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Using a Transection Paradigm to Enhance the Repair Mechanisms of an Investigational Human Cell Therapy

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Abstract

One promising strategy in cell therapies for Parkinson's disease (PD) is to harness a patient's own cells to provide neuroprotection in areas of the brain affected by neurodegeneration. No treatment exists to replace cells in the brain. Thus, our goal has been to support sick neurons and slow neurodegeneration by transplanting living repair tissue from the peripheral nervous system into the substantia nigra of those with PD. Our group has pioneered the transplantation of transection-activated sural nerve fascicles into the brain of human subjects with PD. Our experience in sural nerve transplantation has supported the safety and feasibility of this approach. As part of a paradigm to assess the reparative properties of human sural nerve following a transection injury, we collected nerve tissue approximately 2 weeks after sural nerve transection for immunoassays from 15 participants, and collected samples from two additional participants for single nuclei RNA sequencing. We quantified the expression of key neuroprotective and select anti-apoptotic genes along with their corresponding protein levels using immunoassays. The single nuclei data clustered into 10 distinctive groups defined on the basis of previously published cell type-specific genes. Transection-induced reparative peripheral nerve tissue showed RNA expression of neuroprotective factors and anti-apoptotic factors across multiple cell types after nerve injury induction. Key proteins of interest (BDNF, GDNF, beta-NGF, PDGFB, and VEGF) were upregulated in reparative tissue. These results provide insight on this repair tissue's utility as a neuroprotective cell therapy.

Keywords

cell therapy, tissue-based therapy, peripheral nerve, single nuclei RNA sequencing, neuroprotection

Introduction

The use of human embryonic and neural stem cells has limitations as cell therapies for Parkinson's disease (PD)[1–3]. Obtaining embryonic or fetal cells can be ethically challenging, and embryos for transplantation are not always readily available[1,2,4]. Furthermore, they are not autologous tissues and require the patient to use immunosuppressing drugs. Other stem cell sources include autologous induced pluripotent stem (iPS) cells differentiated into dopaminergic progenitors; however, their use in clinical testing in PD is still in its infancy[5]. In addition, incompletely reprogrammed cells can elicit harmful immune responses[6,7]. A more feasible approach could be to use the body's own repair mechanisms. Autologous tissue, like peripheral nerve, has robust repair capabilities, is readily available, and can be efficiently procured[8,9]. Our strategy is to harness the patient's own reparative peripheral nerve tissue and

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implant it to provide neuroprotection to areas of the brain affected by neurodegenerative disease.

After injury, cells in the peripheral nervous system (PNS) undergo a highly orchestrated transformation to regenerate and re-establish function to the extremities[10–12]. As part of an investigational cell therapy that we are currently trialing, we implanted active reparative peripheral nerve tissue into the substantia nigra of participants with PD. No treatment exists to repair damaged brain cells; thus, our goal was to slow neurodegeneration by implanting reparative living cells into the substantia nigra[8,9,13].

Cell therapy strategies can affect disease progression either by replacing dead or dying cells, or by promoting cell-survival and neuroprotection via secretion of paracrine and neurotrophic factors. Implanting peripheral nerve tissue would not replace sick neurons of the central nervous system, but could provide neuroprotective, anti-inflammatory, anti-apoptotic, and pro-regenerative factors to support dying cells[14–16]. Furthermore, implanting autologous peripheral nerve tissue has the major advantages of being readily obtainable from patients and circumventing host immune rejection. If obtained from a sensory nerve like the sural nerve, the side effects, if any, are mostly paresthesias and hypoesthesia in cutaneous distribution of the nerve[17,18]. In our previous studies, participants have reported that these incidents were not bothersome in the long term[9,13,15].

An ideal cell therapy against neurodegeneration would be robust enough to slow disease progression by providing neuroprotective, anti-apoptotic, and anti-inflammatory support to unhealthy cells while maintaining its potency from harvesting through deployment. Our approach has been to implant a supportive milieu of neuroprotective factors with the expectation that a combination of factors is more durable and effective than using a single neuroprotective factor therapy[19,20]. In addition, we have used peripheral nerve fascicles rather than single cell products (eg, Schwann cells) that have been used in nonclinical rodent and non-human primate animal models[21,22]. To date, definitive trials of single-neuroprotective factor therapy, for example, glial-cell derived neurotrophic factor (GDNF), have shown mixed results in slowing PD progression[19,23–25]. The survival of neural stem cells after transplantation is limited as a proportion of cells die within days after transplantation into the brain[26–28]. Meanwhile, anti-apoptotic factors in the implanted cells could bolster cell survival after implantation[29].

Even though there is a great deal known about the reparative microenvironment of injured peripheral nerves in animal models, data on human peripheral nerve repair and its neuroprotective properties are sparse. To fill this gap in knowledge, we collected human peripheral (sural) nerve tissue before and after transection injury (herein referred to as naïve and reparative for consistency). We used single nuclei RNA sequencing (RNA seq) and immunoassays to generate a profile of the cell types, the RNA expression, and protein content of key neuroprotective factors present in reparative peripheral nerve tissue. The objective of this study is to report our findings of the specific cell types and contents of the reparative human nerve. Our results focus primarily on the properties and contents of the reparative tissue as this is the final product implanted into the brain. This information will provide insight on this tissue's utility as a neuroprotective cell therapy.

Methods

Research Participants

The collection of peripheral nerve tissue was approved as part of a more expansive clinical trial that received approval from the University of Kentucky's Institutional Review Board and was registered at clinicaltrials.gov (NCT02369003). The participants provided written informed consent. Peripheral nerve tissue of the sural nerve was collected from 15 participants before and after sural nerve transection *in situ* for immunoassay studies. The range of differences between naïve and reparative tissue has been previously published by Chau et al[15]. Sural nerve tissue was collected from two additional participants for single nuclei RNA seq studies.

Peripheral nerve tissue samples were collected from 15 participants for immunoassays (mean: 60 years old, range: 51–69 years, assigned birth sex: 9 male/6 female, years since PD diagnosis mean: 10 years, range: 4–17 years). Samples were collected from two additional participants for single nuclei RNA seq (ages were 49 and 60 years old, assigned birth sex: both male, years diagnosed with PD: 7 and 5 years). The time between transection of the nerve (naïve) and collection of regenerating peripheral nerve tissue was 12 and 17 days for participants 1 and 2, respectively.

