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ORIGINAL ARTICLE

Dental Pulp Cell Sheets Enhance Facial Nerve Regeneration via Local Neurotrophic Factor Delivery

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An effective strategy for sustained neurotrophic factor (NTF) delivery to sites of peripheral nerve injury (PNI) would accelerate healing and enhance functional recovery, addressing the major clinical challenges associated with the current standard of care. In this study, scaffold-free cell sheets were generated using human dental pulp stem/progenitor cells, that endogenously express high levels of NTFs, for use as bioactive NTF delivery systems. Additionally, the effect of fibroblast growth factor 2 (FGF2) on NTF expression by dental pulp cell (DPC) sheets was evaluated. *In vitro* analysis confirmed that DPC sheets express high levels of NTF messenger RNA (mRNA) and proteins, and the addition of FGF2 to DPC sheet culture increased total NTF production by significantly increasing the cellularity of sheets. Furthermore, the DPC sheet secretome stimulated neurite formation and extension in cultured neuronal cells, and these functional effects were further enhanced when DPC sheets were cultured with FGF2. These neuritogenic results were reversed by NTF inhibition substantiating that DPC sheets have a positive effect on neuronal cell activity through the production of NTFs. Further evaluation of DPC sheets in a rat facial nerve crush injury model *in vivo* established that in comparison with untreated controls, nerves treated with DPC sheets had greater axon regeneration through the injury site and superior functional recovery as quantitatively assessed by compound muscle action potential measurements. This study demonstrates the use of DPC sheets as vehicles for NTF delivery that could augment the current methods for treating PNIs to accelerate regeneration and enhance the functional outcome.

Keywords: adult stem cells, dental stem cells, neurotrophins, peripheral nerve, neural crest stem cells, scaffold-free

Impact Statement

The major challenges associated with current treatments of peripheral nerve injuries (PNIs) are prolonged repair times and insufficient functional recovery. Dental pulp stem/progenitor cells (DPCs) are known to endogenously express high levels of neurotrophic factors (NTFs), growth factors that enhance axon regeneration. In this study, we demonstrate that scaffold-free DPC sheets can act as effective carrier systems to facilitate the delivery and retention of NTF-producing DPCs to sites of PNIs and improve functional nerve regeneration. DPC sheets have high translational feasibility and could augment the current standard of care to enhance the quality of life for patients dealing with PNIs.

Introduction

Over 1 million people worldwide suffer from peripheral nerve injuries (PNIs) each year, and more than 200,000 peripheral nerve repair procedures are performed annually in the United States.[1,2] Compression or laceration injuries to the nerve can be caused by trauma, disease, or surgical intervention such as tumor resection. The severity of the injury dictates the treatment modality, where more severe cases necessitate surgical intervention. The rate of axon regeneration is a strong indicator of the degree of functional recovery.[1,3] Following treatment, more than 50%

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of patients suffer prolonged repair times and suboptimal functionality.[4,5] Augmenting current treatments of PNIs to accelerate healing and improve the functional outcome would greatly improve patient quality of life.

Neurotrophic factors (NTFs) are a group of growth factors known to enhance axon regeneration and extension.[6] NTFs such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and neurotrophic factor-3 (NT3) have been extensively studied both *in vitro* and *in vivo* for their role in peripheral nerve development and repair.[7] The sustained delivery of NTFs to damaged nerves has been shown to accelerate healing and enhance the functional outcome in animal models.[6,8] Therefore, a significant amount of research has been directed toward developing mechanisms of NTF delivery to sites of PNIs; however, difficulties remain in engineering a system that facilitates the sustained release of these growth factors.[9] To address this challenge, researchers are developing cellular therapies where implanted cells continually produce NTFs. Schwann cells, neural crest-derived glial cells of peripheral nerves, produce significant amounts of NTFs and have been shown to enhance nerve regeneration if infused at the site of injury.[10,11] However, procuring human Schwann cells causes source-tissue morbidity and, additionally, the slow and limited expansion of these cells *in vitro* limits the production of a sufficient number of cells for effective therapeutic use.[12] Alternatively, researchers are proposing delivering cells that are genetically modified to produce NTFs[4,13–15]; however, these cells have been highly manipulated and may therefore pose challenges for clinical translation.

Dental pulp is the soft tissue found at the center of the tooth, which is vascular, innervated, and contains a population of adult stem/progenitor cells that are easily attainable from autologous sources.[16–18] Unlike many of the other commonly studied adult stem/progenitor cell populations, dental pulp cells (DPCs) are embryonically derived from the neural crest, making them an attractive cell source to regenerate other neural crest-derived tissues, including neural tissues.[19–26] DPCs endogenously express NTFs[21,24,27–30] and have been shown to produce these factors at higher levels than bone marrow stromal cells or adipose-derived stem cells,[28,30] a trait attributed to their developmental origins. Additionally, a recent study showed that injection of DPCs into rat spinal cord injury significantly improved axon regeneration and functional recovery, and this effect was further enhanced when the DPCs were cultured with fibroblast growth factor 2 (FGF2).[31] Therefore, engineering a method to deliver DPCs to a site of PNI could be an effective means of sustained NTF delivery, and priming DPCs with FGF2 may enhance the bioactivity of these cells for use in regenerative peripheral nerve therapies.

Cell sheets are a form of scaffold-free tissue engineering that can act as a natural carrier system to deliver and retain cells at sites of injury. Cell sheets are formed by allowing cells to proliferate to confluence and produce endogenous extracellular matrix (ECM) to form a layer of tissue that can be separated from the substrate.[32] This technology has been studied for use in many diverse applications, including cornea, esophagus, heart, periodontal ligament, bone, periosteum, and skin regeneration.[33–39] The cells in the cell sheet can directly facilitate repair of damaged tissues by either depositing new tissue or modulating repair through paracrine signaling.[40] The therapeutic potential of multiple types of cell sheets is already being investigated in human clinical trials validating the safety and feasibility of translating these types of engineered tissues into humans.[41–43]

In this study, we engineered and characterized scaffold-free DPC sheets as a mode of NTF delivery to enhance facial nerve regeneration. We also evaluated the effect of FGF2 on the regenerative potency of DPC sheets. The functional effect of these cell sheets was evaluated *in vitro* and *in vivo* in a rat facial nerve injury model. In this study, we show that scaffold-free DPC sheets present a novel cellular biomaterial that can facilitate sustained NTF delivery to enhance peripheral nerve regeneration.

