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Viral transduction of primary Schwann cells using a Cre-lox system to regulate GDNF expression

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Abstract

Glial cell-line-derived neurotrophic factor (GDNF) is a potent neurotrophic factor known to enhance motor nerve regeneration following its delivery. However, recent studies have determined that extended GDNF delivery to regenerating axons can entrap motor axons at the site of GDNF delivery. This entrapment leads to reduced motor axons available to reinnervate muscle. To address this issue, we designed a cell-based GDNF expression system that can temporally regulate protein expression using an inducible gene excision mechanism to prevent entrapment at the site of expression. To design this system for regulation of GDNF expression, we transduced two lentiviral vectors, one containing a constitutively active GDNF transgene flanked by two *loxP* sites, and the other containing a tetracycline-inducible *cre* transgene along with its constitutively active transactivator, into Schwann cells (SCs). These SCs over-express GDNF, but expression can be suppressed through the administration of tetracycline family antibiotics, such as doxycycline. The engineered SCs produced significantly more GDNF as compared to untransduced controls, as measured by enzyme-linked immunosorbent assay (ELISA). Following doxycycline treatment, these SCs produced significantly lower levels of GDNF and induced less neurite extension as compared to untreated SCs. Engineered SCs treated with doxycycline showed a marked increase in Cre recombinase expression, as visualized by immunohistochemistry (IHC), providing evidence of a mechanism for the observed changes in GDNF expression levels and biological activity. This cell-based GDNF expression system could have potential for future *in vivo* studies to provide a temporally-controlled GDNF source to promote axon growth.

Keywords

Lentivirus; Glial cell line derived neurotrophic factor; candy-store effect; peripheral nerve; drug delivery; cre-lox

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Introduction

Nerve injuries are still common and can have devastating consequences. In the US alone, some 360,000 people suffer upper extremity nerve injuries each year, resulting in over 8.5 million days of restricted activity and nearly 5 million bed/disability days (Kelsey 1997). Peripheral nerve surgery continues to expand and evolve rapidly as our understanding of basic science provides more options for treatment (Boyd et al. 2011). An important factor limiting functional recovery is the considerable amount of time required for nerve regeneration. At a rate of ~1mm a day, it may be months or years before long peripheral axons can re-establish end-organ contact if the injury is proximal (near the cell body) (Fox and Mackinnon 2011; Gordon et al. 2009). Strategies that can improve axonal regeneration and functional recovery after peripheral nerve injury would have a significant clinical impact.

It has become increasingly clear that a cause of poor regenerative outcome is denervated Schwann cells (SCs) in the distal nerve stump. Over time, denervated SCs decrease their growth factor expression and become less supportive of regenerating axons (Gordon 2010; Sulaiman and Gordon 2009). GDNF is a neurotrophic factor expressed by SCs and has been shown to enhance nerve regeneration (Boyd and Gordon 2003; Magill et al. 2010). GDNF expression is up-regulated in the distal nerve stump and in denervated muscles following nerve injury (Hoke et al. 2000; Naveilhan et al. 1997; Trupp et al. 1995). This expression pattern promotes migration and proliferation of SCs at the injury site and promotes regeneration of motor axons toward their end organs (Trupp et al. 1995).

Up-regulation of GDNF is transient (Eggers et al. 2010; Lie and Weis 1998; Zhao et al. 2004), often waning before full re-innervation of target muscles is completed when long distance regeneration is required. In cases of chronic denervation (loss of innervation for prolonged periods of time, >8 weeks in the rat model), GDNF levels in the distal nerve stump are no longer sufficient to stimulate robust axonal regeneration (Boyd and Gordon 2003; Hoke et al. 2002). Exogenous GDNF administration to the site of decreased GDNF expression has been shown to promote axonal extension similar to the endogenous levels found acutely after injury (Boyd and Gordon 2003; Wood et al. 2013a; Wood et al. 2013b).

While previous studies have considered exogenous delivery of GDNF to a nerve injury site using a variety of delivery systems (Barras et al. 2002; Boyd and Gordon 2003; Fine et al. 2002; Wood et al. 2013b), emerging research using viral gene delivery systems show promise as a means for local, long-term drug delivery via cellular expression in peripheral nerve injuries (Hendriks et al. 2007; Tannemaat et al. 2008; Wong et al. 2006). However, prolonged excess GDNF production from transgenic cells at the site of injury has been shown to promote entrapment of motor axons at the site of GDNF expression, an outcome called the “candy-store effect” (Tannemaat et al. 2008). Furthermore, nerve grafts that have been supplemented with GDNF-overexpressing SCs demonstrated increased axon number within the graft, but the nerve distal to the grafts contained fewer axons due to axonal entrapment within the graft (Santosa et al. 2013). These studies suggest that temporally-controlled, cell-based GDNF delivery could provide a therapeutic benefit of increased axons without entrapment.

