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Characterization of the Stability and Bio-functionality of Tethered Proteins on Bioengineered Scaffolds

*IMPLICATIONS FOR STEM CELL BIOLOGY AND TISSUE REPAIR**

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Ting-Yi Wang[‡], Kiara A. F. Bruggeman[§], Rebecca K. Sheean[‡], Bradley J. Turner[‡], David R. Nisbet[§1,2], and Clare L. Parish[‡1,3]

From the [‡]Florey Institute of Neuroscience & Mental Health, The University of Melbourne, Parkville, Australia, 3010 and the [§]Research School of Engineering, The Australian National University, Canberra, Australia, 0200

Background: Tethering proteins onto bioengineered scaffolds enables longterm delivery, however protein stability, release kinetics, and functionality over time remains unknown.
Results: Tethered proteins remain stable and functional for several months, capable of activating intracellular signaling pathways and influencing cell fate.
Conclusion: Tethered proteins are stable and functional long-term.
Significance: Such knowledge may have implications for promoting tissue repair.

Various engineering applications have been utilized to deliver molecules and compounds in both innate and biological settings. In the context of biological applications, the timely delivery of molecules can be critical for cellular and organ function. As such, previous studies have demonstrated the superiority of long-term protein delivery, by way of protein tethering onto bioengineered scaffolds, compared with conventional delivery of soluble protein *in vitro* and *in vivo*. Despite such benefits little knowledge exists regarding the stability, release kinetics, longevity, activation of intracellular pathway, and functionality of these proteins over time. By way of example, here we examined the stability, degradation and functionality of a protein, glial-derived neurotrophic factor (GDNF), which is known to influence neuronal survival, differentiation, and neurite morphogenesis. Enzyme-linked immunosorbent assays (ELISA) revealed that GDNF, covalently tethered onto polycaprolactone (PCL) electrospun nanofibrous scaffolds, remained present on the scaffold surface for 120 days, with no evidence of protein leaching or degradation. The tethered GDNF protein remained functional and capable of activating downstream signaling cascades, as revealed by its capacity to phosphorylate intracellular Erk in a neural cell line. Furthermore, immobilization of GDNF protein promoted cell survival and differentiation in culture at both 3 and 7 days, further validating prolonged functionality of the protein, well beyond the minutes to hours timeframe observed for soluble proteins under the same culture conditions. This study provides important evidence of the stability and functionality kinetics of tethered molecules.

Soluble proteins in their natural physiological environment execute their function and are then degraded by enzymes, oxidation, hydrolysis, and other reactions over relatively short periods of time, thereby losing their original bio-functionality. As such, repeated synthesis and delivery from the local environment is required for ongoing activity (1). When soluble proteins are extrinsically introduced *in vivo* to influence cellular responses (*e.g.* to promote tissue repair or influence disease progression) they are also only present for short periods of time (typically minutes to hours), due to diffusion into the local physiological environment and degradation. Hence methods of ongoing delivery must be employed which typically rely on the use of cumbersome catheters and infusion pumps. Consequently there is increasing interest to develop improved methodologies to enable the stable delivery of molecules and proteins, and to ensure these factors can be administered in temporally and spatially appropriate manners.

Work by us and others has already demonstrated that protein immobilization onto bioengineered scaffolds can provide means to control the localization of biological molecules, create longer lasting stimuli, and can be controlled by way of substrata (scaffold) degradation. We demonstrated that when tethered onto electrospun nanofibrous scaffolds both brain-derived neurotrophic factor (BDNF)[4] and glial-cell derived neurotrophic factor (GDNF) were capable of promoting neural stem cell proliferation and influencing differentiation *in vitro* to a greater extent than culturing cells in the presence of soluble protein (2, 3). Furthermore, we showed that tethered GDNF

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[1] Both authors contributed equally to this work.
[2] Supported by an Australian Research Council Australian Postdoctoral Fellowship and subsequently an NHMRC Career Development Fellowship (APP1050684). To whom correspondence may be addressed: The Australian National University, Canberra. Tel.: +612-6125-7451; E-mail: david.nisbet@anu.edu.au.
[3] Supported by Senior Medical Research Fellowship provided by the Viertel Charitable Foundation, Australia. To whom correspondence may be addressed: Florey Institute of Neuroscience & Mental Health, The University of Melbourne, Parkville, Australia, 3010. Tel.: +613-9035-6526; Fax: +613-9347-0446; E-mail: cparish@unimelb.edu.au.

[4] The abbreviations used are: BDNF, brain-derived neurotrophic factor; E, embryonic day; ED, ethylenediamine; GDNF, glial-derived neurotrophic factor; iGDNF, immobilized glial-derived neurotrophic factor; iGDNF(v), immobilized glial derived neurotrophic factor scaffolds that have been exposed to vortexing; NSC, neural stem cell; PCL, poly ϵ-caprolactone; sGDNF, soluble glial-derived neurotrophic factor.

maintained long-term biofunctionality *in vivo*, supporting the survival, differentiation, and integration of transplanted neural stem cells for up to 28 days (3). Work by others has similarly demonstrated the benefit of tethered proteins. By way of example, photochemically bound nerve growth factor on microporous poly(2-hydroxyethylmethacrylate) was shown to encourage neurite outgrowth of PC12 cells (4); methacrylamide chitosan immobilized with rat interferon-γ promoted neural differentiation of adult neural stem/progenitor cells (5, 6); and tethered epidermal growth factor (EGF) on poly(methyl methacrylate)-*graft*-poly(ethylene oxide) significantly enhanced mesenchymal stem cell (MSC) spreading and survival compared with saturating concentrations of soluble EGF (7).

