the ONS cell based therapeutic approach. This finding was corroborated by the electrophysiological analysis on CMAP measurements, which reflected both the number of axons counted, and their alignment. Mechanical force

analysis and weight evaluation highlighted a reduction in muscle atrophy and increased peak tensile and compressive forces when NGF was included in the treatment.

Ultimately this study has demonstrated that multipotent ONS cells harvested from the nasal mucosa are a viable source of progenitor cells for use in peripheral nerve repair systems. They have shown better results than a cell free NGC across a comprehensive panel of clinical, functional, electrophysiological, and morphological parameters. This study also demonstrates that the environment in which stem cells are implanted is of critical importance in terms of their regenerative effects. We found a collagen outer conduit containing a directional freeze-dried HA-laminin luminal filler to be an optimum delivery system for ONS cells. Furthermore, we found the regenerative effect of the ONS cells was enhanced in the presence of NGF, indicating that a dual stem cell-growth factor treatment strategy is advantageous in peripheral nerve repair models.

CONCLUSION

This study has shown improved clinical and electrophysiological outcomes in ONS cell treated animals. Nerves regenerated across ONS cell loaded NGCs with significantly more axons and the presence of the nociceptive withdrawal reflex in ONS cell treated animals indicated advanced repair. This process was further enhanced in the presence of NGF indicating that a combined cell/growth factor approach may be advantageous for peripheral nerve repair.

REFERENCES

- 1 Uzun N, Tanriverdi T, Savrun FK et al. Traumatic peripheral nerve injuries: Demographic and electrophysiological findings of 802 patients from a developing country. *J Clin Neuromuscul Dis* 2006; 7:97–103.
- 2 Noble J, Munro CA, Prasad VS et al. Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries. *J Trauma Acute Care Surg* 1998;45:116–122.
- 3 Grinsell D, Keating CP. Peripheral nerve reconstruction after injury: A review of clinical and experimental therapies. *BioMed Res Int* 2014;2014:13.
- 4 Bhandari PS, Sadhotra LP, Bhargava P et al. What is new in peripheral nerve repair?. *Indian J Neurotrauma* 2007;4:21–23.
- 5 Pfister BJ, Gordon T, Loverde JR et al. Biomedical engineering strategies for peripheral nerve repair: Surgical applications, state of the art, and future challenges. *Crit Rev Biomed Eng* 2011;39:81–124.
- 6 Splinter R, ed. *Handbook of physics in medicine and biology*. CRC Press, 2010.
- 7 Dezawa M. Central and peripheral nerve regeneration by transplantation of Schwann cells and transdifferentiated bone marrow stromal cells. *Anat Sci Int* 2002;77:12–25.
- 8 Johnson AP, Wood MD, Moore AM et al. Tissue engineered constructs for peripheral nerve surgery. *Eur Surg* 2013;45:122–135.
- 9 Höke A. Mechanisms of disease: What factors limit the success of peripheral nerve regeneration in humans? *Nat Clin Pract Neurol* 2006;2:448–454.

ACKNOWLEDGMENT

This study has received funding from the European Research Council under the European Community's Seventh Framework Programme (FP7/2007-2013) under ERC grant agreement n° 239685.

AUTHOR CONTRIBUTIONS

P.R.: Conception and design, in vitro and in vivo experiments, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript, financial support; T.A.: In vitro and in vivo experiments, collection and/or assembly of data, data analysis and interpretation; A.W.: In vitro experiments, collection and/or assembly of data, data analysis and interpretation; A.R.: In vitro experiments, collection and/or assembly of biomaterials data, data analysis and interpretation; A.M.: In vivo experiments, collection and/or assembly of data, data analysis and interpretation; M.W.: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript; G.P.D.: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript; FJO'B: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript, financial support.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

- 10 Cooke MJ, Vucic K, Shoichet MS. Design of biomaterials to enhance stem cell survival when transplanted into the damaged central nervous system. *Soft Matter* 2010;6:4988–4998.
- 11 Meng X, Leslie P, Zhang Y et al. Stem cells in a three-dimensional scaffold environment. *Springerplus* 2014 11;3:1.
- 12 Delorme B, Nivet E, Gaillard J et al. The human nose harbors a niche of olfactory ectomesenchymal stem cells displaying neurogenic and osteogenic properties. *Stem Cells Dev* 2009 11;19:853–866.
- 13 Au E, Roskams AJ. Olfactory ensheathing cells of the lamina propria in vivo and in vitro. *Glia* 2003;41:224–236.
- 14 Mackay-Sim A. Stem cells and their niche in the adult olfactory mucosa. *Arch Ital Biol* 2010;148:47–58.
- 15 Tabakow P, Jarmundowicz W, Czapiga B et al. Transplantation of autologous olfactory ensheathing cells in complete human spinal cord injury. *Cell Transplant* 2013;22:1591–1612.
- 16 Daly W, Yao L, Abu-rub M et al. The effect of intraluminal contact mediated guidance signals on axonal mismatch during peripheral nerve repair. *Biomaterials* 2012;33:6660–6671.
- 17 McKee KK, Yang DH, Patel R et al. Schwann cell myelination requires integration of laminin activities. *J Cell Sci* 2012;125:4609–4619.
- 18 Chung TW, Yang MC, Tseng CC et al. Promoting regeneration of peripheral nerves in-vivo using new PCL-NGF/Tirofiban nerve conduits. *Biomaterials* 2011;32:734–743.
- 19 Chen Y, Wang D, Wang Z et al. Effect of adenovirus expressing NGF on sciatic nerve