In order to provide GDNF without the undesirable effects of prolonged GDNF delivery, we sought to transduce cells that would constitutively express the protein of interest (GDNF) with a gene excision mechanism allowing us to regulate expression. Here, we describe the use of two lentiviral vectors, one containing a constitutively active GDNF transgene flanked by two *loxP* sites, and the other containing a tetracycline-inducible *cre* transgene along with its constitutively active transactivator, to engineer SCs in which GDNF overexpression can be reduced through the administration of tetracycline family antibiotics, such as doxycycline. The Cre/lox system allows conditional deletion of the genes flanked by *loxP* sites in tissues producing Cre recombinase protein (Sauer 2002; Wachsman and Heidstra 2010), while a tetracycline-inducible system allows conditional gene activation in the presence of doxycycline (Das et al. 2004; Gossen and Bujard 1992; Gossen et al. 1995). The ability to temporally regulate GDNF expression in engineered SCs was determined by measuring GDNF production and biological activity in cell culture.

Materials and Methods

All supplies are from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

Isolation and expansion of SCs

SC cultures were prepared using previously described methods (Kaewkhaw et al. 2012; Morrissey et al. 1991). Briefly, the sciatic nerve was harvested from adult male Lewis rats (Charles River Laboratories, Wilmington, MA). All surgical procedures were performed in strict accordance with the National Institutes of Health guidelines and were approved by Washington University Animal Studies Committee. Harvested nerves were grown *in vitro* in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) or DMEM-D-valine (PAA Laboratories, Piscataway, NJ), containing 10% fetal bovine serum (FCS) supplemented with 100 U mL⁻¹ penicillin/100 µg mL⁻¹ streptomycin, 0.25 µg/mL amphotericin B, 20 µg/mL bovine pituitary extract (FBS), and 5 µM forskolin, for 7 days. These explants were then incubated overnight at 37°C in SC culture medium with 1.25 U/mL dispase (PeproTech, Rocky Hill, NJ) and 0.05% collagenase type IV (PeproTech) before tissue was dissociated and strained. After centrifugation at 400×g for 6 minutes, the pellet was washed in DMEM/10% FBS, then seeded on tissue culture plates coated with poly-L-lysine (pLL) in SC culture medium. On day 6, fibroblasts were complement-killed using a 1:40 dilution of anti-Thy 1.1 antibody in medium (Serotec, Raleigh, NC) and a 1:4 dilution in medium of rabbit complement (Sigma-Aldrich). Cultures were passaged as needed and split 1:2 on pLL-coated plates when they exceeded 80% confluence.

Lentiviral vector construction

Due to the size of required genes, two distinct lentiviral vectors were designed: a GDNF vector and a Cre vector (Figure 1). To produce the GDNF vector, full-length rat GDNF cDNA was cloned into FUIV, a lentiviral expression vector in which gene expression is driven by an ubiquitin promoter, with coexpression of dsRed fluorescent protein via an internal ribosomal entry site (IRES) element, as previously described (Santosa et al. 2013). The GDNF and IRES genes are flanked by two *loxP* sites. The Cre vector is based on the pSLIK lentiviral vector platform (Shin et al. 2006) and consists of two parts: a *cre* gene

under the control of a tetracycline response element (TRE) promoter based on Ptet1 (Gossen and Bujard 1992) and constitutively active third generational reverse transcriptional activator (rtTA3) gene (Zhou et al. 2006) under the control of a ubiquitin promoter. Both viruses were constructed by the Hope Center for Neurological Disorders Viral Vectors Core at Washington University in Saint Louis.

LV-Ubi-loxP-GDNF-IRES-DsRed-loxP—GDNF-IRES-DsRed sequence was amplified by PCR from FCIV-GDNF-IRES-DsRed and inserted into FCIV.FM1 (gift from Dr. Jeff Milbrandt) between BamHI and BsrGI sites, resulting in LV-Ubi-GDNF-IRES-DsRed. Two synthesized LoxP fragments were introduced into LV-Ubi-GDNF-IRES-DsRed with one between AsiI and RsrII and another between HapI and BlnI sites.

