Biomaterials 37 (2015) 242–251

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

# Engineered neural tissue with aligned, differentiated adipose-derived stem cells promotes peripheral nerve regeneration across a critical sized defect in rat sciatic nerve

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ARTICLE INFO

*Article history:*
Received 17 July 2014
Accepted 2 October 2014
Available online 23 October 2014

*Keywords:*
Collagen
Stem cell
Nerve guide
Nerve regeneration
Nerve tissue engineering
Adipose-derived stem cell

ABSTRACT

Adipose-derived stem cells were isolated from rats and differentiated to a Schwann cell-like phenotype *in vitro*. The differentiated cells (dADSCs) underwent self-alignment in a tethered type-1 collagen gel, followed by stabilisation to generate engineered neural tissue (EngNT-dADSC). The pro-regenerative phenotype of dADSCs was enhanced by this process, and the columns of aligned dADSCs in the aligned collagen matrix supported and guided neurite extension *in vitro*. EngNT-dADSC sheets were rolled to form peripheral nerve repair constructs that were implanted within NeuraWrap conduits to bridge a 15 mm gap in rat sciatic nerve. After 8 weeks regeneration was assessed using immunofluorescence imaging and transmission electron microscopy and compared to empty conduit and nerve graft controls. The proportion of axons detected in the distal stump was 3.5 fold greater in constructs containing EngNT-dADSC than empty tube controls. Our novel combination of technologies that can organise autologous therapeutic cells within an artificial tissue construct provides a promising new cellular biomaterial for peripheral nerve repair.



## 1. Introduction

Peripheral nerve injuries lead to pain and significant disability in many affected individuals. Each year approximately 300,000 people of working age in Europe experience a peripheral nerve injury (PNI) [1]; of these less than 50% regain full function after treatment [2,3]. For peripheral nerve damage that results in gaps greater than approximately 3 cm, the current clinical gold standard treatment is the nerve autograft. Many new therapeutic strategies for improving nerve repair are being developed in basic, pre-clinical and clinical trials. Results have shown that guidance conduit structures and living cells are essential for the repair of larger nerve gaps to provide trophic support and recreate the environment provided by the nerve autograft [4–9].

Among the strategies being developed for peripheral nerve repair, the combination of tissue engineering and stem cell technologies represents a powerful approach for generating artificial nerve tissue that could be used in the clinical setting. We recently developed effective technology for the production of engineered neural tissue (EngNT), an aligned cellular biomaterial for nerve repair [10]. The technology involved Schwann cell self-alignment within a tethered collagen hydrogel, followed by a stabilisation process to form robust, aligned cellular sheets. EngNT containing aligned Schwann cells was used within a nerve repair conduit to promote neuronal regeneration across a critical sized defect in the rat sciatic nerve [10]. This approach differs from other tissue engineering approaches because the cells and the extracellular matrix align as a consequence of integrin-mediated cell-generated forces acting within a constrained hydrogel environment [11]. The result is an artificial tissue containing aligned Schwann cells distributed evenly throughout an aligned collagen hydrogel matrix. A key factor limiting the translation of this and other cellular tissue-

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http://dx.doi.org/10.1016/j.biomaterials.2014.10.009

engineered constructs towards clinical application is the source of Schwann cells. Autologous human Schwann cells would need to be derived from invasive nerve biopsies and sufficient cell numbers for regeneration would only become available after a lengthy expansion time *in vitro* [12]. Schwann cell-like cells derived from stem cells are therefore a more attractive source because they can potentially be obtained from the patient for use in an autologous therapy [13]. Autologous cells are generally considered to be more readily accepted by the patient because they do not provoke an immune reaction [7,14].

