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Glial Cell line-derived Neurotrophic Factor Effects on Dental Pulp Cells and Osteoblast-like Cells

by

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ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) is a growth factor promoting survival, proliferation and differentiation of neural crest cells. Neural crest cells play an important role within mesenchymal tissues during dental pulp and calvarial bone development. GDNF also has a role within non-neuronal tissues and is expressed during dental development. GDNF null mutations prevent the formation of the mineralised hard tissues of the tooth. The hypothesis for this study was that GDNF affects mesenchymal dental pulp cells (DPC), promoting the regenerative responses of mineralised tissues. This study utilised cell culture models to investigate the direct effects of GDNF on the proliferation and differentiation of dental pulp cells, bone marrow mesenchymal stromal/stem cells (BMSC) and calvarial osteoblasts. This research demonstrated that these culture models expressed GDNF and its receptors GFRα1 and RET. GDNF was shown to directly stimulate DPC and osteoblast-like cell proliferation and differentiation. Moreover, GDNF was cytoprotective when DPCs were cultured under conditions reflecting aspects of inflammation, which may occur during repair. These conditions included supplementation with the pro-inflammatory cytokine TNFα and culture under serum-starved conditions. It is proposed that GDNF may play an important regulatory role in dental pulp homeostasis and bone metabolism.

For Erin, Shayla and Zena

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ABBREVIATIONS

AA	ascorbic acid or Vitamin C
ADM	adrenomedullin
AKT	acutely transforming retrovirus AKT8 in rodent T cell lymphoma (also known as protein kinase B)
AlP	Alkaline phosphatase
Apaf-1	apoptotic peptidase activating factor 1
ART	artemin
ATM	ataxia telangiectasia mutated
Bad	Bcl-2-associated death promoter
Bak	BCL2-antagonist/killer 1
Bax	BCL2-associated X protein
Bcl	B cell leukaemia
BDNF	brain derived neurotrophic factor
β-gp	β-glycerophosphate
BH3	Bcl-2 Homology (BH) domain 3
Bid	BCL-2 Interacting Domain
BMP	bone morphogenic proteins
BMSC	bone marrow mesenchymal stromal/stem cells
BSP	bone sialoprotein
casp	caspase
CB	Calbindin
Cbfα1	Core binding factor 1
CGRP	calcitonin gene related peptide
Chk2	CHK2 checkpoint homolog (*S. pombe*)
Cn	nerve cranial nerve five

CNTF	ciliary neurotrophic factor
Cyt	cytochrome
D	dentine
DEX	dexamethasone
DMP-1	dentine matrix protein-1
DNA	deoxyribonucleic acid
DPP	dentine phosphoprotein
DPSC	dental pulp stem cells
DSP	dentin sialoprotein
DSPP	dentin sialophosphoprotein
E	embryonic day
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorting
FADD	fas-associated protein with death domain
FGF	fibroblast growth factor
G1	gap phase 1
G1/S	gap phase1/synthesis phase
GAL	galanin
GDNF	glial cell-line derived neurotrophic factor
GFRα	GDNF family receptors alpha
GPI	glycosyl phosphostidylinositol
GRB2	growth factor receptor-bound protein 2
HGF	hepatocyte growth factor
HIF1α	hypoxic inducible factor 1alpha
HOX	Homeobox

HtRA2	HtrA serine peptidase 2
IAPs	inhibitors of apoptosis
IDE	inner dental epithelial
IGFI	insulin like growth factor I
IL-6	interleukin 6
JNK	c-Jun, N-terminal kinase
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAP2-positive	microtubule associated proteins 2
MAPK	mitogen activated protein kinase
MDM2	Mdm2 p53 binding protein homolog (mouse)
Met	c-met transforming gene proto-oncogene encoded
MGIF	murine glial cell-derived neurotrophic factor inducible transcription factor
MSC	mesenchymal multipotent/stem cells
mTOR	mammalian target of rapamycin
NCAM	neural cell adhesion molecule
NCC	neural crest cells
NF200	neurofilament 200kDa
NFκB	nuclear factor kappa B
NGF	nerve growth factor
NGF-β	nerve growth factor beta
NP	neuropeptides
NPY	neuropeptideY
NT-3	neurotrophin 3
NT-4/5	neurotrophins 4/5

NTN	neurturin
OB	odontoblast layer
Ob	osteoblast
Oc	osteoclast
OCN	osteocalcin
ON	osteonectin
OP	osteopontin
p19ARF	p19 alternative reading frame
$p75^{NTR}$	low affinity nerve growth factor receptor
PAX	paired box genes
PDGF	platelet derived growth factor
PDK	pyruvate dehydrogenase kinase
PI3-kinase	phosphoinositide 3-kinase
PI-PLC	phospholipase C
PKC	protein kinase C
PLC-y	phospholipase C-gamma
PP2A	protein phosphatase 2A activator, regulatory
PSP	persephin
PTEN	phosphatase and tensin homolog
RAF	v-raf-1 murine leukaemia viral oncogene homolog 1
RAS	rat sarcoma protein
RET	rearranged during transfection
RTK	tyrosine kinase receptor
sCNS	sympathetic central nervous system
SH3	Src homology 3
Shh	sonic hedgehog

SN	subodontoblastic nerve plexus
SOS	son of sevenless
SOX	SRY (sex determining region Y)-box
SP	substance P
Src	sarcoma virus protein
STAT	signal transducer and activator of transcription
S.mutans	*Streptococcus mutans*
t	trabaeculae
TCF-4	T-cell factor 4 (also known as TCF7L2)
TGF-β	transforming growth factor beta
TIEG	TGF-beta-inducible early-response gene
TNFα	tumour necrosis factor alpha
TrKA	tyrosine kinase receptor A
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide
Wnt	wingless-type MMTV integration site family

CHAPTER 1 INTRODUCTION

Factors regulating the development of mineralised tissues may also be involved in the subsequent repair and regeneration of the tissue. The primary focus of this research was to investigate the possible roles of Glial Cell line-Derived Neurotrophic Factor (GDNF) in the regulation of non-neuronal cells that form mineralised tissues, by using odontoblast-like and osteoblast-like cell culture models.

1 GDNF and Neurotrophic Growth Factors (NTGF)

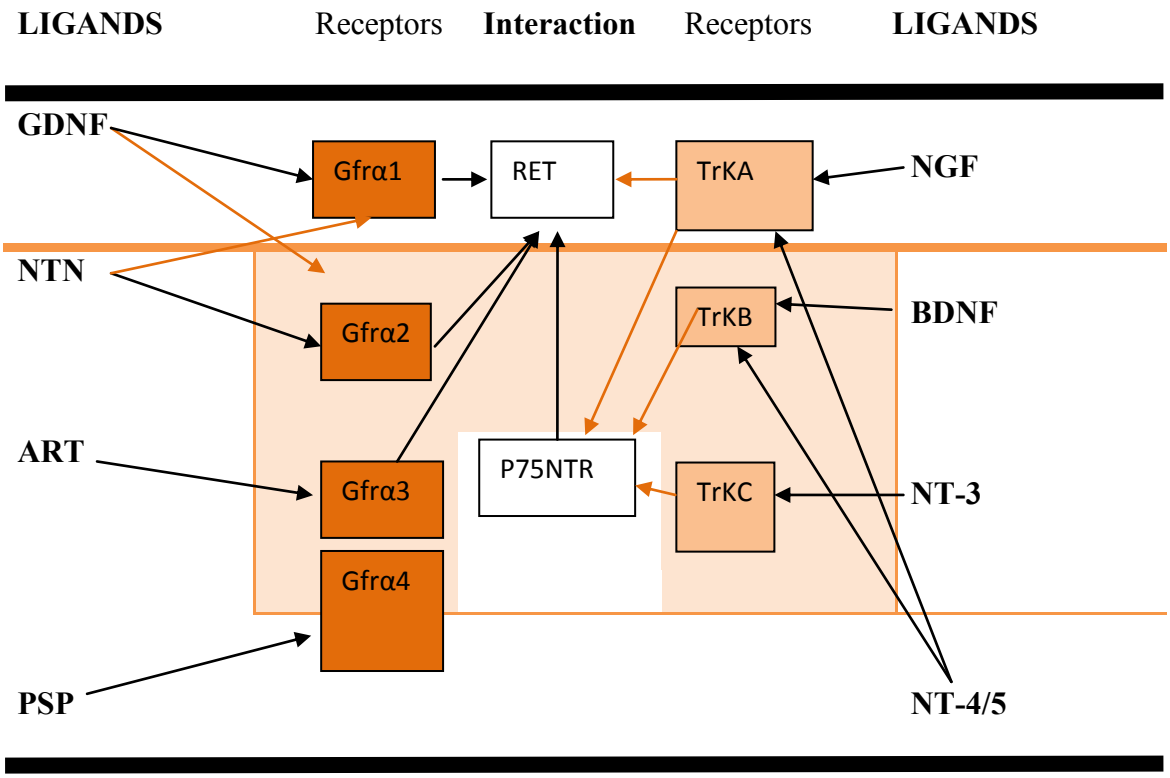
GDNF was first discovered in the medium of the B49 glial cell line and was identified as a survival factor for dopamine secreting neurons (Lin *et al*. 1993). It is secreted as a pro-form that is cleaved to give the mature growth factor, which is a dimeric protein of approximately 33-45 kDa (Lin *et al.* 1994; Green 2002). GDNF belongs to the cysteine knot growth factor super-family and the locations of most cysteine residues within GDNF are homologous to those within the TGF-β2 (transforming growth factor-beta2) structure (Chen *et al.* 2000). The TGF-β family of proteins along with other major classes of proteins such as BMPs (bone morphogenic proteins) are considered to be members of the NTFG family. The GFLs (glial family ligands) consists of GDNF, Neurterin, Artemin and Persephin and are regarded as a part of the larger NTGF family which also includes the neurotrophins; Nerve Growth Factor (NGF) and Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 3 (NT-3) and Neurotrophins 4/5, (NT-4/5) (Levy *et al*. 2005; Freund-Michel and Fossard 2008; Sariola and Saarma 2003). GFLs and NTGFs are known survival factors for neurones (Airaksinen and Saarma, 2002; Hirano et al. 2000; Bromberg and Wang 2009) and includes groups such as the neurokines (e.g. IL-6) which can function during early

repair and development (Nibali *et al.* 2012; Heinrich *et al.* 2003). Family members such as IL-6 are also known to mediate oral pathologies and diseases of bone and joints (Nibali *et al*. 2012; Nishimoto and Kishimoto 2006; Kishimoto 2010).

Neurotrophins have been shown to stimulate the proliferation of numerous cell types in culture, including neurones, liver and muscle cells (Kitomitsu 2000; Brodie and Sampson 1987). However, in certain cases *in vitro* actions are not always replicated *in vivo*, for instance, *in vitro* NGF stimulates the proliferation of muscle cells whereas *in vivo* within damaged muscle, it appears not to have the same effect (Brodie and Sampson 1987; Menetrey 2000). NGF affects cell survival and apoptosis via binding to p75NTR and TrKA receptors (Schor 2005; Bredesen *et al*. 2005; Mizuno 2007).

1.1.1 GDNF receptors and signalling

GDNF dimers elicit intracellular signalling by binding to the GDNF family receptors alpha (GFRα1) (glycosyl phosphostidylinositol (GPI) linked subunits) and RET (Rearranged during Transfection) (Figure 1). RET dimerises, forming the core of the complex with outer GFRα1 dimers (Airaksinen and Saarma 2002; Sariola and Saarma 2003; Naughton *et al.* 2006). RET is a tyrosine kinase receptor (RTK) and can also be classified as a dependence receptor that is able to initiate opposing signalling cascades, i.e. either survival or apoptosis (Goldschneider and Mehlen 2010; Bredesen *et al.* 2005; Mehlen 2005; Del Rio *et al*. 2007). RET binding is common to all GFL's however, these molecules bind more selectively to the GFRα receptor isoforms (Figure 1).



KEY

Most specific binding →

Non-specific binding →

Figure 1 Receptor interactions between neurotrophins (NT) and glial family ligands (GFL). GDNF = glial cell-line derived factor**; NTN** = neurturin; **ART**=Artemin; **PSP**=Persephin; **NGF** = nerve growth factor; **BDNF**= (brain derived neurotrophic factor); **NT-3**= (Neurotrophin 3); **NT-4/5**= (Neurotrophins 4/5).

GDNF binding to RET and GFRα1 modulate cell survival, apoptosis, proliferation and differentiation via activated intracellular signalling cascades (Figure 1.1). GDNF activation of RET dimers, results in autophosphorylation of the RET receptors (Jing *et al*. 1996; Sariola and Saarma 2003; Manié *et al*. 2001) and leads to activation of Ras, PLC-y (PKC), Ras/MAPK, JNK and PI3/AKT intracellular signalling cascades (Airaksinen and Saarma 2002; Coulpier *et al.* 2002; Manié et al. 2001) (Figure 1.1) (Airaksinen and Saarma 2002; Airaksinen *et al.* 1999).

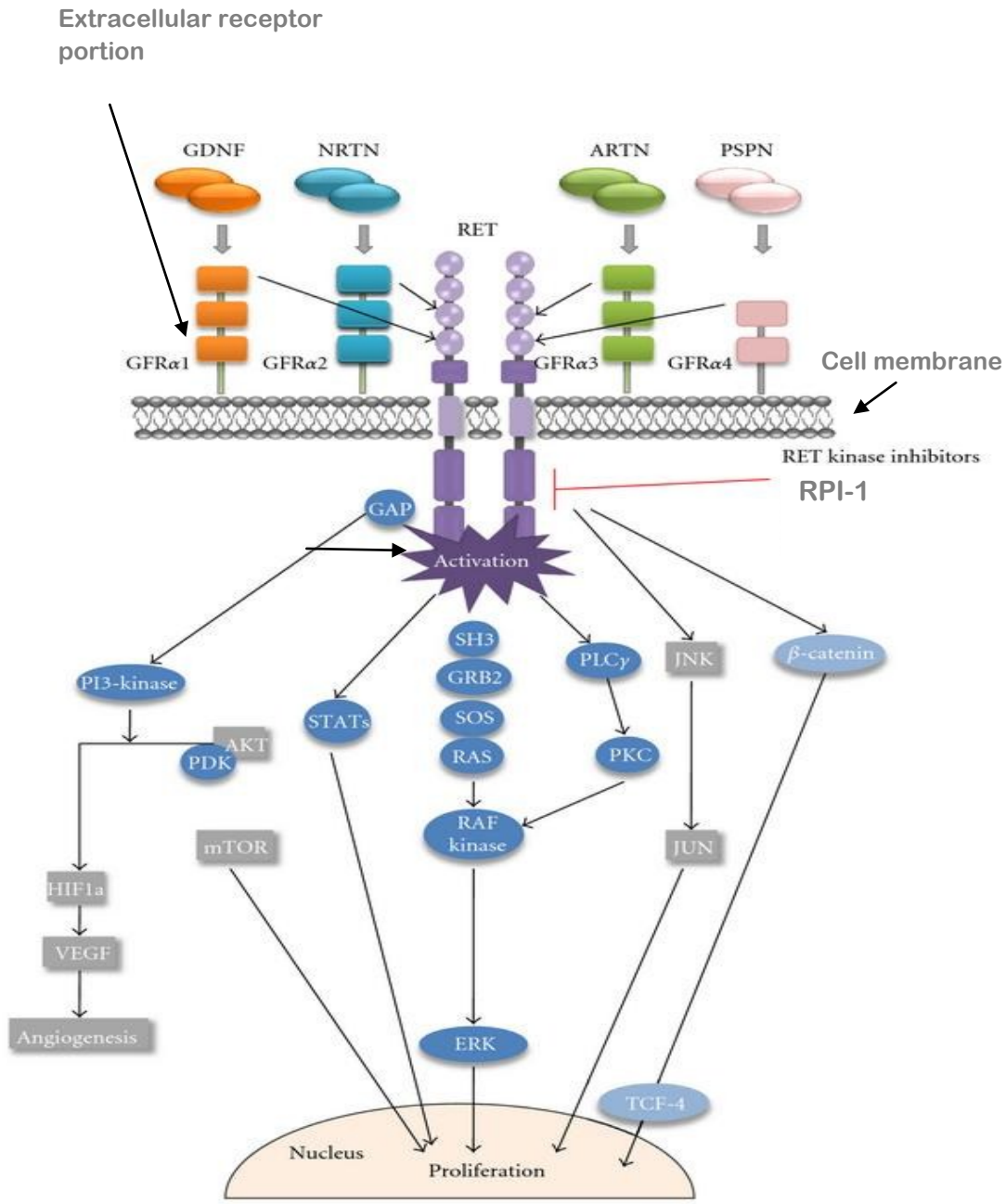


Figure 1.1 Intracellular signalling pathways activated by Gfrα/RET phosphorylation (adapted from Prazeres *et al.* 2011). Abbreviations; **PI3-kinase,** phosphoinositide 3-kinase; **PDK,** pyruvate dehydrogenase kinase; **HIF1α**, hypoxic inducible factor; **VEGF**, vascular endothelial growth factor; **AKT**, acutely transforming retrovirus AKT8 in rodent T cell lymphoma or protein kinase B; **mTOR**, mammalian target of rapamycin; **STAT**, signal transducer and activator of transcription; **SH3**, Src homology 3; **GRB2** =; **SOS,** son of sevenless; **RAS**, rat sarcoma protein; **RAF,** v-raf-1 murine leukemia viral oncogene homolog 1; **ERK**, extracellular signal-regulated kinase; **PLC-y**, phospholipase C-gamma; **JNK**, c-Jun, N-terminal kinase; **TCF-4**, T-cell factor 4 (also known as TCF7L2).

Interestingly the GFRα1 receptor may also be present as a soluble isoform and can subsequently activate signalling in a paracrine manner (Airaksinen and Saarma 2002; Sariola and Saarma 2003). This type of signalling involving soluble GFRα1 with RET may sustain GDNF effects (Manié *et al.* 2001). Furthermore, the GDNF-GFRα1-RET complex may bind in a cell-to-cell manner, whereby one cell may express RET and the other GFRα1 (Sprinzak *et al*. 2010). Poteryaev *et al.* (1999) found that GDNF and Gfrα1 binding could occur independently of RET binding resulting in activation of cAMP, MAPK and PLC-y intracellular signals. Later it was also determined that GDNF signalling through GFRα1 without RET activates Src, a tyrosine kinase receptor (Brown and Cooper 1996) and GDNF also activated Met, the Hepatocyte growth factor (HGF) receptor, present on tumour cells (Sariola and Saarma 1999; Gao and Vande Woude 2005). Popsueva *et al.* (2003) indicated that direct Met and GDNF interactions did not however occur within the kidney and therefore these findings suggest that RET-independent actions of GDNF may be cell-type or tissue specific. NCAM (neural cell adhesion molecule) is also a GPI linked receptor that may act as a GDNF co-receptor mediating the mitogenic effects of GDNF within Sertoli cells (neural crest derived cells) of the testes independently of RET (Yang and Han 2010). NCAM is expressed within the developing dental pulp, however, unlike RET-GDNF-GFRα1 complexes, GDNF and NCAM expression were not found to be overlapping during tooth development (Obara *et al*. 2002) (see Table 1.2 and Figure 1.4). NCAM is expressed by differentiated osteoblasts of human cranial bones (Marie *et al*. 2002) and during long bone development, although NCAM expression appears more prominent during cartilage formation compared with osteoblast formation (Hall and Miyake 1995). GDNF signalling may also be dependent on cells being in contact with the extracellular matrix

(ECM), i.e. requiring cell surface-associated heparan sulphate glycosaminoglycans (Lindfors 2006; Sariola and Saarma 2003). RET protein structure contains cadherin-like and fibronectin binding motifs that are also observed within other adhesion molecules indicating that RET may couple the cytoskeleton to the ECM (Kuma *et al.* 1993; Anders *et al*. 2001; Gumbiner *et al*. 2005).

Interestingly GFRα receptors are much more widely expressed in the nervous system compared with RET receptors (Trupp *et al.* 1997). Okragly and Haak-Frendscho (1997) reported on levels of GDNF expression within the organs of postnatal mice, finding that higher concentrations are found within the brain compared to the kidney and other organs. The importance of the role of the GDNF receptor complex in neuronal and non-neuronal tissue development is highlighted by observation of the phenotypes which occur as a result of genetic mutations in GDNF and its receptors (Table 1).

Gene	Phenotype	Reference
RET	Null mutation results in death at birth or shortly after. Lack of neural crest derived neuronal innervation of gut. Kidneys fail to fully develop or may be absent.	Schuchardt *et al.* (1994)
GFRα1	Null mutations die 24 hours after birth. Reduction in neurons of the colon. No kidneys develop.	Enomoto *et al.* (1998); Garcès *et al.* (2000)
GDNF	Mutations cause megacolon (or Hirschsprung's disease in humans). Null mutations die at birth or shortly afterwards. Lack of neural crest derived neuronal innervation of gut. Dentine and enamel of the teeth fail to develop.	Pichel *et al.* (1996) De Vicente *et al.* (2002)

Table 1. Disease/dysfunction resulting from gene mutations in GDNF and its associated receptors in mice [adapted from Airaksinen *et al.* (1999) and Schor *et al.* (2005)].

1.1.2 GDNF, cell proliferation, survival and apoptosis

GDNF acts as a pro-survival factor for a large number of cell types and is able to exert mitogenic and differentiation effects (Weisenhofer *et al.* 2000; Ng *et al*. 2009; Peterson *et al*. 2004; Toshifumi *et al*. 2005; Baldassarre *et al.* 2002 Meng *et al*. 2000; Park *et al*. 2005; Wu *et al*. 2005 Insua *et al.* 2003; Hofmann *et al*. 2005; Mwizerwa *et al.* 2011). Changes in cell cycle phases are linked with differentiation (Ohnuma *et al.* 2001; Buttitta and Edgar 2007) and studies have identified certain points within the cell cycle that can be modulated by GDNF signalling, in particular G1 entry and G1/S phase via $p27^{kip1}$ and cyclin A regulation (He et al. 2008; Vitagliano et *al.* 2004; Baldassarre *et al*. 2002).

Apoptosis plays an important role during tissue development, differentiation, maintenance and repair as it allows excess or damaged cells to be safely removed (Elmore 2007). For instance, during neuronal development excess cells are generated in response to neurotrophins and those not receiving growth factor stimulation subsequently apoptose (Yuan and Yankner 2000). Apoptosis and survival are controlled by a balance between activators (pro-apoptotic proteins) and inhibitors (anti-apoptotic proteins). Activation of the apoptotic process involves caspases-3, -6 and -7 and these are termed effector or executioner caspases and are responsible for initiating both the degradation of DNA and the end stages of apoptotic cascades (Nicholson 1999; Elmore 2007). Two major pathways of apoptosis are discerned and include the intrinsic or mitochondrial pathway, whereby activators of apoptosis are released from the mitochondria, and the extrinsic pathway, where apoptosis is induced following receptor binding (Ashe and Berry 2003; Elmore 2007). Both processes ultimately result in DNA

and cytoskeletal degradation, nuclear fragmentation and the formation of apoptotic bodies (Elmore 2007) (see Figure 1.2).

The extrinsic pathways leading to apoptosis can be influenced by growth factors (see Figure 1.2, pg 9). GDNF and other growth factors can function as pro-survival factors influencing the expression of anti-apoptotic factors such as Bcl family members (Ghribi *et al.* 2001; Pugazhenthi *et al.* 2000). However, the intracellular domain of RET has a caspase-3 cleavage site, which in the absence of GDNF signalling may be cleaved to initiate apoptotic cascades (Bordeaux *et al*. 2000). GDNF signalling via RET activates the AKT/P13 and Ras pathways that regulate cyclin D and stimulates cell cycle progression, also GDNF stimulation suppresses BAD activity and up-regulates IAPs (inhibitors of apoptosis) (see Figure 1.2) (Neff *et al.* 2002; Drosten *et al.* 2004; Airaksinen and Saarma 2002). Similarly to the RET receptor, the TNFα receptor ($p75^{NTR}$ low affinity nerve growth factor receptor or CD271) can also supply both positive and negative signals for survival determined by the activation of transcription factor NFκB that subsequently regulates apoptosis in response to TNFα (Wullaert *et al*. 2007; Liu *et al.* 1996).

1.2 Tissue development and GDNF function

Apoptosis occurs during cell differentiation within the developing calvarial bones in order to prevent premature closure of the adjoined skull bones and this process of cranial osteoblasts is proposed to be regulated by FGF-2 (beta-fibroblast growth factor) and BMP-2 (Marie *et al.* 2002). Similarly, a wave of apoptosis has been reported during tooth development that coincides with the differentiation of odontoblasts (cells that form mineralized tissue of the tooth (see section **1.2.1** and **1.2.4**)

(Mitsiadis *et al.* 2008) although the growth factors regulating this process have not been reported. Organ development is often subject to reciprocal interactions, involving epithelial tissue transmitting inductive signals (growth factors) and a mesenchymal tissue being competent to respond to the signal by returning an inductive signal (Gurdon 1992; Thesleff *et al.* 1995; Zhang *et al.* 2005). In line with these reports, abnormal GDNF expression and hence signalling between epithelial and mesenchymal tissues within the developing lung has been linked with decreased levels of apoptosis and subsequent

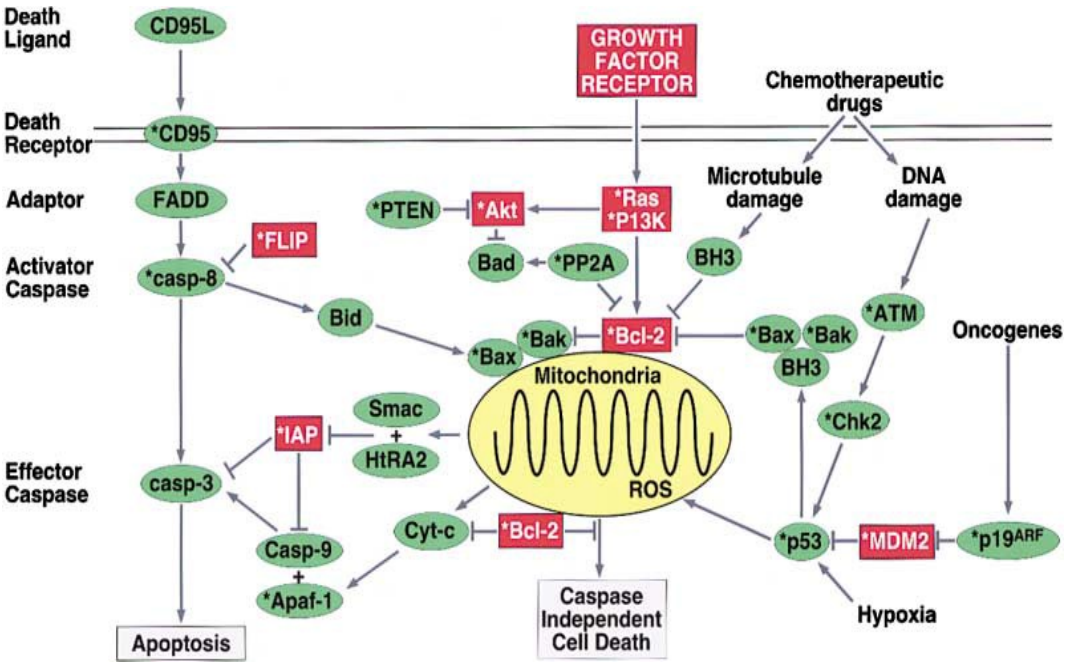


Figure 1.2 Schematic diagram detailing the signalling pathways involved during apoptosis that may be regulated by growth factors such as GDNF (from Johnstone *et al.* 2002). Component molecules in red inhibit apoptosis while those in green promote apoptosis. Abbreviations used: FADD, Fas-Associated protein with Death Domain; Apaf-1, apoptotic peptidase activating factor 1; Bid, BCL-2 Interacting Domain; Smac, diablo homolog ; HtRA2, HtrA serine peptidase 2; Bax, BCL2-associated X protein; Bak, BCL2-antagonist/killer 1; Bad, Bcl-2-associated death promoter; Bcl-xL/Bcl-2 associated death promoter; PTEN, phosphatase and tensin homolog; PP2A, protein phosphatase 2A activator, regulatory; BH3, Bcl-2 Homology (BH) domain 3; ATM, ataxia telangiectasia mutatedChk2, CHK2 checkpoint homolog *(S. pombe)*; MDM2, Mdm2 p53 binding protein homolog (mouse); $p19^{ARF}$, p19 alternative reading frame, casp, caspase; cyt, cytochrome.

malformation of the organ structure (Fromont-Hankard *et al.* 2002). Epithelial-mesenchymal signalling is a prominent process during tooth development and data indicates that GDNF may play a role in such reciprocal interactions (Vainio and Lin 2002; Fromont-Hankard *et al.* 2002; Airaksinen 1999; Nosrat *et al.* 2002; Lukko *et al.* 1997; Nosrat *et al.* 1998; Fried *et al*. 2000).

Organ and tissue development reportedly occurs due to the actions of morphogens (growth factors) forming concentration gradients that specify cell fates within the embryo (Gierer and Meinhardt 1972; Green 2002). Notably GDNF as well as FGF, TGFs and activin (a NTGF) have been identified as morphogens involved in tissue development (Armelin 1973; Todaro *et al.* 1981; Vale *et al*. 1986; Slack *et al*. 1987; Smith *et al.* 1990; Green and Smith 1990; Green 2002). These growth factors subsequently influence a range of transcription factors such as HOX (Homeobox) genes, PAX (Paired box genes) and SOX [SRY (sex determining region Y)-box genes] gene families, specifying cell fates. Interestingly GDNF regulates SOX10 transcription within neural crest cells (NCC), and SOX10 with PAX3 also regulate the transcription of RET (Lang *et al.* 2000, 2003; Linnarsson *et al.* 2001; Wu *et al.* 2008).