Materials and Methods

Dental pulp cell isolation

Similar to previously described studies,[44,45] dental pulp was isolated from healthy, human third molars collected at the University of Pittsburgh, School of Dental Medicine, within 24 h of routine extraction. This use of human teeth in this study is not considered human subject research because the teeth were discarded surgical waste and the samples were deidentified by surgical staff before transfer to the research investigators as approved by the University of Pittsburgh Institutional Review Board (PRO11080044 to Center for Craniofacial Regeneration Director and collaborator Dr. Charles Sfeir). The use of human DPCs in these experiments was also approved by the University of Pittsburgh Human Stem Cell Oversight Committee (18-001). The teeth were cracked open and pulp was removed, minced, and enzymatically digested in 3 mg/mL collagenase (EMD Millipore) and 4 mg/mL dispase (Worthington Biochemical) for 1 h at 37°C. The digest was then passed through a 70-μm cell strainer and the obtained DPCs were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco), 20% fetal bovine serum (FBS; Cat. No. S11550, lot No. A17004; Atlanta Biological), and 1×penicillin and streptomycin (P/S; Gibco). Upon reaching 80% confluence, the cells were passaged and cryopreserved. DPCs from passage 2 to 3 were used to engineer cell sheets.

Fabrication of scaffold-free cell sheets

DPCs were plated onto wells of 6-well plates at an initial seeding density of 200,000 cells per well in growth medium (GM) consisting of DMEM, 20% FBS, 50 μg/mL L-ascorbic acid (Sigma-Aldrich), and 1×P/S. Cell sheets were formed with ±5 ng/mL fibroblastic growth factor 2 (PeproTech). The culture medium was replenished on the DPCs every 2–3 days for a period of 10–12 days.

Quantification of cell number in dental pulp cell sheets

Scaffold-free, engineered DPC sheets were digested in trypsin-EDTA (Gibco) at 37°C for 5–7 min. The detached cells were counted using a standard hemocytometer utilizing trypan blue to determine cell viability.

Quantitative real-time polymerase chain reaction

RNA was collected from DPC sheets or subconfluent DPCs using the QIAGEN RNeasy Mini Kit, following the manufacturer's instructions. Quantitative real-time polymerase

chain reaction (qPCR) was performed with the TaqMan PCR Kit (Applied Biosystems) using primers for human BDNF, GDNF, and NT3 and GAPDH as the housekeeping gene (TaqMan Gene Expression Assays). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay

At the time of cell sheet formation, the cell sheet culture medium was replenished with 2 mL of fresh GM for BDNF and GDNF protein detection or 1 mL of GM for NT3 detection. Following 48 h, this conditioned medium (CM) containing the DPC secretome was collected. CM was centrifuged at 2000 rpm for 5 min to separate cellular debris; the supernatant was collected and stored at −80°C. Uncultured GM ± FGF2 was used as the control sample. The amounts of BDNF, GDNF, and NT3 proteins present in the DPC sheet CM were measured using enzyme-linked immunosorbent assay (ELISA) kits for human BDNF (PicoKine™ ELISA Kit EK0307; Boster Biological Technology), human GDNF (PicoKine ELISA Kit EK0362; Boster Biological Technology), and human NT-3 (RayBiotech). The ELISAs were performed following manufacturer's protocol. NTF concentrations were extrapolated using standard curves generated using standards provided by the manufacturer.

Assessment of neurite outgrowth in SH-SY5Y neuroblastoma cells

Similar to a previous study,[27] SH-SY5Y (ATCC CRL-2266) neuroblastoma cells were plated on poly-L-lysine-coated eight-well chamber slides for 24 h in culture medium comprising α-MEM/F12, 10% FBS, and 1 × P/S. Neuronal induction was achieved by adding 10 μM retinoic acid (ACROS Organics) to the culture medium for 48–72 h. The neuronal cells were then cultured with DPC sheet CM or control GM for 6 days, and formation of neurites in response to CM or GM was assessed. To block the effects of NTFs in the CM, TrK B receptor blocker for BDNF, TrK C receptor blocker for NT3 (R&D Systems), and the neutralizing antibody against GDNF (R&D Systems) were added to the conditioned media at a concentration of 5 μg/mL. This type of inhibition assay has been reported to be an effective method for evaluating the functional effects of NTFs.[29,30,46]

Immunostaining was performed on formalin-fixed, treated SH-SY5Y cells using a primary antibody against tubulin βIII (mouse monoclonal antibody, clone: TUJ1, usage: 1:500; BioLegend) with a secondary antibody of Alexa Fluor goat anti-mouse-546 IgG (usage: 1:500; Invitrogen) to visualize neurites, and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Images were captured with a Nikon ECLIPSE Ti microscope. Neurite extensions of SH-SY5Y neuronal cells were manually quantified with ImageJ software. Cell extensions twice the length of their cell body were considered as neurites. The percentage of neurite-positive cells and range of neurite lengths were quantified.

In vivo *implantation of a DPC sheet on rat facial nerve crush injury*

All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Protocol No. 17121275). RNU immunocompromised rats (Charles River) were anesthetized through intraperitoneal injection of 40 mg/kg ketamine and 5 mg/kg xylazine. A preauricular incision was made in the rat face to expose the facial nerve. The buccal branch of the facial nerve was crushed for 20 s using a vessel clip exerting a pressure of 10 g (World Precision Instruments). In the experimental group, a DPC sheet was then wrapped around the injury and secured with a single knot of a 9-0 nylon monofilament suture ($n = 19$). In control samples, lacking a DPC sheet, the crush site was also marked with a suture ($n = 13$). The wound was closed using a 5-0 resorbable gut suture. Before implantation, the DPCs were fluorescently labeled using Vybrant DiO (Invitrogen), a lipophilic dye, to identify and localize DPCs following explantation. After 3 weeks, animals were anesthetized, underwent compound muscle action potential (CMAP) measurements to assess nerve function, and were then euthanized. The nerves were then dissected and fixed for histological analysis.

Histological analysis

For histological assessment of DPC sheets following *in vitro* culture, formalin-fixed DPC sheets were processed for standard paraffin embedding and sectioned at a thickness of 5 μm. Sections were stained with hematoxylin and eosin (H&E). Immunohistochemical staining was performed using a primary antibody against type 1 collagen (rabbit polyclonal antibody, usage: 1:250; Abcam) and secondary antibody, Alexa Fluor 488 anti-rabbit IgG (usage: 1:500; Invitrogen). DAPI was used to stain nuclei.

