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Retinal ganglion cell polarization using immobilized guidance cues on a tissue-engineered scaffold

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ABSTRACT

Cell transplantation therapies to treat diseases related to dysfunction of retinal ganglion cells (RGCs) are limited in part by an inability to navigate to the optic nerve head within the retina. During development, RGCs are guided by a series of neurotrophic factors and guidance cues; however, these factors and their receptors on the RGCs are developmentally regulated and often not expressed during adulthood. Netrin-1 is a guidance factor capable of guiding RGCs in culture and relevant to guiding RGC axons toward the optic nerve head in vivo. Here we immobilized Netrin-1 using UV-initiated crosslinking to form a gradient capable of guiding the axonal growth of RGCs on a radial electrospun scaffold. Netrin-gradient scaffolds promoted both the percentage of RGCs polarized with a single axon, and also the percentage of cells polarized toward the scaffold center, from 31% to 52%. Thus, an immobilized protein gradient on a radial electrospun scaffold increases RGC axon growth in a direction consistent with developmental optic nerve head guidance, and may prove beneficial for use in cell transplant therapies for the treatment of glaucoma and other optic neuropathies.

1. Introduction

Directing retinal ganglion cell (RGC) axons to extend toward the optic nerve head is a major challenge, both during normal development and also when considering RGC replacement therapy in injury or disease. During development, RGC axons in the retinal nerve fiber layer (RNFL) are directed to the optic nerve head through the optic nerve to targets in the brain, by several soluble and matrix-associated signals including ephrins, netrin-1, slit-1 and 2 and heparan sulfate. Disruption of any these factors can lead to improper guidance within the RNFL and optic nerve hyperplasia [1–3]. Netrin-1, a soluble protein responsible for guidance within the retina but also for branching and targeting in the cortex [4,5], has been shown to be expressed in the developing [6] and adult optic nerve, as well as in a response to injury such as axotomy [7] with the guidance factor regulated temporally through expression of the receptor DCC [8]. This protein has been of particular interest because it is sufficient on its own to guide RGC axons in vitro [9]. Relevant to our studies, guidance of RGCs by Netrin-1 can still be modulated by other signaling pathways owing to the activation of specific integrins [10] capable of reversing the attractive nature of Netrin-1 on fibronectin to a repulsive nature on laminin [11].

Can similar guidance cues be incorporated into tissue engineering approaches being investigated for retinal repair? Recently, transplanted RGCs have been observed to project dendritic processes into the inner plexiform layer [12], and stem cells transplanted directly onto the optic nerve head are able to project a process through the optic nerve head [13]. However, transplanted cells located away from the optic nerve head were unable to extend their axons radially as occurs with endogenous RGCs in the normal retina. In order to direct the axons of transplanted cells, nanofiber scaffolds created by electrospinning have been used to give a radial physical guidance cue for axon extension for transplanted RGCs; however, cells grown on this scaffold extended

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neurites either toward or away from the center where the optic nerve would be, or often in both directions. Tissue engineering of the peripheral and central nervous system has incorporated both soluble and immobilized neurotrophic factors through a variety of methods including microfluidic devices, diffusional gradients and chemical immobilization, to direct the neurite outgrowth of neurons in two and three dimensions. Because chemical immobilization creates a fixed protein pattern on a material surface which can be implanted, while soluble gradients cannot be readily translated in vivo [14], methods for protein gradients have been formed through photolithography, either using a photomask [15] or through focusing of light on a actuating mirror [16], microcontact printing [17] and microstamping [18].

Here we describe a method for polarizing the growth of RGCs cultured on an electrospun radial scaffold. By means of photolithography, Netrin-1 will be chemically immobilized in a linear gradient onto a fibronectin-coated scaffold. We will demonstrate that early postnatal primary RGCs cultured on scaffolds containing Netrin-1 show an increase in polarization during the growth of a single axon, better mimicking the growth seen in vivo. We will also demonstrate that cells cultured on scaffolds containing this protein gradient will show an increase in the guidance of the RGCs toward the center of the scaffold, mimicking the growth toward the optic nerve head found in vivo.

2. Materials and methods

All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research with all protocols approved by the Institutional Animal Care and Use Committee of both the University of Miami Miller School of Medicine and the University of California San Diego.

2.1. Electrospinning

Electrospinning was conducted as previously described [19]. Briefly, poly-D,L-lactic acid (PLA, Purac Biomaterials Inc., PDL20) was dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP, Chem-Impex International Inc.) at a concentration of 6.6% (wt./vol.). The PLA solution was pumped by syringe pump (New Era Pump Systems Inc., NE-500) at a continuous feed rate of 2 ml h^{-1} and ionized in a 20 gauge blunt-tipped needle (Hamilton) using a high-voltage power supply (SpellmanHV, 230-30R). Scaffolds were created using a radial collector which was constructed from a 1 mm diameter copper wire acting as the central pin. A plastic cup, 1.8 cm in diameter, was coated around the outside and upper rim with aluminum foil mounted on the central pin, with both the central pin and cup connected to the same ground. Scaffolds were produced at 15 kV with a flow rate of 2 ml h^{-1} and a collecting distance of 12 cm.