During early embryogenesis NCC are generated from ectodermal tissue of the mid- and hind-brain neural folds (Lumsden 1986, 1988; Ruch *et al.* 1995). These pluripotent NCC migrate from the ectoderm throughout the embryo to specific sites where they proliferate and differentiate to form structures within organs and tissues, e.g. odontoblasts of the dentine-pulp complex of the tooth (Gilbert 2000; Nanci 2003; Gronthos and Brahim 2002). Embryonic NCC develop into the adult cranial and sensory neurones (including nocioceptors) (Nanci 2003), sympathetic neurones and parasympathetic neurones (White and Anderson 1999), the periodontal ligament,

alveolar bone, the adrenal medulla, neurones of the gut (Kruger *et al*. 2002) cranio-facial bones, the skull (Nanci 2003), Schwann cells (Stemple 1992; Kaltschmidt *et al*. 2012), glia (Henion *et al*. 2000), spinal ganglia (Lefcort and George 2007), carotid body and aorta (mechanosensors) of the heart (Izal-Azcárate 2008, Gilbert 2000) (Figure 1.3). This ability of ectodermally derived cells to migrate, inhabit and function as mesenchymal cell types is unique to these pluripotent NCC.

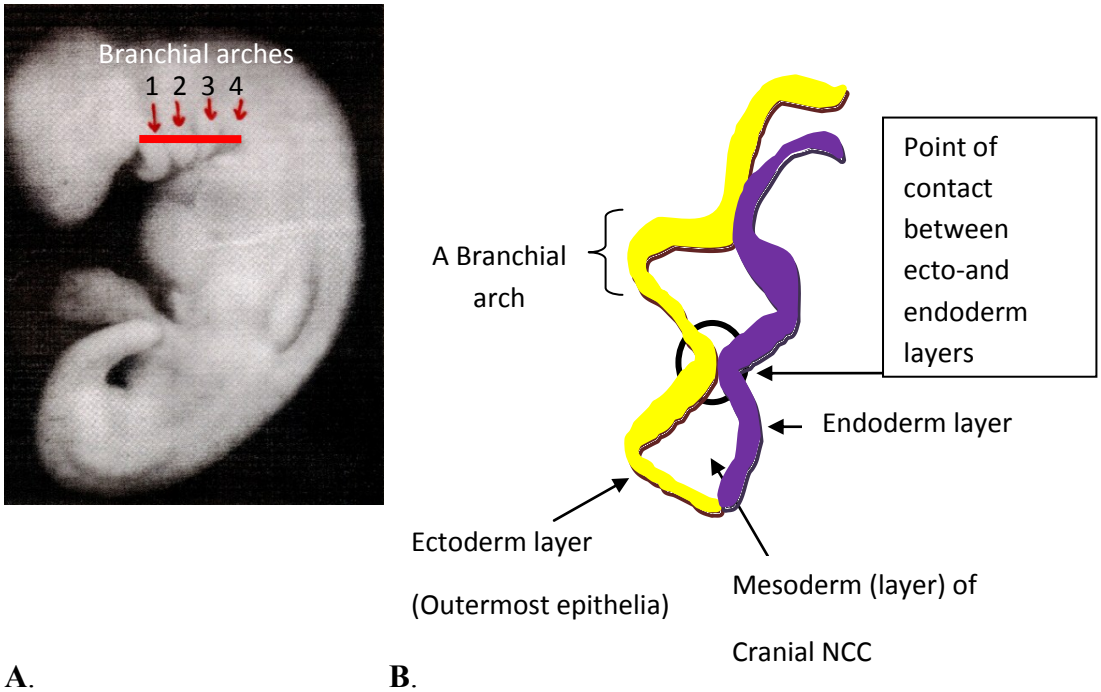
GDNF affects developing NCC derived tissues, influencing cellular proliferation, migration and differentiation throughout the embryo (Britsch *et al.* 2001; Young *et al*. 2001; Natarajan *et al.* 2002; Mwizerwa *et al.* 2011). GDNF is also expressed in postnatal neural crest derived tissues including the dermis (Motohashi 2006; Oshima *et al.* 2010), carotid body of the heart (Izal-Azcárate 2008; Shakhova and Sommer 2010; Miwa *et al.* 2010; Leitner *et al.* 2005), the Sertoli cells of the testes (Sariola and Immonen 2008), the inner ear (Stöver *et al*. 2001; Nam *et al*. 2000; Fransson *et al*. 2010; Minoux and Rijli 2010) and retinal cells of the eye (Hauck *et al.* 2006; Grocott *et al.* 2011). It is now proposed here that GDNF may influence NCC derived dental pulp and bone cell differentiation.

1.2.1 Tooth development

The pathway whereby NCC form odontoblasts begins with cranial NCC migration from the cranial region of the embryo into the mesenchymal tissue of the branchial arches. The first branchial arch (Figure 1.3, pg 12) develops into the jaw and after cranial NCC migration into the branchial arch, the adjacent epithelium thickens and invaginates into the NCC containing condensing mesenchyme (consisting of aggregates of mesenchymal cells) to form a dental placode (Figure 1.4, pg 14). This

developing tooth subsequently proceeds through a series of developmental stages termed bud, cap, bell and crown (see Figure 1.4, pg14).

During the bell stage the condensing mesenchyme develops into the dental papillae and the epithelium forms the dental or enamel organ (Figure 1.4, pg 14) simultaneously re-iterative signals (of growth factors) occur throughout the different stages of tooth development, beginning with initial stimulation from the oral epithelium to the cranial NCC derived mesenchyme (Thesleff and Tummer 2009). The growth factor signalling involved reportedly includes BMPs, FGF, Wnt (wingless-type MMTV integration site family) and Shh (sonic hedgehog) (Thesleff and Tummers 2009).



A. **B**.

Figure 1.3 (A) The gross anatomy of the developing human embryo showing the position of the four branchial arches at the head end of the embryo. Red arrows indicate branchial arch positions. (**B**) **Section through the branchial (pharyngeal arch).** The red line indicates the saggital plane from where the cross section is derived. The branchial arches are shown in sagittal plane as regions of the ectoderm, mesoderm and endoderm. There would appear a mirror image of this structure on the right hand side of the existing image if the whole cross section was shown, from http://rad.usuhs.edu/medpix.

Migration and recombination studies have demonstrated that odontoblasts develop from the NCC derived mesenchyme of the dental papilla and only form following interaction between cranial NCC and branchial arch epithelium (Lumsden 1988; Thesleff *et al.* 1995; Zhang *et al.* 2005). The importance of correct control of cranial NCC migration is highlighted in Di George syndrome where cranial NCC migration is disrupted, subsequently branchial arch structures do not develop normally and tooth development is therefore abnormal (da Silva Dalben *et al.* 2008). Interestingly mutations in VEGF (vascular endothelial growth factor) cause the phenotypic changes seen in Di George patients (Campbell 2003). Notably Tufro *et al.* (2007) reported that VEGF activates RET and induces GDNF up-regulation, indicating an interesting interrelationship between these molecules that may affect craniofacial development. Furthermore, inhibition of GDNF-RET signalling may repress tooth formation (Klein *et al*. 2006; Taketomi *et al.* 2005). Studies have previously shown that GDNF mediates the differentiation of NCC during the development of several other organ systems (Vainio and Lin 2002; Sariola and Saarma 2003).

Odontoblasts differentiate from the cells of the dental papillae and begin producing pre-dentine (unmineralised collagenous ECM of dentine) during the late bell stage. Differentiating dental papilla/pre-odontoblasts also produce proteins such as fibronectin and collagen type III that are associated with the forming basement membrane positioned between the dental papilla and the inner epithelial (IDE) (Smith and Lesot 2001; Tzaifas and Kondados 2010; Ruch *et al.* 1982). Thesleff *et al.* (1978) reported that both mesenchymal dental papilla cells and IDE cells are needed to produce the basement membrane and this interaction initiated odontoblast differentiation.

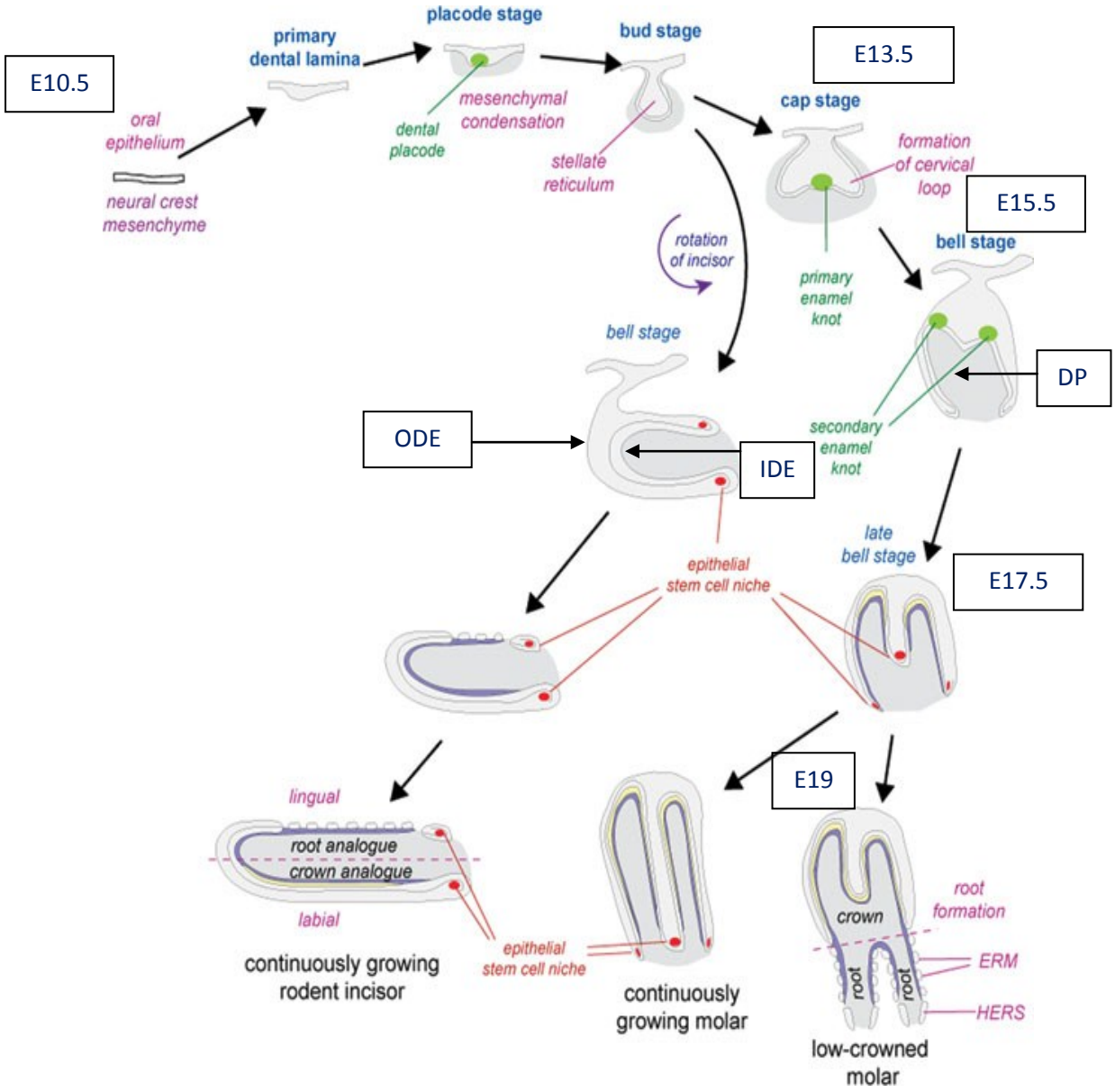


Figure 1.4 Stages of rodent tooth development (adapted from Thesleff and Tummers 2009; Lumsden 1988). **E**=embryonic murine day; **ODE**= Outer dental epithelium; **IDE**= inner dental epithelium; **DP** =dental papilla; **ERM**= epithelial cell rests of Malassez; **HRM**= Hertwig's epithelial root sheath.

The basement membrane proteins fibronectin and decorin have previously been shown to support and signal odontoblast polarisation and differentiation (Ruch *et al.* 1995; Ruch 1998) however more importantly growth factor signalling is considered crucial to this process. In particular, IGF, FGFs (a and b), TGF-βs (-1, -2, -3) and BMP (-2, -4, -6) have been identified as putative factors involved. Other growth factors that may regulate odontoblastic differentiation include NGF, PDGF (platelet derived growth factor) (Yokose *et al*. 2004), HGF (Ye *et al*. 2006), Gdf11 (growth/differentiation factor 11) (Nakashima *et al*. 2002) and ADM (adrenomedullin) (Musson *et al.* 2010).

1.2.2 Primary, secondary and tertiary dentinogenesis

Dentine is located beneath the tooth enamel (Frank 1999) (Figure 1.7 pg 31) and is initially synthesized during development in a process termed primary dentinogenesis. Following tooth eruption dentine is continually formed at the periphery of the dental pulp throughout the life of the tooth (Linde 1989) and this process is termed secondary dentinogenesis (Linde 1989; Sloan and Smith 2007). If the dentine becomes damaged after eruption further dentine may be produced as a replacement in a process referred to as tertiary dentinogenesis (Smith and Lesot 2001; Smith 2001; Tziafas *et al.* 2000).

Odontoblasts have processes which are the first point of contact between the peripheral dentine and dental pulp. Cellular processes from an odontoblasts extend within the dentine tubules along with neighbouring nerve fibres potentially up to the dentine border with the enamel (Frank 1999; Karjalainen and Le Bell 1987; Moss et al. 2005). Consequently, physiological changes within the dentine/enamel are detected by the odontoblast and nerve processes (Chaussain-Miller *et al.* 2006; Smith *et al.* 2001; Smith 2001; Karjalainens and Le Bell 1987) and may stimulate tertiary dentinogenesis.

Physiological changes in the biofilm affect the dentine and enamel. Teeth develop a naturally occurring coating of bacteria termed a biofilm which can become excessively populated by acid producing bacteria, such as *Streptococcus mutans*, that subsequently cause tooth mineral dissolution releasing the components of the dentine/enamel structure (Marsh 2006; Verstraeten et al. 2008). As this dental caries progresses the odontoblast may also come into direct contact with the bacteria or its components. Odontoblasts have been shown to express toll-like receptors (TLR) that bind bacterial surface proteins and these interactions may initiate an immune and inflammatory response within the dental pulp, which may also contribute to tertiary dentinogenesis depending on the severity of the infection (Cooper *et al.* 2010; Berkovitz 2002; Jones and Boyde 1977; Hirao et al. 2009; Botero *et al.* 2006; Horst *et al.* 2009; Durand *et al.* 2006).

Dentine production by existing odontoblasts is termed reactionary dentinogenesis and is a form of tertiary dentinogenesis. Severe disease/trauma may result in odontoblast apoptosis or necrosis (Mitsiadis *et al.* 2008), however neighbouring post-mitotic terminally differentiated odontoblasts cannot divide to produce new daughter cells to repopulate and repair the injury site (Berkovitz 2002; Moss *et al.* 2005). Generation of new odontoblast-like cells, differentiating from mesenchymal dental pulp stem/progenitor cells may occur under appropriate conditions (see section **1.3.1**) and dentine produced during this form of tertiary dentinogenesis is termed reparative dentinogenesis (Smith and Lesot 2001; Sloan and Smith 2007; Shiomi 2000).

1.2.3 Intramembranous and endochondral bone development

Long/limbs bones are formed by endochondral ossification whereby cartilage is formed initially and subsequently replaced by bone produced by osteoblasts. Osteoblasts are differentiated from the mesenchyme surrounding the cartilaginous tissue. However, the flat bone of the cranium develops primarily by intramembranous ossification, whereby osteoblasts directly differentiate from cranial NCC-derived condensed mesenchyme (Figure 1.5, pg 20). Unlike tooth and craniofacial bones, the limb bud mesenchyme is not derived from cranial NCC (LeLièvre and Douarin 1975; Noden, 1988; Chai *et al.* 2000; Jiang *et al.* 2002; Couly *et al*. 1992, 1993; Lumsden *et al.* 1986). In particular, the frontal area of the cranium forms from cranial NCC and the posterior region from mesoderm (Opperman *et al.* 1993, 1996; 2000; Couly *et al*. 1992, 1993; Santagati and Rijli 2003). Thus, due to the differences in cellular origins, the fundamental developmental processes as well as behaviour of osteoblasts within cranial and long bones may be different (Ornitz and Marie 2002; Depew *et al*. 1999) (Mao *et al.* 2006). Indeed Moore *et al.* (2002) noted that fibroblast growth factor -2 (FGF-2) implanted into the developing skull had no detectable effect yet when it was implanted into the developing limb it initiated growth of this structure. Furthermore, null mutation of the BMP receptor type 1B gene resulted in defects in limb bones but not cranial bone formation (Lian *et al.* 2006) underscoring physiological differences between endochondral and intramembranous ossification. Van der Bos *et al.* (2008) also reported differences in levels of collagen, osteopontin and osteonectin within the ECM composition of each bone type.

Little is known about the role of GDNF in bone development and osteoblast differentiation. It is postulated here that GDNF may be involved in cranial bone formation through a direct action on NCC. During intramembraneous ossification mesenchymal cells condense, then differentiate into osteoprogenitors and osteoblasts directly (Hall and Miyake 1992) (Figure 1.5). Osteoprogenitors and pre-osteoblasts secret extracellular matrix (ECM) components (e.g., collagen III, hyaloronan, collagen type I) that may be stimulated by growth factors such as TGF-β and these ECM molecules may play a role in inducing further osteoblast differentiation (Lian and Stein 2003, 2004, 2006). Furthermore, autocrine secretion of BMP-2, BMP-4, and BMP-7 by differentiated calvarial osteoblasts may have further effects stimulating cyto-differentiation, increasing the transcription of bone constituents including osteocalcin (OCN) and bone sialoprotein (BSP) transcripts (Xiao *et al*. 2002; van der horst *et al.* 2002).

The role of GDNF in long bone formation is also unclear. During long bone formation, the proliferation and differentiation of osteoblasts is due to a series of interactions between chondrocytes and a pre-osteoblast mesenchymal region (called the perichondrium) (Vortkamp *et al.* 1998) secreting growth factors such as BMP-2, -4, and -7 (Yamaguchi *et al.* 2000; Vortkamp *et al*. 1998), FGF-7, -8, -17 and -18 (Ornitz and Marie 2002; Ducy *et al.* 1997; Yamaguchi *et al*. 2000). Later osteoblasts differentiate from the mesenchyme (the perichondrium) surrounding the chondrocytes. GDNF mRNA has been detected in mesenchymal tissues surrounding the forming cartilage in femur limb buds at embryonic day 14.5 (Hellmich *et al.* 1996). Furthermore GDNF was secreted by chondrocytes stimulated with TNFα (Kashani *et al*. 2010). Interestingly, HOX11 null mice have no detectable expression of GDNF or the GDNF transcription

factor six2 (Wellik *et al.* 2002; Brodbeck *et al.* 2004). HOX11 functions together with HOX10 (Myers 2008) and HOX9 to regulate digit formation in forelimbs (Xu and Wellik 2011; Goff and Tabin 1997) and HOX11 mutant mice have spine and limb joint abnormalities (Small and Potter 1993; Davis *et al*. 1995). This data suggests that HOX11 is involved in transcription of GDNF during endochondral ossification.

1.2.4 Dentine and bone extracellular matrix

The mineralised matrix of dentine and bone consists of hydroxyapatite crystals [$Ca_5(OH)(PO_4)_3$] that are embedded in a collagenous ECM. The ECM affects the position, orientation, size and growth of the crystal hydroxyapatite in these hard tissues (Boskey 1991). Bone and dentine also contain non-collagenous proteins, including phosphoproteins, dentine matrix protein-1 (DMP-1), dentine sialoprotein (DSP), dentine phosphoprotein (DPP), bone sialoprotein (BSP), osteonectin (ON), osteocalcin (OCN), osteopontin (OPN) and proteins from serum (such as growth factors and albumin) (Smith *et al.* 2011; Hao *et al.* 2009; Huang *et al.* 2008). The acidic non-collagenous phosphoproteins of dentine and bone are suggested to be the sites of nucleation for hydroxyapatite formation on the ECM due to their capacity to bind to collagen and calcium ions (Fujisawa and Kuboki 1991; Houlle *et al.* 1997; Saito *et al*. 1997; Boskey 1991; He *et al*. 2003).

The enzyme alkaline phosphatase (ALP) has been suggested to play an important role in the ECM mineralisation process (Beertsen and van den Bos 1989). It was hypothesized that ALP is involved in the generation of inorganic phosphate ions necessary for matrix mineralisation whilst inhibiting factors that reduce inorganic portions of the ECM (Beertsen and van der Bos 1992; Damek-Poprawa *et al.* 2006).

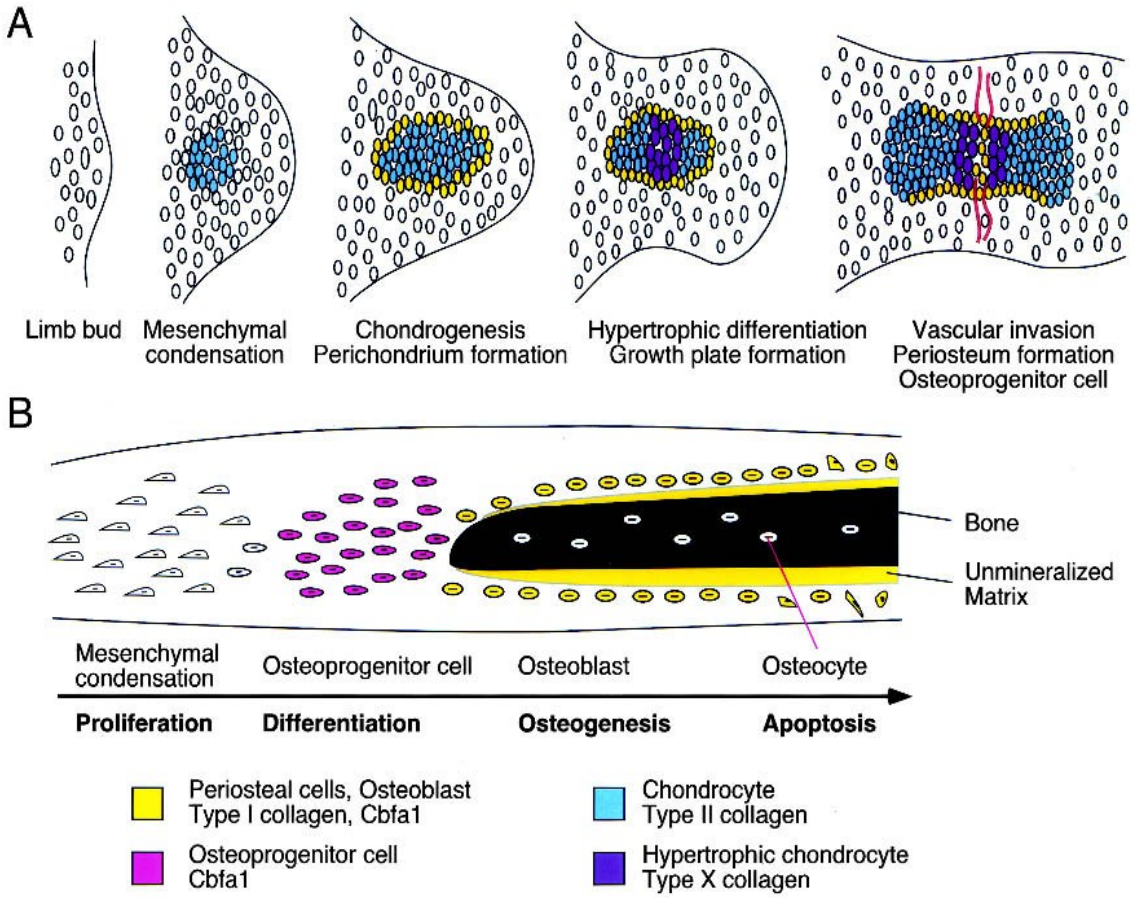


Figure 1.5 Diagrammatic comparison of human endochondral bone formation (A) and intramembraneous ossification (**B**), from Orntiz and Marie (2002). Note the limb bud and mesenchymal condensations initiation of bone development differs, osteoprogenitors (pink) are formed directly from mesenchyme (clear) during intramembranous ossification, these differentiate to osteoblasts (yellow) that later reside on the inner lining surface of the bone facing the bone marrow cavity or in the outermost periosteum lining of the bone surface (**B**), compared to (**A**) endochondral ossification where chondrocytes (light blue) form directly from mesenchyme (clear).

Notably, bone ALP (tissue non-specific ALP or TNSALP) is used as a biochemical and clinical marker for osteoblast activity and total serum ALP is used for biochemical diagnosis of bone diseases (Singer and Eyre 2008; Karjalainens and Le Bell 1987). Recently, mutations in human TNSALP genes and TNSALP null rats have been shown to produce hypophosphatasia a bone disease characterized predominantly by decreased skeletal mineralization (Whyte 2010), indicating ALP is required for the mineralisation

of bone. *In vitro* ALP has also been used as an early, albeit nonspecific, marker for osteogenic cell differentiation and has been suggested to reflect *in vitro* mineralization. During osteoblast differentiation, confluent osteoblast cultures are able to form multilayers which coincide with a decrease in cell proliferation whilst the expression levels of ALP increase; notably as the ECM matures during mineralised nodule formation ALP levels subsequently decrease (Bellows *et al.* 1991; Porter *et al.* 2003; Golub and Boesze-Battaglia 2007). *In vitro* mineralisation can be stimulated by the presence of osteogenic supplements, β-glycerophosphate (β-gp), dexamethasone (DEX) and ascorbic acid (AA or Vitamin C). β-gp is necessary for the formation of mineralised collagenous matrix (Fratzl-Zelman N *et al.* 1998), supplying "active" phosphate for the mineralisation of the collagenous ECM and is also a substrate for ALP (Fratzl-Zelman *et al.* 1998). Collagen synthesis is dependent on the presence of AA (Murad *et al*. 1983; Fratzl-Zelman *et al.* 1998) and DEX is a pleiotropic synthetic steroid that is used as an inducer of osteogenic differentiation *in vitro* and also increases ALP expression (Boskey and Roy *et al*. 2008; Alliot-Licht *et al*. 2005; Cheng *et al*. 1994, 1996).

The "master" gene regulators of transcription during osteoblast differentiation are regarded as Osterix and Cbfα1/RUNX2 (Ducy *et al.* 1997). The processes involved in the regulation of odontoblast differentiation are however less well understood, but, similar transcriptional controls as to those in osteoblasts may be involved in regulating this process (Narayanan *et al.* 2001; Hao *et al.* 2005; Qin *et al.* 2001, 2004, 2007). Interestingly the DSPP (dentin sialophosphoprotein) promoter has putative binding sites for Cbfα1 (Chen *et al.* 2005) and this gene encodes two proteins DSP and DPP, both of which have been associated with odontoblast differentiation and dentinogenesis (Shi 2001).

1.3 GDNF and repair of mineralised tissue

Serum proteins such as albumin, PDGF, TGF-β, bFGF, IGFI and II and VEGF have been identified within the dentine ECM, sequestered there during dentinogenesis (Roberts-Clark and Smith 2000; Linde and Goldberg 1993; Finkleman *et al.* 1990; Kinoshita, 1979). Similarly, during bone development growth factors are also sequestered within the developing mineralised matrix and are involved in osteoclast-osteoblast signalling and the regulation of bone remodelling (Hauschka *et al*. 1986). These signalling molecules may also be present in the soft extracellular stromal/mesenchymal matrix postnatally. Bone remodelling repairs small areas of damaged bone as a part of the normal homeostatic postnatal mechanism, controlling bone size, strength and density. However, research investigating postnatal repair processes of mineralised tissues has suggested larger repairs utilise mechanisms that mimic the developmental processes forming mineralized tissues (Robling *et al.* 2006; Ferguson *et al.* 1998, 1999; Vortkamp *et al.* 1998 Tsiridis and Giannoudis 2006; Silkstone *et al.* 2008; Smith and Lesot 2001; Simon *et al.* 2009, 2010). Several growth factors such as BMP-2, PDGF, VEGF and TGF-β involved in embryonic tooth development and bone development are already known to influence postnatal dental pulp physiology (Kinoshita 1979; Kato 1988; Lynch *et al.* 1999; Roberts-Clarke and smith 2000; Tziafas et al. 2000; Matsushita 2000; Haddad *et al.* 2003; Kurihara 2003, 2007; Iohara *et al.* 2004; Sloan & Smith 2007; Scheven *et al.* 2009; Hauschka *et al*. 1986).

During injury/infection degradation of the mineralized ECM may liberate growth factors and other proteins previously sequestered there (Chaussain-Miller *et al.* 2006), where they may be bound to carrier molecules which maintain their stability, such as

heparin sulphate, decorin and biglycan (Berkovitz 2002; Karjalainens and Le Bell 1987; Smith *et al.* 2011; Buckwalter and Mankin 1988). The release from the ECM following disease may be key to signalling repair (Smith and Lesot 2001; Hing 2004), by evoking migration, proliferation, survival and differentiation of osteo/odontoblasts, progenitors and stem cells that can regenerate the mineralised tissue (Smith et al. 1990; 2001; Benezra *et al.* 1993; Farges *et al.* 2003; Grassinger *et al.* 2009). However, the existence of GDNF, NGF or other NTGFs among sequestered growth factors within dentine is as yet unreported.