LV-TRE-Cre-Ubi-rtTA3—Cre cDNA sequence was amplified by PCR and inserted into pEN-TR-DsRed-PL between two SpeI sites to replace DsRed, resulting in pEN-TRE-Cre. The TRE-Cre fragment was amplified by PCR and then inserted into pSLIK-Ubi-rtTA3 between HpaI and BlnI sites using Gibson Assembly kit (New England Biolabs).

Plasmids and lentivirus production

The packaging cell line, 293T was maintained in DMEM, supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin in 37°C incubator with 5% CO₂. 293T cells were plated at 30–40% confluence 24 h before transfection (70–80% confluence when transfection). Ten µg of lentiviral vector with the appropriate insert, 5.8 µg of pMD-Lg, 3.1 µg of pCMV-G, and 2.5 µg of RSV-REV were cotransfected into 293T cells using the calcium phosphate precipitation procedure. Six hours after transfection, the medium was replaced with the complete medium containing 6 mM sodium butyrate. Culture supernatant was collected 42 h after transfection. The supernatant was passed through a 0.45 µm filter, concentrated by ultracentrifugation through a 20% sucrose cushion, and stored at –80°C until use. Vector titers were determined by transduction of HT1080 cells followed by qPCR assay.

SC lentiviral transduction

Established SC cultures were transduced before passage 7. The day prior to transduction, cells were seeded on pLL-coated 24-well plates at a density of 1.5×10^4 cells/well. On the day of transduction, the number of cells in each well was determined by microscopy. Five random images were taken at 100x using brightfield microscopy with an Olympus IX81 microscope (Olympus) and MetaMorph Premier version 7.6.5.0 (Molecular Devices). The number of cells in each microscope field was counted and divided by the area of the captured image in order to determine an average cell density. This average cell density was then multiplied by the total growth area of the well. This cell number was used to calculate the amount of vector required for a given multiplicity of infection (MOI). Cells were incubated with 2 µg/mL of polybrene (Santa Cruz Biotechnology, Dallas, TX) in SC culture medium for 1 hour at 37°C, after which appropriate vectors at the given MOI were introduced. Cells were then incubated with 2 µg/mL polybrene and vector for 20 hours at 37°C. For multiple transduction events, cells were allowed to rest for 6 hours between events.

Cell proliferation and GDNF vector transduction success

In order to quantify cell survival, the number of cells in each well was determined as described above. Cell counts were normalized to initial cell counts from the same well taken the day after seeding (cell proliferation index) to account for population changes over time. Successful transduction of the GDNF vector was visualized by expression of red fluorescent protein (RFP) reporter in SCs using fluorescent microscopy 3 days following final transduction of vectors. The percentage of cells with RFP expression was quantified by counting the number of discrete fluorescent cells in at least five random captured imaging frames and dividing the number by the total cell count.

GDNF production measurements

Quantification of GDNF production was determined by performing enzyme-linked immunosorbent assay (ELISA) on cell culture medium samples collected daily, using a GDNF ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer instructions. Plates were read using a BioTek EL800 (BioTek, Winooski, VT) plate reader and bundled software. Culture medium was collected daily for GDNF quantification by ELISA starting on day 5 after seeding (2 days after the last transduction event). Cultures were followed until day 7, upon which culture media was collected and cells treated with 5 µg/mL of doxycycline added to the culture media. Control cultures received media as before without doxycycline (sham media exposure). The cells were followed and culture media collected until day 11. Daily ELISA results were normalized to corresponding daily cell counts. Results from days 5, 6, and 7 were averaged to determine mean GDNF production before treatment with doxycycline, while results from days 9, 10, and 11 were averaged to determine mean GDNF production after exposure (or sham exposure) to doxycycline. Day 8 was not included because it represented a transition day, as this day's culture media had been modified to contain doxycycline or sham media. This media would represent GDNF expression during the initiation of Cre recombinase expression, not GDNF expression after the actions of Cre recombinase expression. Previous work with similar tetracycline-inducible systems reported maximum transgene activation at less than 1 µg/mL (Das et al. 2004). Thus, 5 µg/mL is a high dose selected to ensure full Cre activation and expression in all cells that have been successfully transduced with the Cre vector.

