Developing Functionalised Electrospun Scaffolds to Exploit Neural–Stromal Interactions in Wound Healing

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Introduction

Wound healing remains a persistent clinical challenge in surgery, cosmetic procedures, and chronic inflammatory conditions, requiring deeper understanding to support the development of functionalised biomaterials. The peripheral nervous system helps regulate wound healing via neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP), but further research is needed to understand how these influence fibroblasts. Fibroblasts produce extracellular matrix, reorganise dermal architecture, and differentiate into contractile myofibroblasts aiding the proliferation and remodelling phases. This research aims to investigate and define the PNS–fibroblast bidirectional signalling axis to inform the development of a functionalised biomaterial.

The healing of a wound is a highly complicated, multifaceted process which requires communication between a wide range of cell types and vast number of signalling pathways. Current research describes four overlapping phases of wound healing, namely haemostasis, inflammation, proliferation, and remodelling.

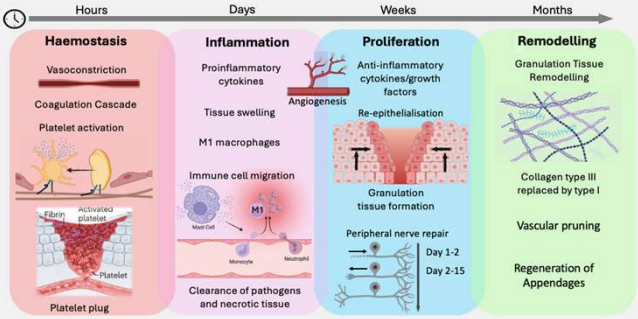


Figure 1: Image depicting the stages of wound healing.

❖ **SP and CGRP** are neuropeptides typically associated with nociceptor pain transmission but have been demonstrated to accelerate wound healing in diabetic and denervated wound healing models. Instead of acting as anti- or pro-inflammatory molecules, there is evidence they regulate the transition between the wound healing phases.

❖ **Electrospinning** is an effective method of biofabrication, capable of creating fibres at the micro and nano scales. Thin fibres are produced when a dissolved substance is passed through an electrified needle, which modifies the minimal free energy structure from a droplet into a cone which ejects a jet of material which undergoes a spiralled whipping effect before being randomly deposited onto the collector.

Aims And Objectives

- ❖ To investigate interactions between neuropeptides and fibroblasts using proliferation, migration and contraction, adhesion, morphology assays as well as mRNA expression of neuropeptide receptors, extracellular matrix proteins and myofibroblast and fibrotic markers.
- ❖ To investigate interactions between primary peripheral neurones and fibroblasts using indirect co-cultures via conditioned media and analysed with immunocytochemistry.
- ❖ Develop and characterise electrospun scaffold functionalised with neuropeptides.
- ❖ Evaluate the wound healing capabilities of the functionalised scaffold, *in Vivo,* using a denervated mouse model and analysed with histology and immunohistochemistry.

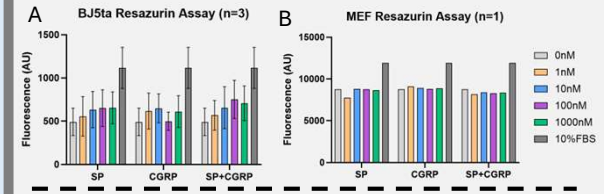
Hypothesis

- ❖ It is hypothesised that an electrospun scaffold can be functionalised with, and act as a delivery system for, neuropeptides that will facilitate a regenerative phenotype in dermal fibroblasts.

In Vitro Investigation of Neuropeptides on Dermal Fibroblasts

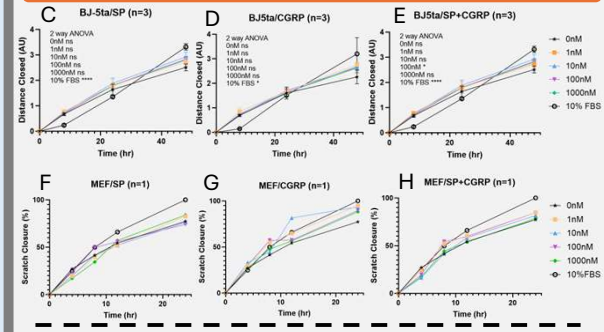
Proliferation Assay

Methods: 96-well plate, 3x10^3 cells/well, 24-hour seed (10% FBS), 6-hour starvation (SFM 0.1% BSA), 48-hour neuropeptide exposure (0.5% FBS, 0.1% BSA).