1.3.1 Mesenchymal stem/stromal cells (MSC)

Mesenchymal stem cells (MSC) have been identified within various mesenchymal tissues including bone marrow, adipose and skin (Zuk *et al.* 2002), kidney (Huang *et al.* 2009), umbilical cord (Lu *et al*. 2006) and muscle (Jankowski *et al.* 2002). Neural crest stem cells within the mesenchyme such as the dental pulp may also be considered as mesenchymal stem cells often referred to as "ecto-mesenchymal". The term stem cell is indicative of the self renewing or colony forming ability of cells along with pluri-/multi-potentiality. MSC may be recruited to sites of injury, migrating and differentiating to form tissue specific cells *in situ* (Koç *et al*. 2000; Crisan *et al.* 2011; Wise and Ricardo 2012; Theise *et al*. 2000; Peterson *et al.* 1999; Barry and Murphy 2004). Distinct MSC populations can be obtained by FACS, however significant work still remains to identify definitive panels of markers for MSC isolation for all tissues (Gronthos *et al.* 2011; Battula *et al*. 2009; Kagami *et al.* 2011; Bühring *et al.* 2007, 2009). The role of mesenchymal stem cells in tissue repair and regeneration has generated a significant amount of interest over the last decade, in particular dental pulp stem cells (DPSC) have now been considered as a potential and promising source

for repair and regeneration of the tooth as well as other tissues (Sasaki *et al.* 2008; Nesti *et al.* 2011; Gandia *et al.* 2008; Arthur *et al*. 2009; Kerkis *et al.* 2006, 2008; Zhang *et al.* 2008). Although the precise identity and tissue localisation of DPSC are not fully known, DPSC share many similarities with bone marrow MSC [(BMSC) bone marrow mesenchymal stem/stromal cells] (Yan *et al.* 2011; Alge *et al.* 2010; Huang *et al.* 2009). DPSC and BMSC are multipotent, able to differentiate along osteogenic, odontoblastic, adipogenic, neurogenic and chondrogenic lineages (Arthur *et al.* 2008; Egusa *et al*. 2005; Sanchez-Ramos *et al*. 2000; Tziafas and Kondonas 2010). Similarly to dentine regeneration, bone can regenerate during repair by osteoblast differentiation from MSC (Hing 2004; Yu *et al.* 2007; Batouli *et al.* 2003). Thus, DPSC and BMSC cultures may be used as model cultures pertaining to study of stem cells and the processes involved in cell differentiation and tissue repair (Aubin 1998, 1999; Frank *et al.* 2002; Zou *et al.* 2008; Wang *et al*. 2010b & c; Gronthos *et al.* 2002; Shi *et al*. 2005; Miura *et al.* 2003, 2004; Couble *et al.* 2000; Woodbury *et al.* 2002; Egusa *et al*. 2005).

The ability of MSC to differentiate along different lineages is influenced by growth factors present within the injured/repairing tissue (Hermann *et al.* 2004; Barry and Murphy 2004; Banfi *et al.* 2000) and MSC themselves also secrete a range of growth factors that further influence the tissue repair and also act in an auto-/paracrine manner (Majumdar *et al.* 1998; Barry and Murphy 2004). Interestingly, DPSC and BMSC cultures produce a range of NTGFs including GDNF which prompted an emerging interest in the use of these cells for neurological disorders and nerve repair (Nosrat *et al.* 2001; Apel *et al.* 2009; Chop and Li 2002; Sasaki *et al.* 2008; Nesti *et al.* 2011). In addition, GDNF has been reported to induce stem cell self-renewal and to be

cytoprotective for MSC of the kidney stimulating proliferation and migration (Shi *et al.* 2008; Meng *et al.* 2000; Gonzalez *et al.* 2009). Therefore elucidation of the basic functions of GDNF that control mesenchymal cell differentiation has potential for broader beneficial applications.

Furthermore, GDNF effects may be co-ordinated with other growth factors such as Wnts, TGF-βs, BMPs or mediators of inflammation, such as TNFα, neuropeptides and neurotrophins, that are up-regulated during mineralised tissue repair (Golub and Boesze-Battaglia 2007; Liu *et al*. 2008; Rawadi *et al.* 2003). Blocking Wnt signalling abrogates ALP activity and results in abnormal tooth development (Qiang *et al*. 2008 Golub and Boesze-Battaglia 2007; Liu *et al.* 2008). Interestingly, Wnt signals down-regulate GDNF, inhibiting stem cell self renewal during spermatogenesis (Tanwar *et al.* 2010) while Wnt11 deficiency decreases GDNF expression within the kidney (Chi *et al.* 2004). TGF-β3 has also been reported to be required for recruiting the GDNF receptor Gfrα1 to the plasma membrane (Peterziel *et al*. 2007; Sariola and Saarma 2003) and TGF-βs are required for the trophic actions of GDNF *in vitro* and *in vivo* (Krieglstein *et al.* 2002; Schober *et al.* 1999). TGF-βs are expressed in odontoblasts (Linde 1989, Tziafas *et al.* 2000) and dental pulp cells, and isoforms I and III increased odontoblast secretory activity, differentiation and repair in rat organ and cell culture models (Sloan *et al.* 2002; Sloan and Smith 1999; Tziafas *et al.* 1998; Nie *et al.* 2006). Moreover TGF-β1 application within pulp capping experiments *in vivo* (Tziafas *et al.* 1998, 2001; 2000; Smith 2001) along with the inhibition of reparative dentinogenesis caused by TGF-β1 anti-sera application in ferrets (Smith *et al.* 1994) indicates TGF-β1's potential important role in promoting dental hard tissue formation. Conceivably, differentiation

and mineralization events which occur during bone and dental pulp repair may involve interactions between TGF-β and GDNF.

TNFα is a cytokine involved in immune and inflammatory responses at sites of disease and injury (Szlosarek and Balkwill 2003; Konisti *et al*. 2012). TNFα inhibits osteoblast terminal differentiation and has been found to be abundantly expressed in many diseases of bone, including rheumatoid arthritis (Boyce *et al.* 2005). Dental pulp cell TNFα expression is up-regulated by bacterial LPS (lipopolysaccharide) and LTA (Lipoteichoic acid) and within infected pulpal tissue (McLachlan et al 2004; Bletsa *et al.* 2006; Keller *et al.* 2010). The role of TNFα *in vivo* during dental pulp inflammation may however have opposing effects on DPC behaviour due to its potential ability to promote repair processes at relatively low concentrations while inhibiting repair processes at high concentrations (Cooper et al. 2010, 2011; Okabe and Matsushima 2006; Paula-silvia *et al.* 2009; Pezelj-Ribaric *et al.* 2002). Notably the GDNF promoter has a binding site for NFκB which is a key transcription factor activated in response to TNFα signalling (Grimm *et al.* 1998) and GDNF expression in Sertoli cells is also known to be up-regulated by TNFα stimulation (Simon et al. 2007; Hofmann 2008). GDNF expression in the brain is also up-regulated during inflammation and repair; a finding similar to that reported for GDNF within other non-neuronal tissues (Eberling *et al.* 2009; Cheng *et al.* 2008; Katoh-Sembah *et al.* 2007; Nam *et al.* 2000; Tsui *et al.* 2006) indicating that GDNF specifically functions during soft tissue injury, infection or repair. GDNF may therefore also influence hard mineralised tissue repair, directly or indirectly via interactions with prominent growth factors and cytokines that regulate these processes.

1.3.2 The role of the nervous system in mineralised tissue repair

Several functions have been ascribed to the up-regulation of NTGF and neuropeptides (NP) released from the nervous system during injury/infection. Such functions include the further stimulation of the nervous and immune systems to remove infection, produce anti-inflammatory factors, modulate pain and perform neuronal repair (Toben *et al.* 2011; Xian and Zhou 2009; Paschalia *et al.* 2011; Lundy and Linden 2004; Wang *et al.* 2003; Pezet and McMahon 2006; Lambrecht 2001). Highly innervated bone (See Figure 1.6, pg 28 for the localisation of nerves) contains numerous sympathetic nervous system fibres that have previously been suggested to regulate haemopoietic cell proliferation and mobilisation from within the bone (Afan *et al.* 1997; Elmquist and Strewler 2005; Chen *et al.* 2002; Marenzana and Chenu 2008; Serre *et al.* 1999; Delgado *et al.* 2004). However, more recently the innervation has been suggested to be involved in bone development (Elmquist and Strewler 2005). This newer more direct role of the sympathetic bone innervation and neuropeptides (NP) on bone formation and putatively on repair was initially demonstrated by adrenergic receptor activation, (Elefteriou 2005, Elefteriou *et al.* 2005; Elmquist and Strewler 2005) is currently under investigation (Lundy and Linden 2004; Sato *et al.* 2007; Goto *et al.* 2007; Natsume *et al.* 2010). However NP are also secreted from non-neuronal tissues during tissue damage and infection and these molecules can stimulate growth of cells within mineralised tissues; for instance VIP (Vasoactive intestinal peptide), and CGRP (calcitonin gene related peptide) are secreted by DPC, furthermore CGRP and SP (substance P) were secreted by DPC *in vitro* after incubation with proteases from *P.gingivalis* (Tancharoen *et al.* 2005). Both CGRP and SP also increased dental pulp

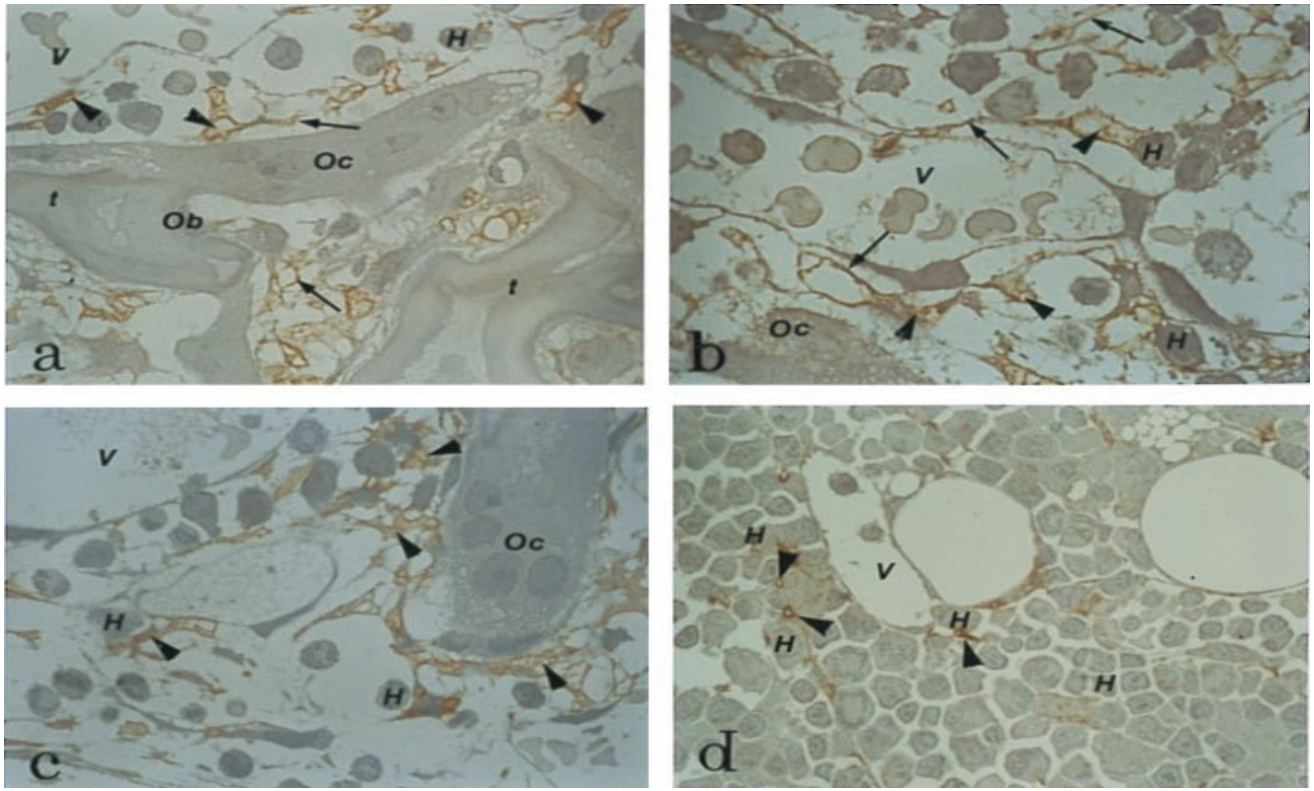


Figure 1.6 Immunohistochemical localization of neurones and nerve markers in long bones of growing rats aged 15-25 days. (a) Metaphysis of neonatal rat femur. NF 200-positive nerve processes (arrows), showing local enlargements (arrow heads), are running along bone trabaeculae **(t)** and vessels **(v).** They are close to bone cells (**Oc**: osteoclast, **Ob**: osteoblast) and hematopoietic cells **(H).** Original magnification: X1000. **(b)** Deep metaphysis of neonatal rat femur at the level of the afferent vessel. Tyrosine hydroxylase is present in these nerve processes (arrows) showing local dilatations (arrow heads) in contact with hematopoietic cells (**H**). **Oc:** osteoclast. Original magnification: X1500. **(c)** Deep metaphysis of neonatal rat femur. MAP2-positive nerve processes showing dilatations (arrow heads) in contact with hematopoietic cells (**H**) and osteoclasts (**Oc**). Original magnification: X1000. **(d)** Diaphysis of 15-day-old rat tibia. NF200 is present in nerve endings (arrow heads) in contact with hematopoietic cells (H). Original magnification: x1000, from Serre *et al.*(1999).

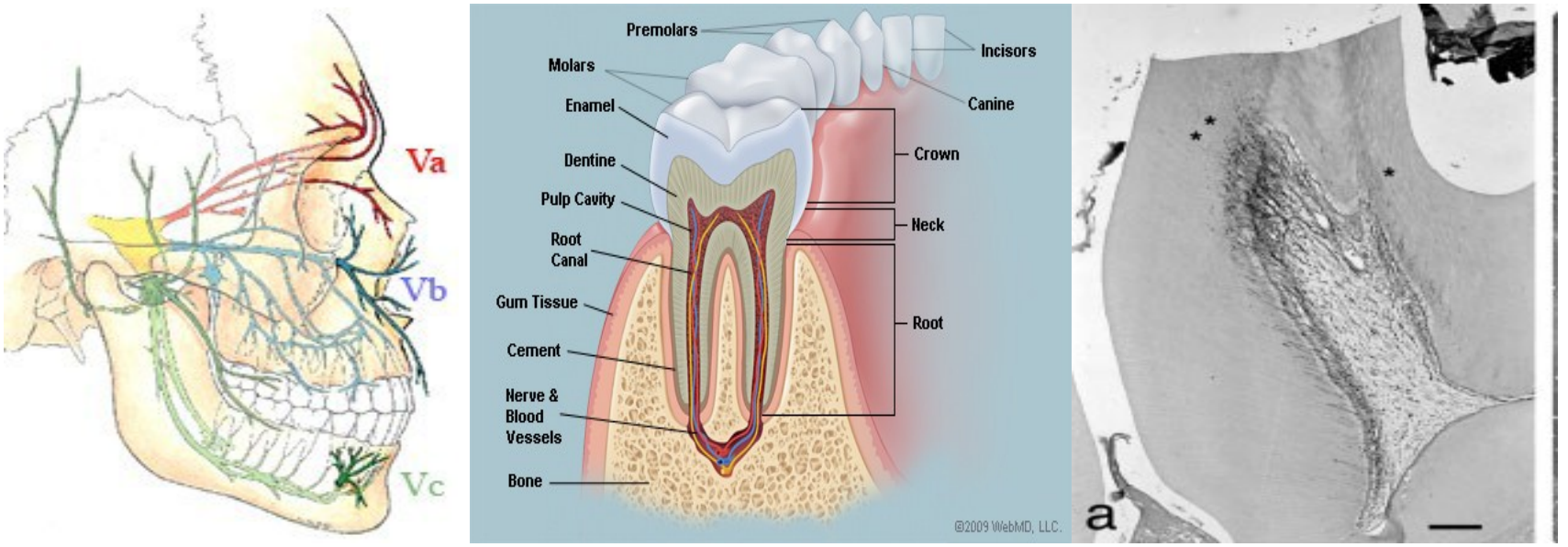
proliferation *in vitro* (Bongenhielm et al. 1995) The NP, ADM (adrenomedullin) is also secreted by many cell types after LPS exposure and stimulates growth of odontoblast-like MDPC-23 cells (Zudaire et al. 2006; So et al. 1996; Musson et al. 2010).Therefore NP and NTGF released from non-neuronal cells of the dental pulp and bone may stimulate both indirect and direct repair of mineralised tissue by activating the immune and nervous systems as well as MSC.

NGF is previously the only NTGF to be investigated regarding direct effects on bone repair and direct application was found to enhance bone regeneration when applied in a fracture model and stimulated the differentiation of osteoblastic cells (Wang *et al.* 2006; Xian and Zhou 2009; Paschalia *et al.* 2011). NGF and its receptor, $p75^{NTR}$, exhibit overlapping expression in polarising/differentiating odontoblasts during odontogenesis and dentinogenesis (see Table 1.2, pg 36) (Maas and Bei 1997; Mitsiadis *et al.* 1992, 1995; Fried 2000). Moreover, recent data also indicates that NGF may also be an important ligand involved in tissue inflammation and repair of the dentine-pulp complex (Woodnutt *et al.* 2000; Shiomi *et al.* 2000; O'Hara *et al.* 2009). Moreover, postnatally NGF was up-regulated on pulp fibroblasts and odontoblasts within injured teeth. Therefore similarly to a direct effect of NGF on osteoblasts, this molecule may conceivably have a more direct function during dental repair by stimulating odontoblast-like cell differentiation (Arany *et al.* 2009; Kurihara *et al.* 2007).

1.3.3 The role of GDNF in mineralised tissue repair

Sympathetic innervation has also been implicated in tooth development and repair (Fried *et al.* 2000; Hildebrand 1995). Indeed the dental pulp is highly innervated and contains sympathetic fibres of the trigeminal nerve that secrete NTGFs and

neuropeptides (Fried *et al.* 2001) (Figure 1.7, pg 31). Similarly to osteoblasts and BMSC within bone, the nerve fibres within pulp are closely associated with odontoblasts and can extend into the dentine (as described above) (Byers *et al.* 2003; Egan *et al.* 1996) (Figure 1.6, pg 28, 1.7, pg 31 and 1.7.1, pg 32) and for animals of lower evolutionary order such as polyphyodont teleost, (fish that continuously produce teeth) nerve fibres have also been implicated in regulating odontogenesis (Hildebrand *et al.*1995). Innervation of the tooth begins during the bud stage of development and this is also at approximately the same time point when the mesenchyme expresses odontogenic potential (Luukko *et al.* 1997). Due to this coordinated timing this has resulted in the suggestion of a role for nerves in the regulation of odontogenesis (Luukko *et al.* 1996; Hildebrand *et al.*1995). However, the location of GDNF and associated receptors appears independent of the presence of the local nerve supply (Hellmich *et al.* 1996; Luukko *et al.* 1996; Lukko *et al.* 1997), indicating that NTGF secretion within the dental papilla may not necessarily emanate from the trigeminal nerve and may originate from the non-neuronal DPC/MSC. Specifically, GDNF, GFRα1 and RET expression are overlapping within sub-odontoblast and odontoblastic regions during late bell stage and early postnatal days when the polarisation and differentiation of odontoblasts occurs (Table 1.2, pg 35) suggesting a role for GDNF during odontogenesis and dentinogenesis. Indeed NGF and GDNF have been suggested to direct the growth of the trigeminal nerve into the dental papilla at the bud stage, however a causative effect of GDNF or NGF has not been definitively demonstrated (Kvinnsland *et al.* 2004; Luukko *et al.* 2008; Lillesaar *et al.* 2003). Moreover, the retrograde transport of NGF and GDNF occurs away from the dental pulp into the main trigeminal nerve (Thoenen *et al.* 1988; Kvinnsland *et al.* 2004).



(i) **(ii)** **(iii)**

Figure 1.7 Innervation of the head and neck region and teeth. (i) The trigeminal nerve is the sensory nerve to the face also called cranial nerve five (Cn V). It has three main, afferent branches from the face; known as Ophthalmic **Va**. Maxillary **Vb** and Mandibular **Vc**.The Vb and c innervates the teeth **(ii)** Schematic showing gross anatomy of the teeth and entry of the nerve fibres into the dental pulp via the root canals **(iii)** Trigeminal nerve density in peripheral pulp and dentine of adult rat molars is greatest near the tip of the pulp horn on one side (**) and is less dense on the other side (*). The asymmetric density decreases towards mid-crown, and the innervation is missing from the dentine at the floor of the crown. Nerve fibres were labelled by radioactive axonal transport and autoradiographic detection (modified from Byers and Kish, 1976; Byers, 1984). Bars = 100 nm. From Byers *et al.* (2003).

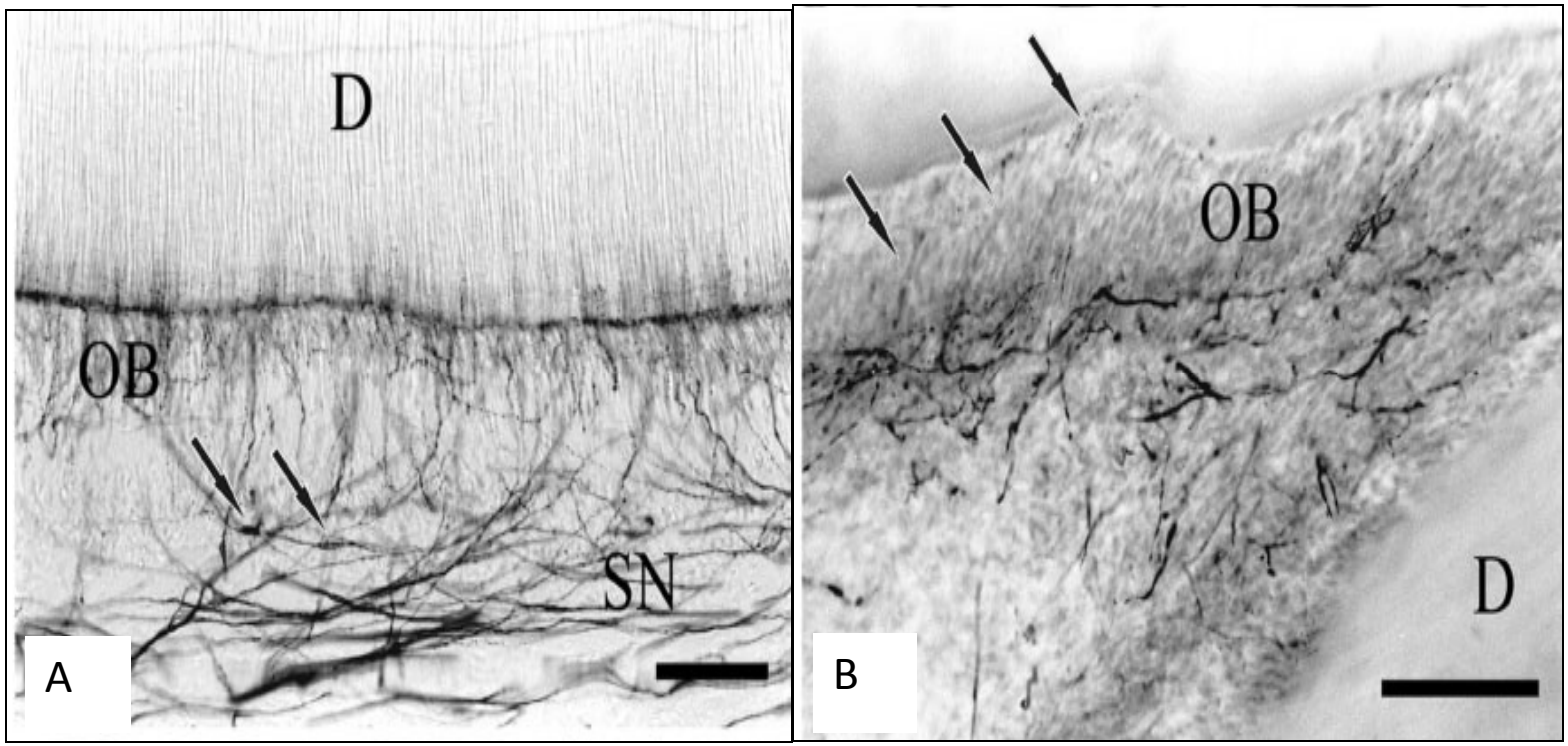


Figure 1.7.1 Demonstration of nerve fibres in (A) human teeth using immunohistochemical staining for p75^{NTR} and (B) rat teeth with immunohistochemical staining for Calbindin (CB). (**A**) The nerve fibres extend from the subodontoblastic nerve plexus (**SN**) towards the odontoblast layer (**OB**). Arrows indicate the immunopositive Schwann cells. Reproduced from Maeda (1996), Bar = 100 nm. (**B**) Thin CB-immunopositive nerve fibers penetrate into the predentin beyond the odontoblast cell layer (**OB**) (arrows) **D**: dentine. Bar = 100 nm, from Miyawaki *et al.* (1997).

GDNF is therefore detected during tooth development (Nosrat *et al.* 1998, 2002; Lukko *et al.* 1997; Fried *et al.* 2000) and up-regulated during repair in other tissues (Eberling *et al.* 2009; Cheng *et al.* 2008). GDNF up-regulation from DPC or MSC may promote repair by modulating nervous and immune systems and mineralised tissue responses. Neither GDNF nor other GFLs have been studied in respect to their direct effects on the functioning within non-neuronal physiology of mineralised tissues until recently (this study; Gale *et al.* 2011; 2012). Indirect evidence however demonstrates that GDNF is likely to be important during tooth development. Indeed GDNF null mice fail to form dentine and enamel, despite trigeminal nerve development being unaffected (De vincente et al. 2002). Interestingly, Waardenburgs syndrome (resulting from Pax3/SOX10 mutations), has a cross over in definition and symptoms with Hirschsprung's disease where ~50% of Hirschsprung cases have inactivating mutations of RET (Tassabehji *et al.* 1993; Wang *et al.* 2010b; Yang *et al.* 2012; Bondurand *et al.* 2007; Pelet *et al.* 1998) and phenotypes of both diseases may present with missing teeth and defects in craniofacial and limb bones (Eigelshoven *et al.* 2009; Bandyopadhyay *et al.* 1999; Zelzer and Olson 2003; Pingault *et al.*1998; Parisi 2002). These data suggest that GDNF-RET expression within the developing dental papilla may reflect the importance of this signalling complex in neural crest derived cells during odontogenesis, dentinogenesis, craniofacial and long bone development.

MGIF (murine glial cell-derived neurotrophic factor inducible transcription factor) is homologous to TIEG (TGF-beta-inducible early-response gene) (Yajima *et al.* 1997; Subramaniam *et al.* 2005; Consales *et al.* 2007; Hefferan *et al.* 2000) and is implicated in osteoblast differentiation. Furthermore upstream of GDNF intracellular signalling, the Lef-1 transcription factor, is reported to stimulate odontoblast and osteoblast differentiation (Yokose and Naka 2010; Hoeppner *et al.* 2011; Miletich and Sharpe 2003) which has a putative consensus site for binding to

the GDNF promoter (Tanwar *et al.* 2010). Taken together, these data further suggest that GDNF may play a role in osteoblast and odontoblast differentiation during development and repair.

1.4. Aims of this study

Hypothesis: GDNF affects non-neuronal/mesenchymal dental pulp cell (DPC) behaviour and may promote the regenerative responses in mineralised tissues.

The aims of the study were to investigate the expression and action of GDNF within *in vitro* culture models of mesenchymal cells capable of forming mineralised tissues. For this purpose, DPC, BMSC and cranial osteoblast-like cells were used.

Research objectives

a) To identify suitable DPC culture models and compare with BMSC and osteoblast-like cultures to allow the study of GDNF action *in vitro*.

b) To study cellular proliferation, differentiation and *in vitro* mineralisation in response to GDNF *in vitro.*

c) To assess the effects of GDNF under conditions reflecting some aspects of the inflammation and infection processes that occur during disease and repair.

STAGES OF TOOTH DEVELOPMENT						
Transcript detected	**Bud stage**	**Cap stage**	**Bell stage**	**Postnatal/neonatal**	**Postnatal**	**References**
GDNF	Facial cartilages; Jaw mesenchyme Dental epithelium	Jaw, mesenchyme, Dental papilla; Dental follicle; Dental epithelium	Dental papilla;Pre-odontoblasts	Pre-odontoblasts, odontoblasts, Sub-odontoblastic mesenchyme; Sub-odontoblastic layer	Odontoblast and Sub-odontoblast region	(Nosrat et al. 2002; Luukko et al. 1997; Nosrat et al. 1998; Fried et al. 2000)
RET	Dental mesenchyme	Dental papilla mesenchyme	Dental papilla mesenchyme	Sub-odontoblastic region and odontoblastic region	Not reported or detected	(Luukko et al. 1997; Nosrat et al. 1997)
GFRα1	Not reported or detected	Dental and outer epithelium	Dental and outer epithelium;Pre-odontoblasts and sub-odontoblastic region	Odontoblasts and Sub-odontoblast region Pre-odontoblast region	Not reported or detected	(Luukko et al. 1997; Nosrat et al. 1997)

Table 1.2 GDNF, RET, GFRα1, NGF and p75NTR transcripts detected within the developing tooth. The data cited above was based on different *in vivo* models; Luukko *et al.* (1997) and Nosrat *et al.* (1997) used murine, Nosrat *et al.* (2002) used human and Hildebrand *et al.* (1995), Byers *et al.* (1990), Mitsiadis *et al.* (1992) used rat dental tissues.