Following explantation, formalin-fixed nerve samples were embedded in Tissue Plus O.C.T Compound (Fisher Scientific) and frozen in liquid nitrogen-chilled isopentane. Cryosections were prepared at a thickness of 5 μm. The sections were stained with H&E, and immunohistochemical staining was performed using a primary antibody, anti-tubulin βIII (mouse monoclonal antibody, clone: TUJ1, usage: 1:100; BioLegend), with a secondary antibody of Alexa Fluor goat anti-mouse-546 IgG (usage: 1:500; Invitrogen). DAPI staining was also performed to detect nuclei.

Images were captured using a Nikon ECLIPSE Ti, ZEISS Scope.A1 AXIO, or Nikon TE 2000 microscope.

CMAP measurements

CMAP measurements were acquired using methods similar to those previously described.[45] Three weeks after surgery, the rats were anesthetized through inhalation of 2% isoflurane. A self-tapping, stainless steel bone screw (Fine Science Tools) was placed in the caudal position of the skull and used as a reference electrode. Hook-type, bipolar stimulation electrodes were placed in the exposed buccal branch of the rat facial nerve proximal to the injury site, and multistrand stainless steel wires (Cooner Wire, AS631) were inserted under the whisker pad between the middle vibrissal rows C and D with the help of a 25G hypodermic needle. The exposed nerve was kept hydrated with phosphate-buffered saline throughout the measurement. One-hertz biphasic stimulation (100 μS each phase, cathodic first, 100 μS interphase delay) of supramaximal intensity at 1500 μA was delivered through the hook electrodes using the Grape Vine Processor and Nano2+Stim Front End (Ripple). The stimulation was continuous for at least 10 s and repeated at least three times. Nerves and muscles were allowed to rest for 1 min in between stimulations. CMAP measurements of the

vibrissal muscle were simultaneously recorded at a 30 kHz sampling rate. CMAP data were processed with MATLAB (MathWorks). The mean CMAP waveform for each stimulation session was averaged from at least 10 individual CMAP responses. Peak-to-peak amplitude for each mean CMAP waveform is defined as the difference between the lowest negative peak and the highest positive peak.

Measurements were made on healthy nerves ($n=7$), nerves with crush injuries lacking DPC sheets (untreated controls; $n=3$), and nerves with crush injuries treated with DPC sheets ($n=7$). Differences in sample sizes across groups are due to unexpected animal deaths during the course of the experiment, likely due to complications associated with the severe immunocompromised nature of these rodents.

Statistical analyses

Data are presented as means ± standard deviations. Statistical comparisons of means for cell number quantification, ELISA, and neurite frequency were determined using Student's *t*-tests or one-way analysis of variance (ANOVA) with Tukey's *post hoc* test. The Brown–Forsythe and Welch ANOVA tests with Dunnett's T3 multiple comparison test were used without assuming equal standard deviations to determine differences in amplitude of CMAP measurements. All statistical tests were performed using GraphPad Prism software and a statistical difference at p-value <0.05 was considered significant.

Results

Formation and structural characterization of DPC sheets following in vitro *culture*

The goal of this study was to develop DPC sheets as an NTF delivery system to enhance nerve regeneration. Human DPCs were plated on standard, six-well, plastic tissue culture dishes, reached confluence 2–4 days after plating, and by 10–12 days in culture, a robust cell sheet was formed that could be simply mechanically detached from the dish and easily handled with forceps (Fig. 1A). These scaffold-free cell sheets could be wrapped around damaged nerves treated with the current standard of care to enhance regeneration (Fig. 1B).

The DPC sheets cultured ± FGF2 are solid tissues comprising layers of cells embedded within an ECM (Fig. 2A, B) that contains type 1 collagen (Fig. 2C, D). Quantification of cellularity showed that the addition of FGF2 doubled the number of DPCs present in the cell sheets (Fig. 2E), and cell viability was close to 100%.

NTF expression by DPCs cultured as cell sheets

NTF messenger RNA (mRNA) expression was evaluated in subconfluent DPCs and DPC sheets ± FGF2; separate samples were generated using DPCs isolated from five independent human donors denoted as human sample (HS) 1–5 (Fig. 3A). Although considerable variability exists across biological samples, overall, culturing DPCs as cell sheets did not result in any generalized negative effects on NTF gene expression. Additionally, culturing DPC sheets with FGF2 did not result in any consistent change in NTF mRNA expression.

While limited trends could be detected in NTF mRNA expression across samples, cell sheets generated by DPCs from different human donors produced similar amounts of NTF protein. ELISA was performed on DPC sheet CM, containing the DPC secretome, to detect NTF protein production; CM was collected from cell sheets formed separately using DPCs isolated from three independent donors, and the results were averaged (Fig. 3B). The total production of BDNF and GDNF proteins was significantly greater

