Supplementary Information

A fusion of minicircle DNA and nanoparticle delivery technologies facilitates therapeutic genetic engineering of autologous canine olfactory mucosal cells

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Supplementary Expanded Experimental

Materials

Cell culture reagents and culture grade plastics were from Invitrogen (Paisley, Scotland, UK), Sigma (Poole, Dorset, UK) and ThermoFisher Scientific (Loughborough, UK). Recombinant Human NRG1-beta 1 (Neuregulin) and Human BDNF Quantikine ELISA kit were from R&D Systems Europe Ltd (Abingdon, UK). Neuromag transfection-grade MPs were from OZ Biosciences (Marseilles, France). The magnefect-nano oscillating 24-magnetic array system was from nanoTherics Ltd (Stoke-On-Trent, UK). mC-DNA vector reagents were from System Biosciences (SBI; Mountain View, CA, USA). mC vector purification kits were from Qiagen (Manchester, UK). All restriction/cloning enzymes were from Promega (Southampton, UK). pmax-GFP plasmid was from Amaxa Biosciences (Cologne, Germany). LIVE/DEAD assay kits were from Invitrogen. Click-iT® EdU Imaging Kits were from ThermoFisher Scientific (Loughborough, UK). Primary antibodies and dilutions used were: the OEC marker low-affinity nerve growth factor receptor p75 (p75), 1:200 (Chemicon, Darmstadt, Germany); the fibroblast marker Fn, 1:400 (Dako, Denmark); GFP, 1:1000 (Thermofisher Scientific); BDNF, 1:500 (Promega, UK). For pmaxGFP transfection, secondary antibodies were from ThermoFisher Scientific (1:400). For mC-BDNF-GFP transfection, secondary antibodies were from Jackson Immunoresearch Laboratories Ltd, Westgrove, PA, USA (1:200). Vectashield mounting medium with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories (Peterborough, UK).

Primary canine olfactory mucosal cell (cOMC) culture

An OEC bank was generated during the clinical trial for OEC transplantation into sites of SCI in companion dogs [1] and used to generate the primary cOMCs used for all experiments. The cells were obtained as part of a protocol reviewed and approved by the Royal College of

Veterinary Surgeons and the Ethical Review Committee of the Department of Veterinary Medicine, University of Cambridge, where the initial study was carried out. Details of recruitment and cell harvest methodology have previously been published [1,2]. In brief, olfactory cells were derived from the lamina propria of the olfactory mucosa from each companion dog with chronic spinal injury recruited into the trial, prior to the autologous transplantation (5 x 10^6 cells per dog) into the sites of injury. Excess cells were then cryopreserved according to previously published protocols for OECs [3] and kept frozen at ultra-low temperature (-162°C in liquid nitrogen) for approximately 48 months before being thawed then expanded in cOMC media composed of DMEM containing 10% fetal bovine serum, forskolin (2μM in dimethyl sulphoxide), neuregulin-1 (20ng/mL), penicillin and streptomycin (both at 10mg/mL), at 37°C in 95% air:5% CO_2.

Magnetofection of cOMCs

Cells (passages 0-11) were seeded (1 x 10^5 cells/mL; 0.6 mL) on to PDL-coated coverslips in 24-well plates. After 24 h, the media in each well was replaced with fresh media (0.225 mL) and returned to the incubator for a minimum of 2 h. To prepare nanoparticle complexes, 88 ng pmax-GFP was mixed with 0.31 μL Neuromag in 75 μL DMEM, corresponding to a Neuromag:DNA ratio of 3.5 μL/μg. After 20 mins, 75 μL complexes were added to each well (controls were DMEM alone) and plates were returned to the incubator and exposed to their respective magnetic field condition for 30 mins. These were: no field, static field (F = 0Hz), and oscillating fields of F = 1Hz and F = 4Hz. Control wells were not exposed to a magnetic field. Magnetic fields were applied using the magnefect-nano oscillating magnetic array system which comprises a 24-magnet array (NdFeB, grade N42; field strength of 421 +/- 20 mT) compatible with a 24 well plate. The frequency (F, Hz), amplitude and duration of each oscillation cycle were programmed using an attached computer and an amplitude of 0.2 mm was used for all experiments which has previously been shown to be optimal for a range of

cell types [4,5]. Cells were then removed from the magnetic array and returned to the incubator for 24 h prior to fixing. The mC-BDNF-GFP transection protocol was performed in an identical manner, using only a single optimum magnetic field condition and control.

mC vector formulation

The mC construct was prepared as previously described [6]. Briefly, 'parental plasmids' are transformed in ZYCY10P3S2T Producer Bacterial Strain, a specifically engineered E.Coli strain which upon addition of arabinose express two enzymes: (i) ΦC31 integrase – which splits the parental plasmid into two circular entities, mC-DNA (size: 4.1 kb), and the bacterial backbone containing SceI endonuclease recognition sequence (size: 4.0 kb) and (ii) SceI endonuclease which degrades the bacterial backbone sequence. This leaves the mC-DNA which was extracted using an Endotoxin-free maxiprep kit (Qiagen, UK).

