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Glial Cell Line-Derived Neurotrophic Factor Promotes Survival and Proliferation of Dental Pulp Cells

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ABSTRACT

This study investigated the effects of glial cell line-derived neurotrophic factor (GDNF) on dental pulp stromal cells (DPCs). Cultures of DPCs expressed GDNF as well as its receptors, GFR α 1 and RET. Addition of recombinant GDNF to cultures in serum-containing medium did not significantly affect DPC growth; however, GDNF dose-dependently increased viable cell number under serum-free culture conditions. Live/dead, lactate dehydrogenase (LDH) and caspase -3,-7 assays demonstrated that cell death occurred under serum-free conditions, and that GDNF significantly reduced the number of dead cells by inhibiting apoptotic cell death. GDNF also stimulated cell proliferation in serum-free conditions, as assessed by the BrdU incorporation assay. The

effect of GDNF was abolished in the presence of specific inhibitors to GFR α 1 and RET suggesting receptor-mediated events. This study also demonstrated that GDNF counteracted TNF α -induced DPC cytotoxicity, suggesting that GDNF may be cytoprotective under disease conditions. In conclusion, our findings indicate that GDNF promotes cell survival and proliferation of DPC and suggest that GDNF may play a multi-functional role in the regulation of dental pulp homeostasis.

Key words: pulp biology, neurotrophic factors, GDNF, TNF alpha, cell survival, cell proliferation.

INTRODUCTION

Neurotrophic factors have been implicated in the development and regulation of dental tissues (Luukko *et al.*, 1997; Nosrat *et al.*, 1998, 2002; Woodnutt *et al.*, Magloire *et al.*, 2001). The glial cell line-derived neurotrophic factor (GDNF) is part of GDNF family of ligands (GFLs) which include neurturin (NRTN), artemin (ARTN) and persephin (PSPN). GFLs are considered to belong to the TGF β superfamily sharing partial amino-acid sequence homology and structural confirmation (see for review Airaksinen and Saarma, 2002). GDNF is a soluble signaling molecule that binds to a specific membrane receptor, the GDNF family receptor α (GFR α 1) which forms complexes with the tyrosine kinase receptor RET or alternative co-receptors such as NICAM eliciting intracellular signals for cell growth and differentiation (Sariola and Saarma, 2003).

GDNF was originally characterized as a potent trophic factor promoting the survival and differentiation of neurons (Airaksinen and Saarma, 2002). GDNF was subsequently shown to be expressed in various tissues outside the nervous system and an important functional role has been recognized in urogenital tissues, in particular relating to kidney

development and spermatogenesis (Sariola and Saarma, 2003). The expression of neurotrophic factors within the adult dental pulp highlighted that GDNF may be involved in neuronal innervations, axon growth and function (Nosrat *et al.*, 1997; Fried *et al.*, 2000; Lillesaar *et al.*, 2001; Luukko *et al.*, 1997)). During tooth development, GDNF and its receptors GFR α 1 and RET are transiently localized in dental organ epithelium and pulpal mesenchyme suggesting a role for GDNF in epithelial-mesenchymal interactions (Hellmich *et al.*, 1996; Luukko *et al.*, 1997; Nosrat *et al.*, 1998)). Interestingly, ultrastructural analysis of molar tooth germs from GDNF-knockout mice revealed that ameloblast and odontoblast differentiation was disrupted suggesting a role for GDNF during tooth cytodifferentiation ((de Vicente *et al.*, 2002)). We postulated that GDNF may be involved in the regulation and maintenance of the postnatal dentin-pulp complex. The aim of this study was to investigate the direct effects of GDNF on dental pulp cells (DPC).

