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NERVE REPAIR WITH ADIPOSE-DERIVED STEM CELLS PROTECTS DORSAL ROOT GANGLIA NEURONS FROM APOPTOSIS

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Abstract—Novel approaches are required in the clinical management of peripheral nerve injuries because current surgical techniques result in deficient sensory recovery. Microsurgery alone fails to address extensive cell death in the dorsal root ganglia (DRG), in addition to poor axonal regeneration. Incorporation of cultured cells into nerve conduits may offer a novel approach in which to combine nerve repair and enhance axonal regeneration with neuroprotective therapies. We examined apoptotic mediator expression in rat DRG neurons following repair of a 10-mm sciatic nerve gap using a novel synthetic conduit made of poly ε-caprolactone (PCL) and primed with adipose-derived stem cells (ADSC) differentiated towards a Schwann cell phenotype or with primary adult Schwann cells. Differentiated ADSC expressed a range of neurotrophic factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and neurotrophin-4 (NT4). Incorporation of either differentiated ADSC or Schwann cells significantly increased anti-apoptotic Bcl-2 mRNA expression ($P<0.001$) in the DRG, while significantly decreasing pro-apoptotic Bax ($P<0.001$) and caspase-3 mRNA ($P<0.01$) expression. Cleaved caspase-3 protein was observed in the DRG following nerve injury which was attenuated when nerve repair was performed using conduits seeded with cells. Cell incorporation into conduit repair of peripheral nerves demonstrates experimental promise as a novel intervention to prevent DRG neuronal loss.

Key words: peripheral nerve injury, dorsal root ganglion, neuronal apoptosis, neuroprotection, rat, conduit.

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Abbreviations: ADSC, adipose-derived stem cells; BDNF, brain-derived neurotrophic factor; dADSC, differentiated ADSC; DRG, dorsal root ganglia; ELISA, enzyme-linked immunosorbent assay; GDNF, glial-derived neurotrophic factor; GGF-2, glial growth factor-2; NAC, *N*-acetylcysteine; NGF, nerve growth factor; NT4, neurotrophin-4; PCL, ε-caprolactone.

Novel approaches are required in the clinical management of peripheral nerve injury because current surgical techniques result in deficient sensory recovery, thereby leading to considerable patient morbidity and socioeconomic impact (Lundborg, 2000; Rosberg et al., 2005). Advanced microsurgical techniques fail to address crucial neurobiological problems such as the extensive cell death in sensory neurons of the dorsal root ganglia (DRG) (Groves et al., 1997; McKay Hart et al., 2002), in addition to insufficient, slow, and misdirected axonal outgrowth at the site of injury (Fu and Gordon, 1997; Dahlin, 2004). Innovative experimental strategies have attempted to address these two problems independently by means of adjuvant neuroprotective pharmacotherapy and tissue-engineered constructs respectively (Evans, 2000; Hart et al., 2008); however, the recent incorporation of cultured cells into nerve conduits may offer a novel approach in which to combine nerve repair and enhanced axonal regeneration with neuroprotective therapies (Mosahebi et al., 2002; Tohill and Terenghi, 2004).

Recently, we demonstrated a novel synthetic conduit made of poly ε-caprolactone (PCL) has experimental promise (Sun and Downes, 2009; Sun et al., 2010). This material possesses many features that would suggest an ideal nerve conduit; in particular it is biodegradable, flexible with sufficient strength for clinical handling, sterilizable, non-toxic, non-immunogenic, and importantly can incorporate supportive cells into its structure. Bioengineered nerve conduits have endeavored to do more than simply provide a pathway for regenerating axons by incorporating growth factor delivery or exogenous cell support (Fine et al., 2002; Xu et al., 2003; Kemp et al., 2008; Novikova et al., 2008). Schwann cells are essential for peripheral nerve regeneration and transplanted Schwann cells seeded in a nerve conduit have been shown to enhance axonal regeneration (Guénard et al., 1992; Mosahebi et al., 2002); however, this has limited clinical application since harvest of autologous Schwann cells requires an invasive nerve biopsy followed by lengthy treatment delays while the cells are cultured and expanded *in vitro* (Guest et al., 1997). As a clinically viable alternative, attention has shifted towards exploiting stem cells which proliferate rapidly and can integrate into the host with immunological tolerance (Tohill and Terenghi, 2004). One of perhaps the easiest to harvest and certainly an abundant source of precursor cells are the recently described adipose-derived stem cells (ADSC) (Gimble and Guilak, 2003; Strem et al., 2005). Undifferentiated precursor ADSC used in a nerve conduit may modestly reduce the atrophy of target muscle; however, the regenerative milieu within the conduit does not

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appear to promote differentiation (Santiago et al., 2009). ADSC have a multi-potent profile and when differentiated to a Schwann cell phenotype they show ability to promote both sensory and motor neurite outgrowth and myelinate neurons *in vitro* (Kingham et al., 2007; Jiang et al., 2008; Xu et al., 2008). Recently we showed that these differentiated ADSC also have the ability to enhance early peripheral nerve regeneration (di Summa et al., 2010, 2011).