Scratch Assay

Methods: 24-well plate, 1.2x10^5 cells/well, 4-hour seed, 12-hour starvation (SFM, 0.1%FBS), P200 scratch, 24-48-hour neuropeptide exposure (SFM, 0.1% BSA).



Transwell Migration Assay

Methods: : 24-well plate, 8um pore insert, 6-hour starvation in flask (SFM, 0.1% BSA), 600ul in well, 200ul 5x10^4 cells in upper chamber, 24-hr incubation, fixed & stained (DAPI)

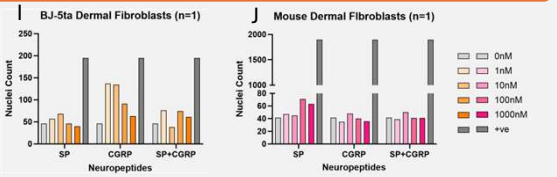
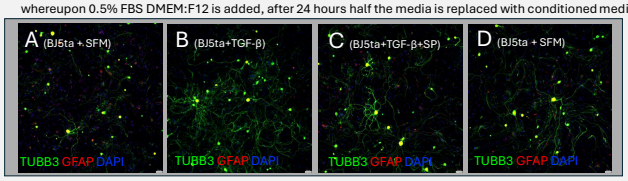


Figure 2: Effect of neuropeptides on BJ5-ta and MEF dermal fibroblasts on proliferation (**A**) and (**B**), 2D tissue culture plastic 'wound closure' (**C-E**) and (**F-I**), and migration through 8um pore transwell (**J**) and (**K**). Scratch and transwell assays were analysed using Fiji. Error bars are standard deviation and 'n' refers to biological repeats. Key words: Substance P (SP), Calcitonin gene-related peptide (CGRP), Mouse Embryonic Fibroblast (MEF), BJ-5ta hTERT immortalised foreskin fibroblast (BJ5ta).

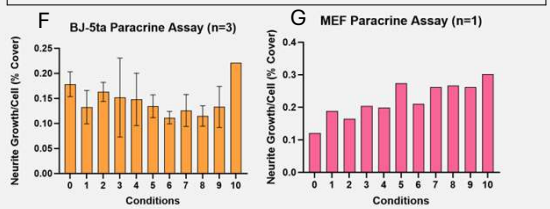
In Vitro Investigation of Fibroblasts Conditioned Media on Primary Murine Trigeminal Ganglia (TG) Cultures

- ❖ This experiment aimed to understand crosstalk between peripheral nerve cultures and dermal fibroblasts by pre-conditioning BJ5-ta fibroblasts using TGF-β, SP and CGRP . Transforming growth factor-β (TGF-β)is commonly used to activate and differentiate fibroblasts into α-SMA$^+$ myofibroblasts, but the effects of SP and CGRP are less defined and how this effects the formation of conditioned media in which to culture TG cells is yet to be explored.
- ❖ Neuronal cultures are obtained by taking mice trigeminal ganglia (TG), dissociating them in 12.5mg/ml collagenase type IV in 10% FBS DMEM:F12 for 90 minutes. These are plated onto a poly-d-lysine and laminin coated coverslip as a 10ul droplet (10% FBS DMEM:F12) and incubated for 2 hours, whereupon 0.5% FBS DMEM:F12 is added, after 24 hours half the media is replaced with conditioned media.



Methodology for Creating Fibroblast Conditioned media											
Conditions	0	1	2	3	4	5	6	7	8	9	10
150k cells seeded in 6 well plate for 48hr	w/o	With Cells (Either BJ-5ta or MEF)									w/o
Washed and preconditions added to cells for 24hr	SFM	(SFM)	TGF-β	TGF-β + SP	TGF-β + CGRP	TGF-β + SP + CGRP	SP	CGRP	SP + CGRP	10% FBS	10% FBS
Fresh media added for 24hr	Washed and fresh SFM 0.1% BSA added to make conditioned media										10% FBS
All media collected, centrifuged, filtered (0.22um pore) and frozen at -80°C.											

Table 1: Methodology for creating fibroblast conditioned media. Numbers correlate to those on graphs (F) and (G).