MATERIALS & METHODS

Dental pulp cell (DPC) cultures

DPC cultures were established from rat dental pulp explants as described previously (e.g., Couble *et al.*, 2000; Huang *et al.*, 2006). In brief, dental pulp was extracted from incisors of 4-6 week-old male Wistar rats and dissected into small ($\sim 5\text{mm}^3$) samples and cultured in tissue culture flasks containing α MEM supplemented with 20% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin and 2.5 $\mu\text{g}/\text{ml}$ Amphotericin B (Sigma Aldrich, UK) in a humidified 5% CO₂ incubator at 37 °C. DPC proliferated from the explants, showing a polygonal stromal/fibroblast-like morphology which

became more cuboidal when reaching confluency. Subconfluent cell cultures were trypsinized with Trypsin/EDTA (Invitrogen/Gibco, UK) and subcultured. DPC at passage 2 to 4 were used for the experiments. DPC were seeded into 96-multiwell plates in α MEM/10% FBS at 5,000 cells/well. After 24 hours, the cultures were replenished with either serum (10% FBS) or serum-free α MEM supplemented with 0.1% bovine serum albumin (BSA). Recombinant human GDNF (rhGDNF, Amgen, Thousand Oaks, USA) was added to the cultures for a further two days. Additional experiments included recombinant human tumour necrosis factor- α (TNF α ; Peprotech, UK) which was co-incubated with 100 ng/ml GDNF in α MEM/10%FBS for 2 days before analysis. For the receptor inhibitor experiments, DPC cultures were treated for 1 hour with different concentrations of phosphoinositide phospholipase C (PI-PLC; Sigma) which blocks signalling *via* GFR α 1 (Krieglstein *et al.*, 1998) , or RPI-1 (Merck/Calbiochem), a specific RET receptor tyrosine kinase inhibitor (Cuccuru *et al.*, 2004) followed by further culture with the respective inhibitors in media with or without GDNF.

Cell number and viability assays

We used the WST1 assay (Roche Applied Biosciences) to assess the number of viable cells (Scheven *et al.*, 2009); the absorbance of the reduced compound was measured at a wavelength of 450 with a reference filter at 630nm using a Biotek plate reader. To distinguish between the number of viable and dead cells in the cultures, we performed a “live/dead” assay using acridine orange (4 μ M) (stains nuclei of live cells) and ethidium bromide (4 μ M) (labels nuclei of dead cells). The numbers of live and dead cells *per*

microscopic field was counted under a Nikon Eclipse fluorescent microscope using 480nm and 520nm filters, respectively.

Cell death and apoptosis assays

The level of cell death in the cultures was determined biochemically using a lactate dehydrogenase (LDH) cytotoxicity assay (Roche, UK). Cell culture supernatants were analysed after a 2-day culture for the presence of LDH. Absorbance was determined at 490/630nm using the Biotek plate reader. To determine the level of cellular apoptosis, we used the Caspase-Glo-3/-7 to determine the enzymatic activities of caspase-3/-7 (Promega); the cleaved luminescent product was measured with a Berthold microplate luminometer.

BrdU cell proliferation assay

Cell proliferation was assessed with the use of a 5-Bromo-2-deoxy-uridine (BrdU) labelling and detection kit (Roche Applied Sciences). In brief, cells were labelled with 10 μ M BrdU for the final hour of the 48-hour culture followed by fixation and immunostaining for BrdU incorporation using a specific anti-BrdU antibody. Cells were counterstained with hematoxylin and the total number of labelled and non-labelled nuclei were counted in 50 independent microscope fields.

Semi-quantitative RT-PCR (sqRT-PCR) analysis: DPC cultures as well as dental pulp and brain extracted from 4-week-old rats underwent lysis in RLT buffer containing β -mercaptoethanol followed by RNA isolation using the RNeasy minikit

(Qiagen, UK). Subsequently, 1-2 µg of DNase-digested total RNA was used for oligo(dT) (Ambion, UK) reverse transcription to generate single-stranded cDNA using the Omniscript kit (Qiagen,UK). Centrifugal filters (Microcon) were used to purify and concentrate resultant cDNA. Both RNA and cDNA concentrations were determined from absorbance values at a wavelength of 260 nm using a BioPhotometer (Eppendorf, UK). sqRT-PCR assays were performed using the RedTaq PCR system (Sigma, UK) and the Mastercycler gradient thermal cycler (Eppendorf, UK). Primers were designed from NCBI mRNA sequences using Primer-3 design software (Table 1).