Axotomy-induced neuronal cell death is mediated by apoptosis, which presents potential targets for neuroprotective intervention. Adjuvant pharmacotherapy in the form of *N*-acetylcysteine (NAC) almost eliminates sensory neuronal death *in vivo* (Hart et al., 2008), and probably mediates its actions at the level of the mitochondria on apoptotic mediators such as Bcl2, Bax, and caspase-3 (Reid et al., 2009). Primary nerve repair alone is only partially neuroprotective and must be performed early (McKay Hart et al., 2002; Ma et al., 2003). The exact mechanism by which surgical repair confers neuroprotection is unknown, although it is believed that a cocktail of neurotrophic factors are exuded by regenerative Schwann cells in the distal stump which then act locally alongside extracellular matrix molecules to guide growth cones, but may also be retrogradely transported to the neuronal cell body and aid in survival signaling (Hall, 2005; Hart et al., 2008). Consequently, it stands to reason that stem cells with the potential to act like Schwann cells in a bioengineered conduit may provide sensory neuroprotection. This study set out to examine apoptotic mediator expression in DRG neurons following peripheral nerve repair with differentiated ADSC-loaded conduits as compared to exogenous Schwann cell-loaded and empty conduits.

EXPERIMENTAL PROCEDURES

Experimental design

We compared three groups of experimental peripheral nerve repair of a 1-cm gap—1) empty PCL conduit; 2) PCL conduit primed with primary adult Schwann Cells; and 3) PCL conduit primed with differentiated ADSC. Outcome measures compared were the gene and protein expressions of apoptotic mediators caspase-3, Bax, and Bcl-2 in the DRG in order to demonstrate the potentially neuroprotective effect of the implanted cells.

Cell culture

Adipose-derived stem cells (ADSC). The animal care and experimental procedures were performed in accordance with the terms of the Animals (Scientific Procedures) Act 1986 and the number of animals used was kept to a minimum. ADSC were isolated from adult Sprague–Dawley rats euthanased by cervical dislocation as described previously (Kingham et al., 2007). Visceral fat encasing the stomach and intestines was carefully dissected and minced using a sterile razor blade. Tissue was then enzymatically dissociated for 60 min at 37 °C using 0.15% (w/v) collagenase type I (Invitrogen, UK). The solution was passed through a 70-μm filter to remove undissociated tissue, neutralized by the addition of Modified Eagle Medium (α-MEM; Invitrogen, UK) containing 10% (v/v) fetal bovine serum (FBS) and centrifuged at 800×*g* for 5 min. The stromal cell pellet was re-suspended in MEM containing 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin solution. Cultures were maintained at sub-confluent levels in a 37 °C incubator with 5% CO_2 and passaged with trypsin/EDTA (Invitrogen, UK) when required.

Schwann cells (SC). Sciatic nerves were removed from adult rats under aseptic conditions. The epineurium was removed using a dissecting microscope. Nerves were cut into 1-mm pieces and placed in a petri dish containing Schwann Cell growth media containing 10-μM forskolin (Sigma, Poole, UK) and 63 ng/ml glial growth factor-2 (GGF-2; Acorda Theraputics Inc, Hawthorne, USA). The nerves were incubated for 2 weeks before the addition of 0.0625% (w/v) collagenase type 4 (Worthington Biochem, Lakewood, NJ, USA) and 0.585 U/mg dispase (Invitrogen, Paisley, UK) for 24 h. The nerves were titrated, filtered through a 70-μm cell strainer and centrifuged at 800 rpm for 5 min. The pellet was resuspended in 5-ml Schwann cell growth media and seeded in a 25-cm^2 flask. The cells were left to incubate at 37 °C/5% CO_2 until they reached confluence and then transferred to a 75-cm^2 flask before transplantation *in vivo*.

Stem cell differentiation

Growth medium was removed from sub-confluent ADSC cultures at passage 2 and replaced with medium supplemented with 1-mM β-mercaptoethanol (Sigma-Aldrich, UK) for 24 h. Cells were then washed and fresh medium supplemented with 35 ng/ml all-trans-retinoic acid (Sigma-Aldrich, UK) was added. A further 72 h later, cells were washed and medium replaced with differentiation medium; cell growth medium supplemented with 5 ng/ml platelet-derived growth factor (PDGF; PeproTech Ltd., UK), 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech Ltd., UK), 14 μM forskolin, and 252 ng/ml GGF-2 (Acorda Therapeutics, USA). Cells were incubated for 2 weeks under these conditions with fresh medium added approximately every 72 h. The expression of the Schwann cell proteins, S100 and GFAP, in differentiated cells was confirmed by immunocytochemistry as previously described (Kingham et al., 2007).