Peripheral Nerve Transection and Tissue Collection

Transection of the peripheral nerve and the tissue collection has been previously described[8,9,13]. Fig. 1 illustrates the naïve and transection injury–induced sural nerve collection approximately 2 weeks after transection. Conventionally, naïve nerve tissue is defined as tissue that had not degenerated. Our naïve tissue had been transected (and flash-frozen after) to remove it from the body, so it is not completely uninjured. However, for the purposes of this study and ease of understanding, we will call this "naïve" tissue at 0 days relative to the "reparative" tissue at approximately 2 weeks.

Briefly, the neurosurgeon identified the neurovascular bundle containing the sural nerve in the ankle and transected it and removed 1 to 2 cm of nerve of naïve tissue (Fig. 1). The nerve tissue was cleaned of loosely external tissues (fat tissue, blood vessels), and individual nerve fascicles (usually 6–10 per patient) were separated manually. The fascicles were cleaned of adherent connective tissue and snap-frozen in centrifuge tubes on crushed dry ice. The fascicles were

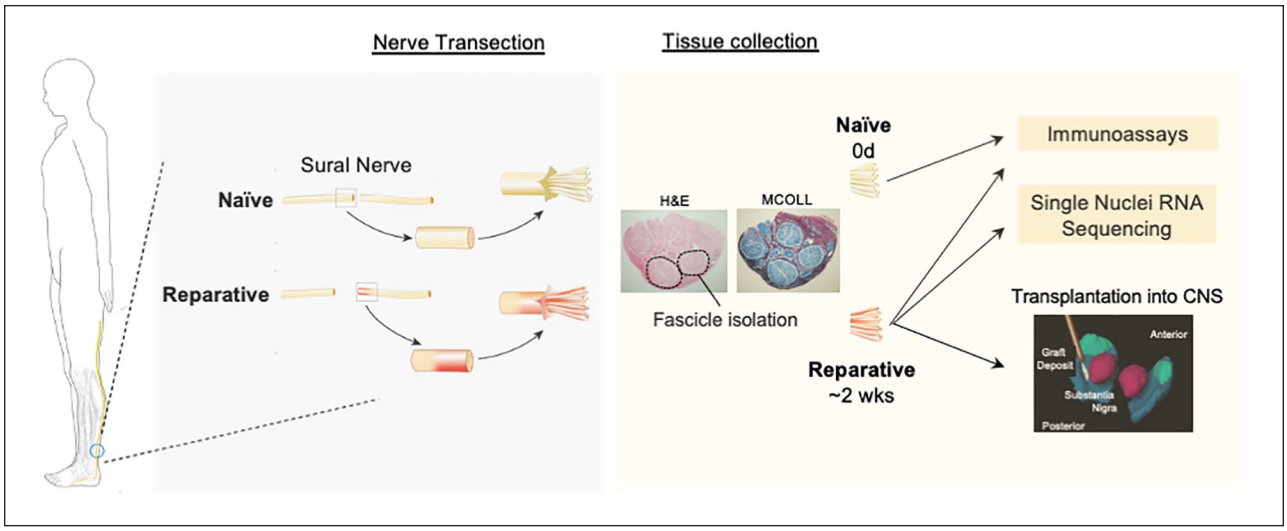


Figure 1. Study overview. This overview illustrates our sural nerve transection approach and subsequent tissue collection of the naïve and reparative nerve tissues. One to two centimeters of nerve was excised, which we called "naïve" nerve tissue. Approximately 14 days after, 1 to 2 centimeters from the distal nerve stump of the same nerve was excised, which we called "reparative" nerve tissue. Cross-sections of reparative sural nerve were stained with H&E (left) and Luxol fast blue (LBF)/MCOLL staining to show myelin and collagen. Individual nerve fascicles were separated, snap-frozen, and used for single nuclei RNA sequencing and immunoassays or implanted directly into the brain as part of our clinical trial. Shown here is a 3D view of reparative nerve fascicles implanted into the substantia nigra in the cell therapy trial. H&E: hematoxylin and eosin; CNS: central nervous system; 3D: three dimensional.

stored in a –80 Freezer for analysis by immunoassays (as in Welleford et al.)[13]. Approximately 2 weeks later, for both analysis and implantation into the substantia nigra for the clinical trial (Fig. 1), the ankle incision was reopened and 1 to 2 cm of the injured peripheral nerve tissue was excised from the distal nerve stump. The individual nerve fascicles were separated, snap-frozen, and stored for assays as described above for the naïve tissue[13]. Transection-injured tissue collection time from dissection to snap-freezing was 32 min for one participant, and 67 min for the other participant. These frozen samples of nerve fascicles were used in single nuclei RNA seq and immunoassays (weight per sample ranged from 40 to 170 mg).

Histology

Reparative peripheral nerve was stained with hematoxylin and eosin (H&E) and MCOLL staining. MCOLL staining distinguishes myelin, collagen fibers, and cells in the peripheral nerve[30]. The nerve was placed in 4% paraformaldehyde solution, then embedded in paraffin blocks for histology[31]. Sections were all taken from within approximately 1 mm from the end of each nerve (as the sections were cut from the terminal end).

Single Nuclei RNA Seq

Single nuclei RNA seq and analysis were conducted by Singulomics Corporation (https://singulomics.com, Bronx, NY). In summary, frozen sural nerve fascicles (1–2 cm each) were homogenized and lysed with Triton X-100 in RNase-free water for nuclei isolation. The isolated nuclei were purified, centrifuged, and resuspended in phosphate buffered saline (PBS) with bovine serum albumin (BSA) and ribonuclease (RNAse) Inhibitor. The nuclei were diluted to 700 nuclei/ul and loaded to 10x Genomics Chromium Controller to encapsulate single nuclei into droplet emulsions following the manufacturer's recommendations (Pleasanton, CA, USA). Library preparation was performed according to the instructions in the Chromium Next GEM 3′ Single Cell Reagent kits v3.1. Amplified cDNAs and the libraries were measured by Qubit dsDNA HS assay (Thermo Fisher Scientific, Wilmington, DE, USA) and quality assessed by BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Libraries were sequenced on a NovaSeq 6000 instrument (Illumina, San Diego, CA, USA), and reads were subsequently processed using 10x Genomics Cell Ranger analytical pipeline (v5.0) and human GRCh38 reference genome with introns included in the analysis. Dataset aggregation was performed using the cellranger aggr function normalizing for the total number of confidently mapped reads across libraries.