Despite evidence for the benefit of immobilized proteins, little attention has been paid to the amount of protein tethered to biomaterial surfaces, stability and longevity of tethered proteins, protein degradation rate, the release kinetics of tethered molecules, activation of intracellular signaling pathway, or the duration of functionality of the protein. Here we investigated the stability of a tethered protein, GDNF, its activation of intracellular signaling pathway, and its functionality using primary cultures isolated from the developing ventral midbrain, enriched with dopaminergic neurons. GDNF has been shown to promote the survival, differentiation, and neurite growth, most notably of dopaminergic neurons *in vitro* and *in vivo* (8, 9). GDNF has additionally been shown to delay degeneration of dopaminergic neurons in Parkinsonian animals as well as promote the survival and integration of these neurons following transplantation into animal models of the disease (10–13). GDNF therefore represents an example whereby prolonged protein delivery could have a significant impact on stem cell populations *in vitro*, disease progression and disease treatment. Our findings demonstrated long-term presence and functionality of GDNF, with no evidence of degradation or leaching, thereby highlighting the potential benefit of tethered molecules in applications such as tissue repair where prolonged exposure to trophic proteins is likely to be of benefit.

EXPERIMENTAL PROCEDURES

Synthesis of Electrospun Scaffolds—Electrospun fibers were produced from polycaprolactone (PCL, Mn 70–90k, Sigma Aldrich), dissolved in a 3:1 (v/v) solution of chloroform (Chem-Supply) and methanol (Chem-Supply), respectively. A home built electrospinner consisting of a syringe pump (KD-100, KD Scientific, Holliston) and an adjustable DC voltage power supply (Model RR 50-1.25R/230/DDPM, Gamma High Voltage Research, Ormond Beach, FL) was employed. Spinning was performed at room temperature using a 20 kV voltage, a 21-gauge needle, a 2 ml/h flow rate, and a 13 cm working distance. The duration of electro-spinning and collection onto a spinning mandrel resulted in two-dimensional (100 um) and three-dimensional (250–300 μm thick) scaffolds, as confirmed by scanning electron microscopy and previously reported (2). After collection, the scaffolds were dried in a vacuum oven at 37 °C overnight (Labec Laboratory Equipment, Australia) and stored in a desiccator prior to use. The scaffolds were then aminolysed by immersion in 0.05 M ethylenediamine (ED, Sigma Aldrich), diluted in 2-propanol (Merck Pty, Australia) for 15 min at room temperature with moderate stirring. The samples were subsequently washed three times with ice-cold distilled water, and sterilized in 70% ethanol for 15 min.

Scanning Electron Microscopy—To validate scaffold structure and assembly, samples were coated with ~2 nm of platinum by sputtering at 20 mA for 1 min. A scanning electron microscope was then used to visualize the morphology of the electrospun materials (Zeiss Ultra Plus FE-S.E., 7.5 μm aperture at 3 kV). A working distance of 3.4 mm and magnification of ×4950 was employed.

Confirmation of Aminolysation—Scaffolds treated with ED (Sigma Aldrich Pty Ltd) were dissolved in 100 μl of tetrahydrofuran and 100 μl of PBS, and reacted with 100 μl of fluorescamine (Molecular Probes, 10 mg/ml in acetone). The fluorescence was detected using a plate reader with ex/em = 390/475–490 nm. For the standard curve amine concentrations included 1.49×10^{-6}, 1.12×10^{-6}, 7.45×10^{-7}, 1.49×10^{-7}, 7.45×10^{-8}, 1.49×10^{-8}, 7.45×10^{-9}, and 0 mol/g of ED in PBS.

Protein Tethering on PCL Scaffolds—For immobilization of GDNF onto PCL scaffolds, 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylic acid 3-sulfo-*N*-hydroxy-succinimide ester sodium salt (sulfo-SMCC, Sigma Aldrich) was used as a cross-linker. A 2.5 mg/ml sulfo-SMCC solution in PBS was gently shaken for an hour at room temperature and then filtered (0.22 mm filter). The PCL scaffolds, after treatment with ED, were immersed in the prepared sulfo-SMCC solution for 2 h at room temperature with gentle agitation, then transferred to a recombinant human GDNF solution (4 mg/ml; R & D Systems) overnight at 4 °C. To ensure that GDNF protein was attached and not simply absorbed onto the scaffolds some scaffolds were vortexed in PBS for minutes following protein immobilization. Amounts of tethered protein, stability, and functionality were compared between: PCL scaffolds (PCL), PCL scaffolds tethered with GDNF (PCL_iGDNF), and PCL scaffolds tethered with GDNF and vortexed (PCL_iGDNF(v)). GDNF was additionally tethered to aminolysed scaffolds in the presence of artificial cerebrospinal fluid (aCSF) to confirm stability and function in a biologically relevant fluid.

