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Non-viral gene delivery transfection profiles influence neuronal architecture in an in vitro co-culture model

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Abstract

Gene delivery from tissue engineering scaffolds can induce expression of tissue inductive factors to stimulate the cellular processes required for regeneration. Transfected cells secrete diffusible proteins that can create local concentration gradients, depending on the number, distribution, and expression level of transfected cells. These gradients are linked to cellular organization and tissue architecture during embryogenesis. In this report, we investigate neuronal architecture and neurite guidance in response to the concentration gradients achieved by localized secretion of a neurotrophic factor from transfected cells. A co-culture model was employed to examine neuronal responses to multiple transfection profiles, which affects the local concentration of secreted nerve growth factor (NGF). Neuronal architecture, as defined by number of neurites per neuron and length of neurites, was influenced by the transfection profile. Low levels of NGF production by few transfected cells produced longer primary neurites with less branching relative to the higher expression levels or increased numbers of transfected cells. Furthermore, for low NGF production by few transfected cells, the growth cone of the axons was marked by longer extensions and larger surface area, suggesting the presence of a guidance cue. Control studies with varying NGF concentrations did not substantially alter the neuronal architecture, further supporting an effect of localized concentration gradients, and not simply the concentration. Mathematical modeling of NGF diffusion was employed to predict the concentration gradients produced by the transfection profiles, and the resultant gradients were correlated to the cellular response. This report connects the transfection profile, concentration gradients, and the resulting cellular architecture, suggesting a design consideration for the application of gene delivery to regenerative medicine.

Keywords

gradient; guidance; neurotrophic factor; gene delivery; dorsal root ganglia (DRG)

Introduction

Gene delivery from biomaterials has been employed to achieve localized expression of proteins for extended times, with many applications in disease treatment and tissue

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regeneration. The biomaterial serves to maintain DNA concentration locally, and sustained release provides prolonged opportunities for cellular transfection. These delivery systems are employed in applications such as gene therapy, the treatment of cancer (Beer et al. 1997; Megeed et al. 2004) or inherited genetic disorders (Dao et al. 1998), and tissue regeneration (Jang et al. 2005). In regenerative medicine, the biomaterial (scaffold) functions to maintain a physical space at a lesion site, while promoting cellular processes (e.g., adhesion, migration) necessary to regenerate healthy tissue (Murphy and Mooney 1999). Gene delivery from the tissue engineering scaffold can induce expression of tissue inductive factors to further stimulate the cellular processes required for regeneration (Shea et al. 1999).

Non-viral gene delivery from tissue engineering scaffolds has been employed to induce expression of tissue inductive factors, such as BMP-4 for bone regeneration (Huang et al. 2005), VEGF for angiogenesis (Jang et al. 2005), and TGF- β 1 for wound healing (Lee et al. 2003). Non-viral vectors are delivered as plasmid or plasmid condensed with cationic lipids or polymers (i.e. complexes) to facilitate internalization and trafficking (Segura and Shea 2001). Vectors are delivered to progenitor cells surrounding or infiltrating the scaffold, and transfected cells behave as bioreactors, secreting the protein of interest. The factors act in a paracrine manner to stimulate adjacent cells to promote the desired responses. Although the duration of expression can influence the response, the number and distribution of transfected cells and the expression level are also critical design considerations (Ozawa et al. 2004). Since transfected cells secrete diffusible proteins, their number, distribution, and expression level determine the concentration profile of inductive factors within the tissue. Concentration gradients of inductive factors are linked to cellular organization and tissue architecture during embryogenesis (Zecca et al. 1996), and tissue regeneration strategies must recapitulate tissue architecture to restore function.

The ability of localized gradients to alter tissue structure has been characterized in the nervous system during development and regeneration. Gradients of neurotrophic factors, such as nerve growth factor (NGF) and neurotrophin-3 (NT-3), guide axon extension *in vitro* (Gundersen and Barrett 1979; Paves and Saarma 1997). The leading edge of an axon, the growth cone, is responsible for gradient detection in the extracellular environment and subsequently directs axons along a specific path (Song and Poo 1999). Non-viral gene delivery provides a method to achieve localized secretion and gradients of neurotrophic factors. However, the manner to deliver genes encoding for neurotrophic factors to direct axon extension is poorly understood. The local concentration at the growth cone is a critical factor, as neurotrophic factors released from an individual cell can alter the structure of nearby neurites on an exquisitely local scale (Horch and Katz 2002).