Immunocytochemistry

DPC were seeded onto multispot microscope slides and incubated for 24h at 37 °C in a humidified 5% CO₂ incubator (20,000 cells/well). Rat glioma C6 cells known to express GDNF as well as GFRα1 and RET (Song and Moon, 2006) were used as positive controls. The adherent cells were fixed with ice-cold acetone for 5 min, then rinsed in phosphate-buffered saline (PBS) containing 1%BSA. Following incubation in 3% H₂O₂ for 30 min (to block endogenous peroxidase) the slides were washed in PBS/1%BSA and incubated in 20% normal goat serum followed by incubation with 2 µg/ml primary polygonal rabbit antibody against GFRα1 (sc10716, SantaCruz) or against RET (sc167; SantaCruz) overnight at 4 °C. The GFRα1 antibody (sc10716, Santa Cruz, USA) specifically binds to the 58-kDa protein, as determined by immunoblotting, while the RET antibody (sc167; Santa Cruz) recognizes the 150- to 179-kDa protein (Pierchala et al., 2006); both antibodies have been validated for use in immunocytochemical staining of cell membrane receptors (Alladi et al., 2010; see also manufacturer's datasheets at <http://www.scbt.com/datasheet-10716-gfralpha-1-h-70-antibody.html>;

<http://www.scbt.com/datasheet-167-ret-c-19-antibody.html>). For controls, the primary antibody was substituted normal rabbit serum. The slides were rinsed in PBS/1%BSA and labelled and stained with biotin-streptavidin-HRP using a Biogenex detection kit (LP000-UL). The slides were counterstained with haematoxylin before examination using a Zeiss microscope.

Data and statistical analysis

Data obtained from the WST-1, LDH and caspase-3/7 assays were corrected for background values and expressed as percentage of controls. Data were analysed by ANOVA with Tukey *post hoc* test.

RESULTS

DPC culture model

RT-PCR analysis showed that GDNF and its receptors GFR α 1 and RET transcripts were present in postnatal dental pulp and DPC cultures (Fig. 1A). Immunocytochemical staining of DPC using specific antibodies against GFR α 1 and RET suggested the presence of these GDNF receptors (Fig. 1B). These data indicated that the cultures provided a suitable model for study of the direct effects of GDNF on DPC.

GDNF stimulates DPSC survival and proliferation

We performed initial experiments to determine the effects of GDNF on cell growth using serum-containing or serum-free cultures. GDNF did not elicit significant changes in the number of viable DPCs over a 2-day culture in medium supplemented with FBS;

however, addition of GDNF to serum-free cultures resulted in a dose-dependent increase in viable DPC numbers (Fig. 2A). We performed experiments to evaluate whether the GDNF-induced increase in cell numbers was due to increased cell survival and/or cell proliferation.

The live-dead assay demonstrated that significant cell death occurred in the control, serum-free DPC cultures as compared to serum-supplemented cultures (Fig 2B). The presence of GDNF significantly increased total cell number, coinciding with a significant decrease in the number of dead cells in serum-free cultures, indicating that GDNF promoted cell survival under these conditions. (Fig. 2B).

To further characterise the effect of GDNF on cell survival, we performed a biochemical cytotoxicity assay to measure LDH release from damaged and dying cells. The results show that GDNF significantly reduced LDH secretion in these cultures (Fig. 2C). To determine if the protective effects of GDNF were due to prevention of cellular apoptosis, we determined cellular levels of caspase-3/7. Results demonstrate that caspases-3/7 levels were significantly reduced in GDNF-treated cultures compared with control cultures, indicating GDNF prevented DPC apoptosis (Fig. 2C).