Adipose-derived stem cells (ADSCs) are an accessible source of adult stem cells that have generated considerable interest as candidates for autologous cell transplantation and they are currently being used in clinical trials for a wide range of indications. Stem and progenitor cells usually make up less than 5% of the total cell population in adipose tissue [15], but this is 2500-fold more than the frequency of such cells in bone marrow [16]. The abundance of ADSCs and the ability to collect large amounts of adipose tissue via liposuction potentially eliminates the need for cell expansion. They are easily accessible in large quantities with little donor site morbidity or patient discomfort. Experimental studies using rat ADSCs have shown that these cells have the ability to differentiate along the glial lineage, making them good potential candidates for use as an alternative to Schwann cells in peripheral nerve repair [17–20]. Rat ADSCs differentiated into Schwann cell-like cells (dADSCs) express a range of Schwann cell proteins, they can promote neurite outgrowth *in vitro* [17–20] and enhance regeneration *in vivo* [21–24]. The regenerative properties of these cells has been attributed to their secretion of neurotrophic factors [25,26], their ability to recruit host Schwann cells to aid the regenerative process [27], their possible direct contribution to myelin formation [24] and their ability to enhance the survival of sensory and motor neurons [28]. Importantly, recent studies indicate that human ADSCs, stimulated by the same protocol as used in the rodent experiments, also have a pro-regenerative phenotype when transplanted into the injured peripheral nervous system [29,30].

The aforementioned studies have used a variety of different types of conduits to deliver the dADSCs to the injury site. Cells have been transplanted in fast resorbing fibrin conduits [21,22,29], synthetic nerve tube conduits [28,31] or naturally occurring decellularised matrices [32]. The aim of this study was to test for the first time whether dADSCs can be used to generate EngNT through cellular self-alignment followed by stabilisation within collagen gels, avoiding the need for construction of aligned scaffolds and subsequent seeding of cells that is common in other tissue engineering approaches [11]. Following characterisation of dADSC morphology and phenotype in EngNT *in vitro*, EngNT-dADSC constructs were tested in a critical sized defect in the rat sciatic nerve that simulates the clinical long gap injury scenario in order to assess their ability to support neuronal growth *in vivo*.

## 2. Materials and methods

All experimental procedures involving animals were conducted in accordance with the UK Animals (Scientific Procedures) Act (1986)/the European Communities Council Directives (86/609/EEC) and approved by the Open University Animal Ethics Advisory Group and the Northern Swedish Committee for Ethics in Animal Experiments.

### 2.1. Isolation, culture and differentiation of adipose-derived stem cells

Fat tissue was harvested from adult rats and the ADSCs were isolated as previously described [19]. Cells were cultured in modified Eagle's medium (α-MEM; Invitrogen, UK) containing 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin solution. The 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. The multi-potent potential of the cell cultures was assessed by ensuring their ability to differentiate along several lineages, as described previously [19]. To differentiate the adipose stem cells into a Schwann cell-like phenotype (dADSCs), growth medium was removed from sub-confluent cultures at passage 2 and replaced with medium supplemented with 1 mM β-mercaptoethanol (Sigma–Aldrich, UK) for 24 h. Then the cells were washed and fresh medium supplemented with 35 ng/ml all-trans-retinoic acid (Sigma) was added. A further 72 h later, the cells were washed and the medium was replaced with differentiation medium containing cell growth medium supplemented with 14 μM forskolin (Sigma), 10 ng/ml basic fibroblast growth factor (bFGF; Pepro Tech Ltd., UK), 5 ng/ml platelet-derived growth factor (PDGF-AA; Pepro Tech Ltd., UK) and 252 ng/ml neuregulin NRG1 (R&D Systems, UK). The cells were incubated for a minimum 2 weeks under these conditions with fresh medium added approximately every 72 h.