Qualitative RT-PCR and immunocytochemical characterization of stem cells

Total RNA was isolated from either ADSC or Schwann cells using an RNeasy™ kit (Qiagen, Sweden) and then 1-ng RNA was incorporated into the One-Step RT-PCR kit (Qiagen) per reaction mix. Primers were produced by Sigma, UK (Table 1). An MJ Research PTC-200 (gradient) cycler was used with the following parameters: a reverse transcription step (50 °C, 30 min), a nucleic acid denaturation/reverse transcriptase inactivation step (95 °C, 15 min) followed by 35 cycles of denaturation (95 °C, 30 s), annealing (30 s, optimized temperature per primer set), and primer extension (72 °C, 1 min) followed by final extension incubation (72 °C, 5 min). PCR amplicons were electrophoresed (50V, 90 min) through a 1.5% (w/v) agarose gel and the size of the PCR products estimated using Hyperladder IV (Bioline, UK). Samples were visualized under UV illumination following GelRed™ nucleic acid stain (BioNuclear, Sweden) incorporation into the agarose. The DNA sequence of each amplicon was confirmed using the Big DyeTM Terminator Sequencing Kit (Applied Biosystems Inc.) and protocol, followed by sequence analysis on the Prism 3100 Genetic Analyser (Applied Biosystems Inc.).

At passage 2–4, undifferentiated or Schwann cell differentiated ADSC (dADSC) were trypsinized and replated on Lab-Tek™ Chamber slides (Nunc-Fischer Scientific, UK) for 48 h. Samples were then fixed in 4% (w/v) paraformaldehyde for 20 min, washed with PBS solution (2×5 min), and then the cells were permeabilized (for neurotrophin antibody staining) using 0.2% (v/v) Triton X-100 for 20 min. After 3×10-min washes with PBS, normal goat and horse serum (5% v/v) was added for 1 h at room temperature. Mouse monoclonal antibodies anti-CD29 (1:100; R&D Systems, UK), anti-CD90 (1:200, BD Biosciences, UK), anti-NGF (1:100,

Table 1. Primer sequences for RT-PCR and annealing temperatures used (°C)

Factor	Forward primer (5'→3')	Reverse primer (5'→3')	°C
CD29	TGGGTCGCTGATTGGCTG	CTCTTCAGTGACTGCAAAAATCG	63.7
CD44	TCATGTTAGAGCATCCGTGC	GGGTTGTACATCATGCCTCC	61.0
CD90	TGAACCCAGTCATCAGCAT	CAGTCGAAGGTTCTGGTTCACC	61.9
CD105	ATCCAACACCATAGAGCTAG	TGGCTGAGGGGACAAGTTC	57.6
NGF	AAGGATCCTGGACCCAAGCTCACCTCA	GAGTGACGTGGATGAGCGCTTGCTCCT	67.0
BDNF	ATGGGACTCTGGAGAGCGTGAA	CGCCAGCCAATTCTCTTTTTGC	66.5
GDNF	TCACCAGATAAACAAGCGGC	TACATCCACACCGTTTAGCG	61.0
NT4	TTCTGGCTCCTGAGTGGGAC	CAGTCAACGCCCGCACATAG	64.9

Autogen Bioclear UK) and rabbit polyclonal antibody anti-BDNF (1:500, Autogen Bioclear, UK) were added and incubated at 4 °C overnight. The following day, after 3×10-min washes with PBS, FITC-conjugated anti-mouse IgG (1:100; Vector Laboratories, UK) for CD29, CD90, and nerve growth factor (NGF) and fluorescent-dye-Cy3-conjugated goat anti-rabbit (1:200; GE Healthcare, UK) for brain-derived neurotrophic factor (BDNF) were applied for 1 h at room temperature in the dark. Next the cells were washed 3×10 min with PBS and the slides mounted with Vectashield with DAPI (Vector Labs, UK). Omission of primary antibodies and incubation with secondary antibodies only was used to confirm the specificity of staining.

Enzyme-linked immunosorbent assay (ELISA)

15,000 dADSC or primary SC were seeded in 200-μl medium (n=4) in a 96-well plate. After 72 h of culture, the medium was analysed by ELISA using a rat beta NGF ELISA kit (RayBiotech Inc., USA) and a ChemiKine™ BDNF sandwich ELISA kit (Chemicon, Sweden) according to the respective manufacturer's protocols. The absorbance was measured at 450 nm using a Spectra Max 190 microplate reader (Molecular Devices, USA). All samples were analysed in triplicate. The quantity of neurotrophic factors (pg/ml) were calculated against standard curves produced using recombinant proteins provided in the kits.