Next, cell proliferation was determined using by the BrdU-incorporation assay. The results showed that GDNF significantly increased the number of BrdU-labeled DPC in serum-free cultures, demonstrating that, along with a pro-survival action, GDNF stimulated cell replication (Fig. 2D).

Receptor-mediated effects of GDNF

To determine whether the GDNF effects described above were mediated via its canonical receptors, we treated cultures with PI-PLC, which cleaves the receptor subunits from their glycosylphosphatidylinositol (GPI)-anchored membrane proteins.

Analysis of the data presented in Fig. 3 demonstrated that PI-PLC abolished GDNF effects on viable cell numbers in serum-free cultures, suggesting an essential role for GFR α 1 in the GDNF effects on DPC viability (Fig. 3A). Moreover, RPI-1, a competitive ATP-dependent RET kinase inhibitor, dose dependently blocked GDNF action, underscoring GDNF dependency on the RET co-receptor (Fig. 3B).

GDNF counteracts TNF α -induced reduction in DPSC number

Finally, this study investigated whether GDNF may have cell- protective effects under conditions that better reflected the pathological environment. For this purpose, DPCs were cultured in serum-containing medium (to obtain an optimal physiological milieu) which was supplemented with TNF α , a pro-inflammatory cytotoxic cytokine up-regulated in pulpitis (McLachlan *et al.*, 2004). The results demonstrated that TNF α dose-dependently decreased DPC numbers; however, cultures supplemented with GDNF showed significantly increased DPC viability as compared with TNF α controls, indicating that GDNF counteracted TNF α -induced cytotoxicity (Fig. 4).

DISCUSSION

The notion that neurotrophic factors are implicated in tooth development and dentin-pulp biology is not surprising due to the cranial neural crest cell origin of dental pulp

mesenchymal stem cells and odontoblasts and the close association of these cells with the pulp neuronal network (Fried *et al.*, 2000; Tziafas *et al.*, 2000). Dental pulp is increasingly gaining attention as a therapeutic tool in nerve repair and regeneration, due to its neurogenic potential and endogenous expression of neurotrophic factors (Nosrat *et al.*, 2001; Lillesaar *et al.*, 2001; Apel *et al.*, 2009). The neurotrophic factor GDNF was shown to be expressed in both ecto-mesenchymal dental papilla as well as inner dental epithelial cells. GDNF is therefore implicated as an important factor controlling epithelial-mesenchymal interactions during tooth development, and in GDNF-knockout mice, ameloblasts and odontoblasts fail to differentiate fully (Hellmich *et al.*, 1996; de Vicente *et al.*, 2002). GDNF and its canonical receptors GFR α 1 and RET are also expressed in dental pulp and (sub) odontoblasts in postnatal teeth, suggesting a role in odontoblast function (Nosrat *et al.*, 1997, 1998; Luukko *et al.*, 1997). This study reports the expression of GDNF and its specific receptors, GFR α 1 and RET in dental pulp and DPC cultures and provides evidence that GDNF is a pro-survival growth factor for DPC *via* interaction with the GFR α 1/RET receptor complex, suggesting that GDNF may have a functional role in the regulation of dental pulp cells.

Our results corroborate the well established role of GDNF as a cell survival factor and regulatory signaling factor for neuronal and non-neuronal cells (Airaksinen and Saarma, 2002; Sariola and Saarma 2003). Serum-free cultures facilitate the study of direct effects on cells without interference of serum factors, but also present a model mimicking pathological conditions involving cellular insult and injury due to trophic factor deprivation (Goyeneche *et al.*, 2006). Serum withdrawal induces toxicity in neuronal cell cultures which is abated by GDNF (Kobori *et al.*, 2006).