Cre recombinase expression

After transduction with GDNF and Cre vectors, SCs were treated with 5 µg/mL of doxycycline for 8 days. Cre recombinase expression was visualized by immunohistochemistry (IHC). At the appropriate experimental endpoint, SCs were fixed with 4% paraformaldehyde (PFA; Polysciences, Warrington, PA) at 37°C for 30 minutes, washed in phosphate-buffered saline (PBS) three times for 5 minutes each, and then permeabilized with methanol at -25°C for 20 minutes. Cells were blocked for 60 minutes in blocking solution consisting of PBS containing 2% Triton X-100, 10% normal goat serum (NGS), and 5% bovine serum albumin (BSA), and then incubated with anti-Cre recombinase antibody (Millipore, Billerica, MA) in PBS at 1:1000 dilution overnight at 4°C. The next day, cells were washed with PBS + 0.1% Tween-20 (PBST) three times for 5 minutes each

before the addition of FITC secondary antibody (Invitrogen). Cells were visualized using fluorescence and brightfield microscopy.

Biological activity (neurite extension) assays

In order to determine the biological activity of the GDNF released from the SCs, dissociated dorsal root ganglia (DRG) were co-cultured in Transwell plates (Corning, Costar) with doxycycline treated and untreated (sham culture media change) SCs. SCs were transduced in 24 well plates using the GDNF and Cre lentiviruses at MOI 20 in culture media as previously described. Twenty-four hours after final transduction, SCs were cultured with media (with or without doxycycline) for 3 days to allow sufficient Cre recombinase expression to regulate GDNF expression. Separately, E8 chick DRG were harvested and dissociated using 0.25% Trypsin-EDTA. DRG were then seeded onto PLL coated 24 well plates at 2.6×10^4 cells/cm² in Neurobasal media with 1x B27 supplement (Life Technologies) for 1 hour to allow for surface attachment before the addition of Transwell plate inserts containing SCs. SCs (with or without doxycycline treatment) were seeded on the semipermeable insert membranes at 1.5×10^4 cells/cm² with fresh SC media (DMEM, 10% FBS, and 1% ABAM). Schwann cells and DRG were allowed to co-culture for 18 h, upon which the inserts were removed and cells were fixed and permeabilized with 4% paraformaldehyde in PBS and 0.1% TritonX-100, respectively. DRG neurite extensions were stained using 1:200 avian neurofilament primary antibody (3A10, University of Iowa DSHB) for 24 h followed by 1:200 Alexa Fluor 555 goat anti-mouse IgG (Life Technologies) for 4 h. Extensions were visualized using a fluorescent light microscope on a 10x objective and images were captured using an Olympus CCD camera. Length of neurite extensions were measured using ImageJ software, where analyzed extensions were greater than one cell body diameter and contained no other neurite crossings. Neurite extension measurements were pooled from 4 culture wells per condition.

Statistical analysis

Data were recorded using Microsoft Excel (Microsoft). Each culture well was treated as an 'n', and experiments were performed with at least one replicate per trial and in duplicate. Statistical analyses were performed using Graphpad Prism version 5 (Graphpad), and significance was considered $p < 0.05$. All data were evaluated with one-way analysis of variance (ANOVA), followed by post-hoc Bonferroni correction methods for comparisons between groups when significance ($p < 0.05$) was present. All results are reported as mean $\pm$ standard deviation except standard error of the mean for biological activity (Figure 6).

Results

Visualization of reporter protein expression in SCs

We first characterized transfection efficiency for the GDNF vector. SCs underwent a single transduction event using GDNF vector and a single transduction event using Cre vector at MOI 10, 20, or 50 for each vector. Transduction success was visualized through the expression of red fluorescent protein reporter (dsRed) associated with the GDNF vector in SCs 3 days following the final transduction event. We observed a greater proportion of cells expressing the red fluorescent protein reporter correlated with higher transduction MOI,

indicating successful transduction and expression of GDNF (Figure 2). No cells exhibited fluorescence prior to transduction.