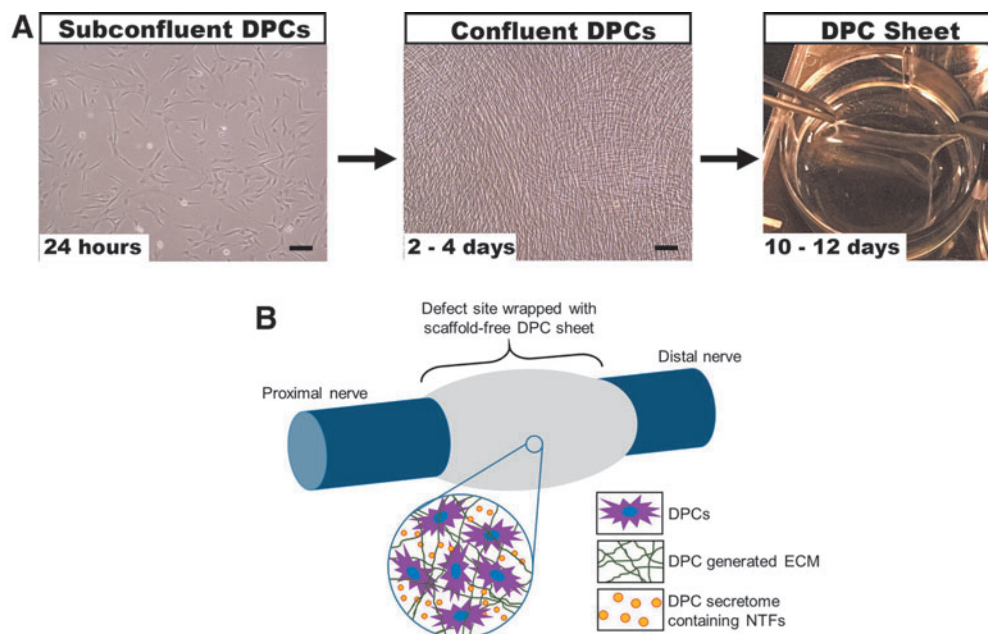
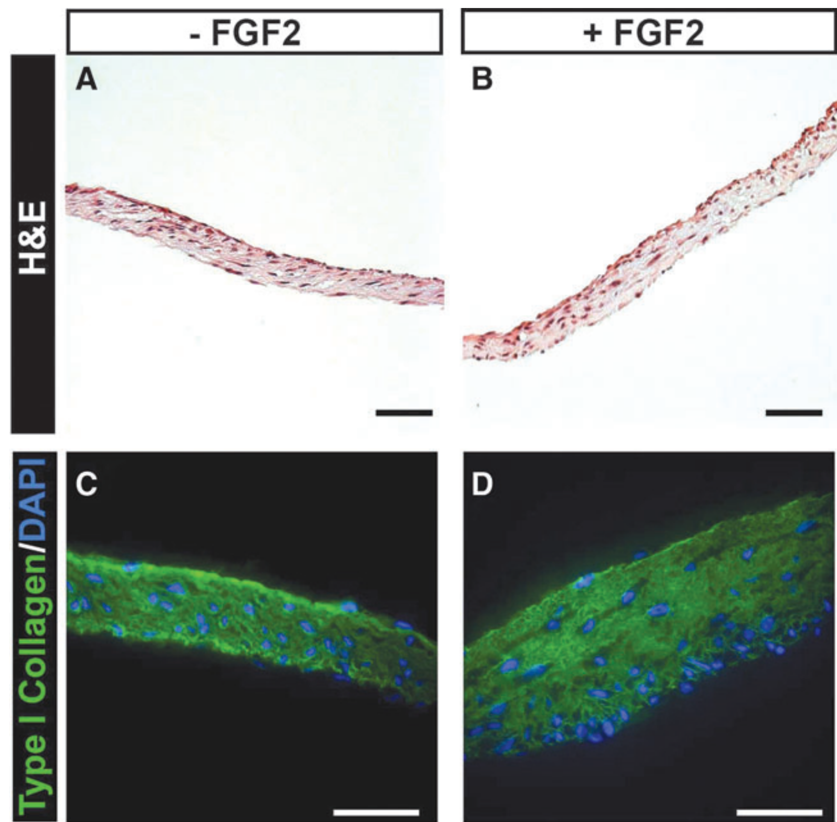


FIG. 1. Formation and potential application of a scaffold-free DPC sheet. **(A)** Timeline of cell sheet formation with representative micrographs of cultured DPCs and a photograph of a fully formed DPC sheet being handled with forceps in a well of a six-well plate. **(B)** Schematic representation of DPC sheet treatment of a damaged nerve. **(A, B)** Scale bars = 100 μm. DPC, dental pulp cell.



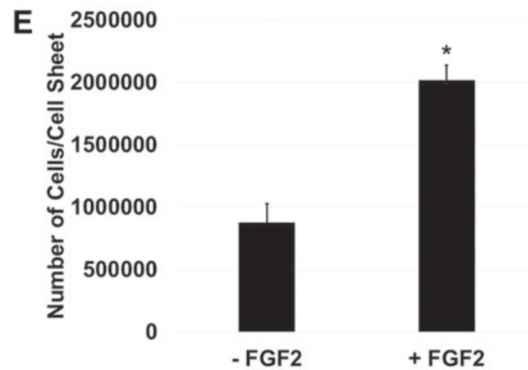


FIG. 2. Structural characterization of DPC sheets. H&E staining of cross-sections of DPC sheets shows that DPC sheets cultured without FGF2 **(A)** and with FGF2 **(B)** are both solid and cellular. Immunostaining of DPC sheets shows that DPC sheets cultured without FGF2 **(C)** and with FGF2 **(D)** contain an ECM comprising type I collagen. Cell sheets cultured with FGF2 have approximately twice the number of DPCs than sheets cultured without FGF2 ($*p<0.05$) **(E)**. Scale bars: **(A, B)** = 100 μm and **(C, D)** = 50 μm. ECM, extracellular matrix; FGF2, fibroblast growth factor 2; H&E, hematoxylin and eosin.

in conditioned media collected from DPC sheets cultured with FGF2 (CM+FGF2) when compared with DPC sheets cultured without FGF2 (CM-FGF2). However, the concentration of NT3 protein was found to be greater in the CM-FGF2 group in comparison with CM+FGF2. Negligible levels of NTF proteins were detected in the control GM ± FGF2.

FGF2 significantly increased total BDNF and GDNF protein production by DPC sheets (Fig. 3B). FGF2 also enhanced the cellularity of these cell sheets (Fig. 2E). Normalizing total NTF production to the average number of cells per DPC sheet indicated that FGF2 treatment did not alter NTF protein expression/cell (Fig. 3C). This indicates that the increased total production of BDNF and GDNF proteins resulting from culturing DPC sheets with FGF2 is likely due to the increased number of DPCs present in these cell sheets. Although FGF2 does not seem to affect NTF expression at a cellular level, by enhancing the cellularity of DPC sheets and thereby increasing total BDNF and GDNF protein production, FGF2 can still positively affect the regenerative potency of these sheets.

Functional evaluation of the effect of DPC sheets on neuroblastoma cells in vitro

DPC sheet CM induced the formation and extension of neurites in neuronally differentiated, SH-SY5Y neuroblastoma cells (Fig. 4). Extensive β-tubulin-positive neurites can be visualized in neuroblastoma cells cultured with DPC CM in comparison with control GM (Fig. 4A–D). Quantitative image analysis verified the presence of a significantly greater number of neurite-bearing cells when the neuroblastoma cells were cultured with DPC CM ± FGF2 in comparison with control GM, and overall, CM+FGF2 induced the greatest number of neurite-positive neuroblastoma cells (Fig. 4E). Additionally, CM ± FGF2 induced the neuroblastoma cells to produce longer neurites than GM ± FGF2