LIVE/DEAD staining

24 h post transfection, media was replaced with 4 μM calcein AM (green fluorescence in live cells) and 6 μM ethidium homodimer-1 (red fluorescence in dead cells) in DMEM. Cells were incubated at 37°C for 15 mins before imaging by fluorescence microscopy. To calculate cellular viability, 3 microscopic fields per experimental condition were taken, with a minimum of 100 cells per field assessed per condition. Number of cells expressing calcein (Live) was expressed as a percentage of the total cells expressing either calcein or EtH (Live + Dead).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (PFA) at 24 h post-transfection to assess transfection efficiency, particle uptake and measures of protocol safety. Fixed samples were

blocked for 30 mins at RT. Blocking solutions differed between experiments involving pmax-GFP transfection (10% normal goat serum and 0.3% Triton-X-100 in PBS) and mC-BDNF-GFP transfection (5% normal donkey serum in PBS-0.3% Triton- X-100). Following this, cells were incubated with primary antibodies (p75 and Fn for Neuromag-GFP transfection; GFP and BDNF for mC-BDNF-GFP transfection) in blocking solution for 24 h at 4°C. Cells were rinsed in PBS before being incubated with appropriate secondary antibodies (2 h, RT). Cells were then mounted using VectaShield mounting medium with DAPI.

Phase and fluorescence microscopy

Phase and fluorescence microscopy of all experiments was performed using an Axio Observer.Z1 equipped with an AxioCam MRm powered by Zen 2 (blue edition) software (Carl Zeiss MicroImaging GmbH, Goettingen, Germany). Images were merged and quantified using ImageJ 1.49v software.

Assessment of particle uptake and transfection efficiency in cOMCs

We have previously validated fluorescence microscopy for analysis of particle uptake and transfection efficiency in other cell types including astrocytes, NSCs and OPCs [7–10]. This allows detailed microscopic evaluation of cell health, including adherence and morphology, and transfection and particle uptake to be conducted at the same time. Three microscopic fields per magnetic field condition were analysed, with a minimum of 100 cells assessed for each condition. Proportions of cells displaying particle uptake were estimated from triple merged DAPI, phase and fluorescent MP images and numbers of GFP expressing cells were calculated from double merged DAPI and GFP images. Immunostaining for copGFP (naturally occurring GFP with low fluorescence intensity) was used in the mC-BDNF-GFP experiments to enhance fluorescence for the purpose of cell counting. To assess

transfection efficiency/success of the mC vector, cells were stained with primary antibodies for GFP and BDNF (see Immunocytochemistry) with GFP positive cells deemed to be transfected. This was assessed using three microscopic fields per experiment, with a minimum of 100 cells assessed for each condition.

Phenotypic characterization of transfected populations

Transfected cells were classified based on immunostaining and morphology and adapted from previous descriptions [11]. Three phenotypic categories were identified: (i) p75+, elongated fusiform bipolar cells, classified as OECs; (ii) Fn+ cells displaying flattened morphology, classified as olfactory nerve fibroblasts (ONFs) [12] and; (iii) uncategorized cells which did not fit either of these categories- this included flattened p75+ cells, unipolar Fn+ cells and small, rounded cells. Three fields per condition were analysed (at least 100 cells per condition).

Assessment of cell viability and proliferation

The proportion of cells displaying pyknotic nuclei (indicating cell death, seen as nuclear shrinkage, fragmentation, and chromatin condensation) was calculated from images taken from evaluation of transfection efficiency, using double merged DAPI and phase images. In order to examine effects of the procedures on cellular proliferation, a Click-iT® EdU (a nucleoside analogue of thymidine incorporated in to DNA during active DNA synthesis) assay was performed according to the manufacturer's instructions. Here, the EdU reagent (1 μM) was added to cells 6 h prior to fixation and cells taking up EdU (proliferating cells) were fluorescently labelled. The proportion of EdU positive cells was estimated using quadruple merged phase, DAPI, GFP and EdU images over three microscopic fields.