In this report, we investigate neuronal responses to varying NGF transfection profiles produced by non-viral gene delivery *in vitro*. We hypothesized that varying the extent of transgene expression and percentage of transfected cells will alter the neurotrophic factor gradients formed by localized expression and secretion. Different transfection profiles were established by altering the type and amount of complexing agent and the amount of DNA administered to HEK293T cells. A co-culture model of transfected accessory cells and neurons was employed to investigate neuron survival and neurite outgrowth, branching, and guidance in response to the different transfection profiles. The gradients that develop from transfected cells were mathematically modeled and correlated to responses observed experimentally. The results obtained from the *in vitro* system addresses a fundamental issue in the application of gene delivery for regenerative medicine.

Materials and Methods

Plasmids and reagents

Plasmid was purified from bacterial culture using Qiagen (Santa Clara, CA) reagents and stored in Tris-EDTA buffer at -20°C . The plasmid pEGFP-Luc has a CMV promoter for expression of a fusion protein consisting of EGFP and luciferase (Clontech, Mountain View, CA), which were used to determine the percentage of transfected cells and the extent of transgene expression respectively. The plasmid pNGF has full length mouse NGF in the RK5 vector backbone with a CMV promoter and was a gift from Dr. Hiroshi Nomoto (Gifu Pharmaceutical University, Japan). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and media components from Invitrogen (Carlsbad, CA) unless otherwise specified.

HEK293T cell culture

The human embryonic kidney cell line (HEK293T) was purchased from the American Type Culture Collection (ATCC) (Manassas, VA). HEK293T cells were maintained in T-75 flasks with media change every 48 h and passage every 60 h in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, and 1% sodium pyruvate at 37°C and 5% CO_2 . For all assays, tissue culture polystyrene wells were precoated with poly-L-lysine (MW 30,000 – 70,000) by incubating a 0.01% solution in wells for 1 h.

Establishment of transfection profiles

HEK293T cells (3×10^4 cells/well) were seeded in 24-wells and cultured for 16 h using conditions described above. Polyethylenimine complexes (polyplexes) were formed by diluting the desired amount of PEI (25 kDa, branched) (N/P 10 or 25) or plasmid (pEGFP-Luc, 0.15 or 0.3 μg /well) in tris-buffered saline, mixing by vortex, and incubating for 15 min. LipofectamineTM2000 complexes (lipoplexes) were formed by diluting the desired amount of LFTM2000 (Invitrogen) (1:1 plasmid:lipid) or plasmid (pEGFP-Luc, 0.5 μg /well) in DMEM, mixed by gentle pipetting, and incubated for 20 min. 16 h after HEK293T seeding, non-viral complexes were added to the culture medium and the cells were incubated for 24 h. After incubation, cultures were assayed for either transfection efficiency or extent of transgene expression. To assay transfection efficiency, cultures were imaged on a Leica inverted fluorescence microscope with a cooled CCD camera (Photometrics; Tucson, AZ) using MetaVue (Universal Imaging; Downingtown, PA) acquisition software for EGFP positive cells, followed by Hoechst 33258 imaging of the cell population. EGFP positive cells and total cells were counted using Adobe Photoshop. To assay extent of transgene expression, cells were lysed and assayed using a luminometer (Turner Biosystems) set for a 3-s delay with signal integration for 10 s. Luciferase levels were normalized to total protein in the sample, quantified by the BCA protein assay (Pierce; Rockford, IL).

Quantification of NGF expression

To assay NGF expression, transfection profiles cultures were performed as described above, with replacement of the pEGFP-Luc plasmid with pRK5-NGF. 24 h after the addition of non-viral complexes, the culture media were analyzed for NGF concentration using a ChemiKine NGF Sandwich ELISA kit (Millipore; Billerica, MA).

Neuronal responses to transfection profiles

A neuronal co-culture model was employed to investigate neuronal responses to the different NGF transfection profiles. NGF transfection profile cultures were performed as described above. 8 h after the addition of complexes, HEK293T cells were rinsed with PBS.