Seurat 4.0.1 was used to further clean and normalize the data. The data from barcodes with mitochondrial genes at a level of $<5\%$ of total gene counts and with a minimum of 1400 UMI counts were retained. Gene read counts were normalized with the Seurat "NormalizeData" function. The top 3000 highly variable genes were identified using Seurat "FindVariableFeatures" function, which were used for principal components analysis (PCA) using Seurat "RunPCA"

function. Clustering was done using Seurat "FindClusters" function based on 11 PCAs. The ElbowPlot test was done to determine the number of PCAs used. Visualization of the cells was performed using the Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) algorithm as implemented by the Seurat "runUMAP" function. Violin plots were graphed as a log2 fold average count for RNA expression in each defined cell cluster through C Loupe 5.0 software (10x Genomics).

Immunoassays for Neuroprotective and Anti-Apoptotic Factors

To quantify the neuroprotective and anti-apoptotic factors of interest present in reparative nerve, tissues were analyzed using enzyme-linked immunosorbent assay (ELISA and multiplex Luminex® immunoassays (Cincinnati Children's hospital flow cytometry core). We analyzed reparative tissues from 15 participants.

Analyte concentrations in the reparative nerve tissue sample supernatants were determined by ELISA according to the manufacturer's protocol. The sources and dilutions used were as follows: neuroprotective factors: cerebral dopamine neurotrophic factor (CDNF; Abcam, Waltham, MA, USA), tissue samples were diluted 1:10; nerve growth factor receptor (NGFR; ThermoFisher Scientific, Carlsbad, CA), tissue samples were diluted 1:2; erythropoietin (EPO) concentrations in the sample supernatants were determined by using MilliplexTM Multiplex kits (MilliporeSigma, Darmstadt, Germany); brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF, beta-NGF), platelet-derived growth factor–AA (PDGF-AA), platelet-derived growth factor–BB (PDGF-BB), platelet-derived growth factor–AB (PDGF-AB), vascular endothelial growth factor (VEGF), GDNF, and neurotrophin-3 (NT-3) were determined by Human Magnetic Luminex Assays (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol; anti-apoptosis factors: nuclear factor erythroid 2–related factor 2 (NRF2) (ThermoFisher Scientific), tissue samples diluted to 1:2; B-cell lymphoma 6 (BCL-6; MyBiosource, San Diego, CA, USA), tissue samples were neat.

Availability of Data and Materials

Data files (.cloupe) can be obtained from the UKnowledge database (https://doi.org/10.13023/bkf8-z725).

Results

Reparative Peripheral Nerve Tissue Contains Transcriptionally Distinctive Cell Types

We used single nuclei RNA seq to identify the cell types present in reparative peripheral nerve tissue based on transcriptional profiling (Fig. 2A). Single nuclei RNA seq data from two participants were aggregated (Fig. 2A). Cell clustering by cell type was reproducible across both participants (Fig. 2B). The data clustered into 10 cell groups and were defined based on characteristic genes in previously published single cell data for each particular cell type (Fig. 2C)[32–41]. –Cell counts were obtained from the single nuclei RNA seq analysis and each cluster's percentage was reported (Fig. 2D). An output of 2425 cells and 2643 cells were used from both participants.

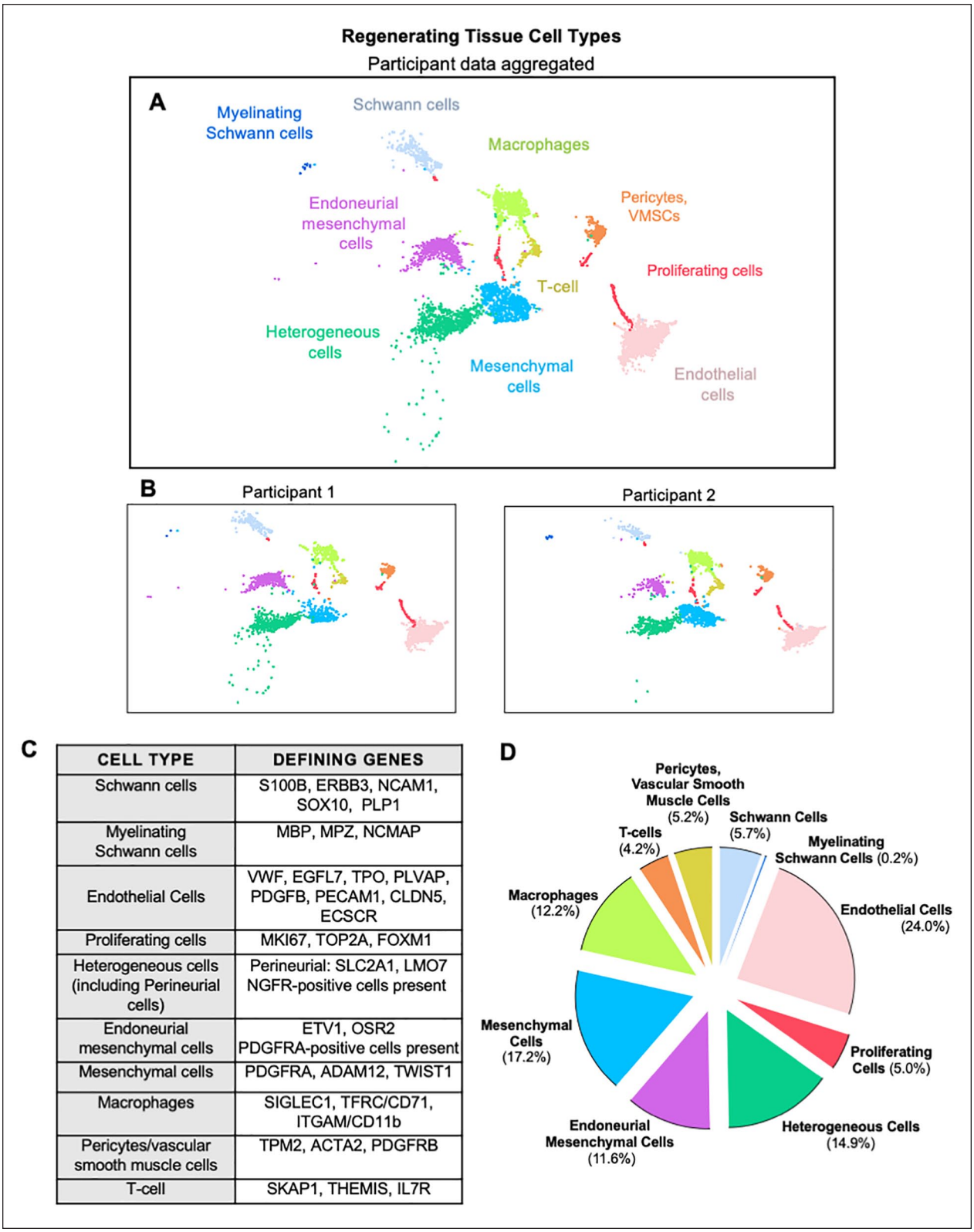
Cell clusters observed included typical Schwann cells (defined by their expression of *S100B*, *ERBB3*, and other lineage-specific genes), macrophages (*ITGAM*), T-cells (*SKAP1*), endothelial cells (*PECAM1*), and pericytes (*ACTA2*) (Fig. 2C). The content of mature (myelinating) Schwann cells was negligible (0.2%). The small number of (myelinating) Schwann cells was an expected finding considering that the nerve transection had a removal of the intermediate segment. The proximal and distal stumps could not physically reconnect, thus could not form axonal regeneration. A proportion of Schwann cells, macrophages, endothelial cells, and T-cells exhibited expression of genes associated with active cell division (Fig. 2A, B, red clusters). The distribution of proliferative cells was coincidentally conserved in the two donors (Fig. 2B).