STAGES OF TOOTH DEVELOPMENT						
Transcript detected	**Bud stage**	**Cap stage**	**Bell stage**	**Postnatal/neonatal**	**Postnatal**	**References**
NGF	Not reported or detected	Dental follicle, outer epithelium	Pre ameloblast, Pre odontoblasts	Not reported or detected	Not reported or detected	(Hilldebrand et al. 1995; Mitsiadis et al. 1992)
P75^{NTR}	Dental epithelium	Enamel epithelium, Dental papilla (transient expression)	Dental papilla (transient expression)/pre odontoblasts	Sub odontoblastic	Sub-odontoblastic	(Byers et al. 1990; Hildebrand et al. 1995; Mitsiadis et al. 1992)

Table 1.2 continued GDNF, RET, GFRα1, NGF and p75NTR mRNA detection within the developing tooth. The data cited above was based on different *in vivo* models; Luukko *et al.* (1997) and Nosrat *et al.* (1997) used murine, Nosrat *et al.* (2002) used human and Hildebrand *et al.* (1995), Byers *et al.* (1990), Mitsiadis *et al.* (1992) used rat dental tissues.

CHAPTER 2 MATERIALS AND METHODS

2.1 Cell isolation

2.1.1 Culture of bone marrow stromal/stem cells (BMSC)

Femurs of male Wistar rats (Charles River Laboratory, UK) aged 4-6 weeks old were dissected out and the muscle stripped from the bones using a scalpel, scissors and forceps. The femurs were then placed into universal tubes containing medium consisting of αMEM (Biosera) supplemented with 10 % FBS (Fetal Bovine Serum), penicillin (100 µg/ml), streptomycin (100 µg/ml) and (2.5 µg/ml Amphotericin B (Sigma-Aldrich). The femurs were sprayed with 70 % ethanol and the ends (epiphyses) were removed using a scalpel blade and were then flushed through with 10 ml medium using a needle (~22gauge) and 20 ml syringe (BD sciences). The bone marrow was collected into a 50 ml centrifuge tube (Falcon). Cells were collected by centrifugation (5804R, Eppendorf) at 800 rpm for 5 mins and pellets were re-suspended in 5 ml of 10 % FBS supplemented αMEM medium. Cell suspensions were seeded into 75 cm^2 flasks (Nunc) containing 10 % FBS supplemented αMEM (Biosera).

2.1.2 Dental pulp extraction

Incisors (upper and lower) were dissected from 4-6 week old male Wistar rats. The extracted teeth were briefly submerged in 70% ethanol for disinfection and then immediately placed into universal tubes containing 20% FBS / αMEM (Biosera) supplemented with penicillin (100 µg/ml) streptomycin (100 µg/ml) and (2.5 µg/ml) Amphotericin B (Sigma-Aldrich). Teeth were placed onto a glass slide within a petri

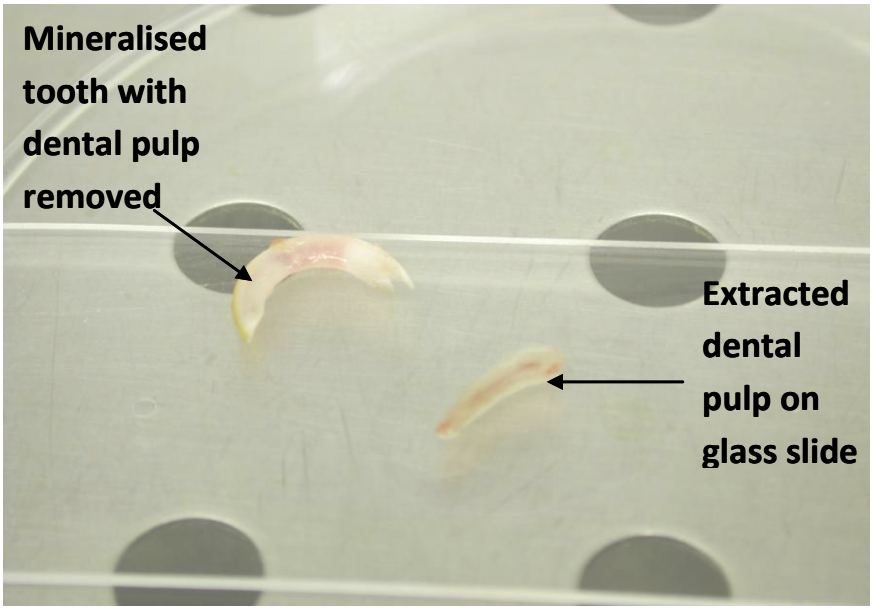
dish and the pulp removed from the tooth using forceps and a scalpel (Figure 2A). Medium was added to the pulp tissue to prevent it from becoming desiccated.

2.1.3 Explant-derived dental pulp cells (DPC)

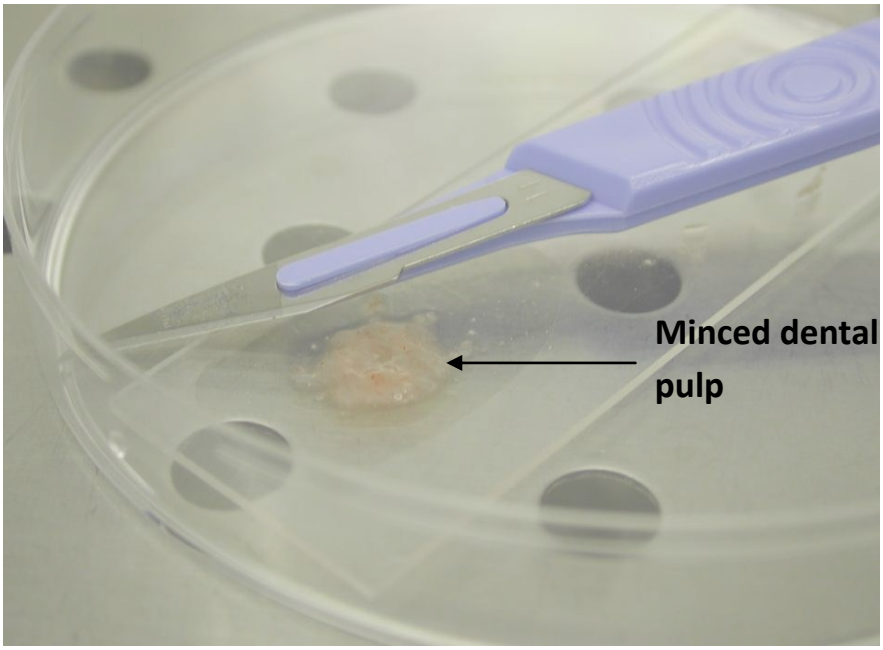
Pulpal tissue was placed onto a sterile glass slide and chopped coarsely to ~ 2mm^2 pieces using a scalpel, prior to transfer to culture flasks containing 1 ml 20 % FBS supplemented αMEM (Figure 2B, pg 42). These explants were gently pressed onto the bottom of the flask using the flat side of the scalpel blade. Cells were incubated overnight in a humidified atmosphere at 37^{o}C with 5 % CO_2 and the following day a further 4 ml of 20 % FBS supplemented αMEM was added to the culture. Medium was changed every 3 days until cultures reached 80 - 90 % confluence.

2.1.4 Enzymatically-derived DPC

Extracted pulp was minced (see Figure 2A, pg 42) using a scalpel and then transferred to a 15 ml centrifuge tube (Falcon) containing 5 ml trypsin [2.5g/l of trypsin in 0.38g/l of EDTA (Invitrogen)] and digested for 30 mins at 37^{o}C. Tissue samples were agitated every 5 mins using a 1 ml Gilson pipette tip to aid dissociation, then after 30 mins, 5 ml 10 % FBS supplemented αMEM was added to halt the action of the trypsin. Subsequently the cell suspension was passed through a 70 μm filter (Millipore) and centrifuged for 5 min at 800 rpm. The supernatant was removed using vacuum aspiration and the cell pellet re-suspended in 1 ml of αMEM and seeded into a 25 cm^2 flask (Nunc). Cells were incubated overnight humidified at 37^{o}C, 5% CO_2 to enable attachment and the following day a further 4 ml of 20% FBS supplemented αMEM was added to the culture. Medium was changed every 3 days until cultures reached 80 – 90 % confluence.



A



B

Figure 2 (A) Dental pulp was extracted from the tooth. (B) Dental pulp was minced using a scalpel blade prior to enzymatic digestion.

2.1.5 Cell lines culture

A C6 glioma cell line of isolated rat glial cells derived from a chemically induced glial tumour by Benda *et al*. (1968), a mouse MDPC-23 cell line isolated from fetal mouse dental papillae (Hanks *et al.* 1998) and the mouse osteoblast MC3T3-E1 cell line established from neonatal calvarial bone of C57BL/6 mice (Wang *et al.* 1999) were established from cryopreserved stocks (original sources of cell lines were ATCC except MDPC-23 cells which were kindly donated by Jacques Nör's Laboratory, University of Michigan, US).

2.1.6 Cell culture

Explant-derived and enzymatically-derived DPC were cultured as above (**2.1.3** and **2.1.4**), MC3T3-E1 cells and BMSC were cultured using the same reagents except 10% FBS (Biosera) was used. MDPC-23 and C6 glioma cells were cultured using DMEM (Biosera); 10% FBS, Penicillin (100 μ/ml) streptomycin (100 μg/ml), L-glutamine (2 mM) (Sigma-Aldrich). All cells were cultured in either 25 cm^2 (primary cells, passage 0-1) or 75 cm^2 flasks (Falcon). Cells were detached upon reaching 80-90 % confluence using trypsin/EDTA. Cell suspensions were transferred to tubes (Falcon) size 15 ml or 50 ml respectively, depending on flask size and centrifuged at 800 rpm for 5 mins to pellet the cells. Supernatants were removed by using a vacuum pump and cells re-suspended in 1 ml of αMEM for counting or subculture. DPC and BMSC were expanded and used at passage 2-4 for subsequent experiments.

2.1.7 Cell counting

A 100 µl sample of cell suspension was mixed with 100 µl Trypan blue (Sigma); the unstained viable cells were counted using a Neubauer haemocytometer slide under a variable relief contrast (VAREL) microscope (Zeiss Axiovert 25). A minimum of three counts per sample were obtained and average cell number was calculated. Cell suspensions were then diluted accordingly and seeded at densities and volumes presented in Table 2, pg 45- 46.

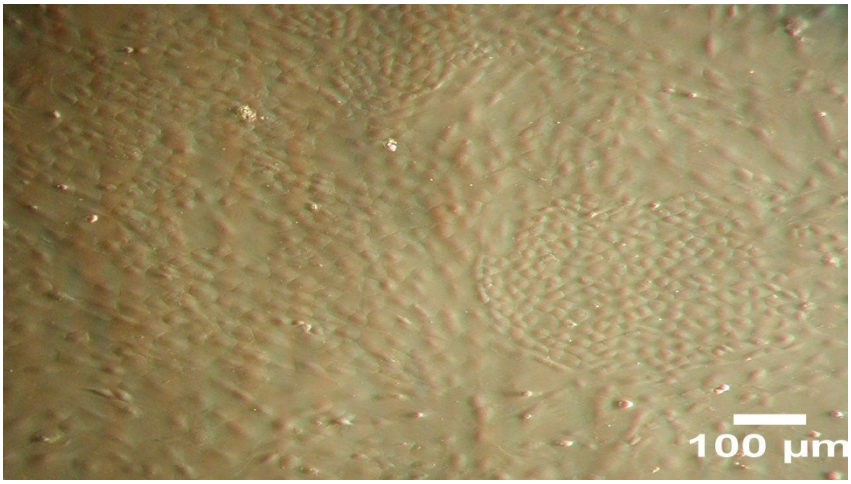
2.1.8 Preparation of serum-free medium

Bovine serum albumin (BSA) supports cell cultures by acting as a buffer, antioxidant and carrier for proteins, lipids and minerals (He and Carter 1992; Nicholson *et al.* 2000). BSA (fraction V, Sigma) was reconstituted with dH_2O to a 1 % solution (w/v), then heat inactivated at 56^{o}C for 30 mins. This solution was filter sterilised using a 0.2 µm filter (Millipore) and stored at -20 ^{o}C prior to use. Serum-free medium consisted of 0.1 % BSA in αMEM, penicillin (100 µg/ml) streptomycin (100 µg/ml) and Amphotericin B (2.5 µg/ml) (Sigma-Aldrich). After seeding into an appropriate tissue culture vessel the cells were incubated in a humidified 5% CO_2 incubator at 37^{o}C.

2.2 Preparation and storage of GDNF

Human GDNF is highly homologous to mouse or rat GDNF (Wang *et al*. 1998). rh GDNF [recombinant human GDNF produced in *E.coli* (Amgen)] was supplied at a concentration of 10 mg/ml and aliquots of 1 µg/ml in 0.1 % BSA/αMEM were stored at -80^{o}C. On thawing GDNF was diluted with medium to the relevant concentrations used for the *in vitro* experiments. Before adding GDNF to the cultures the medium was

replaced with freshly prepared medium, containing either 10 % FBS or 0.1 % BSA supplemented αMEM (serum-free medium). Cells were then cultured for 48 hours, except for the mineralisation assays where cells were cultured for 4 days to obtain confluence before osteogenic conditions were applied. Cells were incubated in a humidified 5 % CO_2 incubator at 37^{o}C, for periods shown in Table 2, pg 45- 46.



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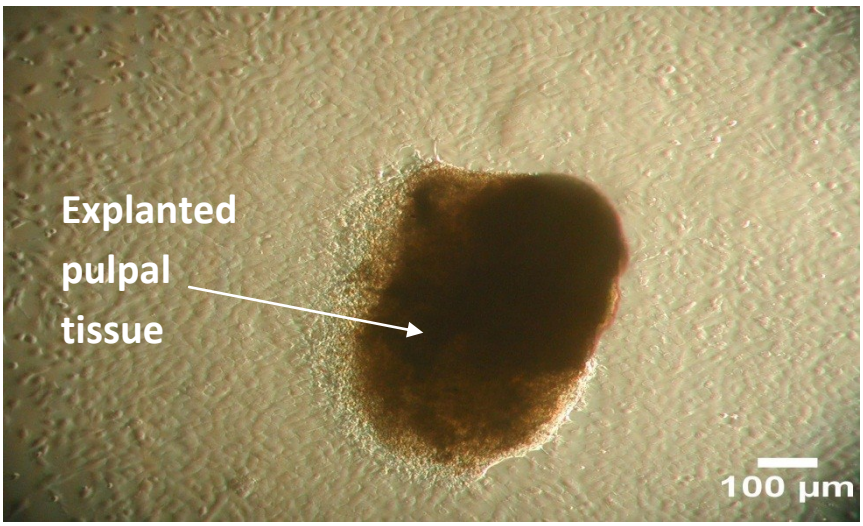


B

Figure 2.1 Phase-contrast images of (A) enzymatically-derived and (B) explant-derived DPC.

2.2.1Preparation and storage of tumour necrosis factor alpha (TNFα)

Human, rat and mouse TNFα are highly homologous, the sequences display 80-89% homogeneity (Kwon *et al.* 1993; Marmenout *et al.* 1985). rh TNFα [produced in *E.coli* (Peprotech)] was centrifuged and reconstituted using 0.1 % BSA/αMEM. 1 µg/ml aliquots were stored at -80^{o}C. On thawing, TNFα was serially diluted using serum-free or serum supplemented medium with or without GDNF (100 ng/ml). The same protocol was used as for the GDNF experimental analysis (section **2.2**; see Table 2, pg 45-46).

2.2.2 Preparation and storage of nerve growth factor beta (NGF-β)

Mouse, rat and human NGF are highly homologous (Hallböök *et al*. 1991; Ullrich *et al.* 1983). NGF has three subunits, alpha, beta and gamma (Baker *et al.* 1974). It is the beta subunit that exerts the majority of the biological effects of NGF (Shooter 2001). rh NGF-β [produced in *E.coli* (Peprotech)] was reconstituted at 100 µg/ml in 0.1 % BSA/αMEM and 1 µg/ml aliquots stored at -80^{o}C. On thawing NGF was diluted in either serum-free or serum supplemented medium and the same protocol was used as for the GDNF experiments (section **2.2**; see Table 2, pg 45-46).

2.2.3 WST-1 viable cell number assay

To determine the number of viable cells the WST-1 assay (Roche) was performed. This assay was originally developed by Ishiyama *et al*. (1993) (see also Scheven *et al.* 2009). In accordance with the manufacturers guidelines 10 µl of WST-1 reagent per 100 µl of medium was incubated with cell cultures for 60 mins at 37^{o}C. In viable, metabolic active cells, WST-1 is converted to form a coloured soluble formazan dye by dehydrogenases. A universal plate reading spectrophotometer (ELx800, Biotek)

was used to quantify the amount of formazan dye formed at 450/630nm. The 630 nm wavelength was used as a reference wavelength, this represents background readings that was subtracted from the test spectrophotometry reading. The linear relationship between the formazan dye product and seeding density (up to 50,000 cells per well) under differing serum conditions was subsequently used to determined the number of viable cells under different experimental conditions.

2.2.4 RET receptor inhibition (RPI-1)

RPI-1 (1, 3-Dihydro-5, 6-dimethoxy-3-(4-hydroxyphenol) methylene)-2H-indol-2-one) (Merck Chemicals Ltd) was used to inhibit RET. RPI-1 was reconstituted to 5 mg/ml in DMSO, subsequently aliquots at 300 μM/0.2 % DMSO/ αMEM were stored at -20^{o}C prior to use. The RPI-1 inhibitor was serially diluted in serum-free medium with or without GDNF (100 ng/ml) and incubated for a further 48 hours with cell cultures.

2.2.5 GFRα1 receptor inhibitor (PI-PLC)

PI-PLC hydrolyses the extracellular portion of the GFRα receptor (and other proteins that are GPI-linked glycosyl-phosphatidylinositol) from the membrane surface (Low 1989). Thus PI-PLC cleaves phosphatidylinositol and this removes the extracellular receptor protein and hence the binding site for GDNF. PI-PLC (Sigma) 62.5 units/ml was stored at 2-8^{o}C. Treatment of the cells was started 1 hour prior to addition of GDNF (to allow adequate time for cleavage of the receptor from the cell membrane). The medium was then removed and replaced with respective concentrations of PI-PLC (as above) in serum-free medium with or without GDNF (100 ng/ml) and incubated for a further 48 hours with cell cultures (see Table 2, pg 45- 46).

Table 2 Summary of experimental assays and conditions used.

Assay Applied	Experiment/Analysis	Culture ware and seeding density used	Duration/time point examined	Detection method	Additional reagents prepared
WST-1 viable cell number	Standard curve determination	96 well plates; $5x10^3$ cells in 200 µl per well	30 mins, 24 and 48 hours	Spectrophotometry at 450/630 nm	0.1 % BSA
WST-1 cell viability	Comparison of serum conditions all cell types	96 well plates; $5x10^3$ cells in 200 µl per well	48 hours, 3, 5 10 days	Spectrophotometry at 450/630 nm	0.1 % BSA
WST-1 cell viability	Effect of GDNF On all cell types	96 well plates; $5x10^3$ cells in 200 µl per well	48 hours	Spectrophotometry at 450/630 nm	GDNF 0.1 % BSA
WST-1 cell viability	Effect of *S.mutans* on DPC and MC3T3-E1 cells	96 well plates; $5x10^3$ cells in 200 µl per well	48 hours	Spectrophotometry at 450/630 nm	*S.mutans*
WST-1 cell viability	Effect of TNFα on DPC and MC3T3-E1 cells	96 well plates; $5x10^3$ cells in 200 µl per well	48 hours	Spectrophotometry at 450/630 nm	TNFα; GDNF; 0.1 % BSA
WST-1 cell viability	Effect of NGF on DPC	96 well plates; $5x10^3$ cells in 200 µl per well	48 hours	Spectrophotometry at 450/630 nm	NGF; 0.1 % BSA
WST-1 cell viability	Inhibition of RET and cleavage of GFRα receptors	96 well plates; $5x10^3$ cells in 200 µl per well	48 hours	Spectrophotometry at 450/630 nm	PI-PLC; GDNF; 0.1 % BSA; RPI-1

Assay	Experiment	Seeding density	Duration/time point	Detection	Additional reagents prepared
ALP	ALP levels for all cell types	96 well plates; $5x10^3$ cells in 200 µl/ well	48 hours	Spectrophotometry at 405/630 nm	Buffer for pNPP tablets
BrdU	Proliferation assay for DPC and MC3T3-E1 cells	12 well plates; $5x10^4$ cells in 1ml/well	24 and 48 hours	Light microscopy. Cell counts calculated using image J cell counter add in.	Buffers; fixative; antibodies; GDNF
Live dead	Number of live and dead cells determined	12 well plates; $5x10^4$ cells in 1ml/well	24 and 48 hours	Fluorescent microscopy. Cell counts	BrdU; Acridine orange; 0.1 %BSA; ethanol fixative
Caspase-3/-7	Levels of apoptosis determined for DPC	96 well plates; $5x10^3$ cells in 200 µl/well	48 hours	Luminometer	0.1 % BSA.
LDH	Levels of lactate dehydrogenases (LDH) determined the levels of cell death for all cell types	96 well plates; $5x10^3$ cells in 100 µl/ well	48 hours	Spectrophotometry at 490/630 nm	0.1 % BSA. Kit preparation
Mineralisation	Levels of calcified ECM for MDPC-23, DPC and MC3T3-E1 cells	48 well plates; $2.5x10^4$ cells at 200 µl/well. 35mm dishes; $5X10^4$	7, 24 and 48 days	Spectrophotometry 450/630 nm	Alizarin Red 2 % solution. Ammonium hydroxide; 0.1 % BSA

Table 2 continued. Summary of experimental assays and conditions used.

2.2.6 BrdU (5-Bromo-2'-deoxy-uridine) assay

Cells were cultured within multiwell plates (see Table 2, pg 45- 46) and after 24 hours medium was replaced with serum-free or serum supplemented medium with or without GDNF (100 ng/ml). After a further 24 or 48 hours the BrdU assay was performed using the 5-Bromo-2'-deoxy-uridine labelling and detection kit II (Roche) according to the manufacturer's guidelines. Cells were grown in 12-well tissue culture plates, incubated with the DNA-intercalating BrdU for 60 mins. The cells were then fixed and fractured to later allow antibody access to the BrdU labelled DNA. The fixation procedure included fixing cells with 70 % ethanol for 20 mins at room temperature and followed by fixation using 50 mM glycine/70 % ethanol at pH 2.0 for 20 mins. Finally cells were fractured by incubating at - 20^{o}C for 20 mins. The DNA labelled 5-Bromo-2'-deoxy-uridine was detected using antibodies and visualised by ALP conjugated secondary antibody reacted with BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidinium in dimethylformamide). Mayer's haematoxylin was used as a counterstain and a variable relief contrast Zeiss Axiovert 25 microscope was used to observe BrdU labelled nuclei that appeared brown/black. BrdU stained and non-stained nuclei were counted in at least nine fields of view for each test condition.

2.2.7 Live and dead assay

Cultures were washed with 500 µl PBS and then ethidium bromide (EthBr) and flurochrome acridine orange (AO) were added (4 µM in PBS) for 5 mins. Dead cell controls were fixed cells using 70 % ethanol/PBS and all fixed (dead) cells appeared orange. EthBr crosses damaged cell membranes and intercalates with DNA and emits

fluorescent light appearing orange. AO binds to nuclear DNA and all live cells take up the AO stain across the cell membrane and nuclei appear green however dead cells with ruptured/leaky membranes take up EthBr and over stain the green AO to appear orange. Cells were visualised and counted using a Nikon Eclipse fluorescent microscope with 480-520 nm filter (530 nm max emission for AO and 590 nm for EthBr). For each experiment three wells were counted per test condition and within each well three fields of view were counted and numbers of live/dead cells were recorded.

2.2.8 Lactate Dehydrogenase (LDH) assay

The LDH Cytotoxicity Detection Kit (Roche) was utilised according to the manufacturer's instructions. After 48 hours samples of cultured cells were exposed to the supplied lysis buffer for 20 mins to act as a positive control. 100 µl of supernatant was removed and incubated with the manufacturers' catalyst and dye reaction mixture for 30 mins at room temperature (RT). After 30 mins the stop reaction mixture was added at 25 µl per well and samples were placed on an orbital plate shaker for 20 seconds. The LDH levels released from damaged cell membranes were then quantified using a multiwell plate reading spectrophotometer (ELx800Biotech) at a wavelength of 490/630 nm.

2.2.9 Caspase Glo 3/7 assay

After 24 hours culture in white walled multiwell plates (Microlite; 7567 Thermoscientific) the medium was replaced and serum-free, serum supplemented and serum-free medium containing GDNF (100 ng/ml) was added to the cultures for a further 48 hours. The Caspase Glo 3/7 assay (Promega) was performed according to the

manufacturer's instructions. Caspases cleave the caspase specific DEVD amino acid sequence within the supplied luminogenic substrate to form a substrate for the luciferase enzyme that is luminescent following cleavage. Luminescence from the reaction was measured by taking continuous readings (0-120 mins) using a multiwell plate luminometer (LB96v Bethold Technologies) and data from the time point of maximum peak emission used for analysis. Plates were incubated within the luminometer at 22^{o}C.

2.3 *Streptococcus mutans (S.mutans)* analysis

Heat inactivated *S.mutans* [(*Streptococcus mutans Clarke 1924 AL*) original source from the ATCC No. 25175] were stored at -80^{o}C at 7.55 x 10^{9}/ml prior to use. On thawing the bacterial suspension was diluted to 5x10^{6} *S.mutans/*ml and further serially diluted to obtain a ratio of 1000, 100, 10 and 1 *S.mutans* per DPC or MC3T3-E1 cell. DPC or MC3T3-E1 cell cultures were seeded at 5x10^{3} per well prior to addition of bacteria in serum supplemented medium with or without 100ng/ml GDNF. After incubation for 48 hours the WST-1 assay was performed to quantify viable cells using a plate reader (ELx800 Biotek).

2.3.1 Mineralisation assay

Cells were seeded into 35 mm dishes under standard culture conditions and medium. After 4 days medium was changed to include osteogenic supplements which consisted of 10^{-7} M dexamethasone (DEX) 50 µg/ml, L-ascorbic acid, 10 mM β-glycerophosphate (Sigma) (Jaiswal *et al.* 1997). This osteogenic medium with or without 100 ng/ml GDNF was freshly prepared prior to use and changed every other day for the duration of the experiments for up to 28 days.

For serum-free mineralisation assays the cells were first seeded into 48 well plates under standard serum conditions for 24 hours. After 24 hours the medium was changed to serum-free medium containing 0.1 % BSA, osteogenic supplements with or without GDNF as described above. L- glutamine is essential for the survival and growth of fibroblasts under low serum conditions (Eagle *et al.* 1955) therefore an additional, 2mM L-glutamine was added to the osteogenic medium. After 4 weeks culture under serum-free osteogenic conditions cultures were stained with Trypan blue to assess the levels of viable cells under the experimental conditions. Culture medium was aspirated and 50 µl Trypan blue: 50 µl PBS was added to each well and viewed immediately. The staining was visualised using an inverted microscope (Zeiss Axiovert 25).

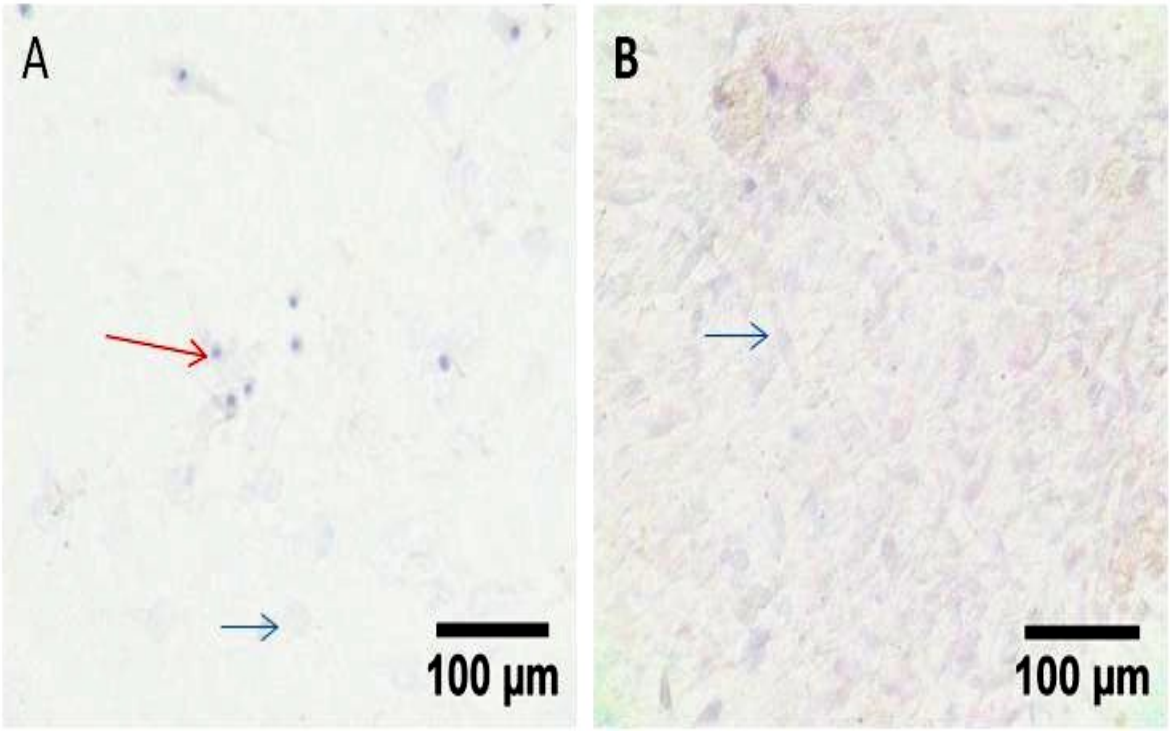


Figure 2.2 Trypan blue staining of DPC in (A) serum-free cultures and (B) serum supplemented cultures after 4 weeks culture with osteogenic supplements. Red arrow shows dead cell identified with Trypan blue staining and blue arrows indicate live cells.