injury in rats. (In Chinese). *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 2009;23:947–953.

- 20 Féron F, Perry C, McGrath JJ et al. New techniques for biopsy and culture of human olfactory epithelial neurons. *Arch Otolaryngol Head Neck Surg* 1998;124:861–866.
- 21 Haugh M, Murphy C, O'Brien F. Novel freeze-drying methods to produce a range of collagen-glycosaminoglycan scaffolds with tailored mean pore sizes. *Tissue Eng Part C Methods* 2010;16:887–894.
- 22 Haugh, M, Jaasma M, O'Brien FJ. The effect of dehydrothermal treatment on the mechanical and structural properties of collagen-GAG scaffolds. *J Biomed Mater Res Part A* 2009;89:363–369.
- 23 Harley BA, Spilker MH, Wu JW et al. Optimal degradation rate for collagen chambers used for regeneration of peripheral nerves over long gaps. *Cells Tissues Organs* 2004;176:153–165.
- 24 Tian W, Hou S, Ma J et al. Hyaluronic acid-poly-D-lysine-based three-dimensional hydrogel for traumatic brain injury. *Tissue Eng* 2005;11:513–525.
- 25 Baptista A, Gomes J, Oliveira J et al. A new approach to assess function after sciatic nerve lesion in the mouse—Adaptation of the sciatic static index. *J Neurosci Methods* 2007; 161:259–264.
- 26 Mukhatyar V, Karumbaiah L, Yeh J et al. Tissue engineering strategies designed to realize the endogenous regenerative potential of peripheral nerves. *Adv Mater* 2009;21:4670–4679.
- 27 Kingham P, Kalbermatten D, Mahay D et al. Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote

neurite outgrowth in vitro. *Exp Neurol* 2007; 267–274.

28 Murrell W, Féron F, Wetzig A et al. Multipotent stem cells from adult olfactory mucosa. *Dev Dyn* 2005;233:496–515.

29 Heckroth J, Graziadei G, Graziadei P. Intraocular transplants of olfactory neuroepithelium in rat. *Int J Dev Neurosci* 1983;1:273–287.

30 Mackay-Sim A, Chuah M. Neurotrophic factors in the primary olfactory pathway. *Prog Neurobiol* 2000;62:527–559.

31 Costa MP, Teixeira NH, Longo MV et al. Combined polyglycolic acid tube and autografting versus autografting or polyglycolic acid tube alone. A comparative study of

peripheral nerve regeneration in rats. *Acta Cir Bras* 2015;30:46–53.

32 de Ruiter GC, Spinner RJ, Malessy MJ et al. Accuracy of motor axon regeneration across autograft, single lumen, and multichannel poly (lactic-co-glycolic acid) (PLGA) nerve tubes. *Neurosurgery* 2008;63:144.

33 Dent EW, Tang F, Kalil K. Axon guidance by growth cones and branches: Common cytoskeletal and signaling mechanisms. *Neuroscientist* 2003;9:343–353.

34 De Boer J, Van Blitterswijk C, Thomsen P et al. *Tissue Engineering*. 2nd ed. Academic Press, Elsevier, 2014.

35 di Summa, PG, Kingham PJ, Raffoul W et al. Adipose-derived stem cells enhance

peripheral nerve regeneration. *J Plast Reconstr Aesthet Surg* 2010;63:1544–1552.

36 Matthes SM, Reimers K, Janssen I et al. Intravenous transplantation of mesenchymal stromal cells to enhance peripheral nerve regeneration. *BioMed Res Int* 2013;2013:573169.

37 Georgiou M, Golding JP, Loughlin AJ et al. Engineered neural tissue with aligned, differentiated adipose-derived stem cells promotes peripheral nerve regeneration across a critical sized defect in rat sciatic nerve. *Biomaterials* 2015;37:242–251.

38 Bellamkonda RV. Peripheral nerve regeneration: An opinion on channels, scaffolds and anisotropy. *Biomaterials* 2006;27:3515–3518.



See www.StemCellsTM.com for supporting information available online.