Dorsal root ganglia (DRG) explants were isolated from 8-day chicken embryos (MI State University; East Lansing, MI), dissociated using methods described previously (Houchin-Ray et al. 2007b), and seeded on HEK293T cultures, with a neuron cell density of 20,000 cells/well in a 24-well plate. Cultures were placed into an incubator and were not disturbed to minimize convection. 48 h after neuron seeding, cultures were stained for the neuron-specific class III β -tubulin by incubating fixed cells in TUJ1 antibody (Covance; Berkely, CA) diluted in 5% normal goat serum (Vector Labs; Burlingame, CA) in PBS for 1 h followed by incubation in TRITC-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch; West Grove, PA) in PBS for 30 min. Cells were counterstained with Hoechst 33258 to visualize cell nuclei. Co-cultures were imaged on a Leica inverted fluorescence microscope with a cooled CCD camera (Photometrics; Tucson, AZ) using MetaVue (Universal Imaging; Downingtown, PA) acquisition software. Neurite length was quantified using the tracing algorithm in the NeuronJ plug-in for ImageJ (Meijering et al. 2004) and normalized to either surface area or sprouting neurons. Primary neurites are those that extend directly from the soma, and branching neurites are all other neurites other than the primary.

To assess growth cone morphology, cultures were performed as described above, with the exception of using poly-L-lysine coated glass chamber wells (Nunc; Rochester, NY), in order to image with high magnification. Co-cultures were imaged on the Leica inverted fluorescence microscope, at high magnification. Growth cone morphology was quantified by tracing the length of the growth cone in ImageJ (NIH) (Zheng et al 1994).

Mathematical modeling of concentration gradients

Mathematical modeling of NGF diffusion was used to predict the concentration gradient surrounding a transfected cell for each transfection profile, using methods described previously (Houchin-Ray et al. 2009). Briefly, Equation 1 describes one-component diffusion in three dimensions in a continuous medium with a term for protein degradation:

$$\frac{\partial C}{\partial t} = D \left[\frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2} + \frac{\partial^2 C}{\partial z^2} \right] - kC \quad (1)$$

where C is the concentration, D is the diffusivity of the protein, and k is the rate constant for protein degradation. The value for the diffusivity of NGF was obtained from published reports ($D = 12 \times 10^{-7} \text{ cm}^2/\text{s}$) (Stroh et al. 2003), and was used in Equation 2 to calculate the effective diffusivity that incorporated reversible binding of the ligand to the accessory cell surface:

$$D_e = \frac{D}{(R+1)} \quad (2)$$

Where D_e is the effective diffusion constant, and R is a dimensionless coefficient, calculated by the equation $S = RC$, where S is the amount of ligand bound to the surface, and C is the amount of soluble NGF (Crank, 1975). The values of S and C were calculated from NGF ELISA data. The culture media was assayed to determine soluble NGF (C), and the culture surfaces were harvested by scraping and assayed to determine bound NGF (S). The rate constant for protein degradation, k, was determined from the reported half-life of NGF ($t_{1/2} = 4.5 \text{ h}$) (Tria et al. 1994), ($k = 0.0029 \text{ min}^{-1}$).

The Crank–Nicolson implicit method was employed to solve numerically the partial differential equation. The initial condition is a zero concentration throughout the culture (Equation 3). The boundary conditions indicate a flux (q), which is determined from the protein production rate, from an individual transfected cell (Equation 4), and no flux boundary conditions elsewhere.

$$C(x, y, z, t=0)=0 \quad (3)$$

$$-D \frac{\partial C}{\partial z} (x=x_{trans}, y=y_{trans}, z=0, t)=q \quad (4)$$

Note that the location of a single transfected cell is defined at $x = x_{trans}$ and $y = y_{trans}$. The average distance between two transfected cells was calculated from experimentally quantified cell confluency and transfection efficiency. The boundaries in the x - and y -directions were defined as the average distance between transfected cells for each condition. The boundary in the z -direction was set equal to 4 mm, the approximate height of the culture medium. The flux, (q) was determined from the protein production rate (p) (pmol/cell/min), which was calculated from NGF ELISA data (pmol/min) while accounting for protein degradation and transfection efficiency (total number of transfected cells). The value q was calculated in terms of pmol/cm³/min, based on the assumption that the volume occupied by a transfected cell was 1000 μm^3 .