Alizarin red stain (ARS) forms salts with calcium, replacing the anions in calcium (Puchtler *et al.* 1969). The pH of the ARS stain was adjusted to pH 4.1 using ammonium hydroxide. ARS was made freshly to ensure pH 4.1 was maintained due to the solubility of calcium deposits at pH 4.5-5 (Puchtler *et al*. 1969). Cultures were washed with 200 µl PBS per well, then fixed with 10 % formalin for 10 mins. Formalin was aspirated. 200 µl of 2 % ARS was added to the cultures at room temperature (RT) and agitated on an orbital plate shaker for 20 mins. The unincorporated ARS was aspirated and the cells were washed gently with PBS, then with dH_2O for 5 mins. The dH_2O was drained by inverting the plate/dish.

For the post staining of 35 mm dishes and 48-well plates, 800 µl and 400 µl of acetic acid (10 %) was added, respectively and incubated for 30 mins RT on an orbital plate shaker. Dishes and wells were scraped and contents placed into centrifuge tubes. Tubes were mixed using a vortex for 30 seconds, heated at 85^{o}C for 10 minutes and after 5 mins cooling on ice, centrifuged at 1500 rpm for 15 minutes. Then 150 µl (for dishes) or 100 µl (per well) of 10 % ammonium hydroxide was added to each sample to neutralise the acid and 150 µl from each sample was added to multiwell plates in triplicate. The concentration of ARS staining per eluted sample was determined against a standard curve using a plate reader at 405/630nm (ELx800, Biotek).

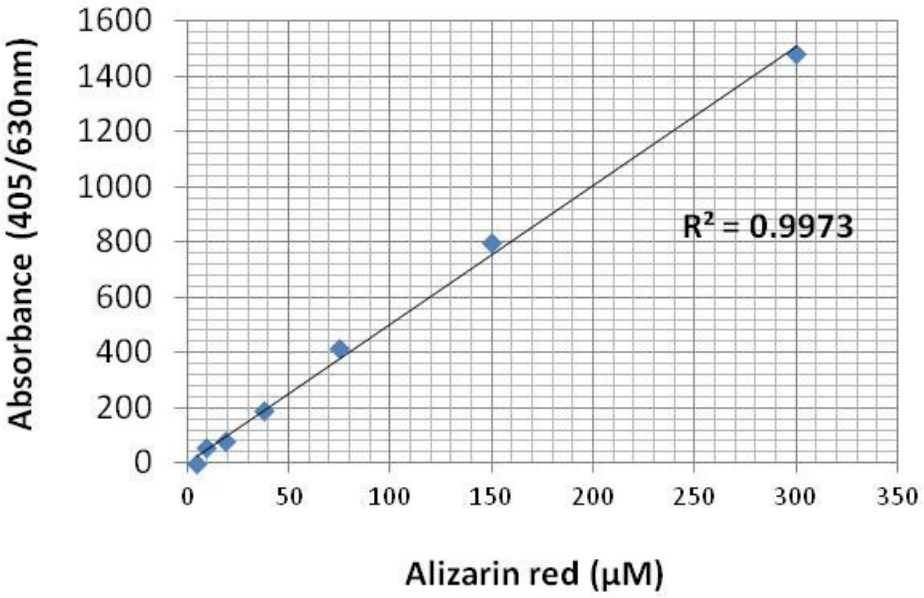


Figure 2.3 Linear standard curve for ARS. Results expressed as average absorbance unit (n=2).

2.3.2 Alkaline phosphatase (ALP) assay

Cells were rinsed using 100 µl PBS per well of multiwell plates (see Table 2.0), then lysed using 0.1 % triton X100. pNPP (*p*-Nitrophenyl Phosphate) tablets were added to DEA buffer to make 1 mg/ml pNPP and incubated at RT for 10 mins. The DEA buffer contains Mg^{2+} and Zn^{2+} ions enabling optimum activity of alkaline phosphatase. The DEA buffer stock at 10X concentration consisted of 500 mg $MgCl_2$, 400 mls dH_2O and 48.5 ml diethanolamine, pH was adjusted to pH 9.8 using concentrated NaOH or HCl. pNPP is hydrolysed by ALP to the yellow coloured p-nitrophenol and its levels were determined using a multiwell plate reader at 405/630 nm.

2.4 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Gene expression was investigated using semi-quantitative RT-PCR (sqRT-PCR) analysis. RNA isolation was performed using the RNeasy mini-kit (Qiagen) with DNase treatment according to the manufacturer's instructions. Dissected tissue samples were initially disrupted using an Ultraturrax homogeniser (Netzgerat T8.01 aboratiechnik) within 700 µl of lysis buffer (10 µl β-Mercaptoethanol per 1ml buffer RLT). 700 µl lysis buffer was added per confluent (80 - 90 %) cultures in 75 cm^2 flasks followed by vigorous pipetting to detach and disperse the cells. After ~ 5 mins 70 % ethanol was added to all samples and two aliquots per sample of the total volume (1400 µl) pipetted into RNeasy mini- column sequentially (the total column volume per aliquot does not exceed 700 µl) and centrifuged for 15 seconds at 10,000 rpm. The flow through was discarded after each centrifugation. All centrifugation steps were performed using an Eppendorf 5415 D centrifuge (Eppendorf, UK). Contaminating DNA was removed by performing the DNase digestion steps; 350 µl RW1 manufacturers buffer was added per column, centrifuged at 10,000 rpm for 15 secs and flow through discarded. 15 µl DNase I (inhibitor) and 60 µl RDD manufacturer's buffer was added per column and incubated at RT for 15 mins. Next 350 µl RW1 buffer added to each column and centrifuged at 10,000 rpm for 15 secs, then flow through was discarded. Subsequently, 500 µl RPE buffer was added to each column within a new collecting tube and centrifuged at 10,000 rpm for 15 secs, then flow through was discarded. This step was repeated except centrifugation took place for 2 mins then columns were placed into new collecting tubes and centrifuged for a further 1 min at full speed. The RNA was eluted by placing the column into an Eppendorf and adding 30 µl RNase free water. RNA was collected by

centrifugation for 1 min and the collected RNA placed onto ice whilst purity and quantity were assessed. Purity and quality of the RNA was determined by measuring absorbance values at 260 nm (A260) and 280 nm (A280) using a Biophotometer (Eppendorf, UK) and by visualisation on agarose gels (see section **2.4.1,** pg 55). An absorbance ratio of between 1.8 to 2.1 and distinct double bands (18S and 28S) of RNA observed on gels signified high purity and quality of the isolated RNA. RNA was stored at - 80^{o}C prior to use.

RNA was reversed transcribed using the Omniscript Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. 50 ng - 1 µg RNA was incubated with 2 µl sterile molecular grade water (Merck) at 65^{o}C for 5 min to denature RNA secondary structure and quenched on ice prior to use. A master mix containing 1X RT buffer, 0.5 mM dNTPS, 1 µM Oligo-dT primer, 0.5 u/µl RNase inhibitor, 0.2 u/µl omniscript reverse transcriptase were combined and added to the RNA solution making a total volume of 20 µl per sample. This reaction mix was incubated in a Mastercycler gradient thermal cycler (Eppendorf, UK) for 60 mins at 37^{o}C and then 95^{o}C for 5 mins.

YM-30 Microcon centrifugal filter devices (Millipore) were used to purify and concentrate resultant cDNA. 20 µl of cDNA in solution was added to 500 µl in molecular biology grade water, transferred to YM-30 microcon assemblies and centrifuged (Centrifuge 5415D, Eppendorf) for 6.5 minutes at 10,000 rpm (Centrifuge 5415D, Eppendorf). The eluate was discarded and this process was repeated until approximately 30 µl cDNA in solution remained on the surface membrane of the filter. The microcon centrifugal filter device was inverted in an Eppendorf tube and centrifuged at 1000 rpm (Centrifuge 5415D, Eppendorf) for 3 mins to collect the cDNA.

The purity and concentration of the cDNA were analysed by determining absorbance readings at 260 nm and gel electrophoresis (see section **2.4.1**). cDNA was stored at -20^{o}C prior to use.

50 ng of the cDNA, primers (25 mM) (see Table 1.0) and REDTaq®ReadyMix™ PCR Mix (with $MgCl_2$) were gently combined and transferred to a Mastercycler gradient thermal cycler (Eppendorf, UK) and amplified using repeated cycles. Reactions were amplified following an initial denaturation step of 5 min at 94 °C, a typical amplification cycle consisted of denaturing 94 °C for 20s, annealing (temperatures shown in Table 2.1, pg 58) for 20 s and extension at 68 °C for 20 s ending with a 10 min extension at 72 °C. Following the designated number of cycles (Table 2.1), 6 µl of the reaction mix was removed. Primers (Table 2.1, pg 58) were designed from NCBI mRNA sequences Primer-3 Primer design software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The PCR products were analysed by agarose gel electrophoresis (see below section **2.4.1**).

2.4.1 Agarose gel electrophoresis and image analysis

Non-denaturing agarose gels (1 % w/v for RNA and 1.5 % w/v for PCR) were generated from lyophilised agarose powder (Helena Biosciences, UK) dissolved in 1X Tris acetate EDTA (TAE) buffer, pH ~8.3, (Helena Biosciences UK). Mixtures were heated in a microwave (Samsung, TDS) and the solution was poured into gel casting trays. Ethidium bromide 50 µg/ml (Sigma) or 1X SYBR gold (Molecular probes) nucleic acid stains along with combs were added to enable nucleic acid visualisation and to form sample-loading wells prior to gel solidification at room-temperature.

Samples of 6 µl per well were added and separated by gel electrophoresis at 50-100V in agarose gels submerged under 1x TAE (Tris-Acetate EDTA) running buffer with the Hyperladder IV (Bioline, UK) as a nucleic acid standard.

Images of RNA and PCR products were visualised by UV illumination and captured using a G:BOX system with the gene tools software (Syngene) used to perform the densitometric analysis. Density values were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene (Robbins and McKinney 1992) control value by dividing target gene sample volume density by respective GAPDH volume density.

2.5 Immunocytochemistry

Cells were seeded onto multispot slides (Hendley) at a density of 1.5 $x10^4$ Glioma cells, 2 $x10^4$ DPC, 2 $x10^4$ MC3T3-E1 cells, or 2.5 $x10^4$ BMSC and incubated for 24 hours in standard serum conditions. Cells were then gently washed with warm (37^oC) PBS/1% BSA, fixed with cold acetone at - 20^oC for 5 mins, followed by quenching of endogenous peroxidase using 3 % H_2O_2/PBS for 30 minutes. After washing with PBS/1 % BSA, the slides were "blocked" with 20 % goat serum/1 % BSA/PBS for 1 hour to prevent non-specific antibody binding. Primary antibodies against Gfrα1 (sc10716, polyclonal rabbit; SantaCruz) or against RET (sc167, polyclonal rabbit; Santa Cruz) at 2 µg/ml/1 % BSA/PBS and controls of normal rabbit serum (Sigma) 14 µg/ml/1 % BSA/PBS or 1 % BSA/PBS were added and incubated for 1 hour at RT. The substitution of 1 % BSA/PBS in place of primary antibody was used to determine the specificity of the secondary antibody (shown in Figure 2.4 pg 59).

Biotinylated secondary antibody (link) was added at 1/200 dilution in 1 % PBS/BSA and incubated for 1 hour at RT. Next streptavidin-HRP (label) was added 1/200 dilution and incubated for 30 minutes at RT. The SuperSensitive™ Link-Label IHC Detection System (Biogenex LP000-UL) was used to detect and visualise the antibody labelling. DAB solution (0.01g 3,3-diaminobenzidine tetrahydrochloride plus 25 µl H_2O_2 in 20 mls PBS) was added until a golden brown colour developed, subsequently, the slides were counterstained with Mayers haematoxylin for 5-15 mins. Cells were then dehydrated through sequential steps in 100 %, 95 % then 70 % alcohol and placed into xylene and mounted using XAM (BDH Laboratory). Stained cells were examined under a variable relief contrast (VAREL) microscope (Zeiss Axiovert 25)

2.6 Statistical analysis

Statistical and data analyses were performed using Microsoft Excel and Minitab (version 15 and 16). Statistical analyses comparing groups were undertaken using the Student's t test and in the case of more than two groups regression/ANOVA with Tukeys posthoc test. A P-value of < 0.05 was considered to be significant.

Gene	Primer sequence (F-forward; R-reverse) 5'➔3'	T_m	Accession no.	Product	Cycle No.
GAPDH	F-CCCATCACCATCTTCCAGGAGC;R-CCAGTGAGCTTCCCGTTCAGC	60	NM_017008	473bp	21-27
GDNF	F-GAGGAATCGGCAGGCTGCAGCTG; R-GATACATCCACATCGTTTAGCGG	60	NM_019139	364bp	33-38
rRET	F-TCAGGCATTTTGCAGCTATG; R-TGCAAAGGATGTGAAAGCAG	62.5	NM_001110099.1	393bp	35-45
Gfrα1	F-AATGCAATTCAAGCCTTTGG; R-TGTGTGCTACCCGACACATT	60	NM_012959.1	218bp	33-43
$P75^{NTR}$(NGFR)	F-CAAAGGACGGATTTCCTGAG; R-AGCTCCTGGGGAGGAAAATA	60	NM_012610	323bp	37
CD105	F-TTCAGCTTTCTCCTCCGTGT; R-TGTGGTTGGTACTGCTGCTC	60	NM_001010968	325bp	41-45
CD44	F-TGGGTTTACCCAGCTGAATC; R-CTTGCGAAAGCATCAACAAA	60	NM_012924.2	392bp	33-37
PCNA	F-TTGGAATCCCAGAACAGGAG; R-CGATCTTGGGAGCCAAATAA	60	NM_022381.2	390bp	30-33
p27kip1	F-ACGGTTCCCCGAATGCTGGC; R-CCCCCACCCAAGTTGCTTCTCT	60	NM_031762.3	483bp	30-42
IL-6	F-TGTGCAATGGCAATTCTGAT; R-GAGCATTGGAAGTTGGGGTA	60	NM_012589	312bp	33
TNFα	F-TCCGGCGGTGTCTGTGCCT; R- CGGGGCAGCCTTGTCCCTTG	60	NM_012675	369bp	35
BSP	F-ATGGAGATGGCGATAGTTCG; R-TCCACTTCTGCTTCTTCGTTC	60	NM_012587.2	307bp	34-35
Cfbα1	F-GCCGGGAATGATGAGAACTA; R-GGACCGTCCACTGTCACTTT	60	NM_053470.2	200bp	35

Table 2.1 Details of primers and assay conditions for PCR analysis. T_m indicates annealing temperature ^oC, rRET, rat RET primer sequences.

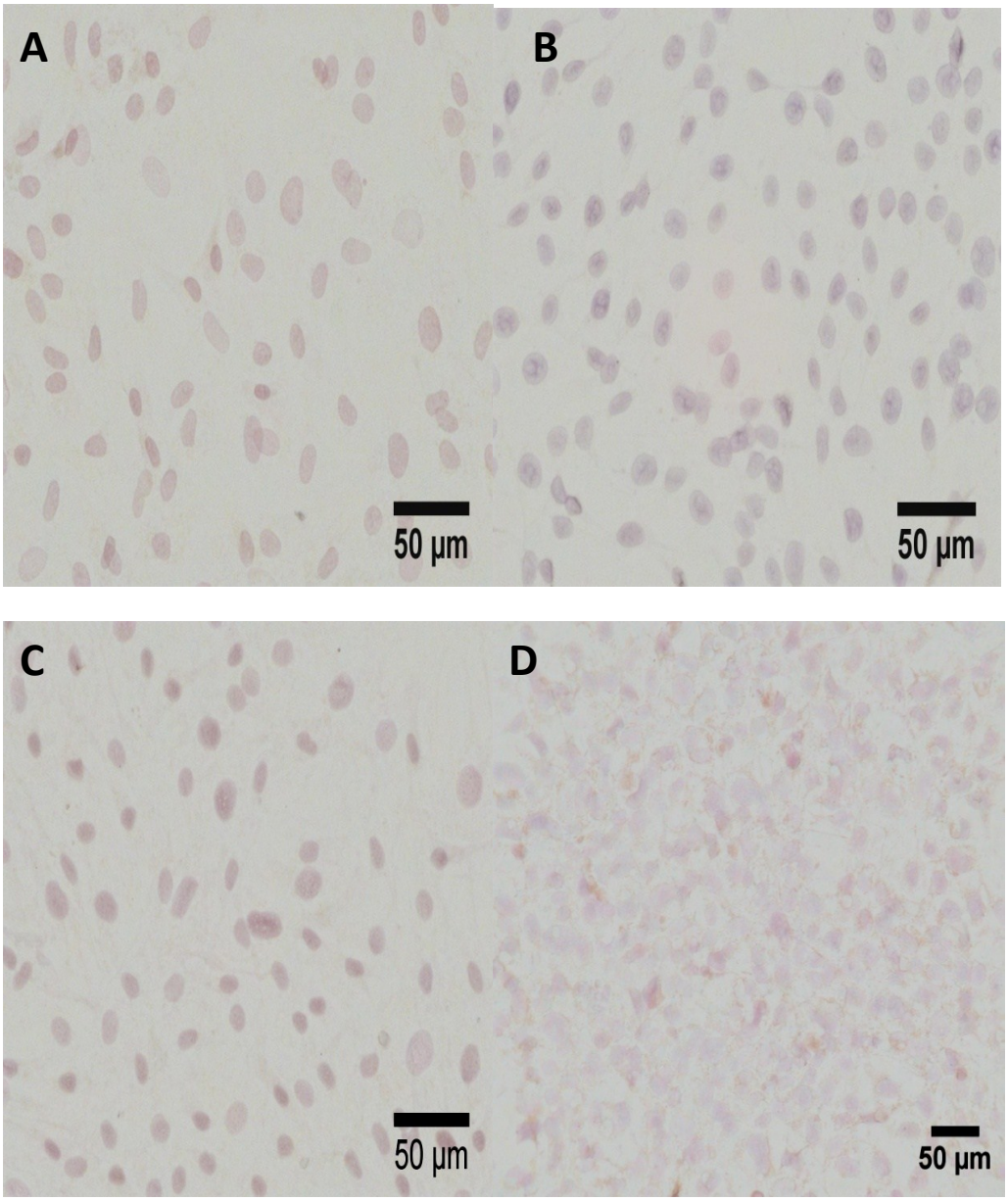


Figure 2.4 Secondary antibody controls for immunocytochemical staining: 1 % BSA/PBS was substituted for primary antibody. (A) Explant derived DPC **(B)** C6 glioma cells **(C)** BMSC **(D)** MC3T3-E1 cells.

CHAPTER 3 RESULTS

Characterisation and analysis of culture models

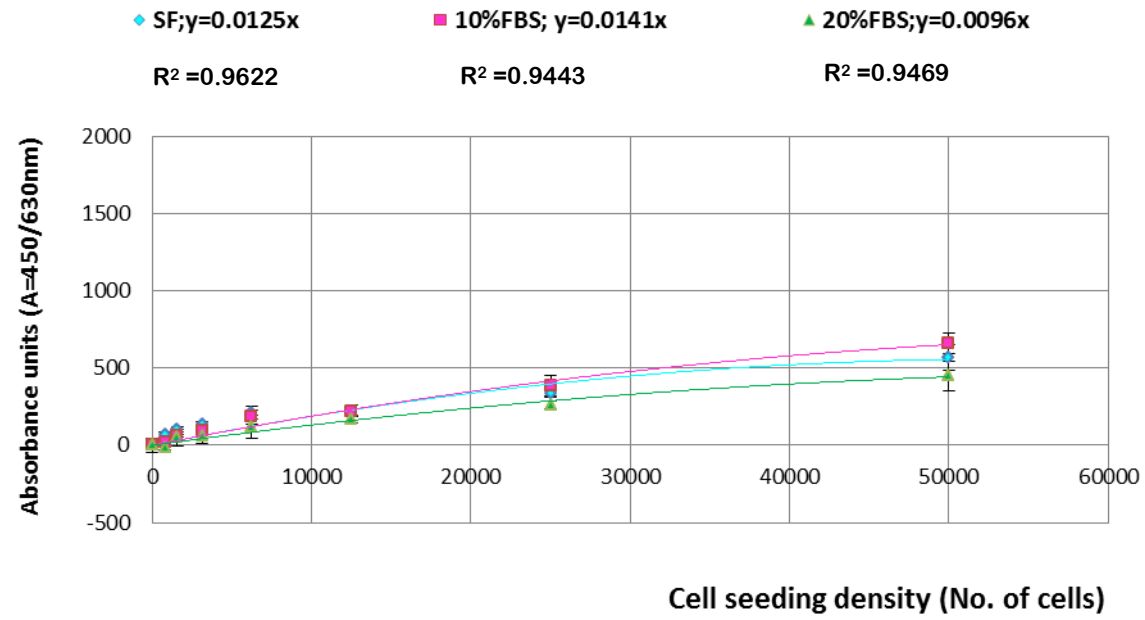
3 Characterisation of cell cultures

The primary aim of this study was to investigate the affect of GDNF on DPC behaviour. For this purpose, different cell models were used; (i) explant-derived and (ii) enzymatically-derived models of DPC were compared to (iii) BMSC along with (iv) osteoblast-like MC3T3-E1 cells. The viability of these cell types following exposure to different culture conditions along with the transcriptional profile and expression of GDNF receptors, RET and GFRα1 were assessed.

3.1 DPC, BMSC and MC3T3-E1 growth curve analysis under varying culture conditions

Two *in vitro* DPC culture approaches were established and compared, these were explant-derived (Couble *et al.* 2000) and enzymatically-derived culture (Gronthos *et al.* 2000, 2002). DPC were cultured in plastic multiwell plates using 10 %, 20 % serum, or serum-free conditions at various cell seeding densities and analysed using the WST-1 assay to determine the number of viable cells. Standard curves obtained using the WST-1 assay demonstrated a linear relationship between seeding density and the absorbance of the reduced WST-1 product for both DPC culture models, BMSC and MC3T3-E1 cells (Figures 3 and 3.1).

A



B

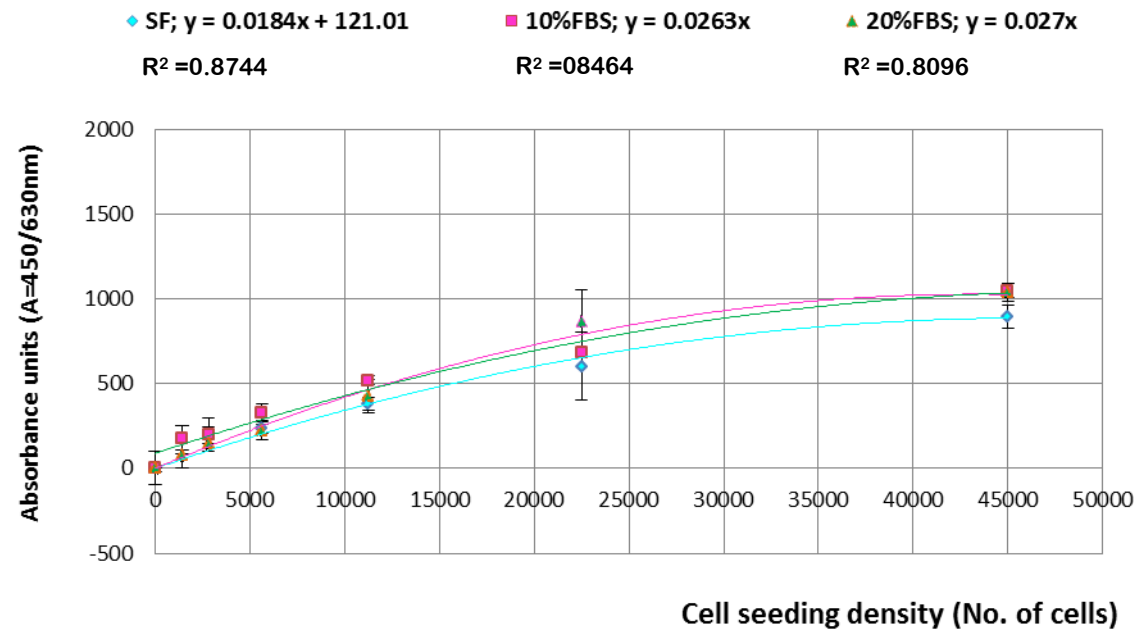


Figure 3 Correlation between number of cells seeded and WST-1 formazan product absorbance. Standard curve for **A**) explant-derived DPC and **B**) enzymatically-derived DPC in serum-free (SF), 10 % FBS and 20 % FBS serum supplemented conditions. R^2 values indicates a linear relationship between seeding density and WST-1 product absorbance (Mean ± SD; n=3).

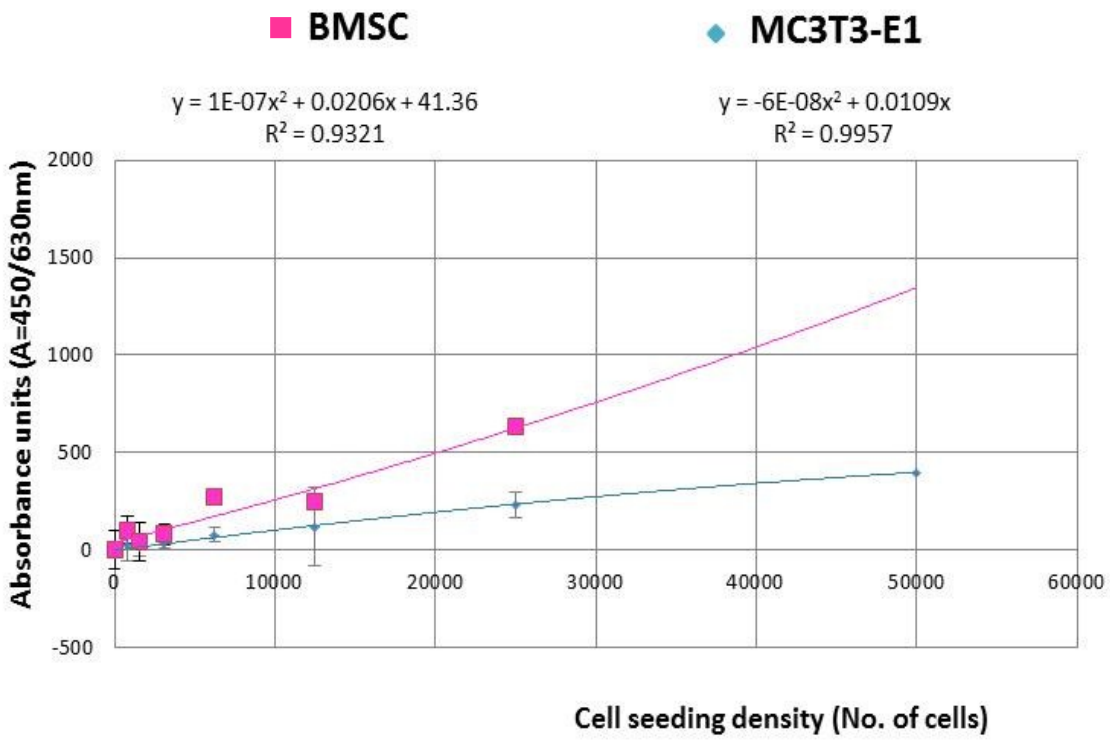


Figure 3.1 Correlation between the number of cells seeded and WST-1 formazan product absorbance. Standard curves for BMSC and MC3T3-E1 cells. Cells were seeded at a range of densities in 10 % serum supplemented medium. R^2 values indicates a linear relationship between seeding density and WST-1 product absorbance (n=3, Mean ± SD).

3.1.1 Comparison of growth curves for explant-derived and enzymatically-derived DPC under serum supplemented conditions after 48 hours

Cells were cultured in 10 % serum supplemented medium for 48 hours to determine whether changes in cell number remained within a range detectable by the WST-1 assay at the 48 hour time point. A relationship between seeding density and absorbance was apparent at 48 hours of culture (Figure 3.1.1). For future experiments an optimal seeding density of 5000 cells per well (observed at 3.69x just below mid-point

on the line graph) was selected to allow decreases and increases in cell number after 48 hours to be within a measureable range for the assay.