Different than what we had expected, a large proportion of the cells were mesenchymal cells including a cluster of mesenchymal cells expressing stem cell–associated genes (*TWIST1*[37], *PDGFRA*[37], and *ADAM12*) and endoneurial mesenchymal cells (Fig. 2C, D). These endoneurial mesenchymal cells were defined by *ETV1*[32,40] and *OSR2*[33,40,41], and were positive for the mesenchymal marker, *PDGFRA* (Fig. 2C). Another unexpected result was that one cell cluster contained a heterogeneous mix of cells including perineurial cells with the expression of markers *SLC2A1*[32] and *LMO7*[42], and possibly repair Schwann cells (*NGFR*)[37].

Reparative Peripheral Nerve Tissue Shows RNA Expression of Neuroprotective Factors 2 Weeks After Nerve Transection

In this study, we aimed to identify a select group of neuroprotective factors in the regenerating tissue and localize the RNA expression to specific cell types (Fig. 3). This differs from our previous work in the whole nerve tissue in which Welleford et al. had identified gene expression of neuroprotective and anti-apoptotic factor pathways[13]. UMAP plots illustrate the cell-type expression of the neuroprotective factors of interest: *BDNF*, *EPO*, *CDNF*, *GDNF*, *NGF*, *PDGFA*, *PDGFB*, and *VEGFA* (Fig. 3A). The accompanying violin plots show the relative RNA expression level (log2 average) and frequencies for each cell type in the regenerating peripheral nerve tissue (Fig. 3A).

Even though certain factors (*GDNF*, *CDNF*, *EPO*) did not show much RNA expression, we included these data as we compared them with their protein level expression. Repair Schwann cells are a source of neuroprotective factor release



CELL TYPE	DEFINING GENES
Schwann cells	S100B, ERBB3, NCAM1, SOX10, PLP1
Myelinating Schwann cells	MBP, MPZ, NCMAP
Endothelial Cells	VWF, EGFL7, TPO, PLVAP, PDGFB, PECAM1, CLDN5, ECSCR
Proliferating cells	MKI67, TOP2A, FOXM1
Heterogeneous cells (including Perineurial cells)	Perineurial: SLC2A1, LMO7 NGFR-positive cells present
Endoneurial mesenchymal cells	ETV1, OSR2 PDGFRA-positive cells present
Mesenchymal cells	PDGFRA, ADAM12, TWIST1
Macrophages	SIGLEC1, TFRC/CD71, ITGAM/CD11b
Pericytes/vascular smooth muscle cells	TPM2, ACTA2, PDGFRB
T-cell	SKAP1, THEMIS, IL7R

Figure 2. Reparative peripheral nerve tissue contains several cell types including regenerating cells. (A) Aggregate of single nuclei RNA sequencing from two participants. Ten unique cell type clusters were present in the reparative tissue. (B) Data from two participants show similarity in their cellular profiles. (C) The data clustered into 10 cell groups and were defined based on characteristic genes previously published for each particular cell type. (D) Each cell cluster's percentage of total number of cells is reported.