In this study, GDNF was shown to inhibit DPC death induced by serum deprivation, suggesting that GDNF may play a cytoprotective role in dental pulp homeostasis during stress conditions and pulpal necrosis. Furthermore, the current paper offers the first evidence that GDNF is able to block cytotoxic effects on DPC by the pro-inflammatory cytokine TNF α , highlighting a possible protective role of this neurotrophic factor in pulpitis. TNF α is able to elicit various cellular responses, depending on cell types and conditions; in particular, TNF α 's cytotoxic effects are widely documented as involving apoptosis-related pathways mediated by its main TNF receptor TNFR1 (Shen and Pervaiz, 2006). The cytotoxic effects of TNF α found in our DPC cultures may involve induction of cell death combined with an inhibitory effect on cell proliferation. This finding corresponds with a previous study describing the cytoprotective effects of GDNF in an adrenal cell line undergoing TRAIL (TNF α -related apoptosis-inducing ligand)-induced cell death (Murata et al., 2006). Interestingly, TNF α induced production of GDNF by astrocytes and glioma cells possibly *via* the NF κ B binding present on the human GDNF gene promoter, suggesting a regulatory "protective" feedback loop involving GDNF in response to inflammation (Appel *et al.*, 1997; Woodbury *et al.*, 1998). The enhanced levels of GDNF found in gingival crevicular fluid from patients with chronic periodontitis emphasize the potential involvement of GDNF in the pathophysiology of dental tissues (Sakai et al., 2006).

Apart from its effect on cell survival, GDNF was shown here to be a mitogen for DPCs. This is not a very surprising finding as it is well known that the cellular repertoire involved in pro-survival signaling is closely associated with cell cycle control processes (Maddika *et al.*, 2007). Our results raise the possibility that GDNF may a role in MSC

recruitment and proliferation during dentine repair (Shi *et al.*, 2008). GDNF is able to bind extracellular proteoglycan heparin sulphate chains (Rider, 2006), indicating the likelihood that GDNF produced by odontoblasts is sequestered within dentin (Nosrat *et al.*, 1997, 1998). Indeed, preliminary findings from our laboratory using an antibody array technology have demonstrated that GDNF was detected within human dentine matrix extracts (Thompson *et al.*, unpublished observations).

This suggests that odontoblast-secreted GDNF can be sequestered within the dentin could be released from the matrix upon injury or disease. It is noteworthy that some of the neurotrophic effects of GDNF require the presence of TGF β which induces the translocation of GFR α 1 to the plasma membrane (Krieglstein *et al.*, 1998; Peterziel *et al.*, 2002). However, a limitation of the current study is that it is not possible to conclude which particular dental pulp cell type(s) responded to GDNF. The DPC cultures cannot be considered homogenous, although, due to the nature of the culture method (explant-outgrowth of cells), the DPCs generally displayed a morphologically similar polygonal fibroblast/stromal-like cell appearance. Previously, DPCs have been shown to express several mesenchymal stem cell features and the capability to differentiate along different mesenchymal lineages, including osteogenic and odontogenic lines (see also Couble *et al.*, 2000; Huang *et al.*, 2006). Considering that GDNF and its receptors are expressed in postnatal dental pulp as well as (sub)odontoblasts (Luukko *et al.*, 1997; Nosrat *et al.*, 1997; this study), it is plausible to speculate that GDNF acts upon cells of mesenchymal origin, including those of the odontoblast lineage, and that GDNF in concert with other local signaling factors such as TGF β 1 may control cell viability and recruitment during reparative processes within the dentin-pulp (Tziafas *et al.*, 2000; Woodnutt *et al.*, 2000; Magloire *et al.*, 2001).

In conclusion, this study demonstrates that GDNF promoted cell survival and proliferation of DPC under serum-starved or pro-inflammatory conditions. We propose that GDNF may have multi-functionality within the dentin-pulp complex, acting as both survival factor and mitogen during tooth injury and repair. Further studies are warranted to evaluate the role of GDNF in dental pulp homeostasis and its potential in novel therapeutic strategies for dental pulp repair and tissue regeneration.