Enzyme-linked Immunoabsorbent Assay—Enzyme-linked immunoabsorbent assays (ELISA) were performed as described previously (3). In brief, PCL scaffolds, + GDNF attachment, were incubated in 1 mg/ml of goat anti-GDNF antibody (R & D Systems) containing 5% donkey serum in PBST (PBS containing 0.05% Tween-20) for 2 h at room temperature. The scaffolds were washed three times in PBST before being immersed in anti-goat horseradish peroxidase (HRP, 1:2000 in PBST solution containing 2% donkey serum) for 1 h. After three washes in PBST, scaffolds were placed in a 96-well plate where the bound HRP activity was assessed by color development using TMB microwell peroxidase system (R & D Systems). 30 μl of 1 M HCl was added to each well to stop the reaction, and the absorbance (at 450 nm) was measured with a microtiter plate reader (SpectraMax). A standard curve for GDNF was performed (0–10,000 pg/ml) from which the amount of bioavailable tethered GDNF protein per scaffold could be determined and compared across treatments. To determine the stability of tethered GDNF over time, scaffold samples were collected immediately

after protein attachment (day 0) and at 3, 7, 14, 28, and 120 days after attachment and storage in PBS and aCSF.

To assess whether tethered proteins were susceptible to proteolytic degradation of tethered proteins we treated iGDNF scaffolds with 0.25% trypsin (Invitrogen) for 10 min and compared the amount of GDNF present on PCL to non-trypsin-treated iGDNF scaffolds. Collected scaffolds and supernatant were stored at −80 °C until determination of protein levels by sandwich ELISA.

BET Surface Area Analysis of PCL Scaffolds—Brunauer-Emmett-Teller (BET) surface area analysis was performed using a micromeritics TriStar II surface area and porosity instrument to determine the surface area of the scaffolds (14), and subsequently the amount of GDNF tethered onto the PCL surface. 45 mg of untreated polymer scaffold was loaded into a dry 0.95-cm diameter sample tube and degassed under vacuum (0.15–0.3 mbar) at room temperature for 7 days on a micromeritics VacPrep 061 sample degas system. The degassed sample mass was determined to be 43 mg. The sample tube was then loaded into the instrument for adsorption analysis of nitrogen gas in a liquid nitrogen bath. The specific surface area was determined to be 3.0050 m^2/g. The weight of the punch biopsies (0.6 cm in diameter) on which GDNF was attached, and cells cultured, was 0.80 mg so the absolute surface area was $0.0008\ g \times 3.0050\ m^2/g = 0.0024\ m^2 = 24\ cm^2$.

Immunoblotting—The dopaminergic neural stem cell line, SN4741, was cultured in DMEM, 10% FBS, L-glutamine (2 mM), penicillin/streptomycin (50 units/ml), and glucose (0.6%). For analysis of intracellular GDNF signaling, 100,000 cells were seeded onto 12-well plates or PCL scaffolds. At 70% confluency the cells were changed into serum-free media with or without GDNF (soluble or immobilized). A standard curve of GDNF treatment (0, 3, 10, 30, 100 ng/ml), using the same cell line, was performed in parallel to quantify the levels of GDNF activity. Cells were stimulated with soluble GDNF (sGDNF, 30 ng/ml) for 60 min or 3 days, and tethered GDNF (iGDNF) for 1 day or 3 days prior to being lysed in ice-cold buffer containing 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% protease inhibitor mixture (Sigma), 50 mM NaF, and 0.2 mM Na_3VO_4 for 20 min on ice. Lysates were centrifuged at 14,000 rpm for 20 min at 4 °C to collect supernatants. Protein was quantified using the bicinchoninic acid assay kit (Pierce) using bovine serum albumin standards.

Protein (50 μg) was electrophoresed through 12.5% SDS-polyacrylamide gels and transferred to Immobilon PVDF-FL membrane (Millipore). Membranes were blocked with 5% skim milk in Tris-buffered saline with Tween-20 (TBST), pH 8.0, for 30 min and incubated with mouse pErk1,2 (1:2,000, #9106, Cell Signaling Technology) and rabbit total Erk1,2 (1:1,000, #9102, Cell Signaling Technology) antibodies in 3% BSA in TBST overnight at 4 °C. Blots were washed 3× in TBST for 10 min and incubated with IRDYe 680 and 800CW-conjugated secondary antibodies (1:10,000) followed by 3× washes in TBST for 10 min and detected using the Odyssey Classic infrared imaging system. Membranes were then stripped and re-probed with mouse anti β-actin (1:5000, Sigma). Blots were quantified by taking the ratio of pErk/Erk bands and subtracting background intensity.