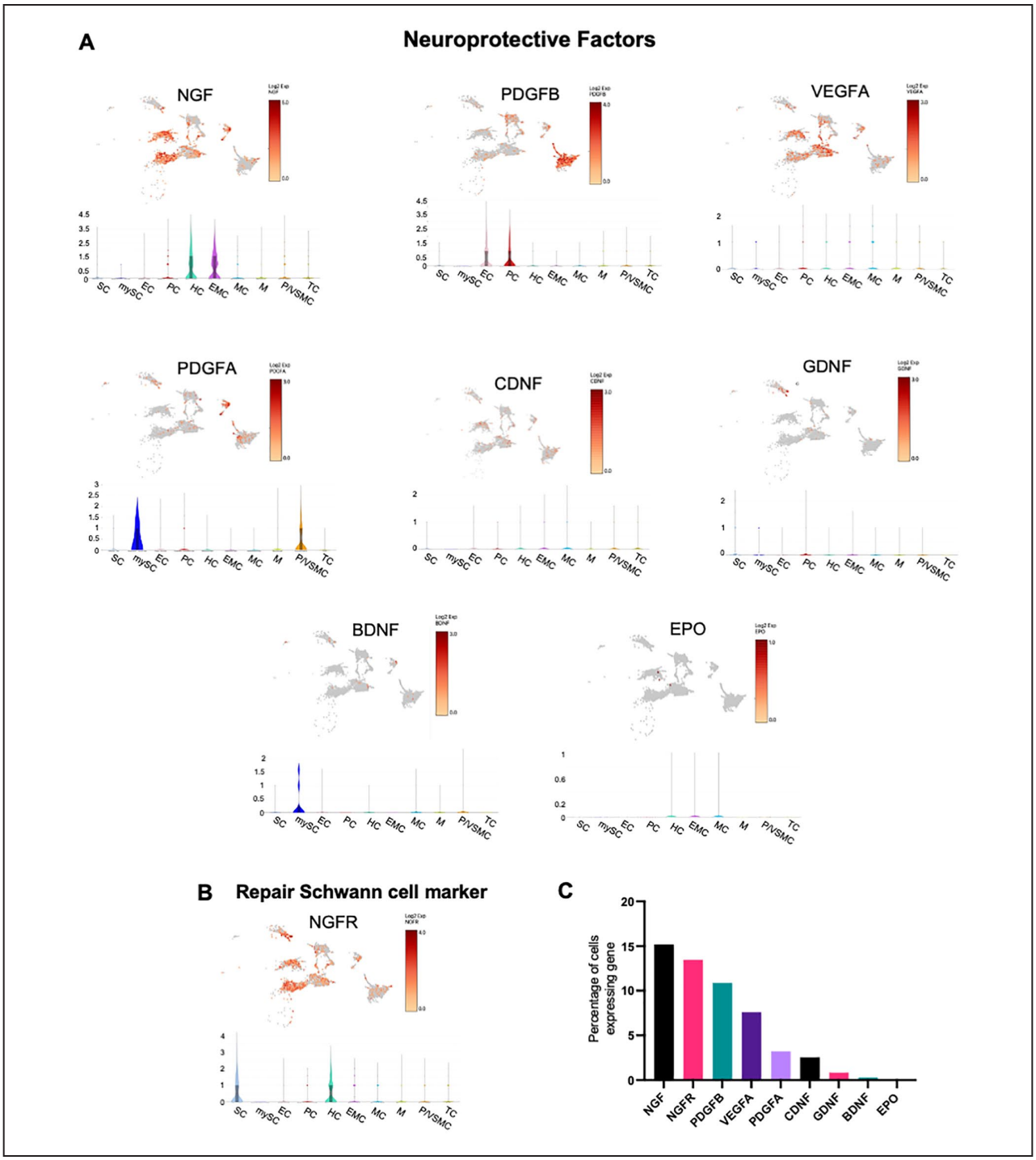


Figure 3. Reparative peripheral nerve tissue shows RNA expression of neuroprotective factors 2 weeks after nerve transection. (A) Single nuclei RNA sequencing UMAP plots show cell-type expression of the neuroprotective factors of interest: *NGF*, *PDGFB*, *VEGFA*, *PDGFA*, *CDNF*, *GDNF*, *BDNF*, and *EPO*. The accompanying violin plots show the relative RNA expression level (log2 average) and frequencies for each cell type in the reparative peripheral nerve tissue. *NGF*, *PDGFA*, *PDGFB*, and *VEGF* were localized to more than one cell type. *NGF* was localized to the heterogeneous cell cluster and endoneurial mesenchymal cells, *NGFR* was localized to Schwann cells and the heterogeneous cell cluster, *PDGFA* was localized to myelinating Schwann cells and pericytes/vascular smooth muscle cells, *PDGFB* was highly expressed in endothelial cells. Cell type key: SC: Schwann cell; mySC: myelinating Schwann cells; EC: endothelial cells; PC: proliferating cells; HC: heterogeneous cells; EMC: endoneurial mesenchymal cells; MC: mesenchymal cells; M: macrophages; PC/VMSC: pericytes/vascular smooth muscle cells; TC: T-cells. (B) Repair Schwann cells are a source of neuroprotective factor release. *NGFR* is an abundantly expressed repair Schwann cell marker that was found in the Schwann and heterogeneous cell clusters. (C) Percentage of

Figure 3. (continued)

total cells expressing each key neuroprotective factors. Note that the x-axis maximum is 20%. Data obtained from $N = 2$ participants. BDNF: brain-derived neurotrophic factor; CDNF: cerebral dopamine neurotrophic factor; GDNF: glial-cell derived neurotrophic factor; NGF: nerve growth factor; NGFR: nerve growth factor receptor; PDGFA: platelet-derived growth factor–A; PDGFB: platelet-derived growth factor–B; VEGFA: vascular endothelial growth factor A; EPO: erythropoietin; UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction.

in reparative nerve. We included the most abundant repair Schwann cell marker expressed in the tissue, *NGFR*[37] to understand the relative location and presence of repair Schwann cells in the tissue (Fig. 3B).

Factors such as *NGF*, *PDGFA*, *PDGFB*, and *VEGFA* were localized to more than one cell type (Fig. 3A, violin plots). *NGF* was localized to the heterogeneous cells cluster and endoneurial mesenchymal cell cluster, *PDGFA* was localized to myelinating Schwann cells and pericytes/vascular smooth muscle cells, *PDGFB* was highly expressed in endothelial cells and proliferating cells. Among the key neuroprotective factors, *NGF* (15.2% of cells) and *PDGFB* (10.9% of cells) were the most widely expressed factors (RNA) across cells of the reparative nerve tissue while *GDNF* (0.8%), *BDNF* (0.3%), and *EPO* (0.04%) were the most limited in expression (Fig. 3B). *NGFR* was expressed in 13.5% of cells (Fig. 3B, note that the x-axis maximum is 20%).

Reparative Peripheral Nerve Tissue Shows RNA Expression of Anti-Apoptotic Factors Across Multiple Cell Types

The anti-apoptotic factors, *NFE2L2 (NRF2)*, *BCL2*, *BCL2L1 (Bcl-xl)*, and *MCL1* were expressed broadly and robustly in many of the cell types. Violin plots show the relative expression across cell types (log2 average) (Fig. 4A). Among the anti-apoptosis factors, *NFE2L2* (36.3% of cells) and *BCL2* (28.6% of cells) were the most widely expressed factors across cells of the reparative peripheral nerve tissue while *BCL6* (16.9%) and *MCL1* (14.5%) were the most limited in expression (Fig. 4B, note that the x-axis maximum is 40%).

Protein Content of Neuroprotective Factors and Anti-Apoptotic Factors

To measure the protein content of neuroprotective and anti-apoptotic factors in reparative peripheral nerve tissue, we conducted immunoassays for several proteins we had characterized with single nuclei RNA seq (Figs. 2–4). The mean protein concentration (and SD) in reparative peripheral nerve tissue samples is summarized in Fig. 5.