2.2. Protein crosslinking

Recombinant Netrin-1 (R&D Systems) or BSA-FITC (Sigma) was crosslinked onto laminin- or fibronectin-adsorbed PLA scaffolds through three methods (Fig. 1): direct chemical coupling, UV-initiated chemical coupling with the crosslinker initially attached to the soluble protein, and UV-initiated chemical coupling with the crosslinker initially attached to the scaffold.

Direct chemical coupling was achieved by activating the carboxylic acid groups of the adsorbed laminin or fibronectin with 500 µl of a 1 mg ml^{-1} *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC, Thermo Scientific) solution for 15 min followed by the addition of 500 µl of a 2 mg ml^{-1} sulfo-*N*-hydroxysuccinimide (NHS, Thermo Scientific) solution, to increase the aqueous stability of the activated carboxylic acid. Primary amine groups from 500 µl of a 0.1 mg ml^{-1} drop of protein reacted with the activated acid groups to form covalent crosslinks.

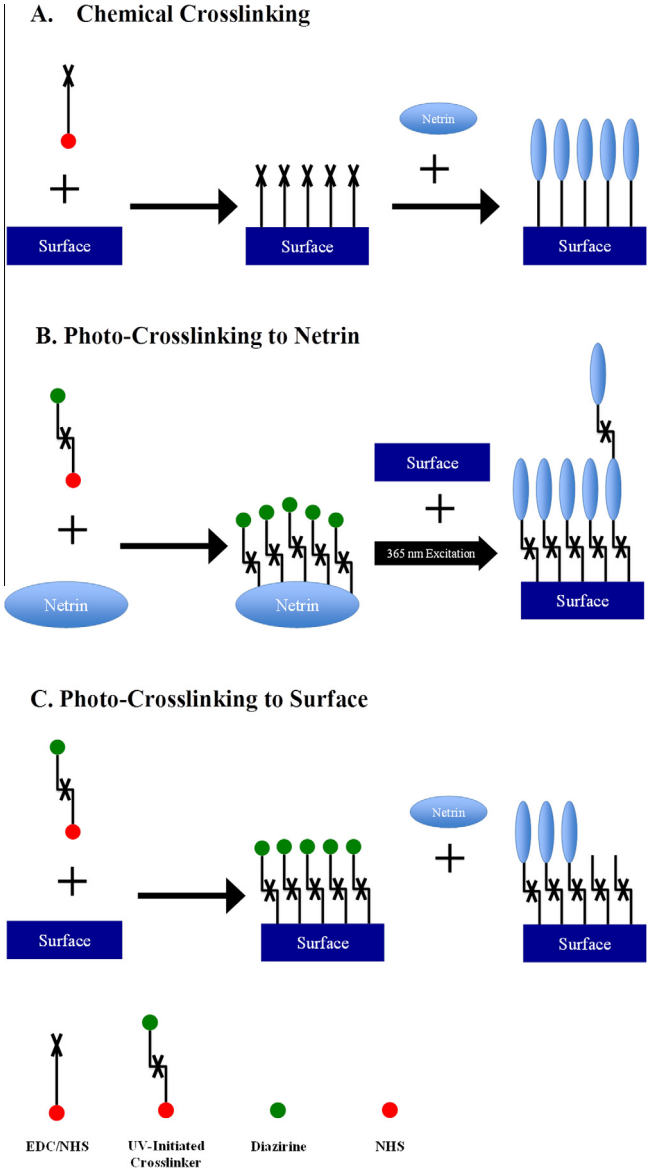

Fig. 1. Netrin-1 immobilization strategies. Netrin-1 was immobilized to the surface of radial scaffolds through three different methods: chemical crosslinking using EDC and NHS reacted first with the scaffold and then the activated scaffold with Netrin-1 (A); UV-crosslinking by reacting the bifunctional UV-crosslinker first with the scaffold surface through its NHS subunit and then with Netrin-1 through the diazirine subunit when activated with 365 nm light (B); and UV-crosslinking by reacting the bifunctional UV crosslinker first through its NHS subunit with Netrin-1 and then with the scaffold surface through the diazirine subunit when activated with 365 nm light (C). While the use of the UV-crosslinker offers more control for the formation of a gradient, reacting the crosslinker first with the surface (B) can lead to a lower concentration of immobilized Netrin-1 should the surface be activated with UV light when the protein is not a favorable distance or should multiple bonds be made to a single protein, while reacting crosslinker first with the Netrin-1 protein can lead to polymerization of the protein should they react with other Netrin-1 proteins prior to reacting with the scaffold surface.

For UV-initiated crosslinking samples with the crosslinker initially attached to the soluble protein, 500 µl of a 0.1 mg ml^{-1} protein solution was reacted overnight at 4 °C with 2 mg ml^{-1} of the diazirine crosslinker sulfosuccinimidyl 6-(4,4'-azipentanamido)hexanoate (Thermo Scientific). The solution was added to a

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