Microdissection and Culturing of Ventral Midbrain Progenitors—All procedures were conducted in accordance with the Australian National Health and Medical Research Council's published Code of Practice for the Use of Animals in Research, and experiments were approved by the Florey Neuroscience Institute animal ethics committee. C57BL/6 mice were housed on a 12 h light/dark cycle with *ad libitum* access to food and water. Cells used for *in vitro* culturing were obtained from mice that were time mated overnight, with visualization of a vaginal plug on the following morning taken as embryonic day (E) 0.5. Ventral midbrain (VM) tissue was isolated at mouse embryonic day 11.5 (E11.5).

Pregnant mice (E11.5) were anesthetized with isoflurane prior to cervical dislocation. The collected embryos were immersed in chilled L15 medium (Invitrogen), the brains removed and ventral midbrain microdissected. Subsequently the tissue fragments were incubated in 0.1% DNase and 0.05% trypsin (in magnesium and calcium free Hank's buffered saline solution, HBSS) for 15 min followed by three gentle washes in HBSS. Finally, the tissue fragments were dissociated in N_2 media consisting of a 1:1 mixture of F12 and MEM supplemented with 15 mM HEPES buffer, 1 mM glutamine, 6 mg/ml glucose (Sigma-Aldrich), 1.5 mg/ml bovine serum albumin, and 1% N2 supplement (all purchased from Invitrogen). Cells were seeded at a density of 175,000 cells/cm^2 onto either poly-D-lysine-coated coverslips (in the presence or absence of soluble GDNF, 30 ng/ml) or PCL scaffolds (+ immobilized GDNF) and incubated at 37 °C in 5% CO_2 for 3 and 7 days. After 3 days and 7 days, the cells were fixed with 4% paraformaldehyde for 20 min, washed, and stored in PBS containing 0.025% sodium azide until the time of immunocytochemistry.

Immunocytochemistry—Fixed cultures were incubated overnight in the following primary antibodies (diluted in 0.3% Triton X and 5% donkey serum): mouse anti-β tubulin (TUJ1, 1:1500, Promega, neuronal marker), and rabbit anti-tyrosine hydroxylase (TH, 1:400 Pelfreeze, rate-limiting enzyme in dopamine synthesis and marker of dopaminergic neurons). Cultures were then washed for 10 min in PBS before the secondary antibodies were subsequently added and incubated for an hour at room temperature. Secondary antibodies (1:300 in PBS containing 0.3% Triton X and 2% of goat/donkey serum) were as follows: DyLight 488 donkey anti-rabbit (Jackson ImmunoResearch), DyLight 549 donkey anti-mouse (Jackson immunoResearch). After washing in PBS, Hoechst (1:1000 in PBS, nuclei marker) was applied for 5 min, followed by two washes. The samples were then slide mounted (Dako) and imaged using a fluorescence microscope.

Statistical Analysis—For all ELISAs, Western blots, and cell cultures ≥3 independent experiments were performed with three coverslips or scaffolds included for each condition in each experiment. For assessments of cell viability and differentiation, 10 fields of view per coverslip or scaffold were imaged using a Zeiss200 inverted microscope (images captured at 20x magnification). All data are expressed as mean ± S.D. Student's t-tests or one-way ANOVAs with Tukey post-hoc tests were used to show significant differences between groups with the level of significance set at 0.05.