Preparation of PCL conduits

PCL conduits were prepared as previously described (Sun and Downes, 2009). Briefly, 3% (w/v) of PCL (Sigma Aldrich, USA) was dissolved in dichloromethane (Fisher Scientific, UK) and spread evenly on degreased borosilicate glass cover slip. Following complete solvent evaporation, the films were treated in 10-N NaOH for 1 h with gentle shaking and washed in distilled H_2O. Films were cut into rectangular sheets and rolled around a 16-G intravenous cannula (Abbocath®, Abbott Ireland, Republic of Ireland), which standardized the internal diameter of the conduits at 1.6 mm, more than 1.5 times the diameter of rat sciatic nerve, thus allowing space for post-injury swelling. Conduits were sealed by controlled heating while still mounted on the cannula and then sterilized using UV radiation. 2×10^6 dADSC or Schwann cells suspended in 50-μl medium were injected into the PCL conduits which were then incubated at 5% CO_2, 95% humidity and 37 °C for 1 day prior to surgical implantation.

Surgical procedures and groups

Surgical procedures were performed under isoflurane general anesthesia, on young adult male Sprague–Dawley rats (180 g–220 g). Animals were allocated into three groups: empty PCL conduit (n=4), PCL conduit primed with primary adult Schwann cells (n=4), and PCL conduit primed with differentiated ADSC (n=4). All animals underwent sciatic nerve transection, and a 10-mm nerve gap was created at the level of the mid-femur. Under microscope vision, the proximal and distal nerve stumps of the transected nerve were secured 2 mm within the PCL conduit using four interrupted 9−0 Ethilon epineurial sutures (Ethicon, USA). The conduit had been cut to 14-mm length in order to maintain a 10-mm gap between the nerve stumps. The wound was closed in layers and post-operative analgesia was given as 4 μg buprenorphine intramuscularly. The animals were caged in a temperature and humidity controlled room with a 12-h light/dark cycle, and food and water provided immediately. After 2 weeks survival, animals were killed by cervical dislocation. Both ipsilateral and contralateral L4 and L5 DRG were harvested carefully but rapidly and flash frozen in liquid nitrogen, for later RNA extraction as above.

Quantitative RT-PCR

100 μg total RNA from ipsilateral L4/5 DRG was converted into cDNA for qRT-PCR. Contralateral L4/5 DRG was used to act as non-axotomized controls. qRT-PCR was performed with a Rotor-Gene 6000+HRM (Corbett Life Science, Australia) using SYBR® Green fluorescence master-mix (SABiosciences Corporation, MD, USA) and analysed using Rotor-Gene 6000 Series Software version 1.7.61 (Corbett Life Science, Australia). Primers were pre-designed by Superarray—genes of interest were Bcl-2 (Acc. No. NM_016993, Cat. No. PPR06577A), Bax (Acc. No. NM_017059, Cat. No. PPR06496B), and caspase-3 (Acc. No. NM_012922, Cat. No. PPR06384A). All reactions had been optimized to work under the same conditions—initial denaturation/HotStart DNA polymerase activation: 95 °C for 15 min; PCR cycles: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s repeated for 40 cycles. One PCR run was performed for each named gene and contained a standard curve, generated from serial dilutions of testicular tissue cDNA over four orders of magnitude; all experimental samples were assayed in triplicate. A negative control assay was always included where cDNA template was replaced with RNase-free water. From the standard curves described above, the C(t) values for the three genes of interest were used to calculate mRNA levels (arbitrary units) in each sample. Confirmation of the amplified products was established by performing a melting curve analysis: 95 °C for 1 min, 65 °C for 2 min, then 65–95 °C, reading every 0.2 °C, holding for 1 s between reads.

Western blotting

Lysates were prepared from control (non injured) and ipsilateral L4/L5 DRG using buffer containing 100-mM PIPES, 5-mM MgCl, 20% (v/v) glycerol, 0.5% (v/v) Triton X-100, 5-mM EGTA, and protease inhibitors (Sigma, UK). Lysates were incubated for 15 min on ice and then subjected to two freeze-thaw cycles prior to analysis of protein content using a commercially available protein assay kit (Bio-Rad, UK). In order to generate the required quantity of protein for Western blot analysis, DRG from four animals in each group were pooled and then 45-μg protein was prepared per sample, combined with Laemmli buffer and denatured at 95 °C for 5 min. Proteins were resolved at 120 V on 17% sodium dodecyl sulfate-polyacrylamide gels. Following electrophoretic transfer to nitrocellulose, membranes were blocked for 1 h in 5% (w/v) non-fat dry milk in TBS-Tween (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% (v/v) Tween), and then incu-

bated overnight at 4 °C with either rabbit anti-caspase 3 (1:500; Cell Signaling Technology, UK), rabbit anti-bcl-2 (1:200; Santa Cruz Biotechnology Inc., USA), or rabbit anti-bax (1:200; Santa Cruz Biotechnology Inc., USA) antibodies. Following 6×5-min washes in TBS-Tween, membranes were incubated for 1 h with HRP-conjugated secondary antibody (goat anti-rabbit 1:2000; Cell Signaling Technology, UK). Membranes were washed as previously and treated with ECL chemiluminescent substrate (GE Heatlthcare, UK) for 1 min and developed by exposure to Kodak X-Omat light-sensitive film. Antibody was stripped from the membranes using 100-mM glycine pH 2.9 and the blots re-probed with mouse anti-actin antibody (1:1000; Millipore, UK) as a loading control.