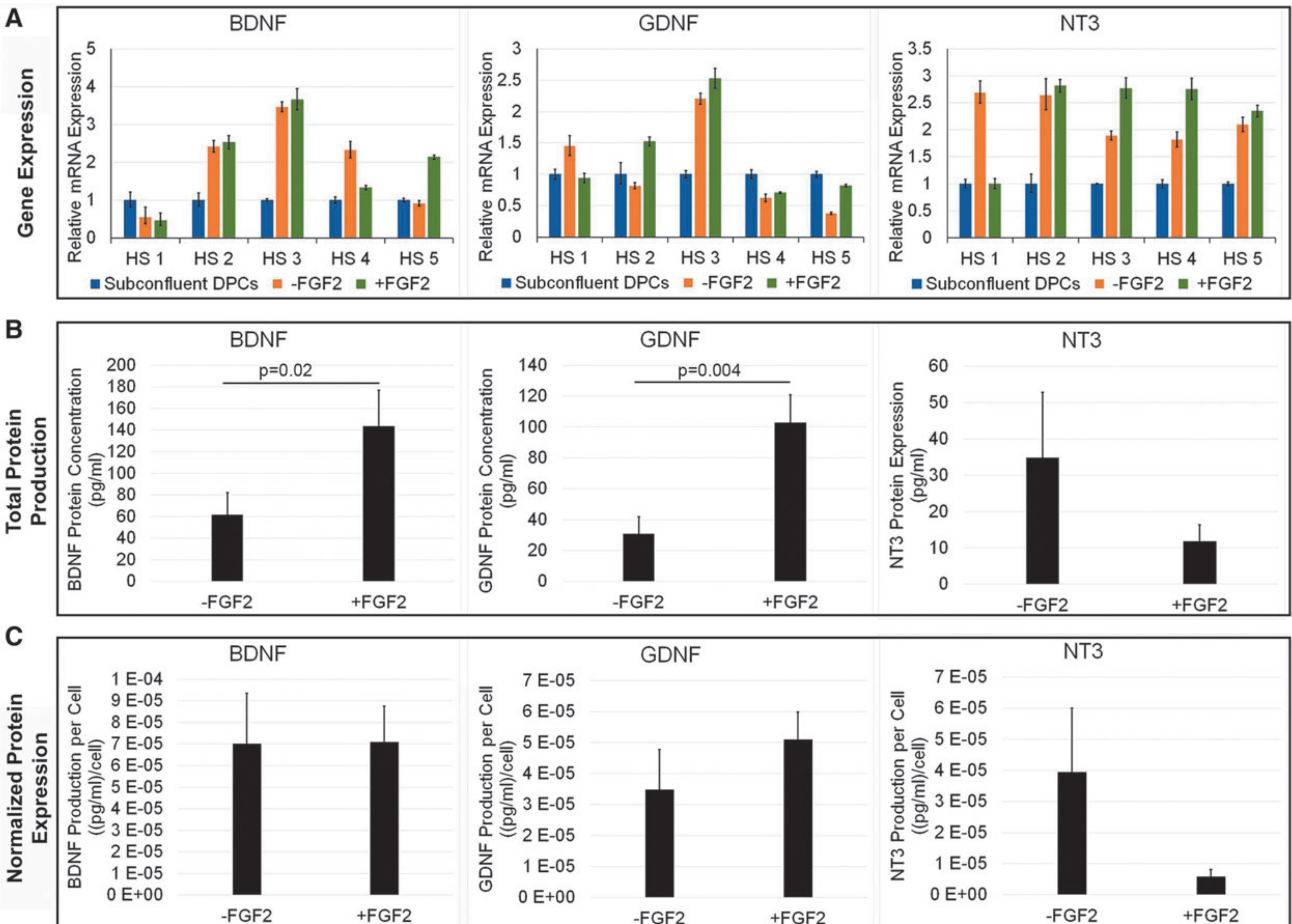


FIG. 3. NTF expression by DPC sheets. **(A)** qPCR analysis of the NTF gene expression by DPCs cultured as cell sheets ± FGF2 relative to subconfluent DPCs. To assess biological variability, mRNA was extracted from cell sheets generated from cells isolated from five different human donors; each human sample is denoted as HS. *Error bars* denote standard deviation across technical replicates. **(B)** ELISA of total NTF protein concentration in CM collected from DPC sheets ± FGF2. The data represent averages of NTF protein expression by three separate sets of DPC sheets produced by cells isolated from different human donors. *Error bars* represent standard deviation across biological replicates. **(C)** NTF protein expression normalized to cell number. The total NTF production as shown **(B)** was normalized to the average number of DPCs present in the cell sheets. *Error bars* represent standard deviation across biological replicates. CM, conditioned medium; ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; NTF, neurotrophic factor; qPCR, quantitative real-time polymerase chain reaction.