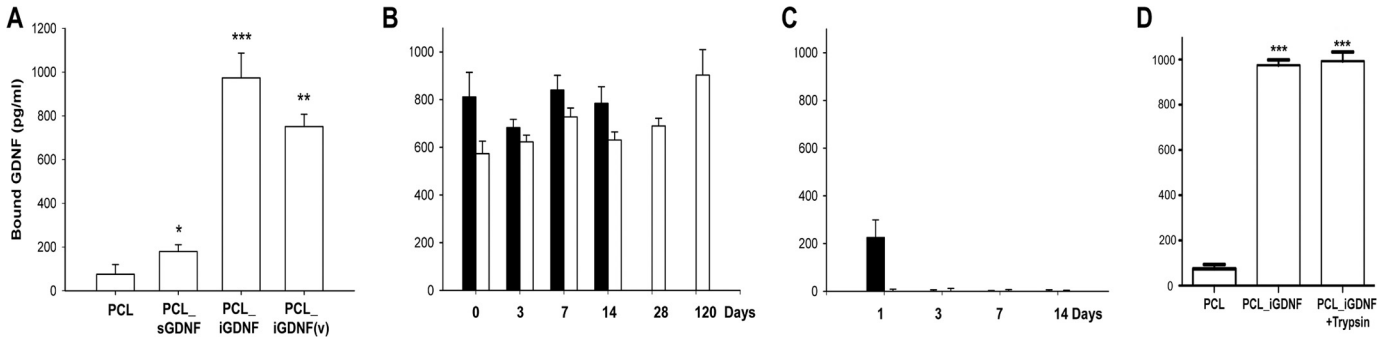

FIGURE 1. **Confirmation of protein tethering and stability on electrospun nanofibers.** *A*, immobilization of GDNF on PCL scaffolds (iGDNF) significantly increases the presentation of protein in culture. The majority of protein was covalently attached as reflected by vortexing to remove excess absorbed protein (iGDNF(v)). *B*, amount of GDNF presents on scaffolds at 0, 3, 7, and 14 days after attachment without vortexing (*black bars*) and at 0, 3, 7, 14, 28, and 120 days after attachment with vortexing (*white bars*). No significant decrease was observed in the amount of GDNF on the scaffolds over time, indicative of protein stability. *C*, small amount of GDNF was absorbed onto the scaffolds at the time of tethering, which leached from the biomaterial within the first day, and subsequently degraded. *D*, treatment of iGDNF scaffolds with trypsin had no effect on the amount of GDNF protein present compared with iGDNF alone, indicating that tethered GDNF was resistant to proteolysis. Data represent mean + S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; one-way ANOVA with Tukey post-hoc test.

RESULTS AND DISCUSSION

Because of the high surface area to volume ratio, tuneable surface chemistry and biomimetic environment, electrospun scaffolds have been applied as a delivery system of biological molecules to influence cell behavior *in vitro* and *in vivo* (15, 16). As proof of principle, we previously demonstrated that the three-dimensional structure of electrospun PCL scaffolds could be exploited to deliver neurotrophins BDNF and GDNF *in vitro* to influence cell survival, proliferation, and differentiation (2, 3). Furthermore, implantation of GDNF functionalized scaffolds promoted the engraftment of neural transplants for up to 28 days (3). However in these former studies, and others like it exploring the benefits of tethered proteins, there remains insufficient knowledge pertaining to the amount of immobilized protein on the scaffold surface, the stability of the tethered protein, activation of intracellular signaling pathway, or the biofunctionality of the molecules over time. In the present study we examined the stability of immobilized GDNF on electrospun PCL scaffolds over 120 days (at 37 °C) and the biological effects on primary neurons in cultures. Specifically, we examined the ability of tethered GDNF protein to activate intracellular signaling pathways and to promote survival and dopaminergic differentiation of ventral midbrain cells for up to 7 days in culture.

Confirmation of Protein Immobilization and Maintained Presentation without Degradation—The electrospun PCL scaffolds were treated with ethylenediamine (ED) to produce amine groups on the fiber surface for protein (GDNF) attachment via a crosslinker, succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) (2). The amount of fluorescamine on the scaffolds after aminolysation was measured to be 1.2×10^{-11} mol/g. Subsequent GDNF attachment onto the scaffolds (24 cm^2), via SMCC crosslinking, was confirmed by ELISA. Results showed significant levels of GDNF on the scaffold (PCL_iGDNF; 975 + 115 pg), compared with PCL scaffolds alone (75 + 45 pg; reflective of background readings) or in the absence of the SMCC crosslinker (PCL_sGDNF, 180 + 30 pg; reflective of physically absorbed GDNF into the scaffold), Fig. 1*A*. Furthermore we confirmed the benefit of utilizing 3D scaffolds, with significantly more (5-fold) GDNF attachment that observed on 2D PCL electrospun scaffolds (data not shown). In light of BET analysis, demonstrating a total surface area of 24 cm^2 for the 0.6 cm diameter PCL scaffold inserts used for protein attachment, and a total of 975 + 115 pg GDNF on the scaffold, we were able to estimate a total of 41 pg of tethered GDNF per cm^2 of PCL scaffolds.

To confirm that the majority of the protein was tethered, and not absorbed onto the scaffolds, we vortexed the scaffolds that had been immobilized with GDNF to shake off any protein embedded but not tethered to the PCL fibers. Under these conditions, no significant difference was seen in GDNF levels on PCL_iGDNF and PCL_iGDNF with vortexing (PCL_iGDNF: 975 + 115 pg, and PCL_iGDNF(v): 650 + 60 pg, respectively, Fig. 1*A*), indicating that the majority of the protein was covalently attached.

To examine the stability of tethered GDNF on scaffolds, the immobilized scaffolds were immersed in PBS for up to 120 days, with PCL scaffold samples collected at day 0 (*i.e.* upon completion of protein immobilization), day 3, 7, 14, 28, and 120 days and supernatant collected at days 1, 3, 7, and 14 days. All samples were stored at −80 °C prior to performing ELISAs. Importantly, we confirmed that freezing of scaffolds had no effect on the stability of the protein, with no significant difference observed in the amount of tethered protein at day 0 from fresh *versus* frozen PCL_iGDNF samples (975 + 115 pg and 810 + 100 pg, respectively, Fig. 1, *A* and *B*). Examination of PCL_iGDNF scaffolds, with or without vortexing, showed no significant difference in GDNF concentration over time (day 0, 3, 7, 14, 28, or 120 days), demonstrating that the protein remained tethered on the scaffold without degradation for at least 120 days (Fig. 1*B*) and thereby highlighting the potential application of these scaffolds for long-term protein presentation. Tethered GDNF stability was additionally confirmed in artificial cerebrospinal fluid (aCSF), with no difference in the level of GDNF present at 1 and 7 days, or from stability in PBS conditions (data not shown). Under these various culturing conditions, the degradation of covalently attached proteins is likely to occur through surface erosion of the electrospun scaffold. Hence, based upon our previous work, we anticipate pro-