Statistics

GraphPad Prism 4© software (GraphPad Software, San Diego, CA, USA) was used to calculate statistics. One-way ANOVA test followed by Bonferroni's Multiple Comparison test was used to determine differences in gene expression between the empty PCL conduit group, the Schwann cell-primed PCL conduit group, and the differentiated ADSC-primed PCL conduit group. All data were expressed as mean±SEM. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Stem cell characterization

Qualitative RT-PCR and immunocytochemistry showed that ADSC expressed a range of stem cell surface markers including CD29, CD44, CD90, and CD105 (Fig. 1A). All cultures were routinely tested for their multi-potent differentiation capacity as previously published (Kingham et al.,

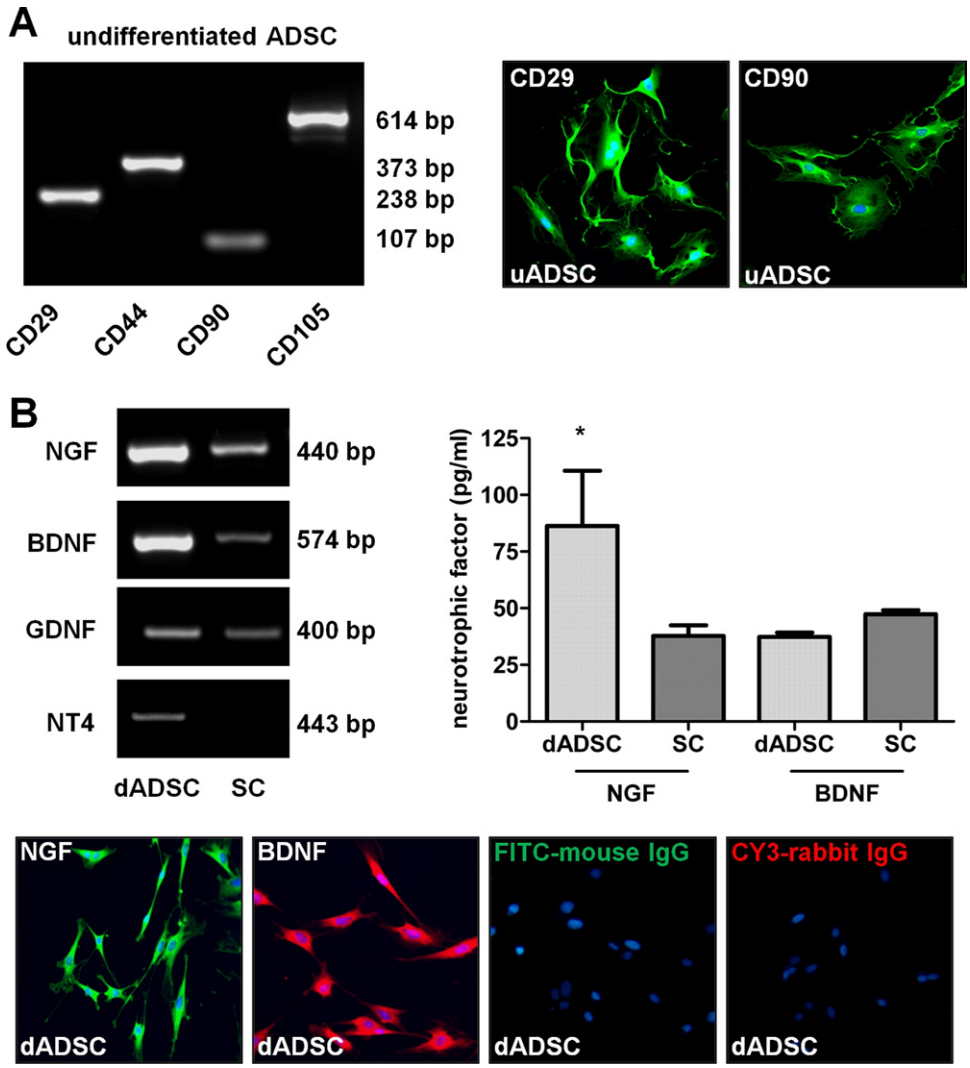

Fig. 1. Characterization of cells for transplantation. (A) RT-PCR shows expression of transcripts for the stem cell surface markers CD29, CD44, CD90, and CD105 in cultures of undifferentiated ADSC (uADSC). Size of amplicon is shown in base pairs (bp). Immunocytochemistry shows CD29 and CD90 protein expression (green) in uADSC. (B) Differentiated ADSC (dADSC) express nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and neurotrophin-4 (NT4) transcripts. A similar pattern of expression was observed in Schwann cell (SC) cultures except for the absence of NT4 transcript. Amplicon size is indicated in bp. Immunocytochemistry shows expression of NGF (green) and BDNF (red) protein in dADSC. Omission of primary antibodies and incubation with secondary antibodies (FITC-mouse IgG or CY3-rabbit IgG) only confirmed the specificity of staining. NGF and BDNF were detected in cell culture supernatants; dADSC secreted significantly more NGF compared with primary SC ($P<0.05$).

2007). Two weeks after differentiation towards a Schwann cell-like phenotype approximately 50% of the differentiated cells showed intense staining with S100 and GFAP antibodies consistent with our previous reports (Kingham et al., 2007). Prior to transplantation, dADSC were analysed for expression of various neurotrophic factors. NGF, BDNF, and glial-derived neurotrophic factor (GDNF) mRNA were detected in dADSC and Schwann cell cultures and neurotrophin-4 (NT4) mRNA was found in dADSC but not Schwann cells (Fig. 1B). Immunocytochemistry confirmed the expression of NGF and BDNF at the protein level (Fig. 1B). Furthermore, NGF and BDNF were detected in the cell culture supernatants indicating that the endogenous protein was secreted. Differentiated ADSC secreted significantly ($P<0.05$) more NGF than primary Schwann cells (Fig. 1B) whereas the level of BDNF secretion was similar between the two cell types.