Cell proliferation following transduction in SCs

We characterized the effect of multiple transduction events for each vector on the proliferative response of the SCs. SCs underwent 1 transduction event using GDNF vector and 1 transduction event using Cre vector at MOI 10, 20, or 50 for each vector. By assessing cell number for up to 5 days following the transduction events, we observed an inverse relationship between transduction MOI and cell proliferation (Figure 3A). Regardless of MOI, viral transduction events led to decreased cell proliferation compared to untransduced SCs. SC groups transduced with GDNF MOI 10 + Cre MOI 10 and GDNF MOI 20 + Cre MOI 20 both demonstrated moderate proliferation over time, where the cell proliferation index had significantly increased from day 5 compared to day 2. However, cell proliferation was significantly blunted in the GDNF MOI 50 + Cre MOI 50 group, as there was no significant difference between cell proliferation indices between days 2 and 5. Additionally, cell proliferation for this group was reduced compared to the alternative transduction groups (MOI 10 and 20) between days 3 through 5.

To further characterize why 2 transduction events at a high MOI (50) resulted in severely limited cell proliferation, cells were transduced with GDNF vector twice or CRE vector twice. The groups: GDNF MOI 50 + Cre MOI 50, GDNF MOI 50 + GDNF MOI 50, or Cre MOI 50 + Cre MOI 50, all showed similar proliferation characteristics (Figure 3B). Therefore, this data suggests that multiple transduction events at high MOIs can lead to limited SC proliferation following the transduction events regardless of transgene identity. It also suggests that vector identity was not a factor effecting cellular proliferation. Based on these results, we eliminated the GDNF MOI 50 + Cre MOI 50 transduction protocol from further experimentation, as a future goal will be to produce high SC numbers needed for transplantation *in vivo*. Furthermore, as transduction efficiency was low for the GDNF MOI 10 + Cre MOI 10 group (Figure 2), we selected the GDNF MOI 20 + Cre MOI 20 transduction protocol for further studies. The GDNF MOI 20 + Cre MOI 20 group provided the best balance of cellular proliferative rate (cell proliferation index) and transduction efficiency.

GDNF production by SCs

Engineered SCs were quantified for GDNF protein production by ELISA. In order to regulate expression of GDNF from the SCs, we evaluated GDNF production in engineered SCs after treating SC culture medium with doxycycline. SCs were transduced once with GDNF vector and once with Cre vector each at an MOI of 20. Before treatment with doxycycline, SCs produced ~225–250 pg GDNF per 10,000 cells (Figure 4). Following treatment with doxycycline, GDNF production by SCs (Dox) was significantly decreased compared to cells not treated with doxycycline (Sham). In contrast, there was a trend but no statistical difference in GDNF production in SCs not treated by doxycycline (Sham) before and after a sham culture medium change, simulating doxycycline addition ($p>0.05$). Additionally, the treatment of cells with doxycycline to produce Cre recombinase did not affect cellular proliferation compared to untreated cells (data not shown). The reduction in

GDNF protein secretion following doxycycline treatment (Dox) represents a temporal mechanism to regulate the dose of GDNF from a cell-based delivery system.

Cre recombinase expression induced in SCs

To understand the GDNF reduction in doxycycline treated SCs, we performed immunohistochemistry to identify Cre recombinase expression. Cre recombinase protein expression was qualitatively evaluated by IHC in engineered SCs treated (or untreated) with doxycycline (5 µg/mL) for 8 days (Figure 5). We observed rare Cre recombinase-expressing cells in the untreated group (Figure 5A) and abundant expression in the doxycycline-treated group (Figure 5B). The positively stained SCs demonstrate temporal regulation of Cre using doxycycline. The robust expression of Cre recombinase due to doxycycline treatment serves to potentially explain our previously observed decrease in SC GDNF production after doxycycline treatment (Figure 4). Specifically, expression of Cre recombinase may lead to GDNF gene excision via a recombination event between the loxP sites that flank it, as intended by our 2-vector designed system.

Biological activity of GDNF produced by SCs

DRG neurite extension is dose-dependant for GDNF, where the dose modulates extension (Wood et al. 2009). To assess the biological activity of GDNF being produced by the engineered SCs, we treated engineered SCs (MOI 20 with GDNF and Cre vectors) with doxycycline (or sham media) for 3 days. We then seeded these SCs in Transwell plate inserts, where DRG were seeded at the bottom of the plates 1 hour prior to the addition of SCs. These Transwell plates separate the DRG and SC but allow factors released (GDNF) from the SCs to diffuse to the opposite chamber containing the DRG. SCs were co-cultured with the DRG for 18 h to permit adequate neurite extension for analysis.