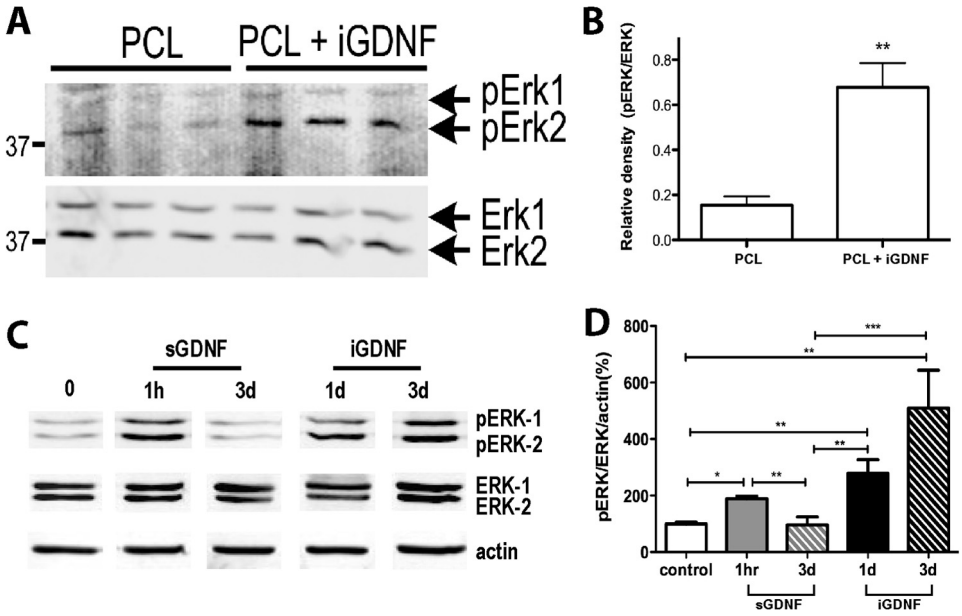

FIGURE 2. **Phosphorylation of intracellular ERK confirms functionality and longevity of tethered GDNF.** *A*, culturing of SN4741 neural cells on PCL+iGDNF, but not PCL alone, resulted in phosphorylation of intracellular Erk determined by immunoblot analysis, indictive of intracellular signaling in response to GDNF presentation. *B*, ratio of phospho-ERK/total ERK level. Tethered GDNF results in a 4.4-fold increase in pERK/ERK level compared with culturing on PCL. *C* and *D*, phospho-ERK levels were significantly elevated after 1 h of stimulation of SN4741 cells with soluble GDNF, but returned to basal levels within 3 days. By contrast culturing cells on iGDNF resulted in maintained elevated pERK levels (at 1 and 3 days in culture). Data represent mean + S.E. *, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$, Student's *t* test and one-way ANOVA.

teins are likely to remain present in culture in excess of 12 months (17).

Results from the supernatant revealed that any absorbed protein leached from the scaffold within 24 h (225 + 75 pg, Fig. 1*C*), and was comparable to the difference observed between iGDNF (PCL_iGDNF: 819 + 100 pg, Fig. 2*B*) and vortexed iGDNF (PCL_iGDNF(v): 515 + 50 pg, Fig. 1*B*). Interestingly, GDNF was only marginally detectable in the supernatant at 3, 7, and 14 days, indicating that the protein measured in the supernatant after 24 h had likely degraded, and thereby further highlighting the benefit of protein tethering for biological applications.

Under biological setting proteases are responsible for the degradation of soluble proteins. To determine whether immobilized proteins may be susceptible to proteolytic degradation we treated iGDNF scaffolds with the protease trypsin and compared the amount of GDNF present to non-trypsinized iGDNF scaffolds. Results showed no significant difference in the amount of GDNF present following trypsin treatment, indicating that our tethered proteins were resistant to proteolysis (Fig. 1*D*).

Here we demonstrate the ability to covalently tether GDNF onto the surface of electrospun PCL scaffolds using the sulfo-SMCC protein crosslinking reaction. This linking is dependent on the protein of interest possessing sulfhydryls (thiols, -SH), which readily react with the maleimide group within the sulfo-SMCC at pH 6.5–7.5. However, it is still possible for maleimides to react with amines, such as those found on the N terminus of a protein or peptide (18). At pH > 7.5 the reactivity of maleimides to amines begins to increase and as our reactions were conducted in PBS (pH 7.4) the same chemistry can be used to tether a protein that does not possess a free sulfhydryl group, although the reaction will be slower. However, it should be noted that hydrolysis of the maleimide group is a possibility during such a conjugation. In this regard, the ability to tether a particular protein may therefore be dependent on the structure of the protein to be tethered (*i.e.* the binding affinity of the maleimide). Furthermore, while it may be possible to attach a number of different proteins using this approach it will also be important to ensure that such crosslinking does not interfere with the cellular accessibility of the protein surface. In this regard it may be necessary to assess other crosslinkers for protein attachment.