Discussion

In this report, we focused on the final product that is implanted into the brain in our clinical trial, the reparative peripheral nerve. We identified the cell types in the peripheral nerve tissue that is used for implantation. Furthermore, this work details the distribution of neuroprotective and anti-apoptotic factors within these cell types, and their protein concentrations. In animal models, multiple cell types contribute to peripheral nerve repair, notably repair Schwann cells[43–46], endothelial cells, and immune cells such as macrophages[47–49]. In the two single nuclei RNA seq subjects with PD in this study, the major cell types in reparative human peripheral nerve tissue were consistent and reproducible (Fig. 2). Understanding the contents of this investigational cell therapy is a critical step in optimizing the product's survival and its ability to neuroprotect vulnerable neurons. We collected samples from same-subject *in situ* peripheral nerve degeneration. It is not typically feasible to collect tissues before an injury has occurred[50]; thus, this type of information has not been available in humans before.

Actively Regenerating Nerve Tissue Serves as a Vehicle for Neuroprotective Factors

In this study, we show a clear upregulation of neuroprotective factors. Interestingly, the results showed low mRNA expression for certain neuroprotective factors including *BDNF*, *GDNF*, *VEGF*, but the protein content was highly expressed. Low mRNA expression but high protein expression may mean that the mRNA was already translated into protein at this time point.

To provide the putative beneficial factors found in reparative peripheral nerve tissue requires the delivery of the collective content of peripheral nerve tissue and not a single cell-type in isolation[51,52]. The combination of cell types has the benefit of including all of the neuroprotective, growth, pro-regenerative, cell survival factors, and anti-inflammatory factors, available from living reparative nerve tissue to support of degenerating cells in the central nervous system (CNS)[13,15] (Fig. 6).

The reparative human peripheral nerve is living tissue, as it is freshly dissected from the participant's ankle and immediately implanted into the brain (Fig. 1). For how long the neuroprotection continues after implantation remains unclear. We speculate that there is a persistent delivery of these factors up to a point to support degenerating cells. In previous reports of participants that had received this implantation in preliminary, open-label trials, we observed an improvement in Unified Parkinson's Disease Rating Scale (UPDRS) part III motors scores at 12 months[9,53]. Furthermore,

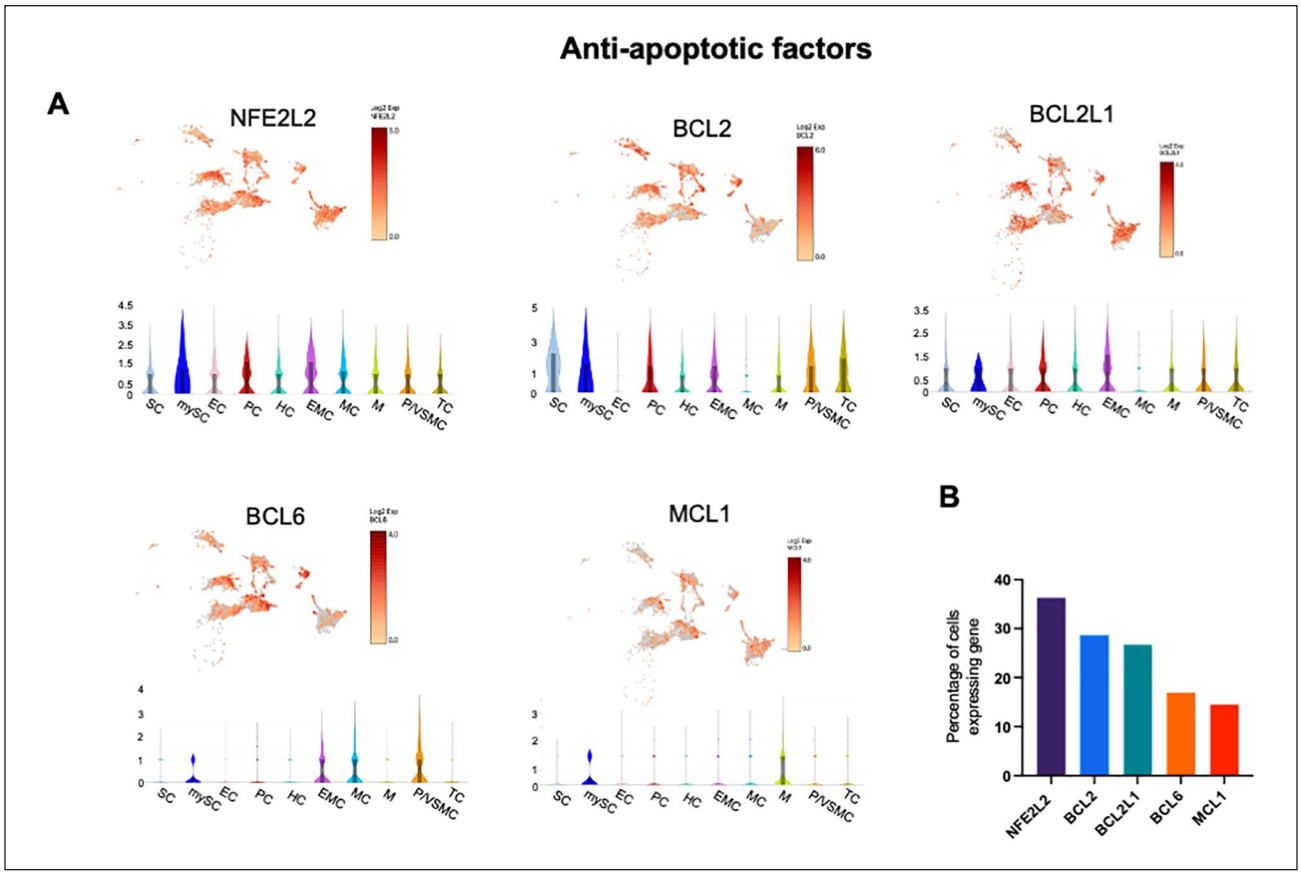


Figure 4. Reparative peripheral nerve tissue shows RNA expression of anti-apoptotic factors across multiple cell types. (A) Anti-apoptosis factors were expressed broadly and robustly in many of the cell types of the reparative nerve for factors of interest: *NFE2L2 (NRF2)*, *BCL2*, *BCL2L1 (Bcl-xl)*, *BCL6*, and *MCL1* of reparative peripheral nerve tissue. Violin plots show the relative expression across cell types (log2 average). Cell type key: SC: Schwann cell; mySC: myelinating Schwann cells; EC: endothelial cells; PC: proliferating cells; HC: heterogeneous cells; EMC: endoneurial mesenchymal cells; MC: mesenchymal cells; M: macrophages; PC/VMSC: pericytes/vascular smooth muscle cells; TC: T-cells. (B) Percentage of total cells expressing each anti-apoptotic factors. Note that the x-axis maximum is 40%. Data obtained from $N = 2$ participants.

in unpublished results from our group, post-mortem sections of a participant who had been engrafted with reparative peripheral nerve tissue to the midbrain 33 months earlier showed immunoreactivity to NGFR present in the area around the engraftment. This suggests the presence of Schwann-like glia or repair Schwann cells[37] which could be a source of neuroprotective factor release and repair. Future studies should be designed to measure secretion levels from reparative peripheral nerve tissue to analyze this tissue beyond the implantation site.