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Tables and Figures

Gene	Primer sequence	Accession no.	Product	T _m
GAPDH	F-CCCATCACCATCTTCCAGGAGC R-CCAGTGAGCTTCCCGTTCAGC	NM017008	450bp	60
GDNF	F-AGAGGAATCGGCAGGCTGCAGCTG R-AGATACATCCACATCGTTTAGCGG	NM019139	337bp	60
RET	F-TCAGGCATTTTGCAGCTATG R-TGCAAAGGATGTGAAAGCAG	NM001110099	393bp	62.5
GFR α 1	F-AATGCAATTCAAGCCTTTGG R-TGTGTGCTACCCGACACATT	U59486	218bp	60

Table 1. PCR primer sequences and annealing temperatures (T_m)

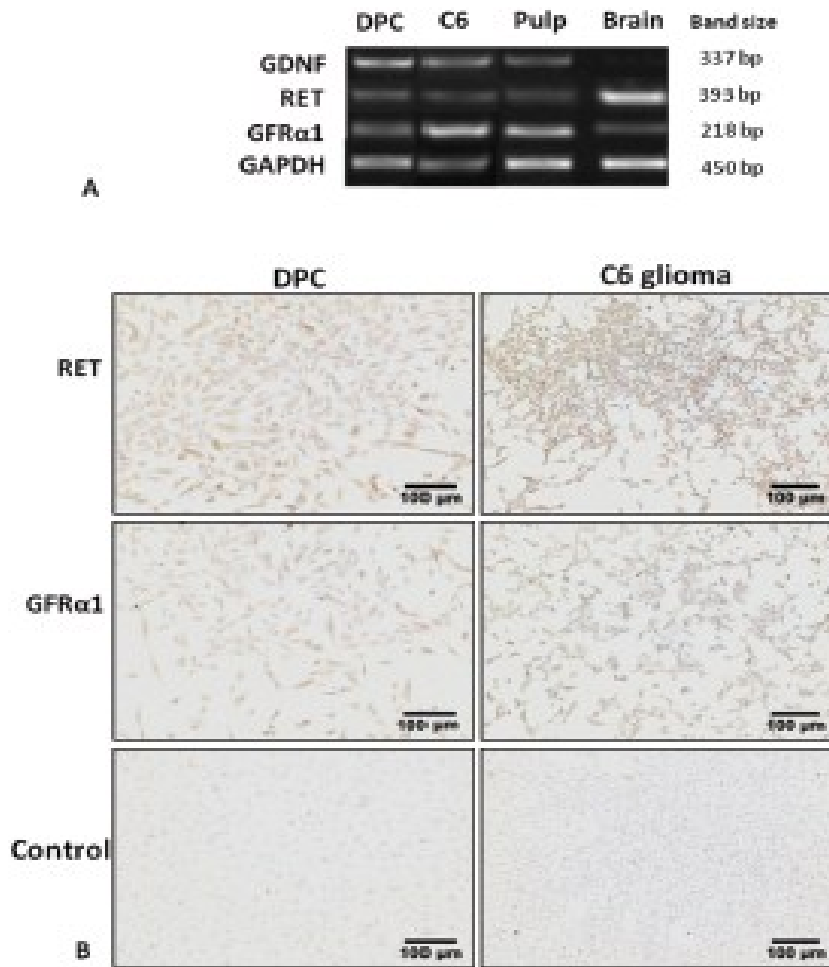


Figure 1: A. RT-PCR gel images showing transcripts of GDNF and its receptors GFR α 1 and RET in dental pulp mesenchymal cells (DPCs), dental pulp and brain. B. Immunocytochemical staining of DPSC for GFR α 1 and RET. Non-immune rabbit serum without specific primary antibody was used as control.