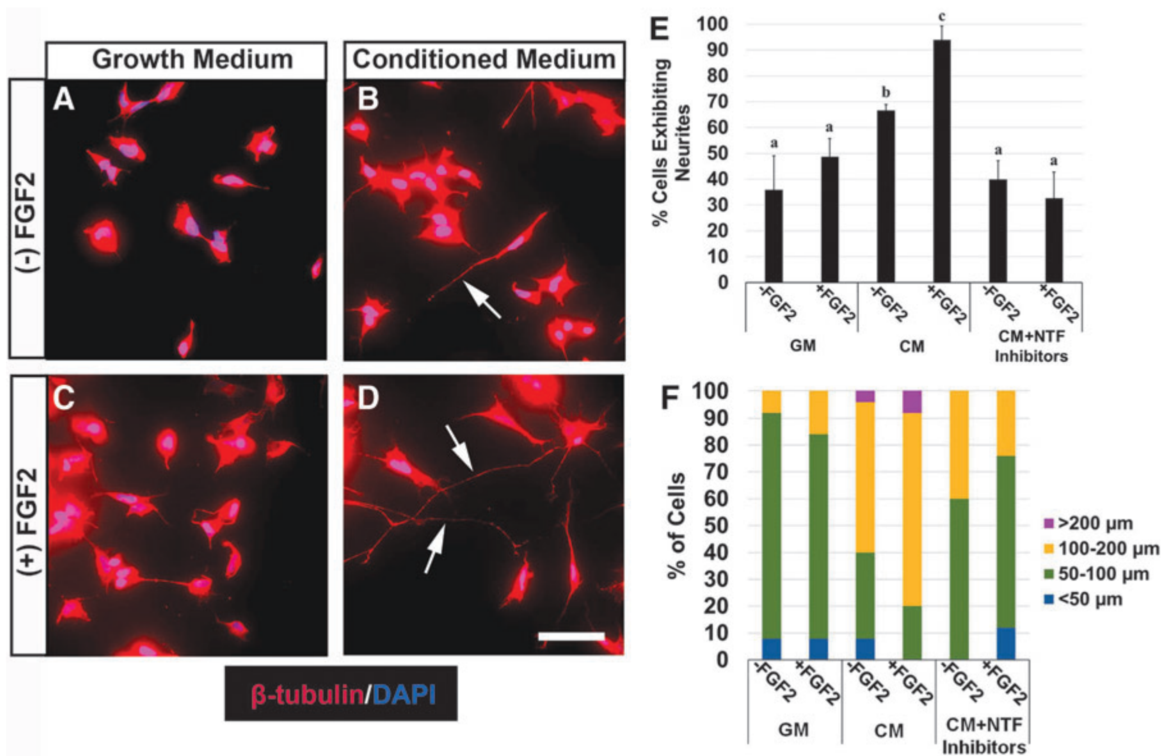


FIG. 4. Functional effect of DPC sheets on neuronally predifferentiated, SH-SY5Y neuroblastoma cells *in vitro*. β-Tubulin (*red*) immunostaining shows neurite extensions in SH-SY5Y cells cultured with GM ± FGF2 and DPC CM ± FGF2 **(A–D)**, images show more and longer neurite extensions (*arrows*) in CM ± groups. Quantification of the percent of SH-SY5Y cells exhibiting neurites is significantly increased when cells are cultured with DPC CM, this effect is reversed by functional inhibition of NTF (significant differences lie between *bars* with *different letters*, $p < 0.05$) **(E)**. DPC sheet CM ± FGF2 induces SH-SY5Y cells to have longer neurites than cells cultured in GM, and this effect is reversed by the functional inhibition of NTF **(F)**. **(A–D)** Scale bar = 50 µm. GM, growth medium.

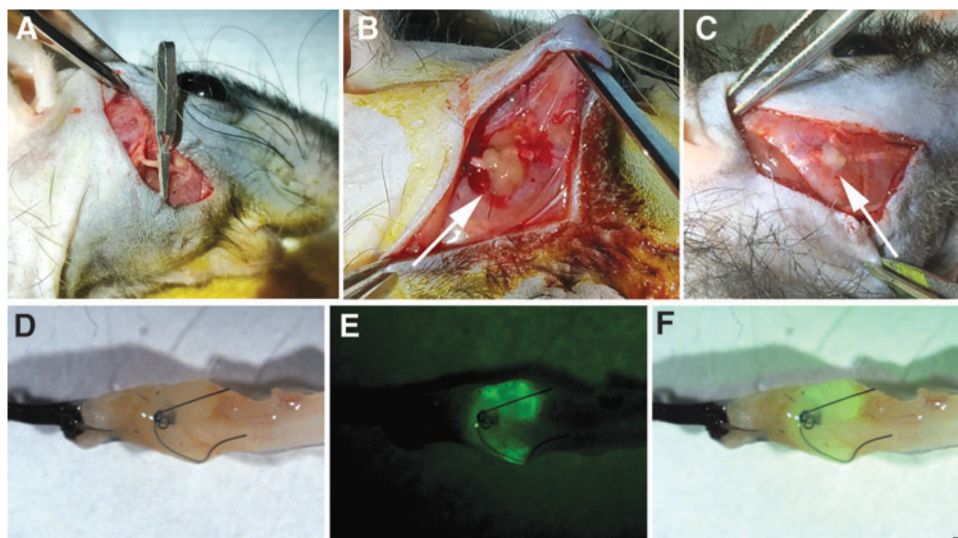


FIG. 5. Images of implantation of a DPC sheet around a facial nerve defect in a rat and the explant after 3 weeks. **(A)** Crush defect was created in the buccal branch of a rat facial nerve using a vessel clip. **(B)** A DPC sheet (*arrow*) is wrapped around the defect site. **(C)** The DPC sheet (*arrow*) remains at the defect site 3 weeks after implantation. **(D)** Bright-field image of dissected nerve explant with the DPC sheet wrap. **(E)** Fluorescent image of the dissected nerve explant showing DPCs (*green*) fluorescing in the DPC sheet wrap. **(F)** Merged image of **D** and **E**.

(Fig. 4F). Both of these functional neurogenesis effects induced by DPC sheet CM (increased number of neurite-positive cells and the formation of longer neurites) were reversed by the addition of NTF inhibitory molecules, indicating that DPC CM has a functional effect on neuroblastoma cell activity through the production of NTFs.

Implantation of DPC sheets on rat facial nerve injuries

The regenerative effects of DPC sheets were evaluated on rat facial nerve crush injuries. Because crush injuries have an intrinsic healing capacity, they present an appropriate injury model to determine if a treatment can accelerate