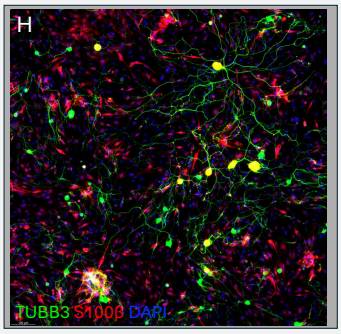
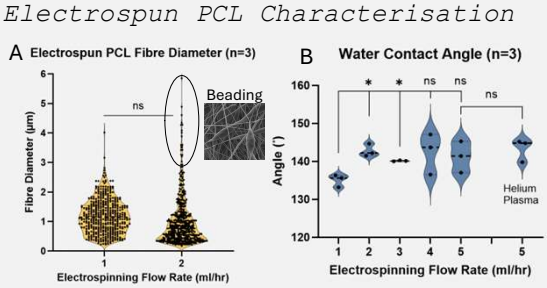


Figure 3: Fibroblast to TG culture paracrine assays. (**A-D**) is images of neuronal cultures which were grown in different BJ-5ta condition media (see in white), imaged using immunocytochemistry and (**E**) is a quantification of these images using Fiji, p<0.01**. This experiment was expanded to include CGRP and both BJ-5ta fibroblasts (**F**) and MEFs (**G**), **table 1** corresponds number to conditioned media. (**H**) is an image of a neuronal culture. Error bars are SD.
Key words: Trigeminal ganglia (TG), Beta III tubulin (TUBB3), S100 calcium binding protein b (s100B), Glial fibrillary acidic protein (GFAP)

Electrospun PCL Characterisation



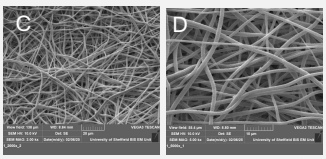


Figure 4: Electrospun scaffolds were created using a 10% PCL solution dissolved in DCM and DMF on a horizonal electrospinning at 24kV and 20cm. (**A**) is a violin plot depicting the effect of electrospinning flow rate on fibre diameter, the elongation of the plot is due to beading, as shown in the image. (**B**) Is a violin plot of a water contact angle assay, which shows the hydrophobicity as a measure of the water droplet angle. SEM image of PCL scaffolds at x2000 (**C**) and x5000 (**D**)
Key words: polycaprolactone (PCL)

Conclusions

- ❖ Overall, the effects of neuropeptides on dermal fibroblasts appear small but detectable. This may be due to the effects of low serum, which was done to establish the sole contribution of the neuropeptides, and so future work will reattempt these experiments using higher a higher FBS content to recreate more physiological conditions. The BJ5-ta cell line is hTERT immortalised, which may affect G coupled protein receptor activity, reducing the effects. The MEFs will require further optimisation to avoid contamination from other cell types, a ceiling affect and stress induced by SFM conditions.
- ❖ The BJ-5ta paracrine assay seems to show a mild inhibition of neurite outgrowth, however, this will need to be repeated as there was a concern with over confluence when creating the conditioned media. The MEF to TG paracrine assay looks more promising and physiologically apt by being mouse-to-mouse.
- ❖ 1ml/hr electrospinning rate appears to be the most uniform and hydrophilic, and so will be used in future experiments.

Future work

- ❖ Optimisation of cell starvation and FBS content.
- ❖ Adhesion assays which will prime the cells with neuropeptides in suspension and assess attachment to TCP and collagenor fibronectin coated TCP, as well as assess integrin expression using western blots and qPCR.
- ❖ Contraction assays which will involve seeding cells into a collagen hydrogel and imaging over time as the hydrogel shrinks.
- ❖ Morphology assays will be used to assess the shape and spreading of dermal fibroblasts and will be used in conjunction with the adhesion assays (2D) and contraction assays (3D) which will be fixed and undergo cryostat and immunocytochemistry.
- ❖ All *in Vitro* wok will be repeated using TG conditioned media.
- ❖ qPCR will quantify mRNA expression of α-SMA, collagen type I, neuropeptide receptor, integrin and others if time permits.
- ❖ Further work with electrospun polycaprolactone scaffolds will explore modifications to facilitate and stabilise the incorporation of neuropeptides by means of plasma treatment and neuropeptide encapsulated PGLA electrospraying.