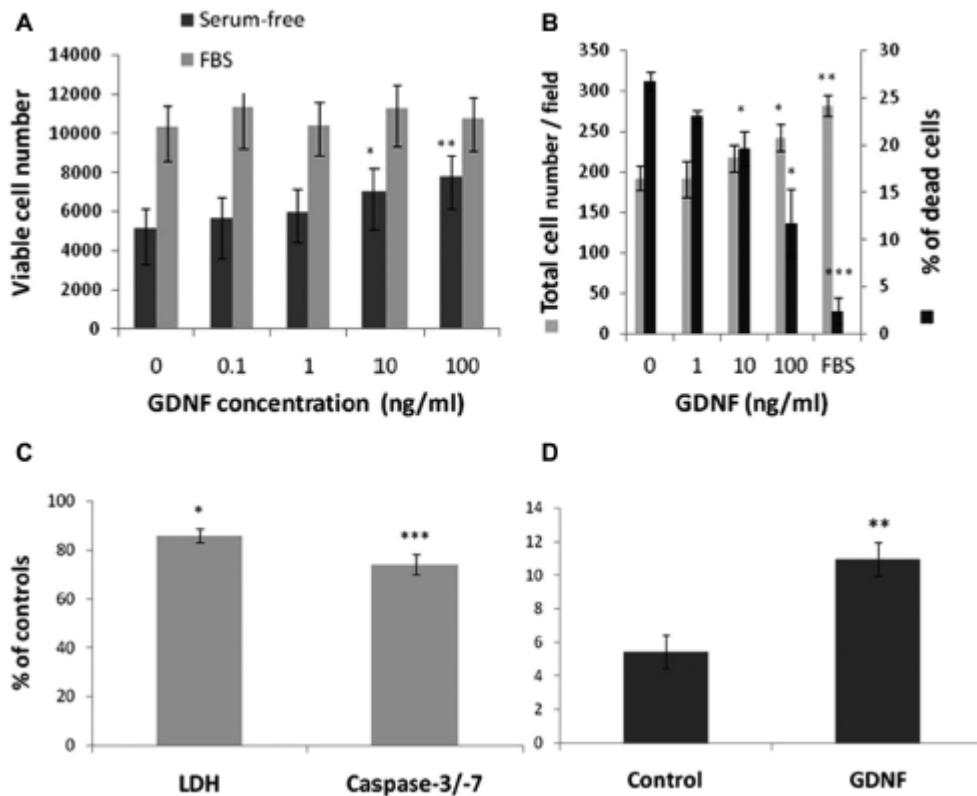


Figure 2. **A.** Effect of GDNF on viable cell number in serum-supplemented or serum-free cultures as assessed by WST-1. Results are expressed as percentage of controls (mean \pm SD; n=4). **B.** Effect of GDNF on the number of dead cells as determined by the live/dead assay. Results show percentage of dead cells (mean \pm SD; n=3) **C.** Relative LDH and caspase-3/7 levels after 2-days culture in serum-free medium cultures supplemented with 100 ng/ml GDNF (mean \pm SD; n=3). GDNF-treated DPC cultures showed significantly reduced LDH and caspase-3/7 levels indicating GDNF-induced cell survival effects involves at least in part an anti-apoptotic effect by GDNF **D.** BrdU incorporation after 2-day DPC culture in serum-free medium. The proportion of cells labeled positively for BrdU (labeling index) was significantly increased in GDNF-supplemented cultures indicating stimulation of replication by GDNF. Statistical differences versus serum-free controls: * $p < 0.05$, ** $P < 0.01$, *** $p < 0.001$.