The Biofunctionality of Tethered GDNF on Cultured Neurons—Next we investigated the bio-functionality of tethered GDNF on a neural stem cell line in culture. Using the dopaminergic cell line (SN4741), known to express the GDNF receptors, c-ret and GFRa1, we examined the ability of tethered GDNF to induce intracellular phosphorylation of ErK1 and ErK2, key components of the GDNF-ErK signaling pathway. Comparable levels of total ErK1 and 2 could be detected in cells cultured on both PCL and PCL_iGDNF. However, the presence of GDNF significantly increased the phosphorylation of ErK (Fig. 2*A*), indicating that tethered GDNF was capable of mediating intracellular GDNF signaling. Quantification of band density revealed a significant (4.4-fold) increase in the ratio of phospho-ErK to total ErK following culturing on tethered GDNF, Fig. 2*B*. Next we examined the duration of functionality of tethered GDNF. While soluble GDNF (30 ng/ml) significantly increased pErK levels after 1 h of stimulation, by 3 days in culture pErK levels were not significantly different to untreated cultures, reflective of degradation of the GDNF protein. By comparison, tethered GDNF resulted in a significant increase in pErK levels at 1 day and was maintained for 3 days in culture (Fig. 2, *C* and *D*). GDNF has been demonstrated to be sufficient to promote cell survival, differentiation, and neurite growth of both rodent and human primary, neural, and pluripotent stem cells (19–26).