Concentration gradient analysis was performed in terms of the absolute concentration gradient (Equation 5) and the fractional concentration gradient (Equation 6):

$$\left. \frac{dC}{dx} \right|_n = \frac{C_{n+1} - C_n}{x_{n+1} - x_n} \quad (5)$$

$$\left. \frac{dC}{C} \right|_n = \frac{C_{n-1} - C_{n+1}}{C_{n-1}} \quad (6)$$

Statistics

Statistical analysis was performed using JMP software (SAS Institute, Cary, NC). Comparative analyses were executed using analysis of variance with Tukey post-tests, at a 95% confidence level. The measurements of neurites were not normally distributed, and the data was transformed prior to statistical analysis. For each experiment, all conditions were performed in triplicate. All experiments were performed in duplicate.

Results

Development of transfection profiles

Three distinct transfection profiles, in terms of transfection efficiency (percent of EGFP expressing cells) (Fig. 1a-c) and extent of transgene expression (luciferase reporter gene levels), were developed (Table 1). The transfection profiles were defined as *profile 1*, *profile 2*, and *profile 3*. Relatively low transfection efficiency was achieved by complexing plasmid

with the cationic polymer, polyethylenimine (PEI) (*profile 1* and *profile 2*), while high transfection efficiency was achieved by complexing plasmid with the cationic lipid, Lipofectamine2000 (*profile 3*). The extent of transgene expression increased 500-fold from *profile 1* to *profile 2* by increasing the N/P ratio (10 to 25) and the amount of plasmid administered (0.15 to 0.30 $\mu\text{g}/\text{well}$), while maintaining relatively low transfection efficiency. *Profile 3* returned a significantly higher extent of transgene expression as compared to *profile 1*, and a marginally higher extent of transgene expression as compared to *profile 2* by increasing the amount of plasmid administered and complexing with Lipofectamine2000.

NGF expression and diffusion

The expression level of the neurotrophic factor, NGF, was subsequently measured for the three transfection profiles. As expected, *profile 1* returned a relatively low NGF concentration after 24 h culture ($0.17 \pm 0.03 \text{ ng/ml}$) as compared to *profile 2* ($4.2 \pm 0.9 \text{ ng/ml}$) and *profile 3* ($40.4 \pm 0.9 \text{ ng/ml}$) (Fig. 2). NGF expression levels were combined with the previously calculated transfection efficiencies to determine the average NGF production rate by a single transfected cell for each transfection profile (Table 2). Transfection efficiencies were also employed to estimate the average distance between transfected cells for each profile.

Localized secretion and diffusion of NGF from a transfected cell results in a concentration gradient around from the transfected cell. To investigate the local concentrations, the values from Table 2 were incorporated into a three-dimensional mathematical model of NGF diffusion from a transfected cell for the three transfection profiles. The maximum NGF concentrations, occurring at the sites of production, were 36 pM (*profile 1*, Fig. 3a), 550 pM (*profile 2*, Fig. 3b), and 2000 pM (*profile 3*, Fig. 3c) at the time of assay, 48 h. The distance between two transfected cells influenced the minimum NGF concentrations, since the diffusion of protein secreted from one transfected cell would encounter protein secreted from the nearest transfected cell. The predicted minimum NGF concentrations were 5 pM (*profile 1*, Fig. 3a), 150 pM (*profile 2*, Fig. 3b), and 1700 pM (*profile 3*, Fig. 3c).

Neuronal responses to transfection profiles

Neuronal response to different microenvironmental concentrations of NGF was subsequently investigated by co-culturing DRG neurons with HEK293T cells expressing NGF according to the three established transfection profiles. Importantly, we have established previously that HEK293T cells alone, without pNGF transfection, do not support neuron survival or neurite extension (Houchin-Ray et al. 2007b). Therefore, any neuronal responses observed can be attributed to NGF production by the transfected cells. Neurons cultured on *profile 1* generally exhibited a unipolar morphology, with primary neurites extending from one end of the cell soma, and few branches (Fig. 4a). Neurons cultured on *profile 2* and *profile 3* exhibited unipolar and bipolar neurite outgrowth, with many branches creating a dense arbor (Fig. 4b, c). Images similar to Fig. 4 a-c were employed to quantify the extent and length of neurite outgrowth, and a representative distribution of neurite lengths for the three profiles is shown (Fig. 4 d-f).