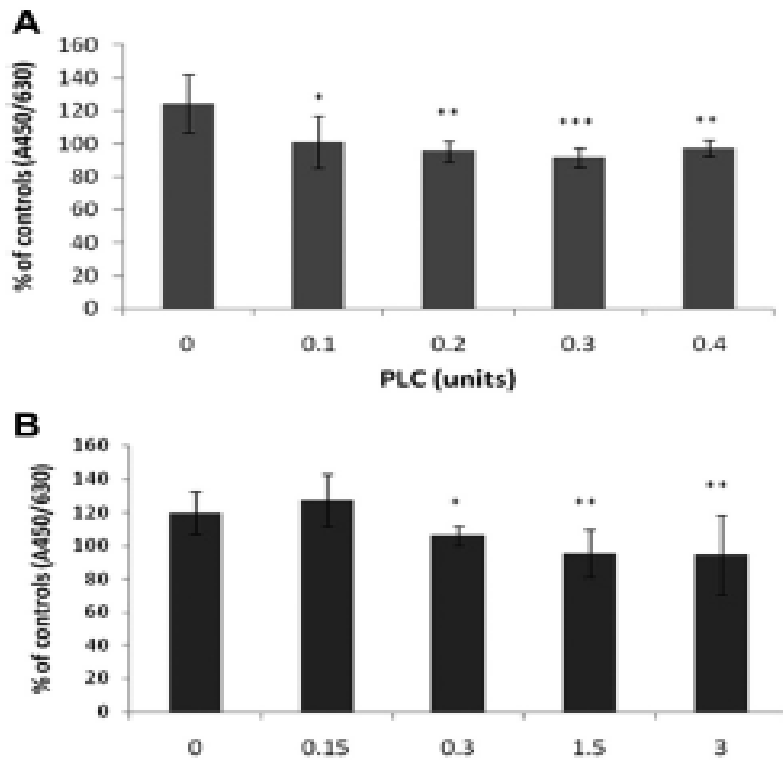


Figure 3: Effects of GDNF receptor inhibitors on GDNF-stimulated DPC cultures. A. Effect of phosphatidylinositol-specific phospholipase C (PI-PLC) on viable cell number as assessed by WST-1 in cultures supplemented with 100 ng/ml GDNF. B. Effects of the RET kinase inhibitor, RPI-1, on viable cell number in 2-day GDNF-treated DPC cultures. Results are percentage of control values as determined by the WST1 assay (mean \pm SD; n=6-8). * $p < 0.05$, ** $P < 0.01$ versus GDNF cultures.

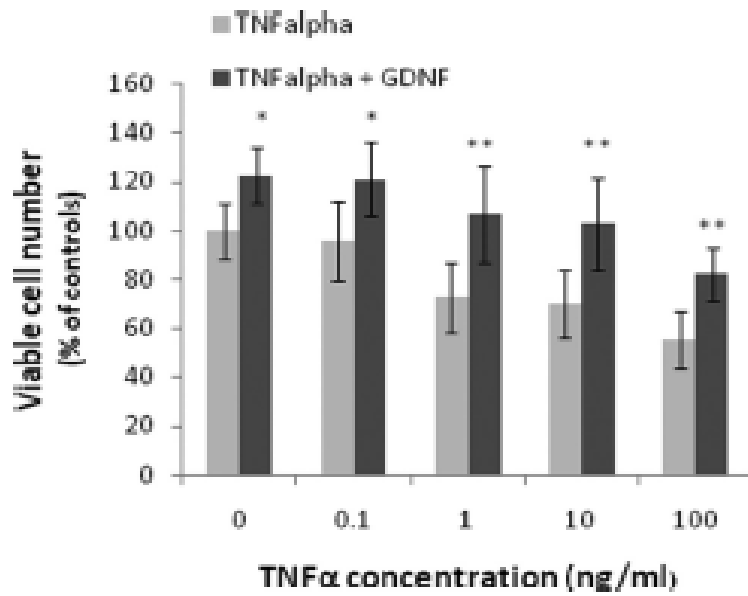


Figure 4: Effects of 100 ng/ml GDNF on 2-day DPC cultures in the presence of increasing concentrations of TNF α . Results are percentage of control values determined by the WST1 assay (mean \pm SD; n=5-6). *P < 0.05, **P < 0.01 *versus* corresponding TNF α cultures.