Even though the anti-apoptotic factors that we highlight in this study are not secreted, their robust expression in the transplanted cells could bolster their own survival after implantation. It is typical to find high levels of anti-apoptotic genes in transected nerves in all cell types. These cells are primed for survival and reprogramming due to the transection[46,54,55]. We used this to our advantage to implant a robust product. A percentage of transplanted neural stem cells die due to the trauma and manipulation of cells days after transplantation into the brain[26–28]. This suggests that cell survival may be supported by a robust expression of anti-apoptotic factors in the implanted cells[29]. In addition, neurotrophic factors are also inherently anti-apoptotic and pro-survival.

Beneficial Cell Types in Active Reparative Tissue

Repair Schwann cells. Much of the work surrounding the reparative cell types in peripheral nerve tissue focuses on repair Schwann cells[46,55,56]. After injury or transection, Schwann cells undergo an extensive reprogramming that transforms the mature myelinating and non-myelinating (Remak) cells into dedifferentiated, repair cells[46,55,56]. Repair Schwann cells release neuroprotective factors to facilitate axonal regeneration[46,56–58]. In the early phase of the response to injury, Schwann cells undergo an epithelial-mesenchymal

Protein	Reparative Nerve Protein Content mean (SD)
BDNF	8.0 (7.7) pg/ml
CDNF	173.7 (98.6) pg/ml
EPO	321.6 (148.2) pg/ml
GDNF	53.2 (36.1) pg/ml
beta-NGF	42.5 (51.3) pg/ml
NGFR	300 (200) pg/ml
PDGF-AA	4.9 (3.0) pg/ml
PDGF-BB	3.7 (2.7) pg/ml
VEGF	6.9 (5.0) pg/ml
NFE2L2	61.2 (22.0) pg/ml
BCL6	1200 (600) pg/ml

Figure 5. Protein content of neuroprotective factors and anti-apoptotic factors. The mean protein concentration (and SD) in regenerating peripheral nerve tissue samples is summarized. BDNF (n = 15 participant samples), GDNF (n = 7), beta-NGF (n = 12), PDGFB (n = 15), VEGF (n = 15), NGFR (n = 14), CDNF (n = 15), PDGFA (n = 13), EPO (n = 13), NFE2L2 (n = 14), and BCL-6 (n = 15). BDNF: brain-derived neurotrophic factor; CDNF: cerebral dopamine neurotrophic factor; EPO: erythropoietin; GDNF: glial-cell derived neurotrophic factor; NGF: nerve growth factor; NGFR: nerve growth factor receptor; PDGFA: platelet-derived growth factor–A; PDGFB: platelet-derived growth factor–B; VEGF: vascular endothelial growth factor.

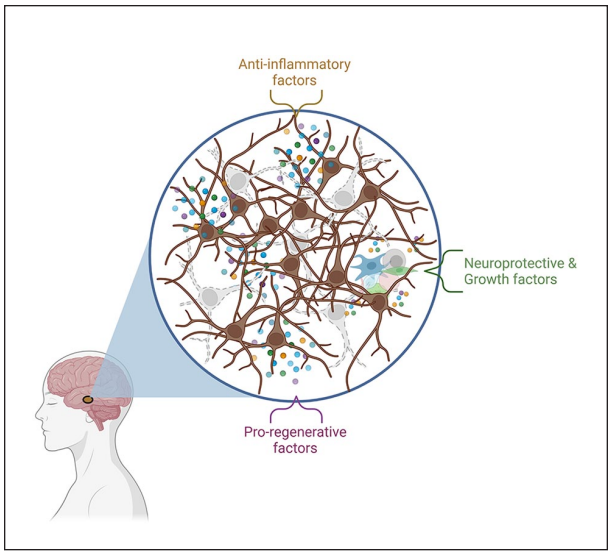


Figure 6. Proposed action of reparative peripheral nerve tissue transplant. Reparative peripheral nerve tissue deployed into the substantia nigra of participants with PD may act in multi-factorial ways with paracrine effects on the surrounding tissue. Anti-apoptotic factors may contribute to graft survival. Through this combination, a diversity of cell-types from regenerating peripheral nerve tissue could provide neuroprotective, pro-regenerative, and anti-inflammatory factors interacting with the degenerating cells in the CNS. Created with Biorender.com. CNS: central nervous system; PD: Parkinson's disease.

transition (EMT)-like process with the upregulation in the expression of stem cell–associated transcription factors such as Sox2, Notch1, and Oct6. This EMT-like process transforms cells to be similar to multipotent stem cells and release neurotrophic factors and support cell survival[36]. In animal models, transplanted Schwann cells have been shown to provide support to dopaminergic cells *in vivo*[21,22].

To contextualize this to our single nuclei RNA seq analysis, the data segregated into a very small population of myelinating Schwann cells (0.2%, Fig. 2) and a larger population of non-myelinating Schwann cells (5.7%). These clusters shown represent the "typical" Schwann cells based on classic marker expression (*MBP*, *MPZ*, *S100B*, *ERBB3*, *NCAM1*, *and SOX10*). We expected the myelinating Schwann cell population to be small as this phenotype is consistently down-regulated after transection[13,15,36]. Likely, the myelinating and non-myelinating Schwann cells transformed into repair cells after transection[44,46,56]. However, our data suggest that repair Schwann cells may be more heterogeneous and widespread in different clusters than the clearly defined Schwann cell clusters shown.

Many clusters beyond the defined Schwann cell clusters may contain repair Schwann cells including the heterogeneous, mesenchymal, and epineurial mesenchymal cell clusters. We observed in these clusters the expression of *NGFR* (a marker for Schwann-like glia cells) that may represent the presence of repair Schwann cells[37]. *NGFR* is highly expressed during development, but expression goes down when the axon is mature[59]. *NGFR* is re-expressed in Schwann cells when there is axon or myelination degeneration[59]. The heterogeneous cell cluster in our analysis shows the most *NGFR*-positivity only second to the non-myelinating Schwann cell cluster; thus, we have interpreted that this cluster may contain repair Schwann cells. We have named this as a heterogeneous cluster due to the presence of other cell types such as perineurial cells and mesenchymal cells (Fig. 2C). Further histology would be needed to confirm the phenotypes.