Differentiated ADSC down-regulate apoptotic gene expression in the DRG

The gene expression of key apoptotic mediators was calculated in the ipsilateral DRG of all animals that had undergone 10-mm sciatic nerve gap repair with PCL conduit.

Caspase-3 mRNA expression. Uninjured contralateral L4/5 DRG served as control, whereupon mean relative expression of caspase-3 mRNA was 33.8±3.2 ($n=4$) (Fig. 2A). Empty PCL conduit repair resulted in an almost threefold increase of caspase-3 mRNA expression of 92.3±10.5 ($n=4$) ($P<0.001$); while in comparison to the empty conduit, both dADSC and Schwann cell-primed PCL conduits significantly reduced caspase-3 expression with relative quantities of 45.2±5.5 ($n=4$) ($P<0.01$) and 55.5±3.9 ($n=4$) ($P<0.01$), respectively. Western blot analysis of control (no injury) and injured DRG samples showed expression of full length (35 kDa) caspase-3 in all samples (Fig. 2B). The large fragment (17 kDa) of caspase 3, resulting from cleavage at aspartic acid 175, was detected in DRG taken from animals which had sciatic nerve lesion and were repaired with an empty PCL conduit. However, it was weakly expressed in the animals treated with PCL conduits filled with either dADSC or Schwann cells, consistent with the down-regulation of caspase 3 mRNA expression observed with PCR.

Bcl-2 mRNA expression. Uninjured contralateral L4/5 DRG served as control, whereupon mean relative expression of Bcl-2 mRNA was 200.3±8.9 ($n=4$) (Fig. 3A). Empty PCL conduit repair resulted in a much reduced Bcl-2 transcript expression of 112.9±3.2 ($n=4$) ($P<0.001$); while in comparison to the empty conduit, significantly increased Bcl-2 expression was observed in both dADSC and Schwann cell-primed PCL conduit repairs with mean expressions of 292.2±9.5 ($n=4$) ($P<0.001$) and 287.6±4.5 ($n=4$) ($P<0.001$), respectively.

Bax mRNA expression. Uninjured contralateral L4/5 DRG served as control, whereupon mean relative expression of Bax mRNA was 44.3±2.5 ($n=4$) (Fig. 3B). Empty PCL conduit repair resulted in a reduced Bax transcript expression of 37.8±0.6 ($n=4$); while in comparison to the empty conduit, significantly reduced Bax expression was observed in both dADSC and Schwann cell-primed PCL conduit repairs with mean expressions of 24.7±1.3 ($n=4$) ($P<0.001$) and 24.1±1.4 ($n=4$) ($P<0.001$), respectively.