Subsequently, neuron survival and neurite outgrowth were quantitatively evaluated. Total neurite outgrowth, normalized to surface area, was significantly higher on *profile 3* ($76.0 \pm 10.1 \text{ cm}^{-1}$) as compared to *profile 1* ($28.9 \pm 5.4 \text{ cm}^{-1}$) and *profile 2* ($19.1 \pm 1.6 \text{ cm}^{-1}$) (Fig. 5a, $p < 0.05$). Additionally, neuron survival was significantly higher with neurons cultured on *profile 3* as compared to *profile 1* and *profile 2* (data not shown). Interestingly, the mean extent of neurite outgrowth per sprouting neuron was greater with neurons cultured on *profile 1* (290.9 μm) as compared to *profile 2* (128.9 μm) and *profile 3* (170.4 μm) (Fig. 5b).

The neuronal architecture was evaluated by quantifying the number of neurites per neuron and the average length of a single neurite for the three transfection profiles. The average number of primary neurites extending from a neuron was 1.2 ± 0.1 for *profile 1*, consistent with the observed unipolar morphology (Fig. 5c). The average number of primary neurites per neuron increased with neurons cultured on *profile 2* (1.5 ± 0.1) and *profile 3* (2.2 ± 0.1), demonstrating a transition to a bipolar morphology (Fig. 5c, $p < 0.05$). The average number of branching neurites per neuron was similar for *profile 1* (0.65 ± 0.05) and *profile 2* (0.69 ± 0.11), and increased with neurons cultured on *profile 3* (1.6 ± 0.1) (Fig. 5c, $p < 0.05$). Additionally, primary neurites were significantly longer with neurons cultured on *profile 1* ($219.8 \pm 14.9 \mu\text{m}$) as compared to *profile 2* ($68.3 \pm 6.2 \mu\text{m}$) and *profile 3* ($58.0 \pm 6.5 \mu\text{m}$) (Fig. 5d, $p < 0.05$). The average length of a branching neurite was also higher with neurons cultured on *profile 1* ($71.2 \pm 13.7 \mu\text{m}$) as compared to *profile 2* ($36.0 \pm 6.6 \mu\text{m}$) and *profile 3* ($26.7 \pm 2.0 \mu\text{m}$) (Fig. 5d, $p < 0.05$).

The source of differences in neurite outgrowth observed by the transfection profiles was subsequently investigated by performing co-cultures without pNGF transfection, but with varying doses of NGF added to the culture medium. Eliminating transfected cells allowed for the investigation of neuronal responses initiated by changes in mean NGF concentration, and not the differences in microenvironmental concentrations of NGF caused by transfection and resultant concentration gradients. Similar to the transfection studies, the total neurite outgrowth, normalized to surface area, increased with increasing NGF concentration (0.10 to 50 ng/ml) (Fig. 6a, $p < 0.05$). Additionally, neuron survival increased with increasing NGF concentration (data not shown). However, the extent of neurite outgrowth normalized to sprouting neurons was similar for all NGF concentrations (0.10 to 50 ng/ml) (Fig. 6b). The average number of primary neurites extending from a neuron ranged between 1.2 and 1.7 for all NGF concentrations, indicative of both unipolar and bipolar morphologies (Fig. 6c). The average number of branching neurites per neuron ranged between 0.35 and 0.70 for all NGF concentrations (Fig. 6c). Additionally, the average length of a primary neurite and a branching neurite were statistically similar for all NGF concentrations (Fig. 6d).

Growth cone morphology

The response triggered by varying transfection profiles was investigated further by characterizing the morphology of the growth cone. Growth cones extending from neurites cultured on *profile 1* exhibited an elongated morphology, marked by long filopodia (Fig. 7a) as compared to growth cones extending from neurites cultured on *profile 2* (Fig. 7b) and *profile 3* (Fig. 7c), which were marked by short filopodia. Quantitative evaluation of growth cone length indicated longer growth cones for neurons cultured on *profile 1* ($25.8 \pm 3.7 \mu\text{m}$) as compared to *profile 2* ($14.1 \pm 1.6 \mu\text{m}$) and *profile 3* ($11.9 \pm 0.4 \mu\text{m}$) (Fig. 7d, $p < 0.05$).