As for other markers that typically characterize repair Schwann cells, they were present in the Schwann and in the heterogeneous cell clusters, but do not segregate as their own defined cluster in the UMAP plot. These markers are *NCAM1*, *NGFR*, and *SOX2* for immature Schwann cells. Mature Schwann cells de-differentiate into a flexible phenotype after injury. Other markers of repair Schwann cells

expressed in our reparative peripheral nerve tissue were *EGR2* and *SHH*, but the presence of these markers were not very high in the tissue. We had expected that Schwann cells would be a bigger proportion of cells in our results[39]. The lower Schwann cell count could be explained by the mature Schwann cells having already de-differentiated into a mesenchymal cell type at 2 weeks.

The connection between mesenchymal cells and Schwann cells should not be overlooked. Clements et al. revealed novel aspects of Schwann cell de-differentiation after nerve transection including the transformation into a mesenchymal phenotype[36]. They found that transforming growth factor (TGF)-beta reprograms the bridge Schwann cells involved in reconnecting the axon into mesenchymal-like cells and a migratory phenotype to drive cells across the wound[36]. Our data revealed sizable mesenchymal cell populations (mesenchymal cluster, endoneurial mesenchymal cluster) possibly due to the de-differentiation of Schwann cells after transection. Furthermore, a marker for mesenchymal cells, PDGFRA in our data was also found in other clusters like the heterogeneous cell cluster suggesting that mesenchymal cells may exist beyond just the clearly defined mesenchymal/epineurial mesenchymal clusters.

Macrophages as reparative and anti-inflammatory. Our data show that macrophages were 12.2% of the total cells 2 weeks after transection, and the fourth largest cluster of cells after transection. Macrophages play dichotomous roles in injury in pro-inflammatory (M1) and anti-inflammatory (M2) ways. M1 macrophages are pro-inflammatory and secrete cytokines, and M2 macrophages are anti-inflammatory and contribute to tissue repair[60]. The switch in the polarization of their phenotype is influenced by their environment. The dual roles of macrophages allow them to contribute in tissue homeostasis such as in injury progression and also tissue repair. Soon after injury, macrophages also play an important role in engulfing myelin and axonal debris[60]. M1 pro-inflammatory macrophages release chemokine ligand 2 (CCL2), inducible nitric oxide synthase, and tumor necrosis factor (TNF)-related apoptosis-inducing ligand. M1 macrophages promote the removal of debris and clearing of apoptotic cells[61]. The many subtypes of M2 macrophages induce anti-inflammatory effects to promote the resolution of inflammation, cell proliferation, growth factor production, tissue repair, angiogenesis, and wound healing[61–63]. Our data did not segregate into M1 and M2 phenotypes; however, transplanting the macrophages in the M2 phenotype could be beneficial to degenerating neurons contributing to tissue repair[61–63].

Data Interpretation

Even as a powerful tool, there are limitations to the single nuclei RNA seq approach[64]. The clusters of cells are generated via automatic bioinformatics analysis and may or may not reveal all the actual cell types as evidenced by anatomical location, function, or immunochemistry. One of the clusters exhibited the heterogeneity of several cell types including perineurial cells, markers for Schwann cells, and mesenchymal cells. More specific histology staining for the characteristic markers of these cell is needed to confirm cell identities.

One concern from our previous whole tissue RNA seq analysis[13] had been the time between excision and freezing of the tissue; but in later proteomic analyses, differences in sample freezing times did not appear to account for proteomic differences[15]. Meanwhile, in the two participants whose peripheral nerve tissue underwent single nuclei RNA seq analysis, the UMAP display of cell clusters were concordant even though there was a greater than 30-min difference (67 vs 32 min) in freezing times for peripheral nerve tissue between participants. Our interpretation is that the profile of the regenerating peripheral nerve tissue is stable under our collection conditions. We recognize that ideally, the freezing time should be reduced further to more definitively maintain the stability of the samples, but based on the current design of the trial and surgery logistics, a shorter freezing time is not practicably possible. Furthermore, the timing of sample collection of reparative peripheral nerve tissue is the actual timing for the peripheral nerve tissue that is implanted in clinical trials, and we detected and measured the concentration of key factors in this product (Fig. 5).

In previous studies[65–67] where embryonic stem cells or embryonic dopaminergic neurons were transplanted into the basal ganglia of participants with PD, the number of cells transplanted has varied across studies, sometimes limited by the availability of embryos and stem cells. That variability has been wide, from transplanting into the putamen 20 µl of embryonic mesencephalic tissue containing dopamine neurons from fragments of aborted embryos[2], to 9861 to 21,552 dopaminergic neurons per putamen side[68], to dopaminergic neurons from one or four donor embryos per side (approximately 30,000 cells per side for one embryo and 70,000–120,000 cells per side from four embryos)[1]. Based on the cell counts reported here, we estimate the delivery of 5000 to 10,000 cells of reparative peripheral nerve tissue per deployment, of course with a different cell composition, and objective, from dopaminergic transplants.

We recognize the use of tissue from participants with PD introduces a concern of neuropathies as people with PD have a higher incidence[69,70]. For the single nuclei RNA seq, one of the participants had no history of neuropathy, and one did have a history of neuropathy. Even with these limitations, this study provides insight into the composition of the reparative peripheral nerve tissue implanted in ongoing clinical trials.

Summary

In this work, we were able to demonstrate the types of cells and the anti-apoptotic and neuroprotective contents that are implanted into our trial participants. This novel reparative

peripheral nerve tissue engraftment may also have immediate utility in other neurodegenerative diseases such as stroke[71–73], traumatic brain injury (TBI), and Alzheimer's disease. We found that multiple cell types in reparative peripheral nerve tissue contribute to the production of a wide array of factors utilized in our goal to alter the progression of PD.

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Ethical approval and consent to participate

The University of Kentucky's Institutional Review Board (IRB) approved the study (clinicaltrials.gov: NCT02369003), and the participants provided written informed consent.

Statement of Human and Animal Rights

This article does not contain any studies on animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

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