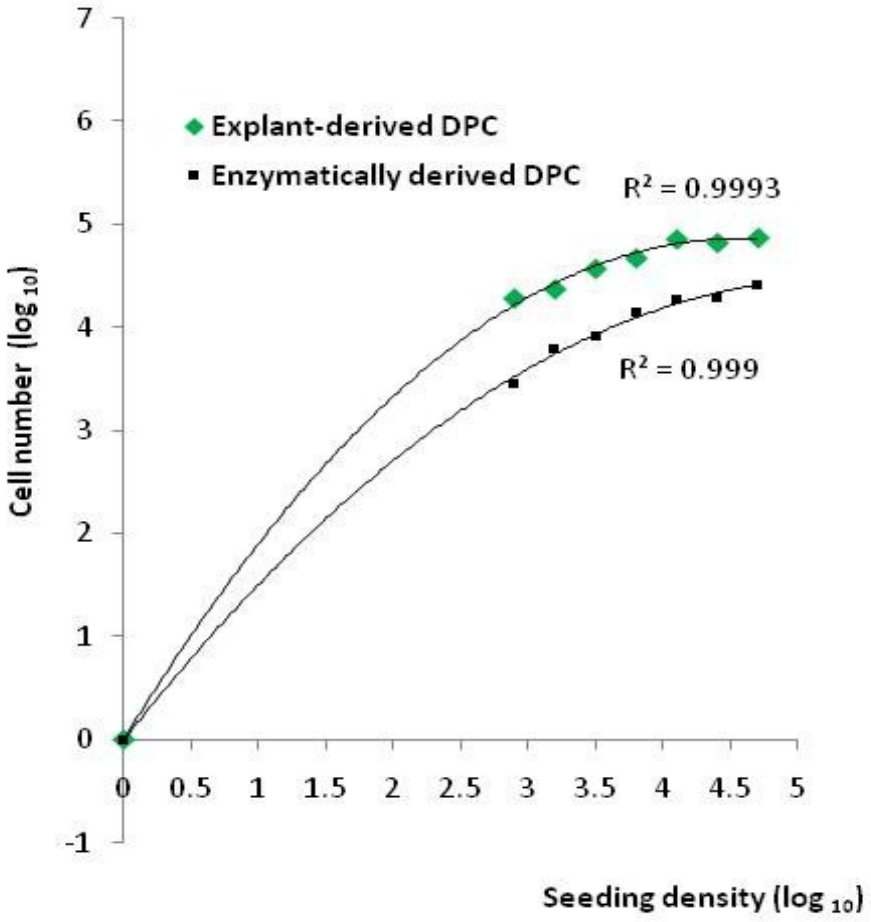


Figure 3.1.1 Number of viable explant and enzymatically derived DPC determined after 48 hours culture in 10% serum supplemented medium. Explant (**A**) and enzymatically (**B**) derived DPC increasing seeding densities increased viable cell number at 48 hours. Line equations for explant derived DPC and enzmatically derived DPC were $y=-0.2339x^2+2.1315x+0.0037$ and $y=-0.1538x^2+1.6631x-0.0046$ respectively. (Explant derived DPC and enzymatically derived DPC number were determined using the formulae from **Figure1**; $y=0.0141x$ and $y = 0.0263x$, respectively).(n=3, Mean ± SD)(ANOVA = 0.0001 all groups were significantly different to zero cells seeded).

3.1.2 DPC, BMSC and MC3T3-E1 cell viability under serum-free and serum supplemented conditions

To characterise this culture model further, the viability of explant-derived DPC were analysed under serum-free culture conditions. Serum is ill-defined, although major protein constituents are known; batches can differ in various bioactive components, including growth factors, which contribute to the survival and expansion of cell cultures (Zolotarjova *et al.* 2008; Gstraunthaler 2003). Notably, serum supplementation of media can mask or modify cellular responses during experiments designed to assess the effects of growth factors (Chase *et al.* 2010; Wagner & Ho 2007). To establish cell culture conditions for the culture models under which the effect of GDNF could later be analysed, the culture of DPC under serum-free conditions was compared to DPC cultured in 10 % serum supplemented medium at both 24 and 48 hours (Figure 3.1.2). Based on the reliability of the explant-derived cultures in terms of numbers of cells obtained per isolation, consistency of cellular morphology, technique reproducibility and technical speed, the further studies described below utilised this approach.

Serum-free DPC cultures exhibited significantly reduced numbers of viable DPC compared to 10 % serum cultures (Figure 3.1.2). DPC number did not increase over time from 24 to 48 hours indicating absence of cell growth under serum-free conditions (Figure 3.1.2).

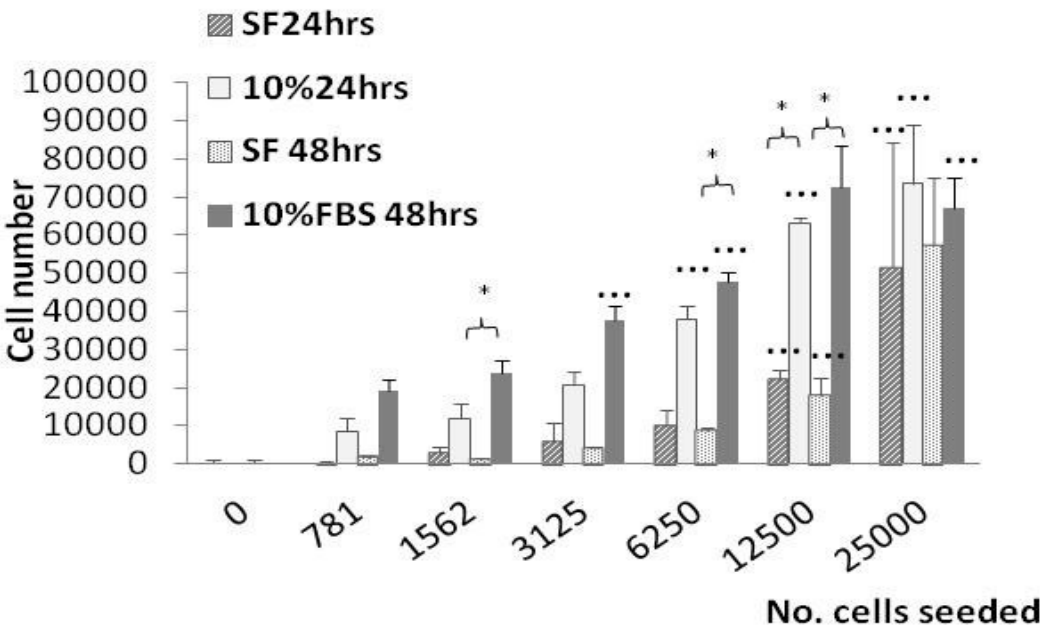
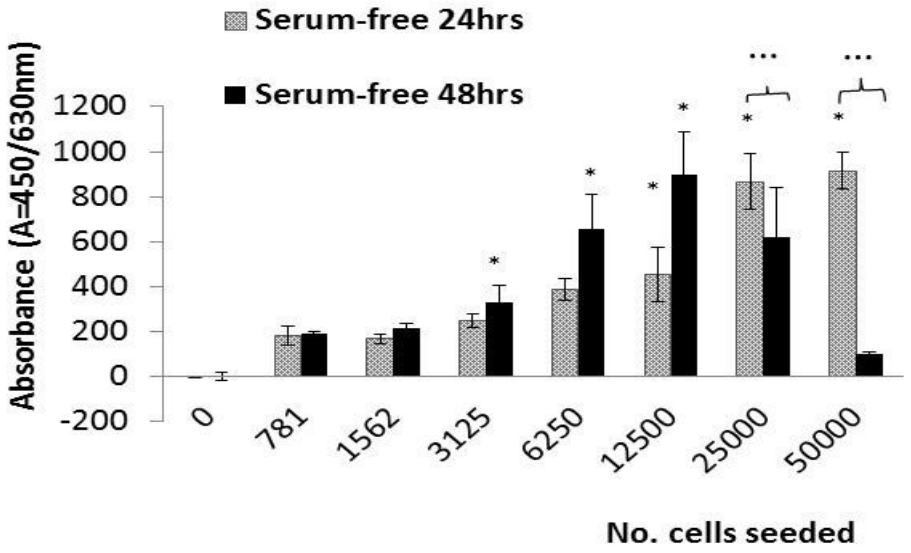


Figure 3.1.2 Effect of serum-free culture on explant derived DPC at 24 and 48 hours culture. Serum-free (SF) culture for 48 hours significantly decreased the number of viable explant derived DPC compared to 10 % serum supplemented (10 %) culture throughout a range of seeding densities (n=3, Mean ± SD;2-way ANOVA * p= 0.003 signifcantly different groups; ¨p= 0.0001 significantly different to respective zero cells seeded).

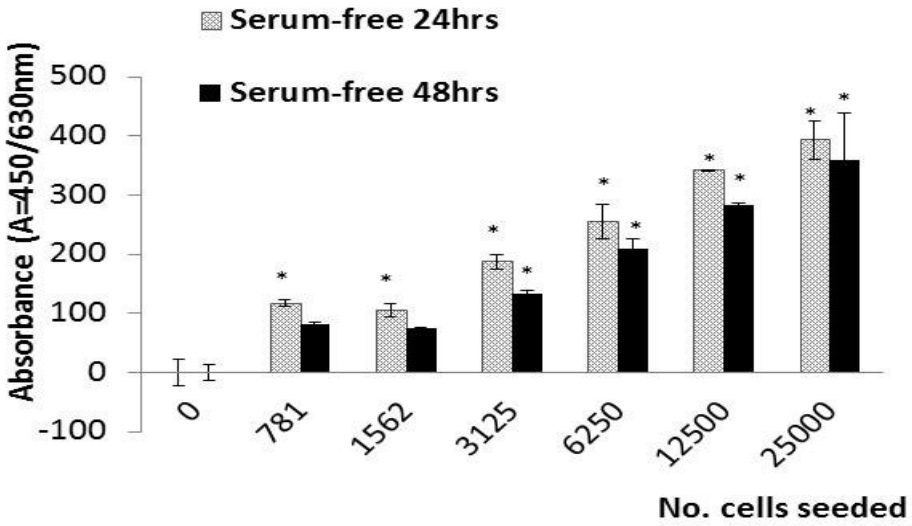
BMSC and MC3T3-E1 osteoblast-like cells are well characterised models used for multipotent mesenchymal and osteoblastic cell differentiation, respectively (Harichandan and Bühring 2011, Jones and McGonagle 2008; Beck *et al.* 2001; Raouf and Seth 2000). While the full repertoire and exact integrated regulatory processes which underpin mineralised tissue formation are not yet completely understood the transcriptional control and intracellular mechanisms of osteoblast differentiation and hence bone formation during development and repair are generally better described within the literature compared with DPC differentiation and dentine formation (Aubin and Heersche 2000; Karsenty 2008; Robling *et al.* 2006; Komori 2005; Simon *et al.*

2009; Smith and Lesot 2001; Mitisiadis and Rahiotis 2004; Thelstaf 2006; Lin and Rosenburg 2011).

BMSC and MC3T3-E1 cultures were therefore used to investigate if differential responses occur between BMSC, MC3T3-E1 cells and DPC when exposed to the same culture conditions. The effect of serum-free medium on viable BMSC and MC3T3-E1 numbers at 24 and 48 hours was evaluated (Figure 3.1.3). The number of viable BMSC and MC3T3-E1 cells did not significantly increase over time following culture in serum-free conditions. This data was comparable to that obtained for the DPC cultures (Figure 3.1.2). Indeed a minimal decrease in viable cell DPC and MC3T3-E1 cell number was apparent after 48 hours under serum-free conditions, although no decrease in BMSC numbers was evident.



A



B

Figure 3.1.3.Effect of serum-free culture conditions on BMSC and MC3T3-E1 cell growth. Number of viable **A)** BMSC and **B)** MC-3T3-E1 (BMSC (n=3), MC3T3-E1 (n=2) Mean ± SD; 2- way ANOVA . * p = 0.001 significant difference from respective control (zero cells seeded) ; ••• p = 0.001 significant difference between groups at 24 and 48 hours.

To further investigate whether serum-free conditions would support long-term differentiation and mineralisation of cultures, the effect of extended culture periods was

also analysed. BMSC cultures were not included in this analysis due to the lack of reduction in viable cell numbers observed at 48 hours compared to 24 hours. Conversely in serum-free culture at seeding densities of 3125 to 12500 cells per well BMSC number increased after 48 hours compared to 24 hour culture (Figure 3.1.3A). Therefore it was on this premise that BMSC could be supported in long term serum-free culture.

During longer-term cultures (up to 10 days), the number of viable DPC and MC3T3-E1 cells in serum-free conditions decreased significantly at day 5 (Figure 3.1.4). DPC numbers remained significantly decreased at day 10 compared to day 3. However at day 10 the decrease in number of viable explant-derived and MC3T3-E1 cells under serum-free conditions was minimal at 16.92 % and 14.43 % respectively.

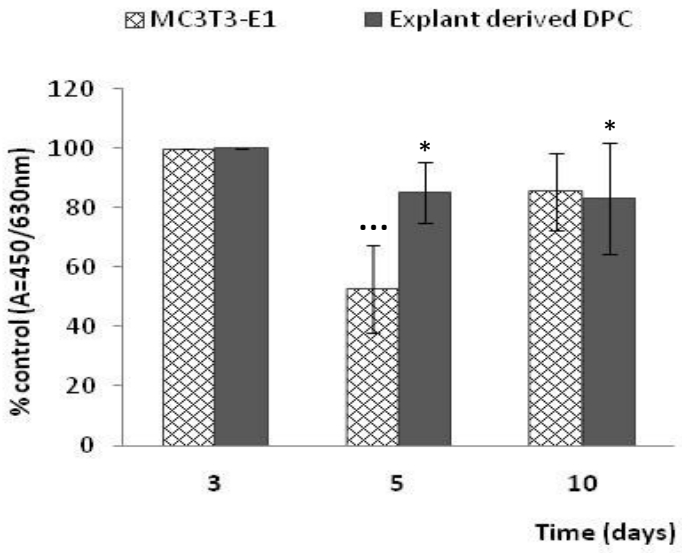
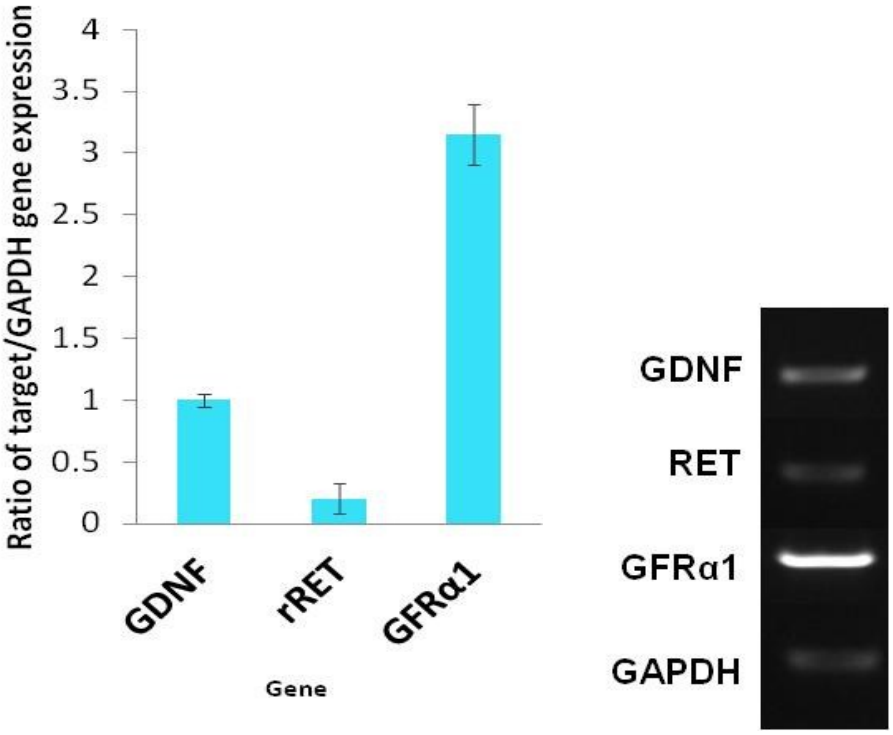


Figure 3.1.4 Effect of serum-free culture on the number of viable explant-derived DPC and MC3T3-E1 cells after 3, 5 and 10 days. Viable MC3T3-E1 cell numbers were signifcantly decreased at Day 5 and viable DPC numbers significantly decreased at Day 5 and 10. Results are expressed as percentage controls (day 3 value). (n = 6, mean ± SD; TTest ...p= 0.000 and *p= 0.05, resepectively).

3.2 Relative gene expression profiles in DPC, BMSC and MC3T3-E1 cultures

3.2.1 GDNF, GFRα1 and RET receptor gene expression within a glial cell line.

To enable the culture models to be screened to detect GDNF and GDNF receptor (GFRα1 and RET) gene expression, RT-PCR conditions were established using RNA from rat C6 glioma cell line, which is reported to express both receptors (Wiesenhofer et al. 2000; Wan and Too 2010). Transcripts for GDNF and its associated receptors, RET and GFRα1, were detected in accordance with this literature (Figure 3.2).



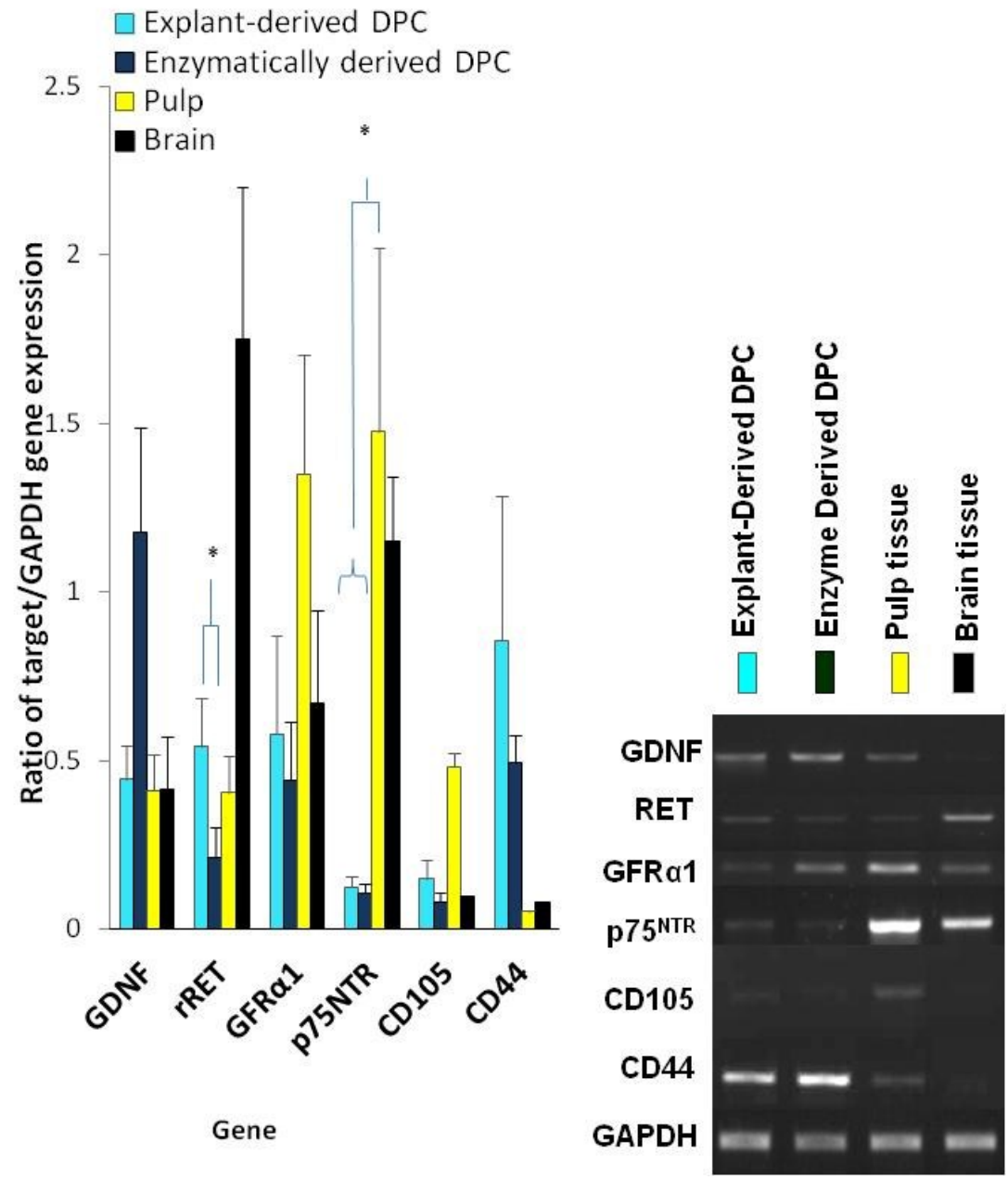
A **B**

Figure 3.2 A) sq-RT-PCR analysis of C6 glioma cell line relative gene expression for the GDNF receptors, RET and GFRα1 and GDNF as analysed by sq-RT-PCR. GAPDH was used as an amplification control. (**B)** Representative gel electrophoresis image of transcripts detected (n=3, Mean ± SD).

3.2.2 Relative gene expression of GDNF, Gfrα1 and RET in DPC cultures

Explant-derived and enzymatically-derived DPC culture models were analysed for the gene expression of GDNF receptors, RET and GFRα1, along with GDNF. The culture models were additionally analysed for the presence of mesenchymal stem cell (MSC) marker transcripts, $p75^{NTR}$ (CD271), CD44 and CD105 (Endoglin) (Pittenger *et al.* 1992, 2008; Jones and McGonagle 2008; Bühring *et al*. 2007; Moscatelli *et al.* 2009) (Figure 3.2.1).

Both DPC models expressed the MSC markers, $p75^{NTR}$, CD105 and CD44 (Huang *et al.* 2009; Strys *et al.* 2011; Bianco *et al.* 2001), indicating the mesenchymal nature of these cultures. GDNF receptor gene expression was evident in both DPC culture models and *in vivo* dental pulp and brain tissue. Gene expression levels of GFRα1 and RET receptor suggested that these receptors may be relatively higher in explant-derived DPC compared with enzymatically derived DPC (Figure 3.2.1).



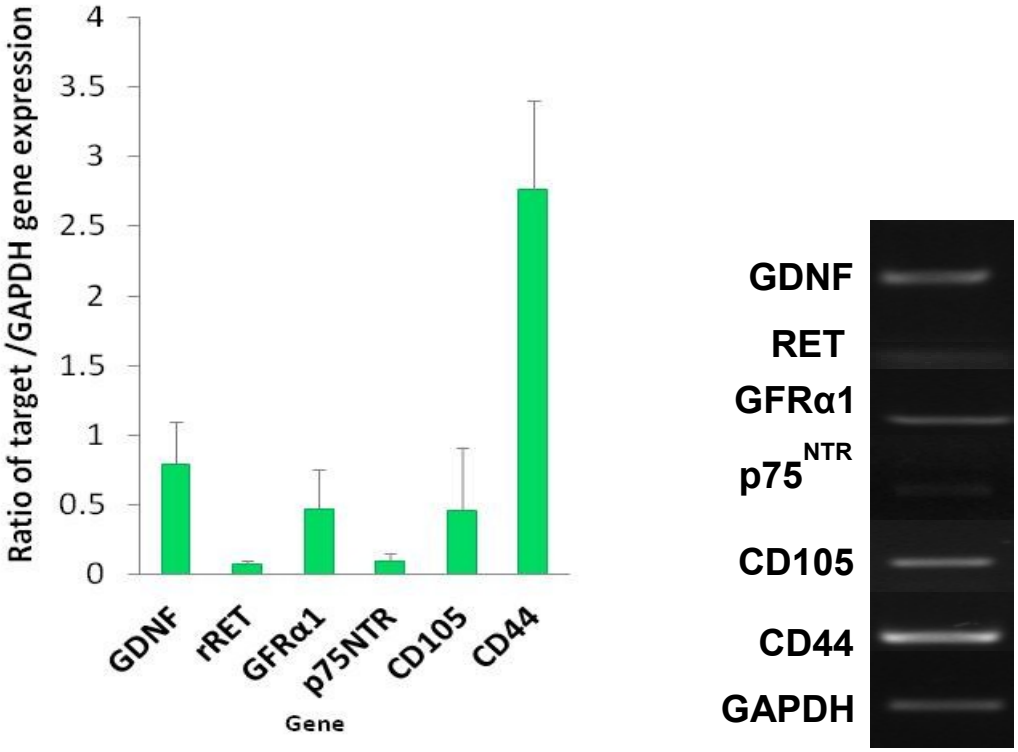
A **B**

Figure 3.2.1 sq-RT-PCR analysis of explant-derived and enzymatically-derived DPC cultures. A) Relative gene expressions of DPC and **B)** representative electrophoresis gel images. Brain tissue RNA extracts were used as controls. GAPDH was used as an amplification control;* $p<0.05$. (n=4, Mean ± SEM (except CD105 and CD44 n=2)).

These data indicated that the GDNF receptors are expressed *in vivo* within pulpal tissue and *in vitro* in DPC, indicating that both DPC culture models may provide suitable model systems for the analysis of the effects of GDNF.

3.2.3 Relative gene expression of GDNF, GFRα1 and RET in BMSC and MC3T3-E1 cell cultures

In vitro cultures of BMSC and the MC3T3-E1 cell line were also investigated for transcript expression of GDNF and its receptors RET and GFRα1, along with that of mesenchymal cell markers. Both GDNF and GFRα1 transcripts along with mesenchymal cell markers were detected within BMSC and MC3T3-E1 cells (Figures 3.2.2 and 3.2.3). Gene expression within BMSC and the MC3T3-E1 cell line indicates that these models are also potentially responsive to GDNF.

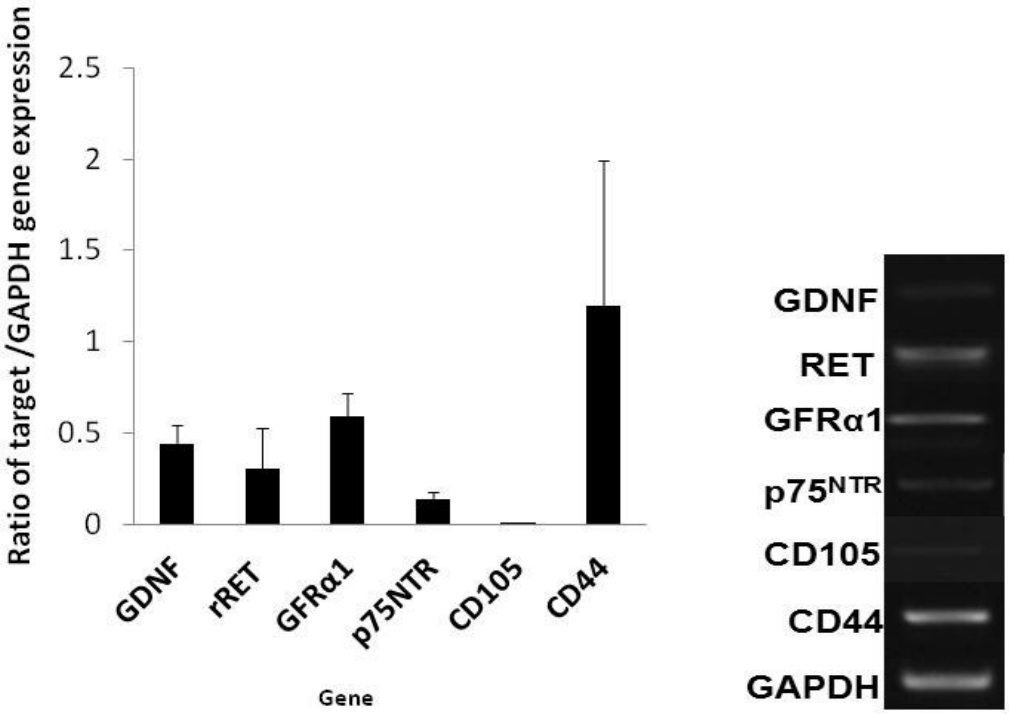


A **B**

Figure 3.2.2 sq-RT-PCR analysis of BMSC relative gene expression for GDNF, RET & GFRα1 receptors along with mesenchymal cell markers A) Graphical representation of relative gene expression levels in BMSC and **B)** representative electrophoresis gel images of transcripts detected for each gene amplified. GAPDH was used as an amplification control. (n=4, Mean ±SE).

3.3 Immunocytochemical analysis of GDNF receptor expression

To substantiate the RT-PCR results and determine the protein expression and localisation of RET and GFRα1, immunocytochemical (ICC) staining was performed using the C6 glioma cell line as control and in explant-derived DPC, BMSC and



A B

Figure 3.2.3 sq-RT-PCR analysis of MC3T3-E1 gene expression for GDNF, and RET & GFRα1 receptors along with mesenchymal cell markers. A) Relative gene expressions of MC3T3-E1 and **B)** representative electrophoresis gel image. GAPDH was used as an amplification control (n=3 except for CD44 where n =2; Mean ±SE).

MC3T3-E1 cell cultures (Figures 3.3-3.3.3). Similar uniform distribution of cytoplasmic staining was visible for the GDNF receptors, RET and GFRα1, within DPC, BMSC and MC3T3-E1 cell cultures. GFRα1 receptor staining of BMSC and MC3T3-E1 cells displayed distinct darkly staining areas within the cytoplasm at levels much elevated above background as seen compared to the control. Specifically, where staining that appeared to be localised towards the nucleus and may therefore possibly indicate that the receptors aybe stored within the endoplasmic reticulum within DPC and MC3T3-E1 cells (Figures 3.3 and 3.3.3).