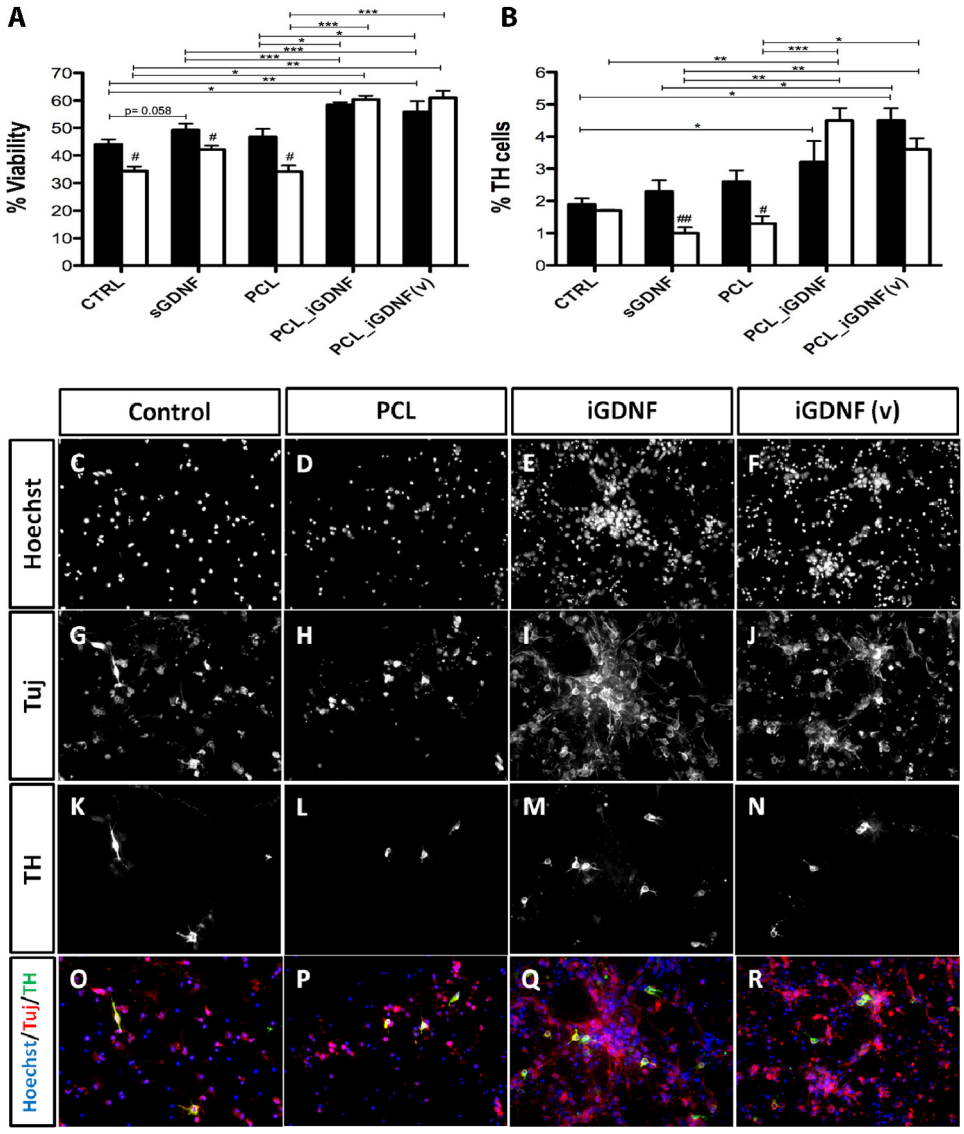

FIGURE 3. **GDNF immobilization enhances cell viability and differentiation.** *A*, GDNF protein tethering, with or without vortexting, significantly increased the viability and (*B*) proportion of tyrosine hydroxylase-immunoreactive (TH+) cells in ventral midbrain cultures, compared with soluble GDNF (sGDNF, at 7 days) and culturing under control conditions (PDL-coated plastic, control or PCL scaffolds, *PCL*). *Black bars*: 3 days *in vitro* (DIV), *white bars*: 7 DIV. Prolonged culturing (7DIV) resulted in a significant decrease in cell viability and TH cells, effects that could be prevented by maintained presentation of GDNF in culture. *C–F*, representative photomicrographs of Hoechst-labeled nuclei, *G–J*, TUJ+ neurons, *K–N*, TH+ dopaminergic neurons, and *O—R*, merged images of VM cells cultured on PDL-coated plastic (control), PCL, PCL with immobilized GDNF (PCL_iGDNF), and PCL with immobilized GDNF and vortexed (PCL_iGDNF(v)). Data represent mean + S.E. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. #, $p < 0.05$; ##, $p < 0.01$, 7 days in culture significantly different from same condition at 3 days in culture ($p < 0.05$). One-way ANOVA with Tukey post-hoc test.

These functions of GDNF have been additionally supported by increased phosphorylation of Erk. However to achieve these effects commonly requires repeated protein delivery, particularly in *in vivo* contexts. Here we demonstrate tethered protein is capable of activating intracellular Erk to a similar magnitude. Importantly however, the temporal expression and ability to maintain signaling (*i.e.* phospho-Erk levels) is sustainable, and may therefore present a superior method to prolong functional protein delivery for *in vitro* and *in vivo* applications.

Finally we examined the ability of tethered GDNF to not only induce an intracellular response, but also provide a prolonged effect on neural progenitors in culture. Here we demonstrate that soluble GDNF (added at the time of cell seeding) increased cell survival (and proportion of TH+ dopaminergic neurons) at 3 days, however viability significantly diminished thereafter to levels not different from untreated controls (Fig. 3, *A* and *B*), presumably reflecting insignificant GDNF levels in the media beyond this time. By contrast, tethering GDNF onto the scaffold surface significantly increased and maintained cell survival. After 3 days in culture, we demonstrated that tethered GDNF significantly improved the viability of VM cells (Hoechst labeled non-pyknotic nuclei) in culture compared with cells cultured on PDL coated plastic (56.37% + 0.96 and 44.04% + 1.78, respectively), or PCL scaffolds alone (46.74% + 2.86), Fig. 3. Similarly immobilized GDNF enhanced the number of tyrosine Hydroxylase immunoreactive (TH+) dopaminergic cells in culture, Fig. 3, *B*, *K–N*. After 7 days, cells cultured in the presence of immobilized GDNF showed no decrease in cell viability or the proportion of dopaminergic neurons compared with 3 days in culture, demonstrating maintained activity of

GDNF, while cells cultured on PCL alone showed significantly reduced viability over time (Fig. 3). These findings demonstrate the benefits of maintained presentation and functionality of tethered protein.

The development of assays to provide long-term presentation of molecules is important for increasing our understanding of physiological processes. Most *in vitro* studies focused on understanding the role of a given molecule in development, adult homeostasis, or disease rely on application of soluble proteins in culture; effects that are rapid and transient and often do not reflect the ongoing presentation of a protein that typically occurs in nature. In addition to providing a more relevant assay to understand these basal and pathophysiological functions, the use of tethered proteins can also aid in the development of new treatments. For example, long term delivery of GDNF *in vivo*, by way of protein tethering onto bioengineered scaffolds, such as microspheres, could be exploited to stall disease progression in PD, or promote the survival and integration of newly transplanted dopaminergic neurons for patients. The development of such protein/molecule tethering technologies will be dependent on ongoing validation of protein/molecule stability and function, using methodologies such as those described here.

CONCLUSION

Protein immobilization on the surface of tissue engineering scaffolds has been investigated for their potential to influence cellular responses *in vitro* and *in vivo*. While the benefits of tethered proteins have been recognized for some years now, there has been a notable lack of research concentrating on their stability and bio-functionality kinetics. Here we demonstrate, by way of GDNF as an example, that tethered proteins on electrospun scaffolds are stable for prolonged periods of time (with no evidence of degradation), activate intracellular signaling cascades, and maintain cellular effects (GDNF influencing cell viability and differentiation). These findings hold significant potential for the use of biomaterials in presenting and maintaining the activity of proteins *in vitro* and *in vivo*, and may impact on the ability to enhance tissue repair.

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Characterization of the Stability and Bio-functionality of Tethered Proteins on Bioengineered Scaffolds: IMPLICATIONS FOR STEM CELL BIOLOGY AND TISSUE REPAIR

Ting-Yi Wang, Kiara A. F. Bruggeman, Rebecca K. Sheean, Bradley J. Turner, David R. Nisbet and Clare L. Parish

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