Discussion

In this manuscript, we employ a range of transfection profiles to investigate neuronal responses to gradients formed around NGF-expressing cells. Transfection efficiency, extent of transgene expression, and the resultant concentration gradients were altered by changing the type and amount of complexing agent and the amount of plasmid delivered. Transfected cells expressing NGF supported neuron survival and neurite outgrowth, with the extent of survival and outgrowth dependent on the mean concentration of NGF in the culture medium. Neurons cultured on *profile 3* extended a higher number of neurites per neuron as compared to *profile 1* and *profile 2*. However, neurons cultured on *profile 1* extended significantly longer neurites as compared to neurons cultured on *profile 2*, *profile 3*, and non-transfected accessory cells with NGF added to the culture medium. Moreover, neurons cultured on *profile 1* exhibited elongated growth cone morphology, marked by longer filopodia, as compared to neurons cultured on *profile 2* and *profile 3*. These findings highlight the

importance of considering both transfection efficiency and extent of transgene expression, rather than the mean concentration of a therapeutic factor, when applying gene delivery for tissue engineering.

The extent of neuron survival and neurite outgrowth was determined by the mean NGF concentration in the culture medium. Neuron survival and neurite outgrowth, normalized to surface area, increased with increasing NGF concentrations in the culture medium, when neurons were cultured on non-transfected accessory cells. Additionally, neurons cultured on *profile 3* exhibited higher levels of neuron survival and neurite outgrowth as compared to *profile 1* and *profile 2*. This difference was directly attributed to the mean NGF concentration in the medium, as NGF is a common neurotrophic factor capable of eliciting survival and outgrowth cues (Levi-Montalcini 1982).

Neuronal architecture, defined by the number of neurites per neuron and the average length of a neurite, was determined by the transfection profile and presumably the resultant NGF concentration profiles. The number of neurites per neuron was significantly higher on *profile 3* as compared to *profile 1* and *profile 2*, but remained unchanged in controls with varying NGF concentrations in the culture medium and neurons cultured in the absence of transfected cells. Moreover, the number of neurites on *profile 3* was significantly higher as compared to the non-transfected/ NGF supplemented control, indicating an effect of the transfection profile. The role of the transfection profile was also observed with the average neurite length, which was significantly higher with neurons cultured on *profile 1* as compared to *profile 2* and *profile 3*, but did not change with non-transfected/ NGF supplementation. Moreover, neurites extending from neurons cultured on *profile 1* were significantly longer than with neurons cultured on non-transfected accessory cells with NGF supplemented medium, regardless of the dose, further indicating that the transfected cells in *profile 1* were eliciting the response.

The NGF gradients forming around transfected cells were investigated through mathematical modeling of the concentration profiles. The three transfection profiles returned varying NGF absolute and fractional concentration gradients, which may drive the differences in the neuronal architecture. The predicted absolute concentration gradient outside a single cell remained consistent over the 48 h time course, and ranged between 50-0.01 ng/ml/mm for *profile 1*, 600-0.5 ng/ml/mm for *profile 2*, and 430-20 ng/ml/mm for *profile 3* (Fig. 8a). The fractional concentration gradient over the approximate width of the growth cone, 20 μ m, varied along the 48 h time course. At $t = 5$ h the fractional concentration gradient ranged between 55-0.2% for *profile 1*, 50-0.1% for *profile 2*, and 9-0.7% for *profile 3* (data not shown), and at $t = 48$ h ranged between 65-0.1% for *profile 1*, 56-0.1% for *profile 2*, and 11-0.2% for *profile 3* (Fig. 8b).

Longer neurites and fewer branches in *profile 1* may suggest the presence of a guidance cue. A guidance cue directs neurites along a specific path with few navigational errors, and therefore decreases neurite pausing and cytoskeletal rearrangements required for branching (Kalil et al. 2000; Mahoney et al. 2005). Growth cone morphology of neurons cultured on *profile 1* also supported the hypothesis that a guidance cue was present. Soluble guidance cues increase filopodial numbers in the direction of the gradient (Zheng et al. 1996), and the highly motile growth cones adopt an elongated morphology (Dent and Kalil 2001). Growth cones extending from primary neurites with neurons cultured on *profile 1* exhibited elongated morphology, characteristic of polarized filopodia, as compared to growth cones from primary neurites with neurons cultured on *profile 2* and *profile 3*, which exhibited shorter filopodia, characteristic of paused neurites (Dent and Kalil 2001).