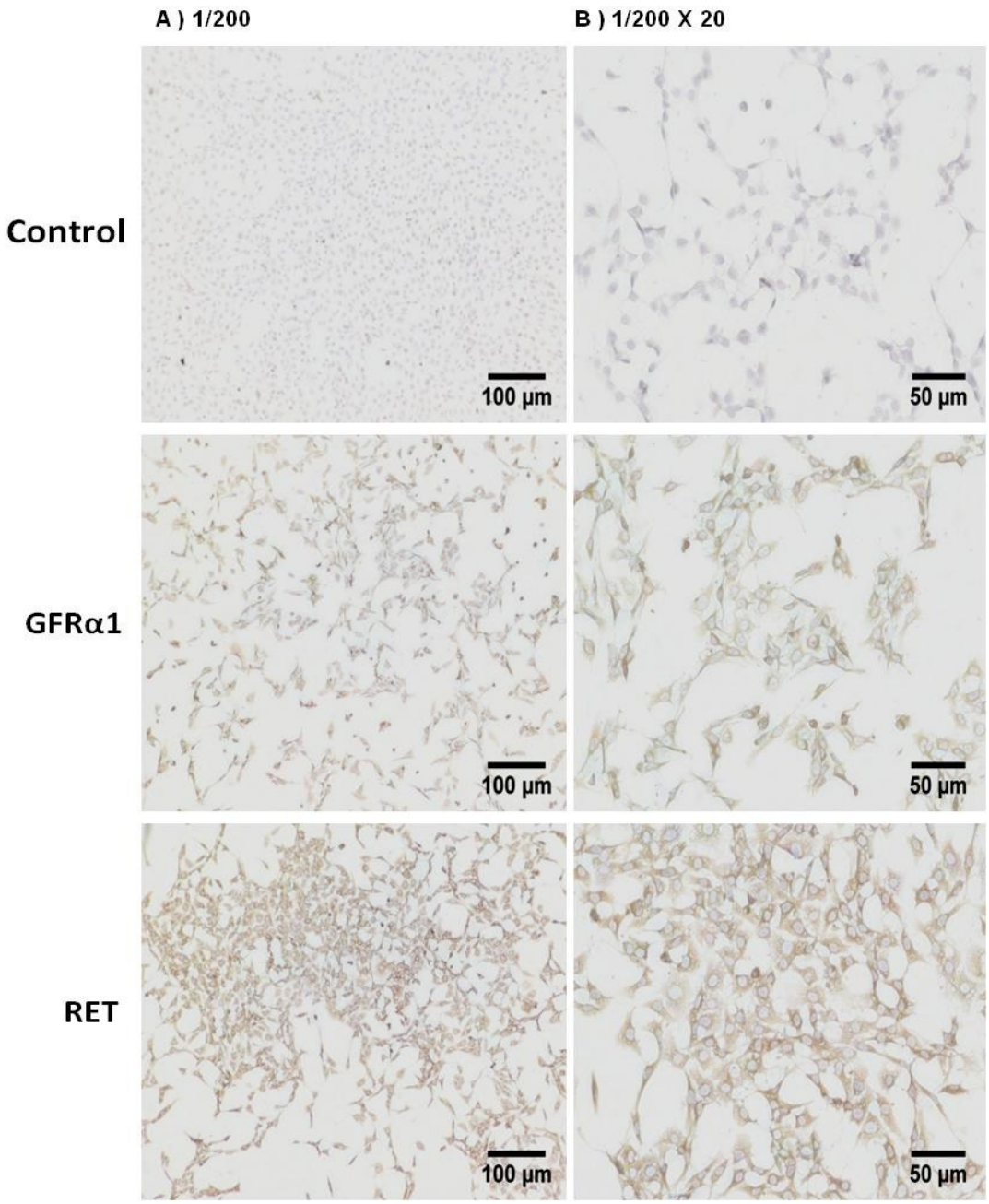


Figure 3.3 C6 Glioma cell cultures stained for the GDNF receptors GFRα1 and RET cultured in 10 % FBS supplemented medium on multispot glass slides. Non-specific/negative staining controls used were normal rabbit serum substituted for primary antibody. The biotinylated secondary antibody was used at 1/200 (column (**A**)) and higher magnification images are shown in column (**B**). DAB and Mayer's haematoxylin were used for visualisation of antibody binding and as a counterstain, respectively. Scale bars represent for (**A)** 100 µm and for (**B**) 50 µm.

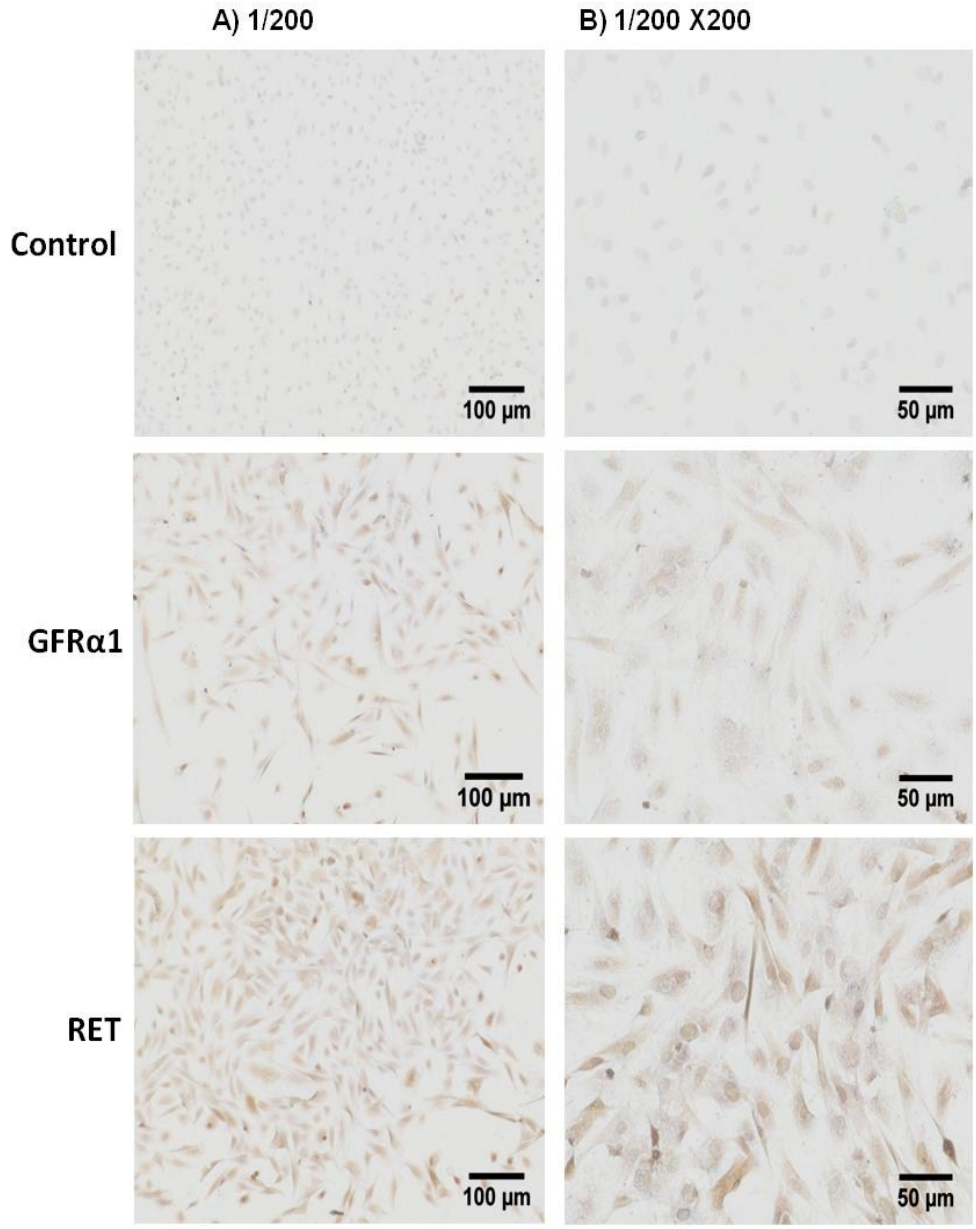


Figure 3.3.1 DPC cultures stained for the GDNF receptors GFRα1 and RET in 10 % FBS supplemented medium on multispot glass slides. Non-specific/negative staining controls used were normal rabbit serum substituted for primary antibody. The biotinylated secondary antibody was used at 1/200 (column (**A**)) and higher magnification images are shown in column (**B**). DAB and Mayer's haematoxylin were used for visualisation of antibody binding and as a counterstain, respectively. Scale bars represent (**A)** 100 µm and for (**B**) 50 µm.

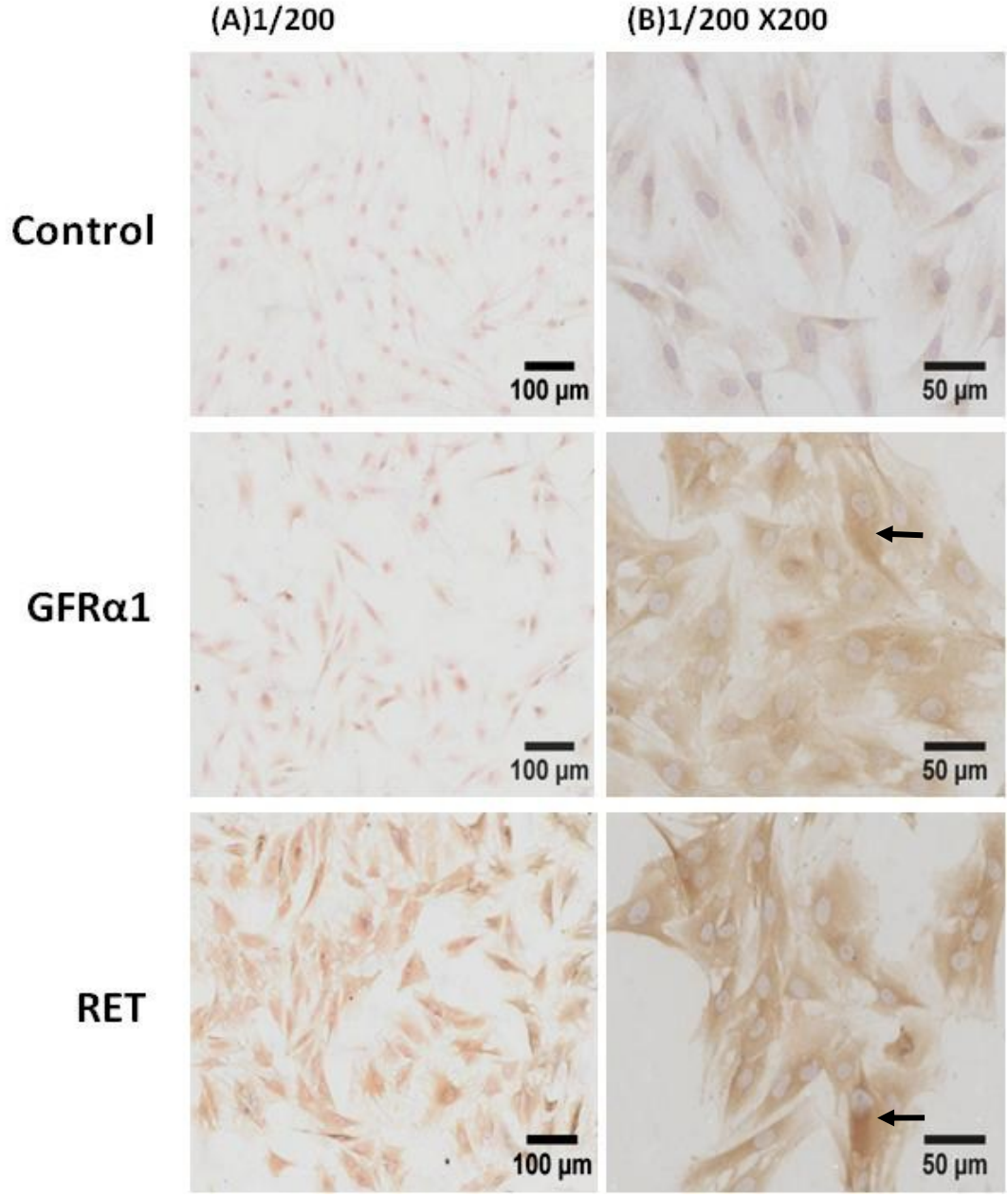


Figure 3.3.2 BMSC cultures stained for the GDNF receptors RET and GFRα1 in 10%FBS supplemented medium on multispot glass slides. Non specific/negative staining controls used were normal rabbit serum substituted for primary antibody. The biotinylated secondary antibody was used at 1/200 (column (**A**)) and higher magnification images are shown in column (**B**). DAB and Mayer's haematoxylin were used for visualisation of antibody binding and as a counterstain, respectively. Scale bars represent (**A)** 100 µm and for (**B)** 50 µm. Arrows depict increased intensity of staining specific to the receptors within cytoplasm compared to weak diffuse non-specific controls (normal rabbit serum).

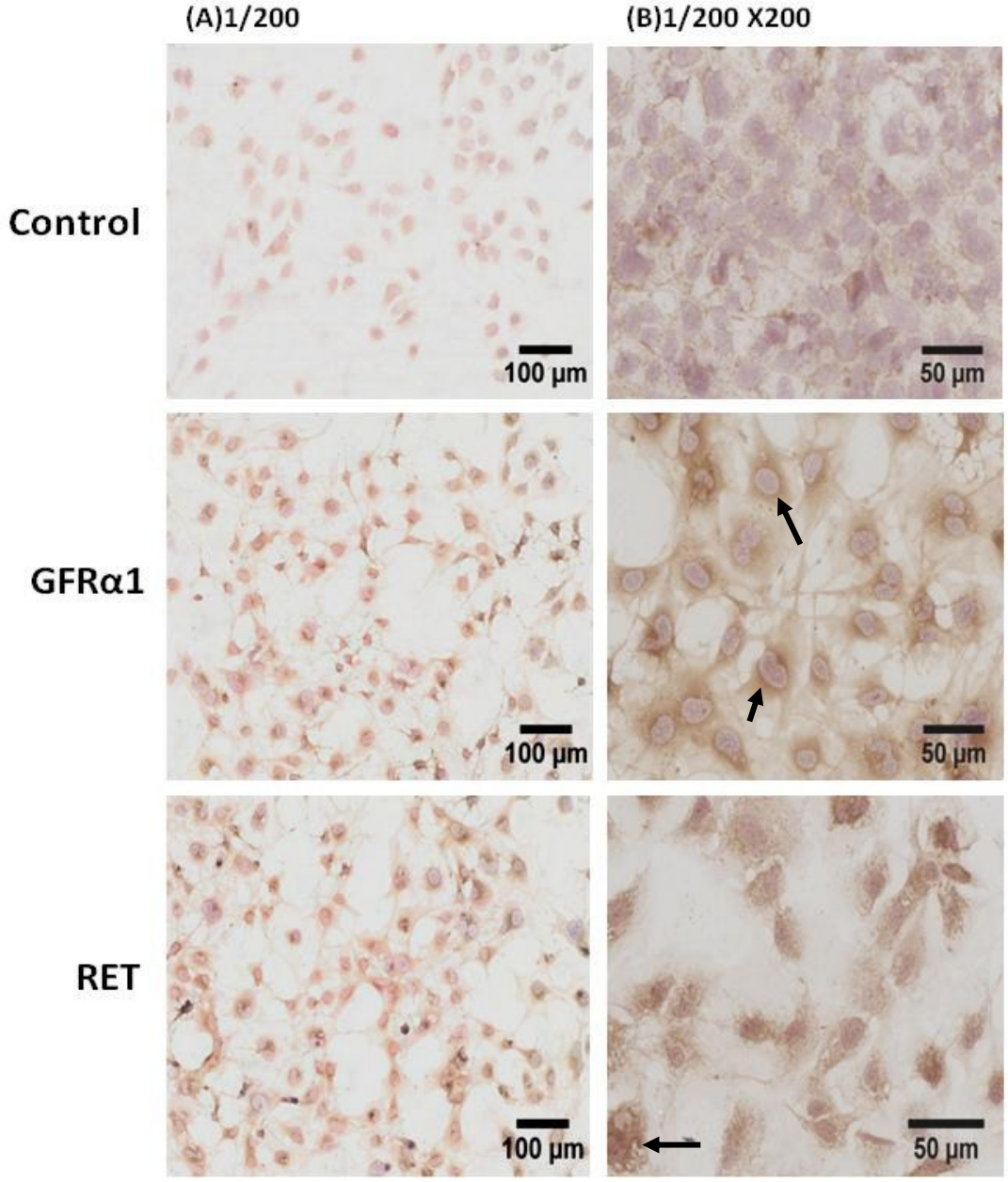


Figure 3.3.3 MC3T3-E1 cell cultures stained for the GDNF receptors RET and GFRα1 in 10% FBS supplemented medium on multispot glass slides. Non-specific/negative staining controls were normal rabbit serum substituted for primary antibody. The biotinylated secondary antibody was used at 1/200 (column (**A**)) and higher magnification images are shown in column (**B**). DAB and Mayer's haematoxylin were used for visualisation of antibody binding and as a counterstain, respectively. Scale bars represent (**A)** 100 µm and for (**B)** 50 µm. Arrows depict increased intensity of staining specific to the receptors within cytoplasm compared to weak diffuse non-specific controls (normal rabbit serum).

CHAPTER 4 RESULTS

GDNF effects on DPC, MC3T3-E1 and BMSC survival and proliferation

4 GDNF effects on viable cell numbers

Standard cell cultures contain serum or additional components and growth factors to support cell viability and growth, whilst serum or growth factor deprivation often results in apoptosis and senescence. Chapter 3 demonstrated that serum-free culture decreased DPC and MC3T3-E1 viable cell numbers indicating growth arrest/increased cell death occurred under these conditions. To study cellular proliferation, cultures in growth arrest are commonly used to detect a response to exogenously applied growth factors. In this study, the direct cellular effect of GDNF was investigated in both serum-free and serum-supplemented cultures.

Changes in viable DPC, BMSC and MC3T3-E1 cell numbers in response to GDNF were measured under serum-free and serum supplemented conditions using the WST-1 assay. Glioma cell cultures express GNDF receptors and have been reported to be responsive to GDNF (*Ng et al.* 2009). Glioma cells were used as a positive control and GDNF significantly increased the numbers of viable C6 glioma cells under serum-free culture (Figure 4).

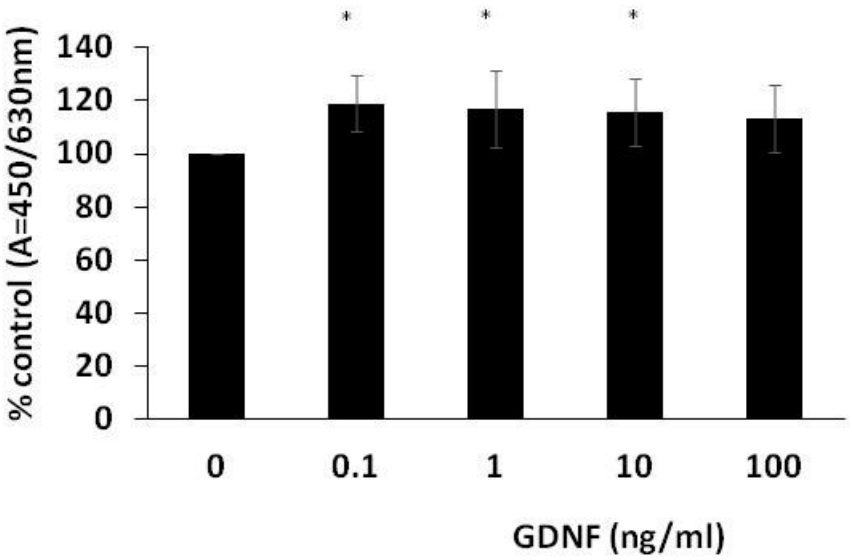


Figure 4 GDNF effect on C6 glioma cell numbers as assessed by WST-1 under serum-free culture conditions after 48 hour culture. Results are expressed as percentage control (0 ng/ml unsupplemented cultures) (n=3, Mean ± SD; *p< 0.005 *versus* control).

The addition of GDNF to serum-free DPC and MC3T3-E1 cell cultures also significantly increased the number of viable cells. Linear regression analysis of individual experiments for (i) DPC resulted in $p < 0.000$- 0.007 with R^2 values up to 0.49 and (ii) for MC3T3-E1 $p>0.000$ with R^2 values up to 0.54. No effect of GDNF was apparent in serum supplemented DPC cultures (Figures 4.1). Interestingly, the number of viable BMSC did not change significantly in response to GDNF addition in serum-free cultures. However a decrease in viable BMSC and MC3T3-E1 cell numbers in serum supplemented conditions was evident at 100 ng/ml GDNF (Figure 4.1.1 and 4.1.2).

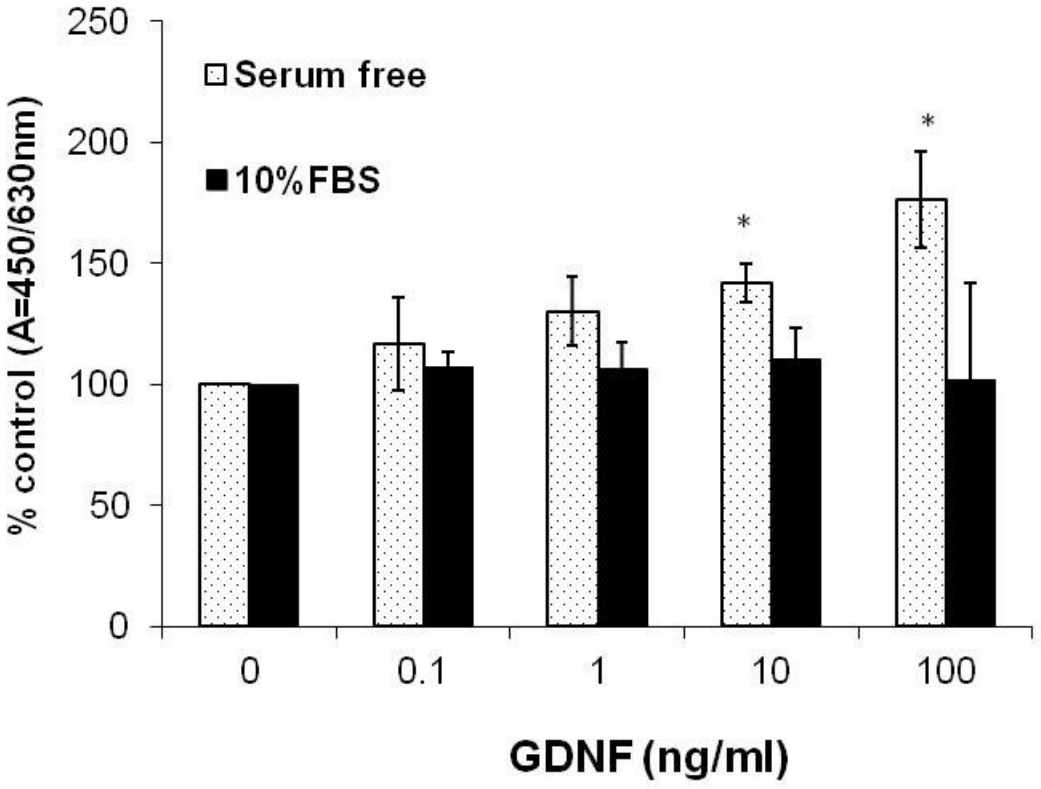


Figure 4.1 GDNF effect on viable DPC number as assessed by WST-1 under serum-free and 10 % serum supplemented culture conditions. Results are expressed as percentage controls (0 ng/ml GDNF unsupplemented cultures) (n=4, Mean ± SEM; *p= 0.0003 *versus* control).

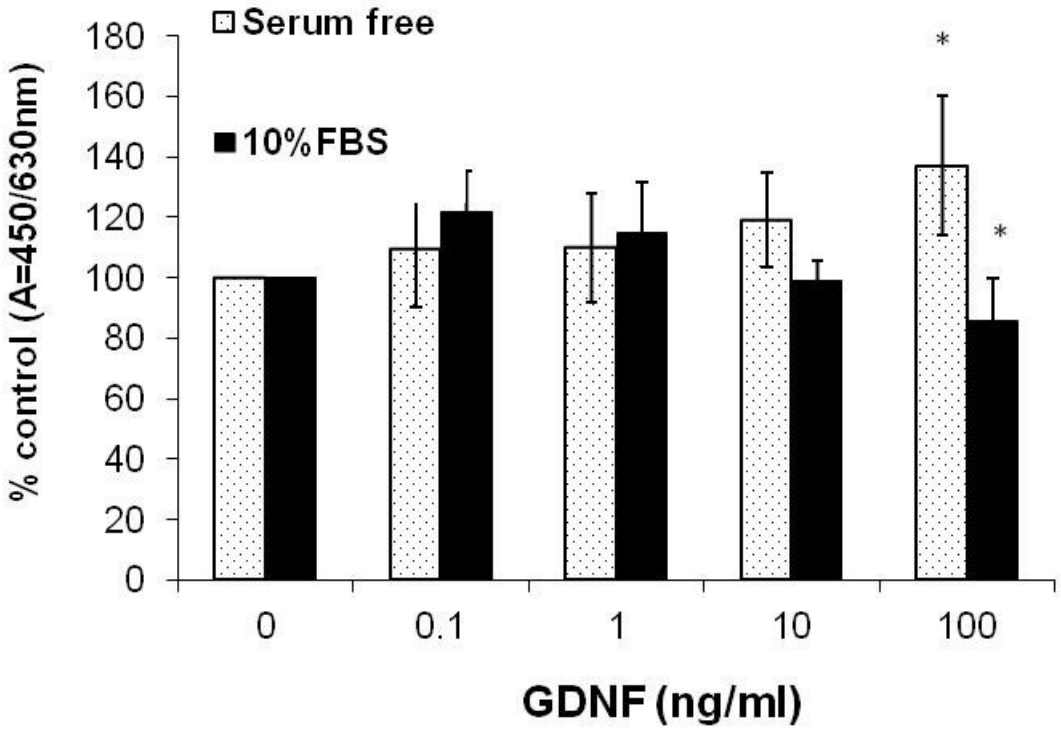


Figure 4.1.1 GDNF effect on viable MC3T3-E1 cell number under serum-free and 10% serum supplemented culture conditions as assessed by WST-1. Results are expressed as percentage of controls (n=4, Mean ± SEM; *p= 0.0003 *versus* control (0 ng/ml GDNF unsupplemented cultures).

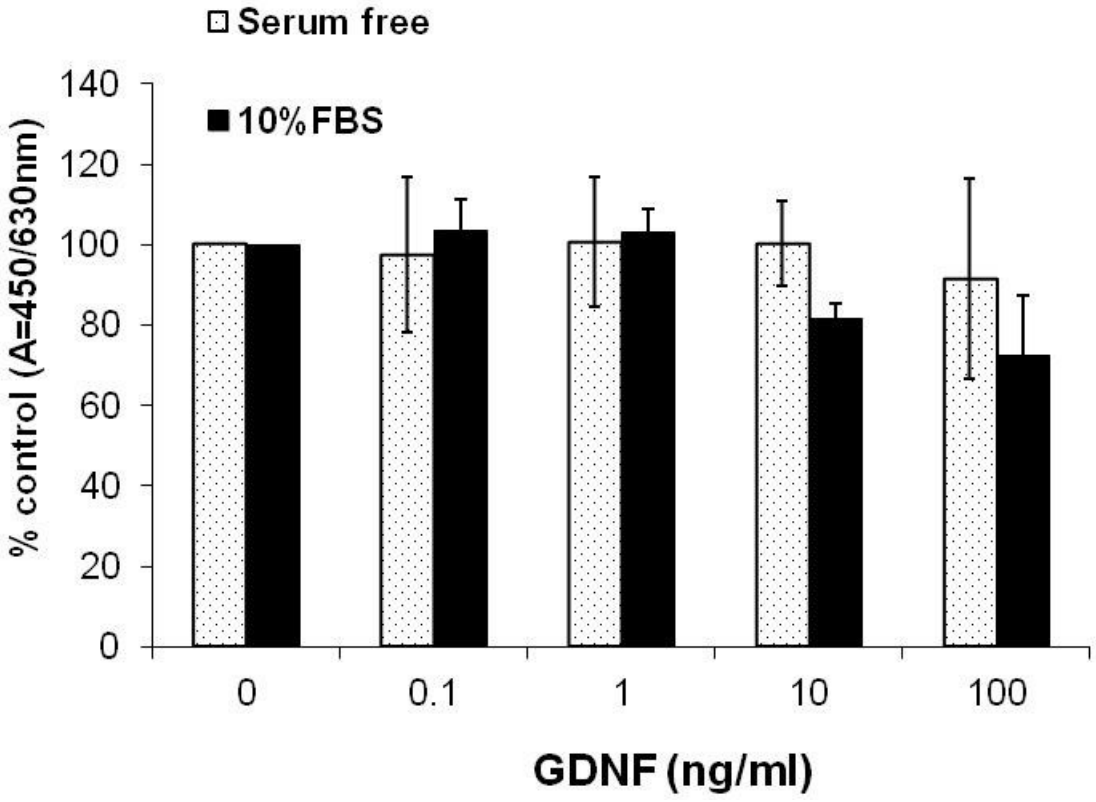


Figure 4.1.2 GDNF effect on viable BMSC number under serum-free and serum supplemented culture conditions as assessed by WST-1 Results are expressed as percentage of controls (0 ng/ml GDNF unsupplemented cultures) (n=4, Mean± SEM).