### 2.2. Immunofluorescence staining of dADSCs

Cells were seeded at a density of 10,000 cells/well in an 8-well LabTek chamber slide and allowed to settle for 48 h before they were fixed for 20 min in 4% (w/v) paraformaldehyde at room temperature. The cells were then washed in phosphate buffered saline (PBS) before the addition of 5% (v/v) normal goat serum (NGS) and 5% (v/v) normal horse serum (NHS) together with 0.1% Triton X-100 (v/v) in PBS for a further 15 min at room temperature. The blocking serum was then removed and the primary antibodies, mouse monoclonal anti-GFAP (Millipore) and rabbit polyclonal anti-S100B (Dako) at respective dilutions of 1:500 and 1:1000 were added and the samples incubated overnight at 4 °C. The cells were then washed in PBS, re-blocked using NGS and NHS before addition of the secondary antibodies, Alexa 350 conjugated goat anti-rabbit IgG (1:50 dilution) and Alexa 488 conjugated goat anti-mouse IgG (1:300 dilution) for 2 h at room temperature. The samples were then washed 3 × 15 min with PBS before repeating the blocking step with NGS and NHS. Mouse monoclonal anti-p75NTR (Abcam, dilution 1:500) was then added and incubated overnight at 4 °C. The cells were then washed in PBS, 3 × 15 min, before a final blocking step in NGS and NHS. Finally the secondary antibody Alexa 568 conjugated goat anti-mouse IgG (1:300 dilution) was added for 2 h at room temperature before final washes with PBS and addition of ProlongGold anti-fade mountant. Images were captured at 200× final magnification using a Nikon Eclipse 90i microscope with a Nikon DS-U2 digital camera. Five random images were recorded from each well and a total of 4 wells for each preparation were imaged. dADSCs were analysed from 2 different rats and these were then used for further *in vitro* studies and parallel *in vivo* transplantations.

### 2.3. Fabrication of EngNT-dADSC

EngNT was prepared according to methods described previously [10]. dADSCs were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with penicillin and streptomycin (100 U/ml and 100 mg/ml, respectively; Sigma), 10% (v/v) FBS and the growth factors used for differentiation as described above. To prepare gels, 1 volume of 10× Minimum Essential Medium (Sigma) was mixed with 8 volumes of type I rat tail collagen (2 mg/ml in 0.6% acetic acid; First Link, UK) and the mixture neutralised using sodium hydroxide before addition of 1 volume of dADSCs suspension (final density $4 \times 10^6$ cells per ml of gel). One ml of this mixture was added to each mould at 4 °C and integrated with tethering mesh at opposite ends before setting at 37 °C for 10 min. Tethered gels were immersed in culture medium and incubated at 37 °C in a humidified incubator with 5% $CO_2$/95% air for 24 h during which time the cells aligned. Aligned cellular gels were stabilised by plastic compression: gels were separated from the tethering mesh using a scalpel, placed on an absorbent paper pad and immediately compressed by loading the gel with 120 g for 1 min during which time fluid was absorbed by the paper pad underneath [33]. The resulting sheets of EngNT were either transferred directly to 24-well plates for *in vitro* experiments, or rolled to form rods (approximately 200 μm diameter × 15 mm length) and maintained in culture medium for up to 24 h prior to *in vivo* experiments.

### 2.4. RT-PCR of cultured dADSCs and EngNT-dADSC

Total RNA was isolated from the samples using an RNeasy™ kit (Qiagen) and one step RT-PCR (Qiagen) was performed using 1 ng of RNA per reaction mix. The samples were loaded into a thermocycler (Biometra, Germany) which was used with the following parameters: a reverse transcription (RT) step (50 °C, 30 min) and a nucleic acid denaturation/RT inactivation step (95 °C, 15 min) followed by 28–34 cycles of denaturation (95 °C, 30 sec), annealing (30 sec) and primer extension (72 °C, 1 min) followed by a final extension incubation (72 °C, 5 min). Forward and reverse primer (all 5′ → 3′) pairs for a variety of glial cell markers and neurotrophic factors were manufactured by SigmaAldrich and are listed in Table 1 together with their annealing temperatures. The reaction product amplicons were electrophoresed at 50 V for 90 min through a 1.5% (w/v) agarose gel and their size was estimated using Hyperladder IV (Bioline, UK). Samples were visualised under ultraviolet (UV) illumination following GelRed™ nucleic acid stain (Bio Nuclear, Sweden) incorporation into the agarose.

### 2.5. Assessment of EngNT-dADSC in co-culture with neurons

Dissociated dorsal root ganglion (DRG) neurons were prepared from adult (200–300 g) Sprague Dawley rats. DRGs were incubated in collagenase (0.125%; Sigma) for 1.5 h at 37 °C then dissociated by trituration and washed twice with 20 ml