All SC-neuron co-cultures resulted in neurite extensions from the DRG (Figure 6). Engineered SCs treated with doxycycline (Dox) extended shorter neurites compared to untreated SCs (Sham). As engineered SCs treated with doxycycline produced less GDNF than untreated SCs (Figure 4), this result suggests that the change in GDNF production has a measurable and sensitive biological effect.

Discussion

Schwann cells play a critical role in nerve regeneration (Araki and Milbrandt 1996; Bunge 1994; Bunge et al. 1986; Levi et al. 1994), and the addition of exogenous SCs to conduits or nerve grafts has been shown to enhance regeneration (Guenard et al. 1992; Santosa et al. 2013). Furthermore, autologous SC transplantation has already entered clinical trials for the treatment of spinal cord injury (Levi 2012; Saberi et al. 2008). Taken together, transgenic SCs altered to express therapeutic proteins are an intriguing prospect for transplantation therapies, as they could serve a dual purpose of cell therapy and drug delivery via growth factor expression. However, the transgenic SCs produced currently require Cre recombinase expression, which has been shown to have toxicity issues due to cryptic loxP site excision (Schmidt-Supprian and Rajewsky 2007). Our transgenic SC system provides a tool to study

spatial and temporally regulated drug delivery and would need further modification before clinical consideration.

Earlier attempts to use transgenic SCs modified to over-express GDNF to enhance nerve regeneration produced mixed regenerative results (Santosa et al. 2013). This use of modified SCs resulted in a frustrating paradox, whereby axon regeneration (axon numbers) appeared robust within the delivery site (nerve graft) but was reduced distal to the delivery site. Further study revealed that entrapment of axons at the site of delivery was the cause for the reduced axon number in nerve distal to the delivery site. Therefore, strategies for GDNF delivery must be carefully regulated to avoid axon entrapment (Blits et al. 2004; Eggers et al. 2013; Eggers et al. 2008; Shakhbazau et al. 2013; Tannemaat et al. 2008). Toward this goal, we engineered transgenic SCs in which GDNF expression can be regulated via activation of Cre recombinase expression through doxycycline administration. Our study (1) establishes a two-vector SC transduction protocol that balances optimization of cell survival and transduction efficiency, and (2) demonstrates proof-of-concept for the ability to regulate transgene expression.

We observed a dose-dependent relationship between SC proliferative rate and dose of lentiviral vector received. As a result, there is a balance between the percentage of cells transduced and proliferative potential of the cells following transduction. Cell counts were used to determine cell proliferation after transduction, and a significant decrease in cell growth (rate) was seen in cells undergoing any transduction event, where essentially no cell growth was observed in the MOI 50 group. This effect was observed regardless of viral vector transduced, suggesting that transduction itself is detrimental to SC growth, rather than GDNF over-expression. Stunted SC proliferation has not been reported in previous studies using lentiviral transduction of SC. However, while others have found no differences in viability between naïve and transduced SCs by both MTT assay and overall outgrowth following a single lentiviral transduction event at MOI 50, doubling time was not immediately followed after the transduction events (Shakhbazau et al. 2012). Additionally, in our study, we tracked cell proliferation for two transduction events rather than one. This additional transduction event may be detrimental to cellular proliferation and merits future exploration.

SCs transduced using the optimized protocol for the GDNF/Cre lentiviral system produced 225–250 pg GDNF per 10^4 cells per day. Previous studies have demonstrated that transplantation of 10^5 – 10^6 SCs is a viable range for successful cell transplantation for nerve (Jesuraj et al. 2011). Injection of 10^6 engineered SCs from our studies could provide a daily dose of ~20 ng of GDNF. Previous studies have determined that a minimum of 100 ng GDNF/day is necessary to promote improved nerve regeneration when GDNF is delivered at the nerve repair site via osmotic pump or microspheres (Boyd and Gordon 2003; Wood et al. 2013a). While our delivery system falls short of this dose, these previous delivery systems released GDNF *outside* the nerve. Our engineered SCs are intended for injection *inside* the nerve or construct, such as a nerve graft, where GDNF release would have better availability to regenerating axons and less protein loss due to diffusion and degradation. As a result, less GDNF may be needed (compared to previous studies) to have an effect on axonal regeneration and functional recovery with our cell-based expression system.