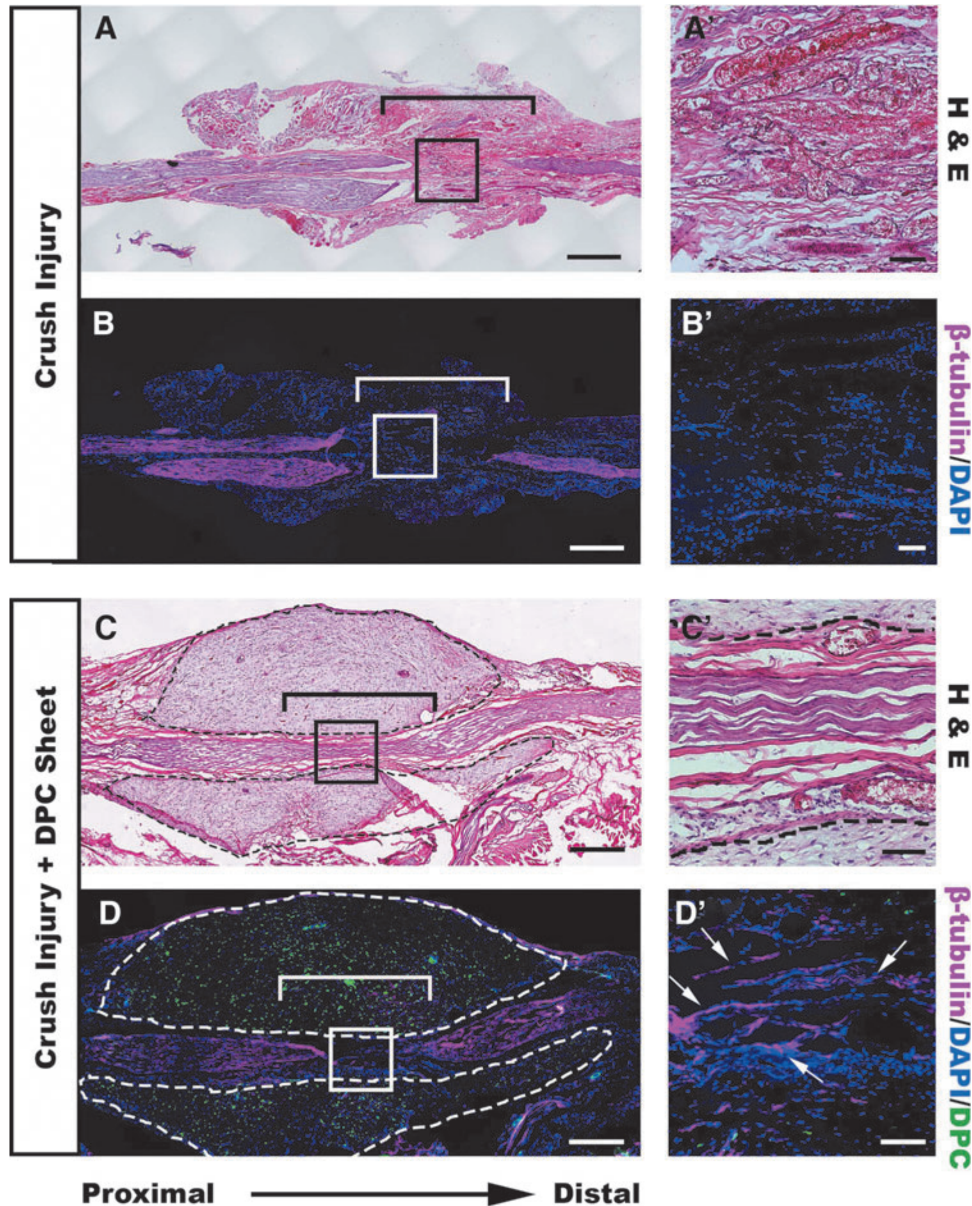


FIG. 6. Histological assessment of nerve explants at a 3-week time point. **(A)** H&E image of a control nerve lacking DPC sheet treatment shows a distinct break in the nerve structure at the defect site and the presence of a highly vascularized tissue lacking any discernable neural tissue, a higher magnified image of the *boxed region* **(A)** is shown **(A′)**. **(B)** β-Tubulin (*magenta*) immunostaining of control explants shows that axons do not extend through the defect region of the nerves lacking DPC treatment, and a higher magnification of the *boxed region* **(B)** is shown **(B′)**. **(C)** In contrast, H&E images of a nerve treated with DPC sheets show the presence of a nerve structure through the defect region, and furthermore, the DPC sheet (outlined by *dashed black lines*) has remained around the defect region of the nerve. Higher magnification image of the *boxed region* **(C)** can be seen **(C′)**, showing more clearly the nerve structure in the defect region; the dashed *black* lines separate the nerve structure and DPC sheet. **(D)** β-Tubulin (*magenta*) immunostaining of the nerve segment treated with DPC sheets shows the presence of axons extending through the defect site, and DPCs (*green*) can be seen in the DPC sheet (outlined by a *dashed white line*). A higher magnification of the *boxed region* **(D)** can be seen **(D′)**, clearly showing β-tubulin (*magenta*)-positive axons (*arrows*) extending through the defect site. The *brackets* **(A–D)** mark the defect region in the nerve. DAPI (*blue*) was used to stain nuclei in the fluorescent images **(B, B′, D, D′)**. Scale bars: **(A–D)** = 500 μm, **(A′–D′)** = 100 μm. DAPI, 4′,6-diamidino-2-phenylindole.

recovery.[3,47–52] The *in vitro* analyses of DPC sheets demonstrated that sheets cultured with FGF2 had increased BDNF and GDNF total protein production and an enhanced functional effect on neuroblastoma cells in comparison with DPC sheets cultured without FGF2. Based on these results, all animal studies were performed using DPC sheets cultured with FGF2.

Crush injuries were created in the buccal branch of a rat facial nerve using vessel clips exerting a specified force (Fig. 5A). DPC sheets were wrapped around the nerve injuries and secured with a single suture (Fig. 5B), and the cell sheets remained secured to the injury site 3 weeks following surgery (Fig. 5C). Before implantation, the DPCs were fluorescently labeled; the cell sheet with fluorescent DPCs can be seen wrapped around the nerve explants (Fig. 5D–F).

Histological evaluation of nerve regeneration in vivo

H&E staining of histological sections of nerve explants shows drastic structural differences between the control nerves and nerves treated with DPC sheets (Fig. 6). The injury site in control explants lacks discernible nerve structure (Fig. 6A, A′), whereas a fascicular structure is present through the injury site of nerves treated with DPC sheets (Fig. 6C, C′). Additionally, the DPC sheet can be seen localized to the nerve injury in the H&E-stained sections of treated explants (Fig. 6C, C′). Minimal β-tubulin-positive axons can be detected in the injury region of control samples, indicating a distinct gap in axon extension through the damaged nerves (Fig. 6B, B′). In contrast, β-tubulin-positive axons can be clearly visualized extending through the injury region of nerves treated with DPC sheets (Fig. 6D, D′). Moreover, the fluorescently labeled DPCs can be seen localized within the cell sheet at the injury site (Fig. 6D, D′). These data suggest that DPC sheets could help maintain the nerve structure and accelerate axon regeneration and extension following nerve injury.

Functional evaluation of nerve regeneration in vivo *through CMAP analysis*

CMAP measurements were performed to evaluate the effect of DPC sheet treatment on nerve functional recovery. Electrical stimulation was applied to the buccal branch of the nerve proximal to the injury and action potential was measured in the vibrissal muscle of the whisker pad of the rats. In comparison with untreated controls, crushed nerves treated with DPC sheets had enhanced electrophysiological functionality, as indicated by the significantly increased CMAP amplitude (Fig. 7). Although at this relatively early time point functionality of these treated nerves is not equivalent to that of healthy nerves, this result shows that DPC sheets accelerate the repair of the damaged nerve and improve the functional outcome.

Discussion

In this study, we have established that DPC sheets can act as local NTF delivery systems to significantly enhance nerve regeneration. DPC sheet treatment was evaluated in a facial nerve injury model; however, we anticipate similar regenerative effects on peripheral nerve recovery throughout the body. We envision that these DPC sheets could be used as stand-alone biomaterials that could augment the current standard of care for treating PNIs to accelerate healing and improve the functional outcome, addressing the major challenges of current methods of treatment. Alternatively, DPC sheets could be used in combination with commercially available nerve conduits or cuffs to provide a bioactive component to improve nerve regeneration. Scaffold-free DPC sheets could be formed using autologous or allogeneic cells and lack exogenous scaffold materials, thereby providing a safe and efficient method to enhance peripheral nerve regeneration.