NGF gradients that arise from transfected cells in *profile 1* may guide neurites, as gradients of NGF direct neurites via a chemotactic mechanism. The absolute and fractional concentration gradients, both of which have been implicated in neurite guidance, formed around transfected cells were within ranges that have previously been shown to guide neurites. The absolute (Fig. 8a) and fractional (Fig. 8b) gradients for all profiles were well above values that have previously been shown to guide neurites with spatial patterns of NGF expression (8-0.5 ng/ml/mm and 8.0-1.5%, respectively) (Houchin-Ray et al. 2008). Although the gradients achieved in all profiles are sufficiently steep to theoretically present a guidance cue, the relatively high NGF expression levels in *profile 2* and *profile 3* may underlie the absence of guidance. Studies of leukocyte chemotaxis suggests that a chemotactic guidance signal is most effective if the mean concentration is kept near the dissociation constant for the ligand's receptor (Devreotes and Zigmond 1988). The dissociation constant for the trkA receptor, the NGF receptor that has been shown to govern growth cone guidance by NGF gradients (Ming et al. 1999), is 0.1 nM (Tse et al. 2007). In *profile 2* and *profile 3*, microenvironmental concentrations reach 0.5 nM and 2 nM, respectively, values above the dissociation constant for the trkA receptor.

The connection between transfection, NGF concentration gradients, and the neuronal architecture observed herein is an important consideration in the application of gene delivery for nerve regeneration. In nerve regeneration, stimulation of neuron survival and neurite outgrowth is critical. However, localized sprouting of axons is not necessarily indicative that neurons have become competent to sustain longer-distance elongation of axons (Smith and Skene 1997). Growing axons must cross the entire length of a lesion to restore function. After peripheral nervous system injury, adult neurons have an inherent ability to switch from a branching morphology to an elongating morphology, extending longer neurites to span an injury site (Fu and Gordon 1997; Smith and Skene 1997). This change in morphology is triggered by differences in gene expression after injury (Caroni 1997; Skene 1989), that do not readily occur in the central nervous system (CNS) after injury (Fernandes et al. 1999), and turning on those genes in the CNS leads to longer axonal extension (Bomze et al. 2001). We have identified that the local concentration gradient promotes elongation and minimal branching without altering gene expression within neurons, since gene therapy to neurons has proven to be difficult (Slack and Miller 1996). Non-viral gene delivery from tissue engineering scaffolds has promoted transgene expression for weeks to months (Jang et al. 2005), and therefore holds promise for providing long-term guidance signals. Recently, gene delivery from spinal cord bridges that contain channels to provide physical guidance achieved transgene expression within a rat spinal cord hemisection injury model (De Laporte et al. 2008). The combination of physical guidance barriers with the proper transfection profiles to guide axons may provide long term and synergistic axon guidance signals within a nerve lesion site (Houchin-Ray et al. 2007a).

The correlation between transfection profiles and neuronal architecture also likely extends to other tissues in which localized gradients influence cellular processes. Ozawa *et al.* (Ozawa et al. 2004) demonstrated that high levels of VEGF secretion by retrovirally transduced myoblasts induced the growth of abnormal blood vessels. Decreasing the number of cells transplanted, which decreased the total dose of VEGF, served to reduce the region in which abnormal blood vessel formation was observed. However, transplantation of cells that were selected for low VEGF expression resulted in the formation of normal, mature vascular structures. The result was hypothesized to occur by the existence of gradients forming around VEGF-expressing myoblasts. Additionally, the migration of various cell types, such as fibroblasts, macrophages, and keratinocytes are guided by gradients of factors, such as PDGF and IL-8, and contribute to the regulation of epithelialization, tissue remodeling, and angiogenesis during cutaneous wound healing (Gillitzer and Goebeler 2001). Controlled

transfection profiles, combined with characterization of concentration gradients, provides a platform to investigate tissue responses to localized expression of therapeutic factors.

Conclusions

In this manuscript, transfection profiles were developed, in terms of transfection efficiency and extent of transgene expression, by altering the type and amount of non-viral complexing agent, and amount of DNA administered. Mathematical modeling of NGF diffusion confirmed that concentration gradients form around NGF-expressing cells in each transfection profile. The NGF gradients produced by transfected cells in *profile 1* were capable of guiding neurites, as evidenced by the average length of neurites and growth cone morphology. We have demonstrated that the extent of NGF expression governs the ability of gradients to guide neurites. The in vitro system presented in this manuscript provides a tunable method to investigate cellular responses to varying transfection profiles, which may influence the architecture of regenerating tissue.