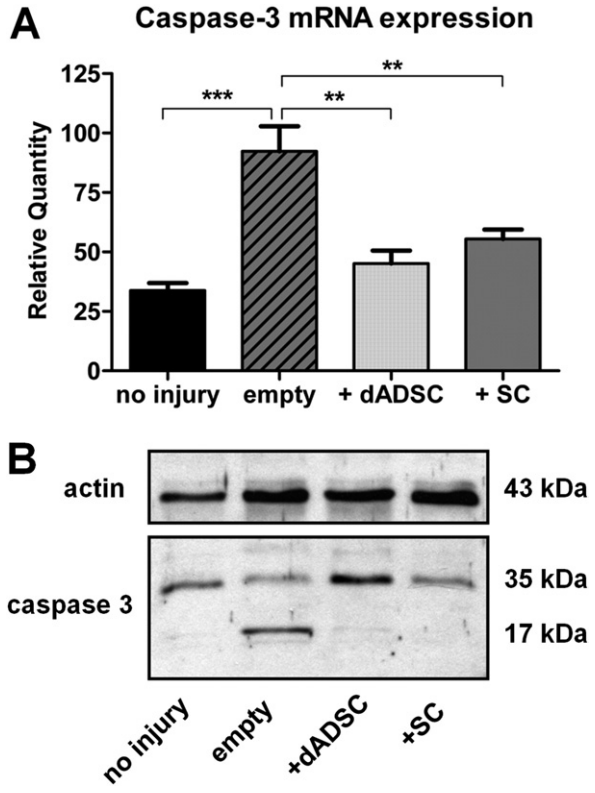

Fig. 2. Caspase-3 mRNA and protein expression in DRG. (A) Caspase-3 mRNA expression was measured using qRT-PCR in the ipsilateral DRG, 2 wks after nerve repair. Expression values are relative to HPRT expression and are therefore in arbitrary units. Contralateral DRG from the empty PCL conduit repair group was used for control measurements (no injury). Nerve injury resulted in a significantly (** $P<0.001$) increased caspase-3 transcript expression in the DRG of animals with empty PCL conduit nerve repairs; while in comparison to the empty conduit, both dADSC and Schwann cell (SC)-primed PCL conduits significantly reduced pro-apoptotic caspase-3 expression (** $P<0.01$). (B) Western blot analysis of caspase-3 shows expression of the cleaved large fragment (17 kDa) in the animals treated with empty PCL conduit. This was barely detectable in animals treated with conduits seeded with cells and uninjured animals. Actin antibody shows equal loading of protein.

Bcl-2: Bax mRNA ratio. The relative protein expression of Bcl-2 compared to Bax is seemingly vital to the survival of the neuron (Gillardon et al., 1996). It may be reasonable to infer that the ratio of transcript levels for these two species may also be of importance. On this basis, we calculated the relative mRNA expression levels of Bcl-2 compared to Bax as a ratio in each experimental animal (Fig. 3C). Non-injured contralateral DRG had a Bcl-2: Bax ratio of 4.55: 1. Empty PCL conduit repair resulted in this ratio decreasing to 2.99: 1. However, with the addition of cells into the conduit repair the Bcl-2: Bax ratio was observed to significantly increase. Specifically, dADSC-primed PCL conduit repairs increased the ratio to