DRG neurite extension has a dose-dependant response due to GDNF delivery (Wood et al. 2009). We demonstrated a difference in DRG neurite extension due to co-culture with doxycycline treated, engineered SCs versus untreated, engineered SCs. We also observed differences in GDNF production in doxycycline treated, engineered SCs as compared to untreated, engineered SCs. Taken together, our results strongly suggest that our engineered SCs are capable of producing GDNF at doses dependant on a doxycycline inducible cue, and these doses have enough distinction to affect biological activity. Thus, we hypothesize that these engineered SCs could be transplanted *in vivo* to provide therapeutic levels of GDNF with the capacity to down-regulate GDNF expression (in the presence of doxycycline) to levels that prevent axon entrapment at the site of delivery.

Conclusions

In conclusion, we have engineered SCs that constitutively over-express GDNF in which expression can be reduced following a temporal cue (doxycycline addition to culture medium). This cue allows for regulation of GDNF expression. This gene excision mechanism can potentially provide the benefits of GDNF delivery for peripheral nerve regeneration while avoiding axonal entrapment at the site of GDNF delivery.

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Figure 1.

Schematic of engineered SC with GDNF and Cre lentiviral vectors. In order to engineer SCs that express GDNF in a manner that can be temporally regulated, two lentiviral vectors were transduced into the SCs. The GDNF vector (top) contained a constitutively active GDNF and reporter fluorescent protein (dsRed) flanked by a pair of loxP sites. The Cre vector (bottom) consisted of a tetracycline-inducible Cre gene which, when activated by binding tetracycline derivatives, results in the theoretical loxP/Cre-mediated excision of the GDNF gene contained between the loxP sites.

Figure 2.

Visualization of red fluorescent protein reporter in SCs that have been transduced with GDNF and Cre vectors at variable MOIs. Images were taken at day 6 after seeding (3 days after the last transduction event). (A) Untransduced controls. (B) SCs transduced with GDNF and Cre vectors at MOI 10 (GDNF 10 + CRE 10). (C) SCs transduced with GDNF and Cre vectors at MOI 20 (GDNF 20 + CRE 20). (D) SCs transduced with GDNF and Cre vectors at MOI 50 (GDNF 50 + CRE 50). Scale bar = 100 μ m. (E) GDNF vector transduction efficiency increased as MOI increased. Shown as mean $\pm$ SD, n=6, * = $p < 0.05$ compared to GDNF 10 + CRE 10, and ^ = $p < 0.05$ compared to GDNF 20 + Cre 20.

Figure 3.

SC proliferation rate follows an inverse relationship with respect to MOI. SCs underwent transduction events using GDNF vector and Cre vector at variable combinations of MOI for each vector. (A) Cell proliferation was reduced as MOI increased. (B) At high MOIs, SC proliferation was severely blunted regardless of transgene identity. Arrows represent timing of transduction events. Cell counts were normalized to the number cells present the day after seeding (before any transduction events). Shown as mean $\pm$ SD, $n=3$ for GDNF 50 + GDNF 50, $n=6$ for all other groups. * = $p<0.05$ compared to untransduced control, ^ = $p<0.05$ compared to GDNF 10 + Cre 10, # = $p<0.05$ compared to GDNF 20 + Cre 20, + = $p<0.05$ compared to GDNF 50 + Cre 50.

Figure 4.

GDNF expression by engineered SCs. (A) Average GDNF production in engineered SCs (GDNF MOI 20 + Cre MOI 20) before and after exposure to doxycycline. Culture medium was collected before and after treatment with doxycycline (5 μ g/mL; Dox) or sham culture medium (no doxycycline; Sham) and measured using ELISA. The addition of doxycycline to culture media resulted in decreased GDNF protein expression compared to prior exposure levels or the untreated group. Data (n=12) represent mean $\pm$ SD and * indicates statistical significance ($p < 0.05$) from doxycycline treated SCs (Dox).

Figure 5.

Cre recombinase expression in engineered SCs. (A) Engineered SCs that have not been treated with doxycycline visually demonstrate little Cre recombinase protein (green). (B) Engineered SCs that have been treated for 8 days with 5 µg/mL of doxycycline demonstrate robust staining for Cre recombinase (Scale bar = 100 µm).

Figure 6.

Effect of engineered SCs on neurite extension *in vitro*. Average neurite extension from DRG was reduced due to co-culture with doxycycline treated SCs (Dox) compared to untreated SCs (Sham). Data (n = 132) represent mean $\pm$ standard error of the mean and * indicates statistical significance ($p < 0.05$) from doxycycline treated SCs (Dox).