ELISA

Cell supernatants were collected 24 h post-transfection and centrifuged to clear cells/cell debris. To determine the BDNF protein concentration in cell supernatants, an ELISA was applied using the Quantikine® ELISA Kit (R&D Systems, UK) according to the manufacturer's instructions. This assay employs the quantitative sandwich enzyme immunoassay technique. Briefly, standards and samples were added to wells pre-coated with a monoclonal antibody specific for human BDNF and incubated for 2 h followed by several washes. Horseradish peroxidase-linked monoclonal antibody specific for BDNF was added to the wells. The substrate was added and color intensity was measured (absorbance at 450 nm) using Glomax Multi Detection System (Promega, UK).

Statistical analyses

For pmax-GFP transfection, all data were analysed by a one-way ANOVA. For mC-BDNF-GFP transfection, data were analysed using an unpaired t-test. In both cases, statistical differences were determined by Bonferroni's multiple comparison test (MCT) using Prism software (version 6.0). Data is expressed as mean +/- SEM and the number of experiments 'n', refers to the number of independent cultures used, each derived from a different companion dog.

Supplementary Results:

Primary cell populations were successfully recovered from cryopreservation and cultured, with good adherence to the substrate surface (**Supplementary Figure 1A**). These cultures were a mixed population of cells derived from the canine olfactory mucosa (cOMCs) with ca. 60% p75+ elongated, bipolar cells classified as OECs and ca. 20% rounded cells positive for fibronectin (Fn), classified as ONFs (**Supplementary Figure 1B-F**). The remaining cells could not be ascribed to either category and were unclassified.

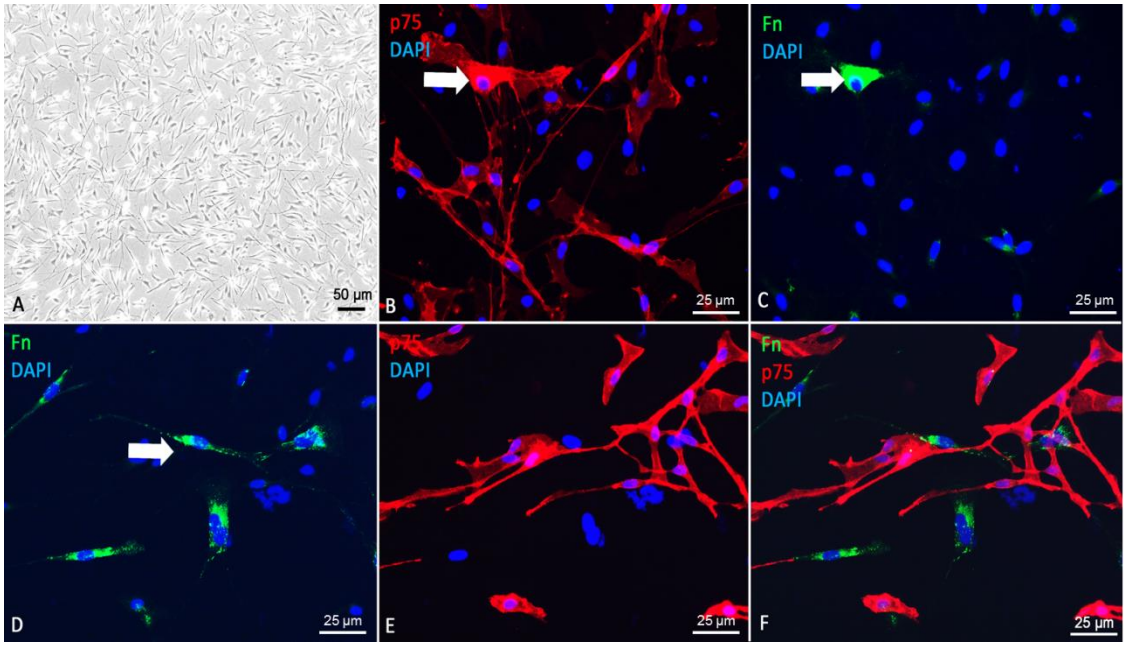

Figure 1: Morphological characteristics of cOMCs. *(A) Representative phase image of cOMC cultures demonstrating that spindle shaped bipolar cells form the majority of the cells. (B – F) Representative immunocytochemical images of cOMCs. (B) Representative fluorescent image of elongated, p75+ cells classified as OECs. (C) Counterpart to (B) showing some p75+ cells also had a broad appearance and stained for both p75 and Fn, therefore were unclassified (arrows point to same cell). (D) Representative fluorescent image of Fn positive cells most of which displayed broad and rounded morphology and were therefore classified as ONFs. Note however that some elongated cells were also Fn positive*

(arrow), although they presented a monopolar morphology, and these cells were unclassified. (E and F) Counterparts to (D) showing (E) single channel p75 staining and (F) the merged image depicting p75+ cells interspersed with ONFs.

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