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Figure 1. Three transfection profiles

EGFP expressing HEK293T cells (green) overlaid with the entire cell population, marked by the nuclei stain Hoechst 33258 (blue), with *profile 1* (a), *profile 2* (b), and *profile 3* (c).

Figure 2. Nerve growth factor expression by different transfection profiles

Quantification of NGF production 24 h after non-viral DNA complexes were added to the culture medium. Values are reported as mean $\pm$ SEM. Significant differences are indicated by different letters ($p < 0.05$).

Figure 3. Predicted concentration profiles

Mathematical model predictions for NGF concentration profiles at 48 h after non-viral DNA complexes were added to the culture medium for *profile 1* (a), *profile 2* (b), and *profile 3* (c). Note that profiles 1, 2, and 3 have concentrations predicted for distances of 0.24, 0.17, and 0.04 mm, which are half of the mean distance between transfected cells for the three profiles. Note that profiles at 24 and 48 hours are similar and may be difficult to visualize due to overlap.

Figure 4. Neurons co-cultured on three transfection profiles

Images of neurons co-cultured on HEK293T cells expressing NGF with three distinct transfection profiles: *profile 1* (a), *profile 2* (b), and *profile 3* (c). Neurons were stained with antibodies to TUJ1 as indicated in the method section. Scale bars = 50 μm. Representative distribution of neurite lengths for *profile 1* (d), *profile 2* (e) and *profile 3* (f).

Figure 5. Quantification of neurite outgrowth from neurons co-cultured on three transfection profiles

Neurite outgrowth and branching was quantified with neurons cultured on HEK293T cells with three distinct transfection profiles. Neurite outgrowth normalized to surface area (a). Neurite outgrowth normalized to sprouting neurons (b). Average length of primary and branching neurites (c). Average number of neurites per neuron (d). Values are reported as mean $\pm$ SEM. Significant differences are indicated by different letters above the bars ($p < 0.05$).

Figure 6. Quantification of neurite outgrowth from neurons co-cultured on non-transfected accessory cells

Neurite outgrowth and branching were quantified with neurons cultured on HEK293T cells with NGF supplemented culture medium. Neurite outgrowth normalized to surface area (a). Neurite outgrowth normalized to sprouting neurons (b). Average length of primary and branching neurites (c). Average number of neurites per neuron (d). Values are reported as mean $\pm$ SEM. Significant differences are indicated by different letters ($p < 0.05$).

Figure 7. Neurite growth cone morphology

Neurite growth cones extending from neurons co-cultured on HEK293T cells expressing NGF with distinct transfection profiles, *profile 1* (a), *profile 2* (b), and *profile 3* (c). Quantification of growth cone length (d). Values are reported as mean $\pm$ SEM. Neurons were stained with antibodies to TUJ1 as indicated in the method section. Significant differences are indicated by * ($p < 0.05$). Scale bar represents 25 μ m.

Figure 8. Predicted NGF concentration gradients

The predicted absolute concentration gradients (a) and fractional concentration gradients (b) from a single HEK293T cell expressing NGF with three distinct transfection profiles at 48 hours. Note that $x = 0$ is at the center of a transfected cell. Note that profiles at 24 and 48 hours are similar and may be difficult to visualize due to overlap.

Table 1

Characterization of three distinct transfection profiles. Values are reported as mean $\pm$ SEM. Significant differences between conditions are indicated by different letters (a, b, c) ($p < 0.05$).

	Complexing Agent	DNA amount ($\mu\text{g}/\text{well}$)	Transfection efficiency (% of total)	Transgene expression (RLU/mg)
Profile 1	PEI, N/P = 10	0.15	1.3 ± 0.3^a	$1.5 (\pm 0.2) \times 10^{4a}$
Profile 2	PEI, N/P = 25	0.30	2.9 ± 0.9^a	$7.3 (\pm 3.2) \times 10^{7b}$
Profile 3	LF2000, 1:1	0.50	40 ± 2.3^b	$3.9 (\pm 0.2) \times 10^{8b}$

Table 2

Calculated NGF production rates and estimated distance between transfected cells for three transfection profiles.

	NGF production rate (pg/cell/min)	Distance between transfected cells (μm)
Profile 1	7.1×10^{-5}	481
Profile 2	9.3×10^{-4}	350
Profile 3	6.8×10^{-4}	96