In this study, we found that DPC sheets produce BDNF, GDNF, and NT3 mRNA and proteins, substantiating previous work reporting the endogenous expression of these

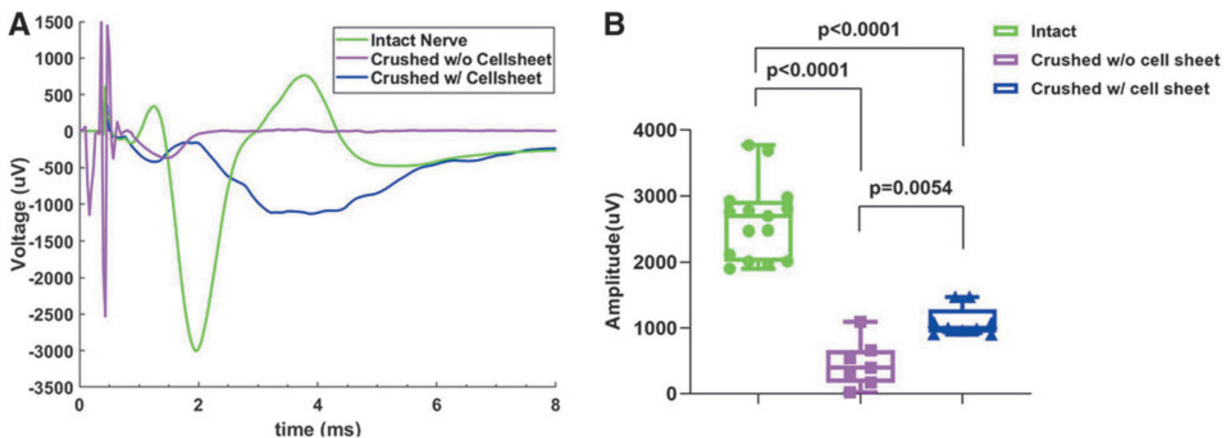


FIG. 7. CMAP analysis of healthy nerves, untreated nerves with crush defect, and nerves with crush defect treated with DPC sheets. **(A)** Average representative CMAP waveforms for three experimental conditions. The sharp peaks within 0–1 ms are the stimulation artifacts and the waveforms within 1–8 ms are the CMAP measurements recorded from the vibrissal muscle. **(B)** The peak-to-peak amplitude of the CMAP for three experimental groups. The crushed groups showed significant amplitude decrease relative to the intact nerve group ($p < 0.0001$). The cell sheet treatment group showed significant amplitude recovery compared with the nontreated group ($p = 0.0054$). CMAP, compound muscle action potential.

NTFs by DPCs.[27,29] We have now further demonstrated that culturing DPCs to confluence to form cell sheets does not negatively affect NTF expression. We observed variability in NTF gene expression in DPC sheets generated using cells collected from different human donors. However, with regard to NTF protein expression, biological sample variability was minimal. We found that DPC sheets produce a substantial amount of NTF proteins, and importantly, this NTF production was sufficient to induce a functional effect on neuronally differentiated, SH-SY5Y neuroblastoma cells. These experiments validate the use of DPC sheets as an NTF delivery system to enhance axon extension.

FGF2 is a multifunctional protein critical for regulating multiple processes during embryonic development, tissue homeostasis, and repair.[53] FGF2 has been shown to have numerous effects on DPCs, including affecting cell proliferation and differentiation.[54] We showed that FGF2 did not largely alter NTF mRNA expression in DPCs cultured as cell sheets. However, DPC sheets cultured with FGF2 comprised significantly more cells, which, in turn, led to increased total production of BDNF and GDNF proteins. Correspondingly, the higher NTF concentration in CM collected from DPC sheets cultured with FGF2 stimulated greater functional effects in neuroblastoma cells *in vitro*. FGF2 did not alter the gene expression for NT3 in DPC sheets; however, a decrease in NT3 protein production was observed. Potentially, NT3 protein expression is further regulated by post-transcriptional modulators affecting the overall release of mature proteins. Alternatively, binding of NT3 proteins to the DPC sheet ECM may also be a contributing factor to the decreased concentration detected in the DPC sheet conditioned media. Recently, Nagashima *et al.* showed that injecting DPCs cultured with FGF2 into a murine spinal cord injury model enhanced functional recovery.[31] Similar to the results presented here, they found that FGF2 did not have an effect on NTF gene expression and speculated that culturing DPCs with FGF2 before *in vivo* injection could have positively affected cell proliferation. The resulting greater number of DPCs may have led to the beneficial regenerative effects of their cellular therapy.

DPC sheets enhanced regeneration in a rat facial nerve injury model *in vivo*. Histological analysis showed that our DPCs remained at the injury site 3 weeks following implantation. A distinct discontinuation of axon extension within the injury site was observed in the facial nerves lacking the cell sheet, but axons clearly extend through the injured region of nerves treated with DPC sheets, suggesting that the DPC sheet accelerates axon regeneration and extension. Importantly, CMAP analysis showed that DPC sheets significantly enhanced functional recovery of injured nerves. It has been documented that rat facial nerve crush injuries are capable of full functional recovery.[47] To observe the accelerating effect of the DPC sheet treatment, histological and functional electrophysiological assessments were performed 3 weeks following surgery since it has been reported that in rat facial nerve crush injuries, significant differences in CMAP amplitude between injured and healthy nerves can still be detected at this time point.[49,51] Accelerating the repair and regeneration is important for gaining full functional recovery, especially for nerve injuries that occur at locations distant to the end organ. DPC sheet treatment showed significantly improved histological outcomes coupled with better functional recovery in comparison with untreated controls, indicating that DPC sheets have significant positive effects on nerve regeneration.

Based on *in vitro* data, we expect that the positive effects of DPC sheets on nerve regeneration *in vivo* were due, in part, to NTF expression by DPCs. However, the DPC sheets could be involved in regulating numerous biological activities. Although the focus of the current work was on NTFs, dental pulp cells secrete various different growth factors and cytokines. We anticipate that DPC sheets may be involved in modulating numerous additional events at the injury site to both enhance nerve regeneration and potentially modulate nerve degeneration, which could widen the scope of this study in future.

In this study, we showed that human DPCs can form robust cell sheets that produce sufficient NTFs to induce a functional effect on neuronal cells *in vitro* and enhance facial nerve regeneration *in vivo*. The cell sheets developed here could be used to augment current standard of care of PNIs to accelerate healing and improve the functional outcome. The accessibility of DPCs from autologous and allogeneic tissues and the already ongoing clinical assessment of scaffold-free cell sheets[41–43] increase the likelihood of translating these DPC sheets to enhance nerve regeneration in humans, thereby improving patient quality of life.

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No competing financial interests exist.

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