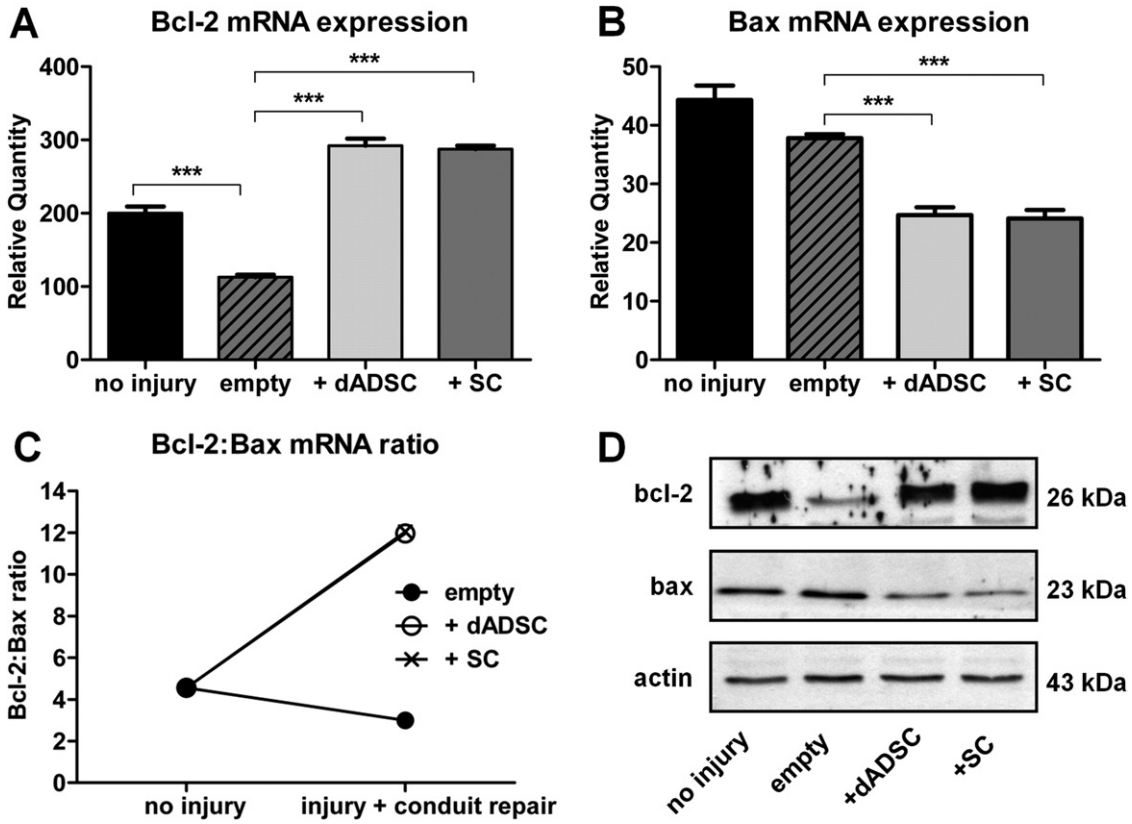

Fig. 3. Bcl-2 and Bax mRNA expression in DRG. (A) Bcl-2 mRNA expression was measured using qRT-PCR in the ipsilateral DRG, 2 wks after nerve repair. Expression values are relative to HPRT expression and are therefore in arbitrary units. Contralateral DRG from the empty PCL conduit repair group was used for control measurements (No Injury). Nerve injury resulted in a much reduced (*** $P<0.001$) Bcl-2 transcript expression in the DRG as seen in the animals with empty PCL conduit nerve repairs; while significantly increased Bcl-2 expression (*** $P<0.001$) was observed in both differentiated ADSC and Schwann cell (SC)-primed PCL conduit repairs. (B) Significantly decreased Bax expression (*** $P<0.001$) was observed in both differentiated ADSC and SC-primed PCL conduit repairs vs. empty conduit used to treat nerve injury. (C) The Bcl-2: Bax mRNA ratio was calculated in each animal and expressed here as a mean of each group. Non-injured contralateral DRG had a Bcl-2: Bax ratio of 4.55: 1. Empty PCL conduit repair resulted in this ratio decreasing to 2.99: 1. However, with the addition of cells into the conduit repair the Bcl-2: Bax ratio was observed to significantly increase. Specifically, ADSC-primed PCL conduit repairs increased the ratio to 11.96: 1, while Schwann cells (SC) increased the Bcl-2: Bax ratio with a similar magnitude of response to 12.05: 1. (D) Western blot analysis of Bcl-2 and Bax show that changes in protein expression levels are consistent with the differences detected by qRT-PCR. Actin antibody was used a loading control.

11.96: 1, while Schwann cells increased the Bcl-2: Bax ratio with a similar magnitude of response to 12.05: 1. Western blot analysis of Bcl-2 and Bax proteins confirmed the changes in expression levels observed with qRT-PCR (Fig. 3D).

DISCUSSION

We have demonstrated here for the first time that differentiated ADSC, when incorporated into a bioengineered nerve conduit significantly decrease apoptotic gene expression in the dorsal root ganglia neurons. Consequently, ADSC therapy could offer a novel means towards neuroprotection following a peripheral nerve injury.

Autologous nerve grafting remains the clinical gold-standard for nerve gap repair and this is thought to be attributable to the presence of Schwann cells and structural extracellular matrix proteins which supports neuronal survival and appropriate axonal regeneration. With recent advances in stem cell biology, bioengineered nerve conduit alternatives now aspire to recreate and augment this regenerative microenvironment. It has been demonstrated that cultured adult syngeneic Schwann cells seeded in synthetic guidance channels support extensive peripheral nerve regeneration with increased myelination (Guénard et al., 1992), and allogeneic Schwann cells seeded in a resorbable poly-3-hydroxybutyrate (PHB) conduit improved axonal regeneration distances significantly at 3 weeks after injury (Mosahebi et al., 2002). Bone marrow stem cells (BMSCs) differentiated into “Schwann cell-like” cells have been demonstrated also to augment peripheral nerve regeneration *in vivo* by improving myelination of axons and increasing regeneration distances (Dezawa et al., 2001; Keilhoff et al., 2006); however, undifferentiated BMSCs may not have the same capacity (Tohill et al., 2004; Keilhoff et al., 2006). Recently we showed that differentiated ADSC could also enhance early peripheral nerve regeneration (di Summa et al., 2010, 2011). While stem cell action at the site of injury may enhance axonal outgrowth, neuronal survival is a prerequisite for regeneration; therefore, action towards preventing neuronal cell death is likely to be of even greater consequence.

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