4.1 GDNF effects on cell proliferation and cell viability

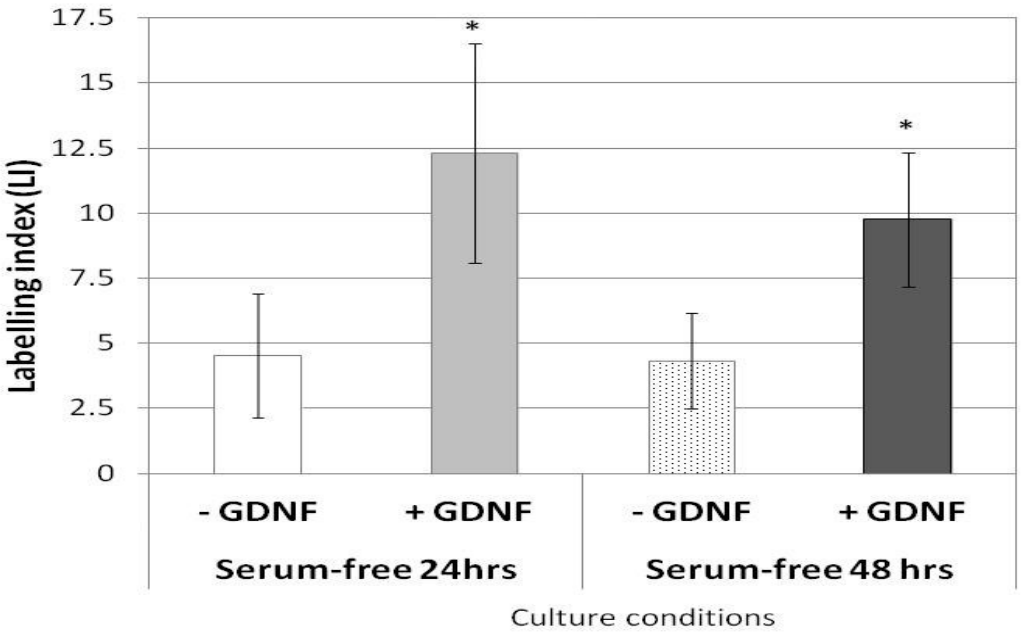
To further investigate whether the increase in viable cell numbers was due to increased proliferation or increased survival, the BrdU assay, and the live/dead assays were applied.

4.1.1 Effect of GDNF on DPC and MC3T3-E1 cell proliferation

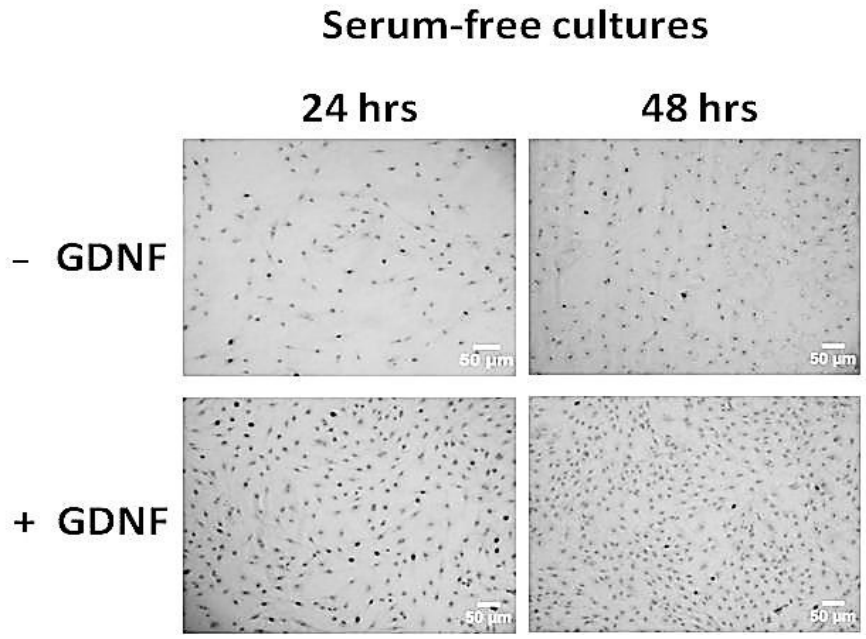
A BrdU incorporation assay was performed under serum-free and serum supplemented conditions for DPC and MC3T3-E1 cells in response to GDNF (100 ng/ml) (Figures 4.2 to 4.2.2). In serum-free media proliferative rates were decreased for MC3T3-E1 cell cultures compared to cultures containing serum as demonstrated by a significantly reduced BrdU labelling index (Figures 4.2.2).

At 48 hours, GDNF significantly increased the number of labelled nuclei in serum-free DPC and MC3T3-E1 cell cultures [BrdU labelled DPC nuclei increased from 4.5 to 9.9 % and BrdU labelled MC3T3-E1 nuclei from 1.3 to 3 % (Figures 4.2 and 4.2.2]). At 24 hours the numbers of BrdU labelled DPC nuclei under serum-free conditions with GDNF was similarly increased compared to serum-free conditions (from 4.5% to 12.2% BrdU labelled DPC nuclei) (Figures 4.2).

Conversely GDNF significantly decreased the number of BrdU labelled DPC nuclei at 24 hours and MC3T3-E1 BrdU labelled nuclei at 48 hours within serum supplemented conditions (for DPC, BrdU labelled nuclei decreased from 23.8 % to 5.68 % and for MC3T3-E1 cells, BrdU labelled nuclei decreased from 8.9 % to 5%) (Figures 4.2.1 and 4.2.2). These data indicate that GDNF increased DPC and MC3T3-E1 cell proliferation in serum-free cultures whilst decreasing mitosis in serum supplemented cultures, albeit at differing time points. BMSC cultures were not included in this investigation because results from the WST-1 assay detected no significant difference in viable cell numbers following the addition of GDNF in serum or serum-free cultures at 48 hours (see Figure 4.1.2).

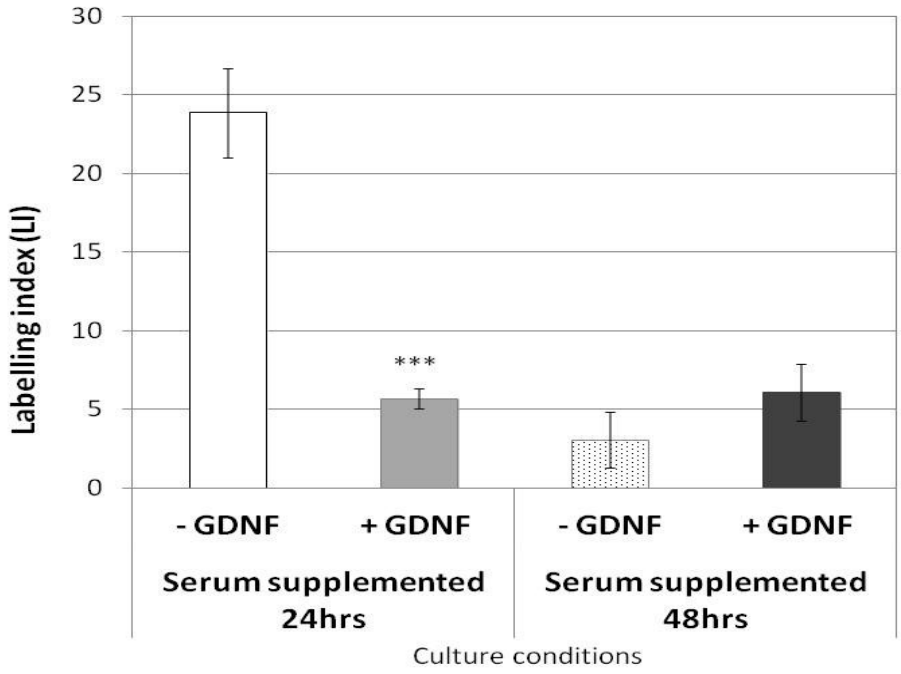


A



B

Figure 4.2 BrdU labelling of DPC at 24 and 48 hours under serum-free conditions. **A.** Labelled number of DPC nuclei under serum-free conditions with and without GDNF addition recorded using Image J software at 24 and 48 h**.** Results are percentage labelled /total nuclei for each condition (labelling index)(**B**. Representative microscope images of stained DPC cultures: BrdU labelled nuclei are shown appearing black and haematoxylin counterstained nuclei appearing grey. (n=3, Mean ± SEM; *$p \leq 0.05$ *versus* 0 ng/ml GDNF unsupplemented control, scale bars represent 50 µm).



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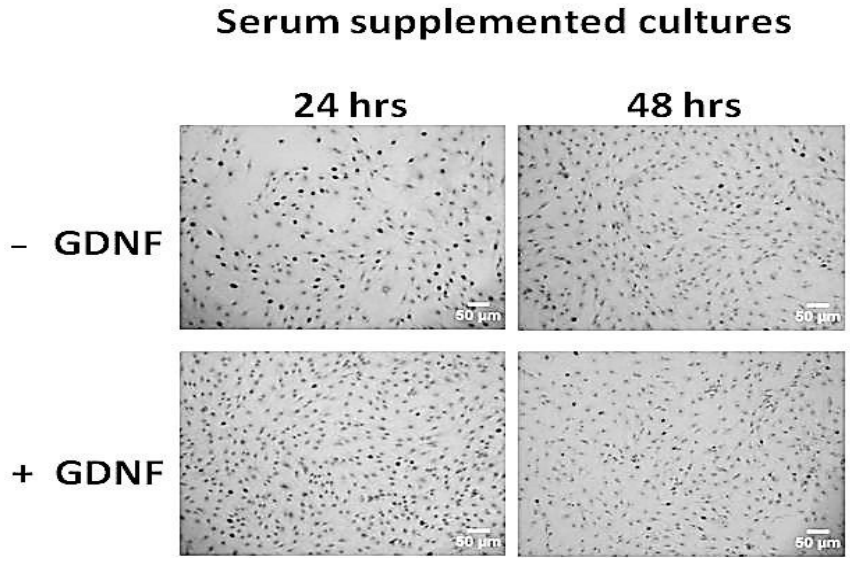
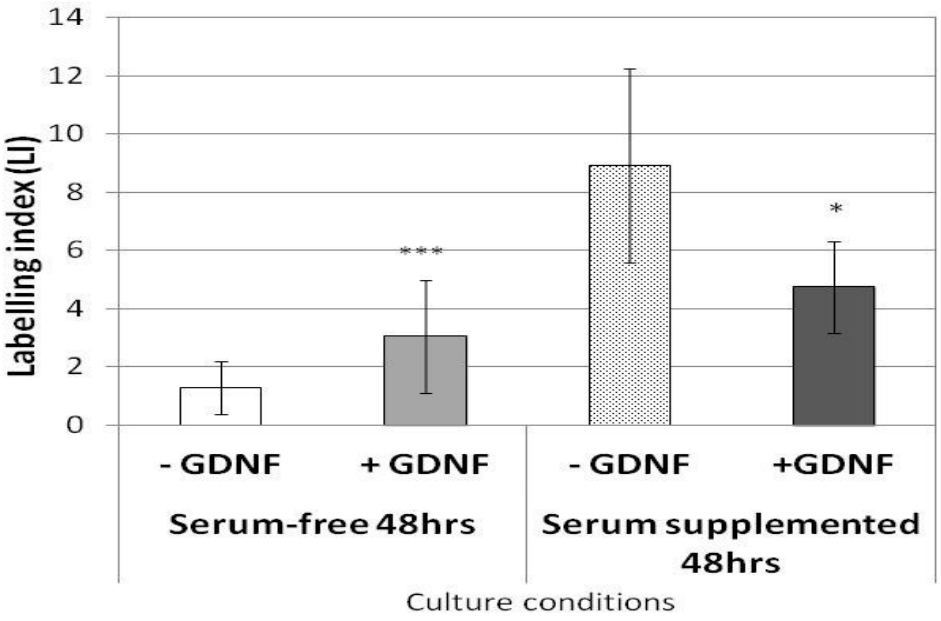


B

Figure 4.2.1 BrdU labelling analysis of DPC at 24 and 48 hours under serum supplemented (10 % FBS) conditions. A. Labelled number of DPC nuclei under serum supplemented conditions with and without GDNF addition recorded using Image J software at 24 and 48 h. Results are percentage labelled/total nuclei (labelling index) for each condition. **B**. Representative images of stained DPC: BrdU labelled nuclei are shown appearing black and haematoxylin counterstained nuclei appearing grey. (n=3, Mean ± SEM; ***p ≤ 0.005 *versus* 0 ng/ml GDNF unsupplemented control, scale bars represent 50 µm).



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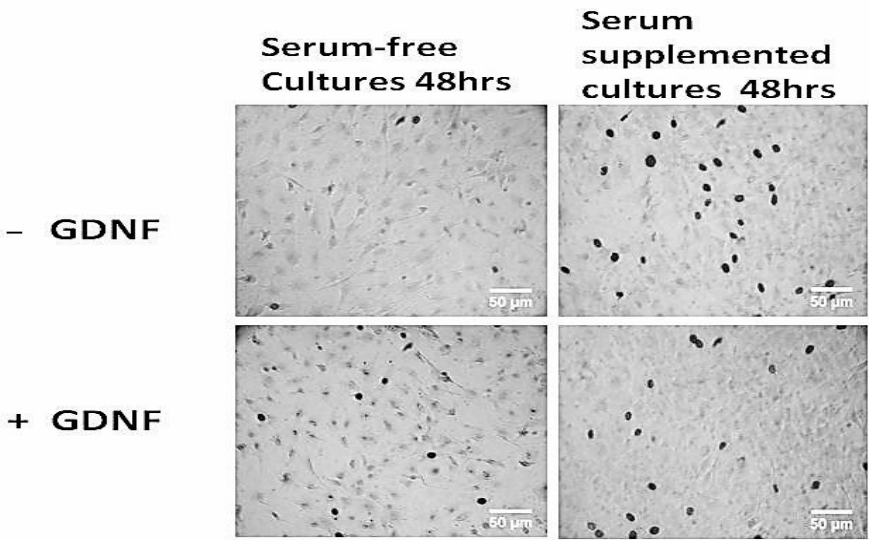


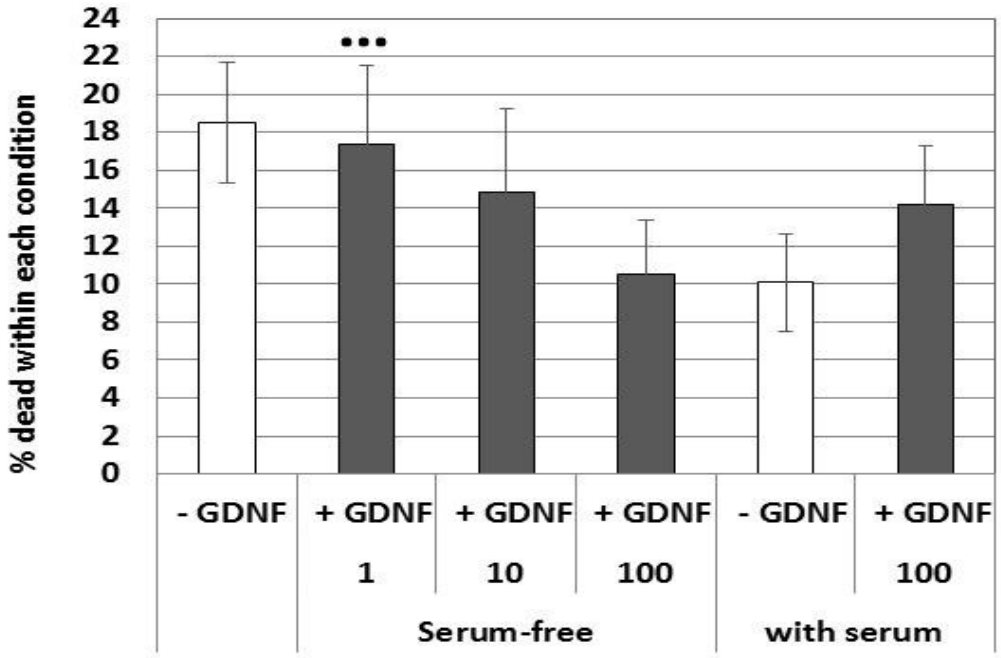
B

Figure 4.2.2 BrDU labelling analysis of MC3T3-E1 cultures at 48 hours under serum-free and serum supplemented (10 %FBS) conditions. A. Labelled number of MC3T3-E1 nuclei under serum-free and serum supplemented conditions with and without GDNF addition recorded using Image J software at 48 h. Results are percentage labelled /total labelled nuclei (labelling index) for each condition **B**. Representative images of labelled MC3T3-E1 cells: BrdU labelled nuclei are shown appearing black and haematoxylin counterstained nuclei appearing grey. (n=2, Mean ± SEM; T-test;*p ≤ 0.05, ***p<0.005 *versus* 0 ng/ml GDNF unsupplemented control, scale bars represent 50 µm).

4.2 Effect of GDNF on DPC, BMSC and MC3T3-E1 cell survival

The live/dead assay was used to assess cell survival in terms of the number of dead and live cells under serum-free and serum supplemented conditions. A range of GDNF concentrations were applied under serum-free conditions while 100 ng/ml GDNF was applied only for serum supplemented conditions. At both 24 and 48 hours the numbers of dead DPC, BMSC and MC3T3-E1cells under serum-free conditions without GDNF addition were all significantly increased compared to serum supplemented cultures (Figures 4.3- 4.3.5). GDNF significantly decreased the percentage of dead DPC, MC3T3-E1 cells and BMSC under serum-free cultures (albeit at differing concentrations and time points) (Figures 4.3.1; 4.3.3-4.3.5). DPC death was significantly decreased at 48 hours at both 10 and 100 ng/ml GDNF supplementation whereas MC3T3-E1 cell death was significantly decreased at 48 hours in response to 1 ng/ml GDNF (Figures 4.3.1;-4.3.3). GDNF at 10 ng/ml also significantly decreased BMSC death under serum-free conditions (Figures 4.3.4 and 4.3.5).

Under serum supplemented conditions there were no significant changes in dead cell numbers with GDNF supplementation, although BMSC consistently demonstrated reduced levels of cell death at 24 and 48 hours in response to GDNF (Figures 4.3.4 - 4.3.5).



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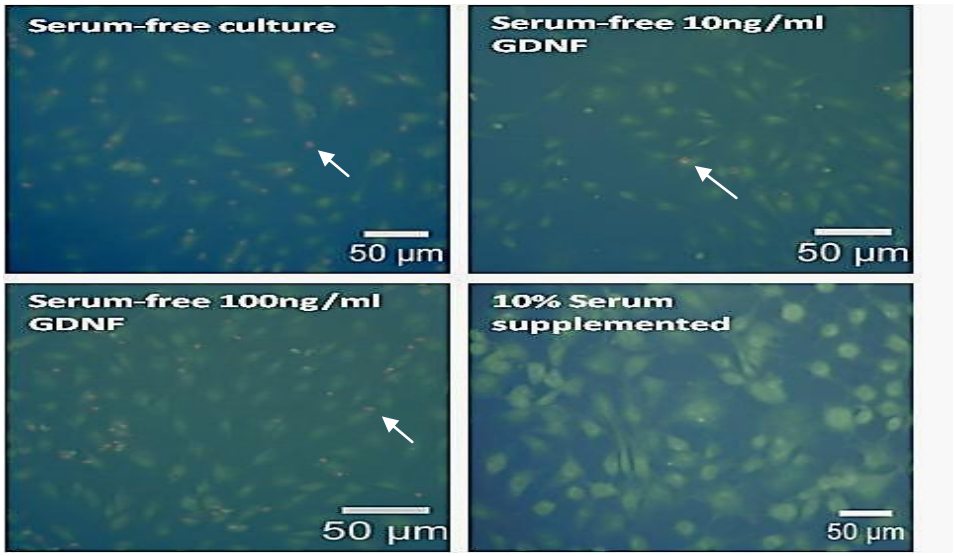
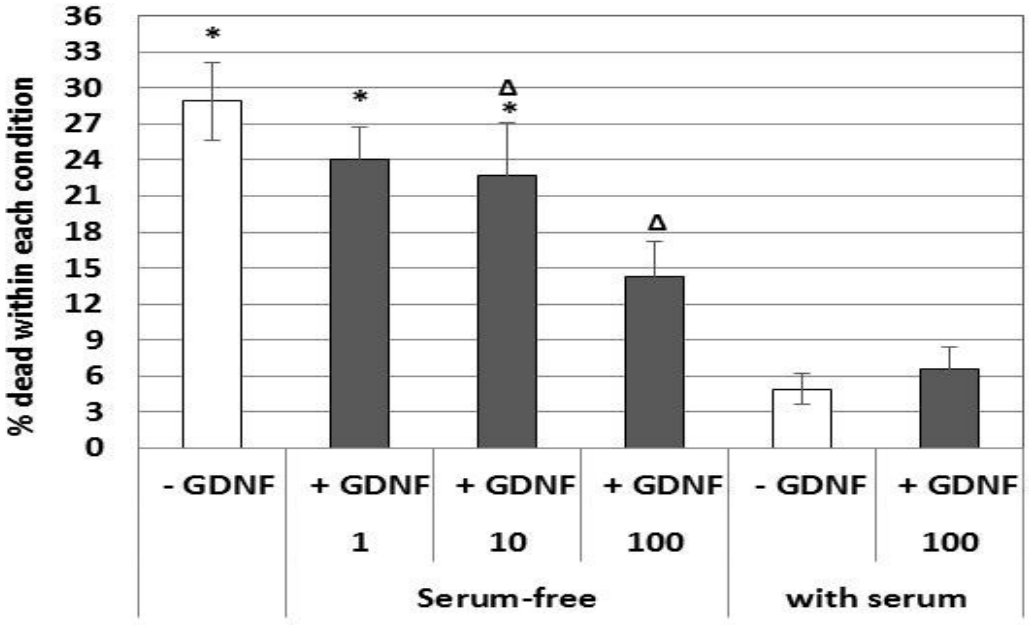


B

Figure 4.3 Live and dead analysis of DPC under serum-free and serum supplemented conditions at 24 hours. A) The percentage of dead DPC within each condition (Serum-free with 0, 1, 10 or 100 ng/ml GDNF and serum supplemented with 0 or 100 ng/ml GDNF). **B**) Representative images of live and dead staining; arrows show non-viable cells. Results are expressed as percentage; dead/total DPC. (n=3-7, Mean ± SEM; ⋯p = 0.0001 *versus* 100 ng/ml GDNF under serum-free conditions).



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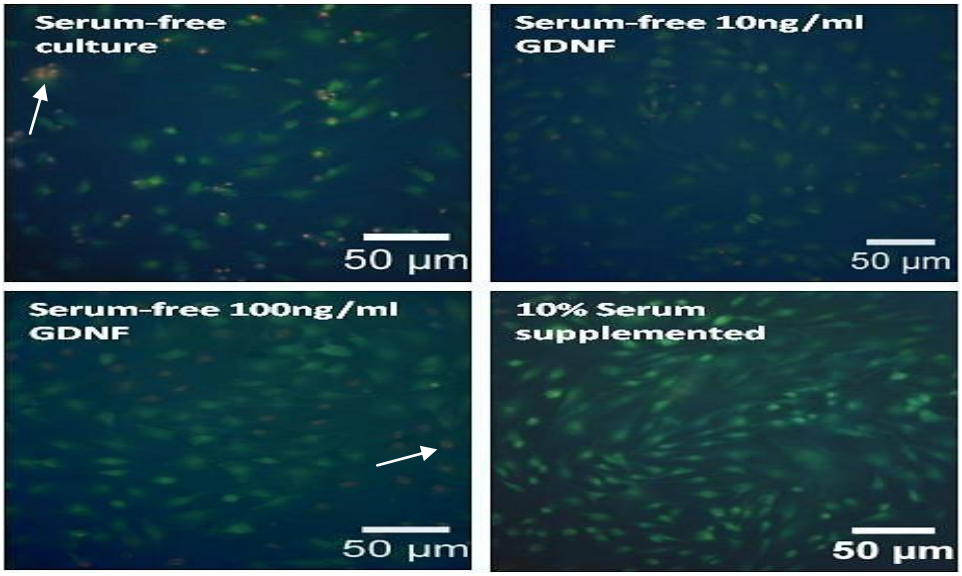
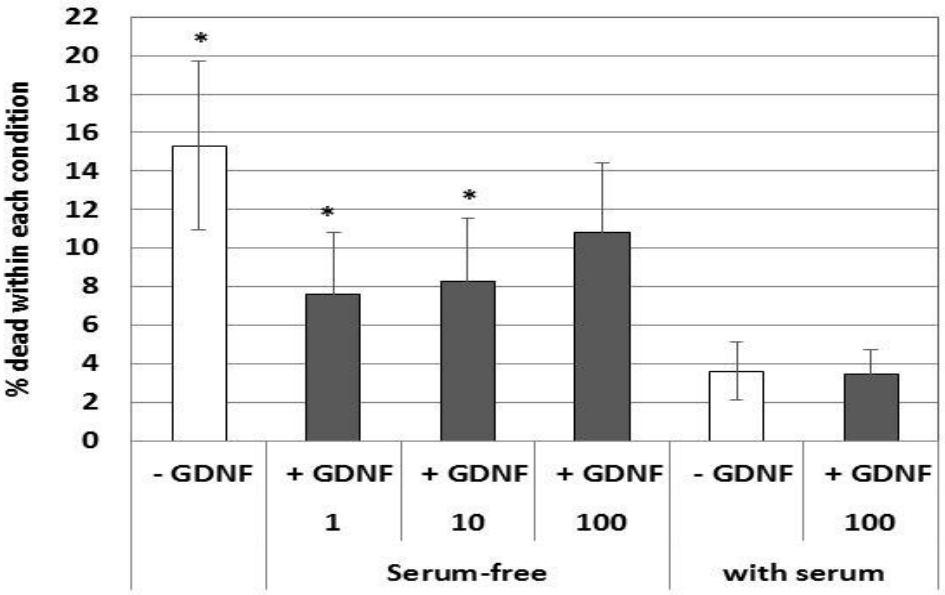


B

Figure 4.3.1 DPC under serum-free and serum supplemented conditions at 48 hours. A) The percentage of dead DPC within each condition (Serum-free with 0, 1,10 or 100 ng/ml GDNF and serum supplemented with 0 or 100 ng/ml GDNF). **B**) Representative images of live and dead staining, arrows show non viable cells. Results are expressed percentage; dead/total DPC (n=3-7, Mean ± SEM; *p =0.0001 *versus* serum supplemented (0 ng/ml GDNF unsupplemented); $^{\Delta}$ p = 0.0001 *versus* serum-free control (0 ng/ml GDNF unsupplemented).



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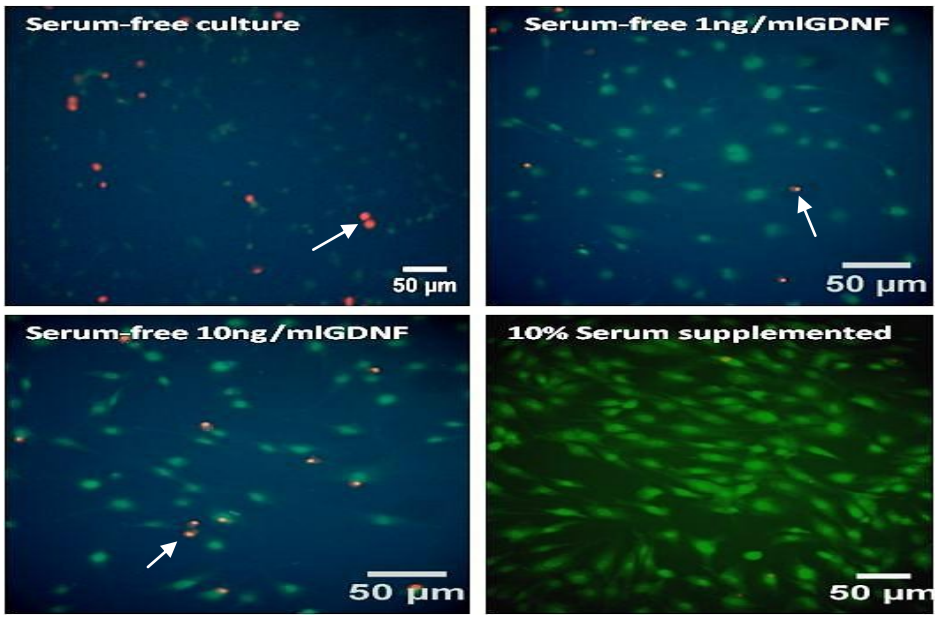
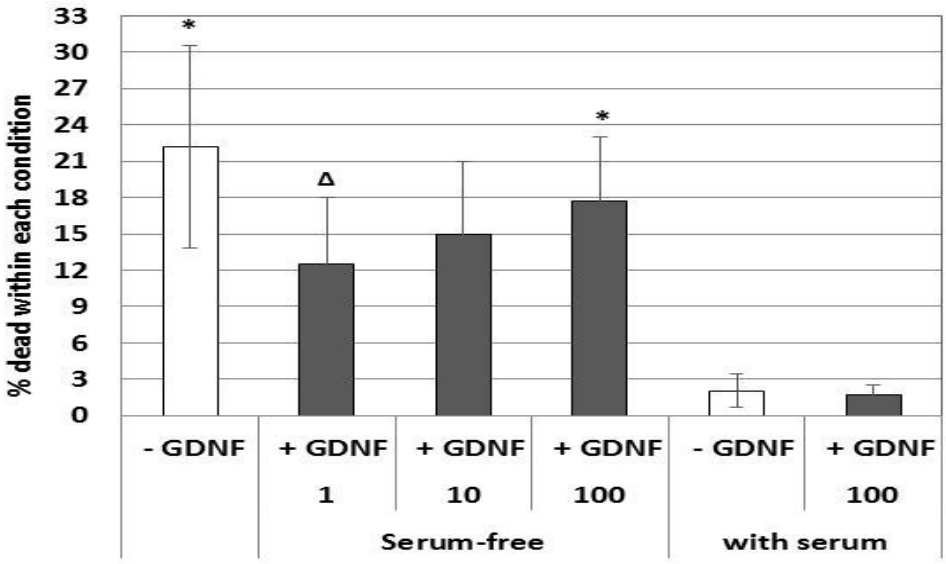


B

Figure 4.3.2 MC3T3-E1 cells under serum-free and serum supplemented conditions at 24 hours. A) The percentage of dead MC3T3-E1 cells within each condition (Serum-free with 0, 1,10 or 100 ng/ml GDNF and serum supplemented with 0 or 100 ng/ml GDNF). **B)** Representative images of live and dead staining, arrows show non viable cells. Results are expressed as percentage; dead/total MC3T3-E1 (n=3-7, Mean ± SEM; *p =0.0001 *versus* serum supplemented (0 ng/ml GDNF unsupplemented).



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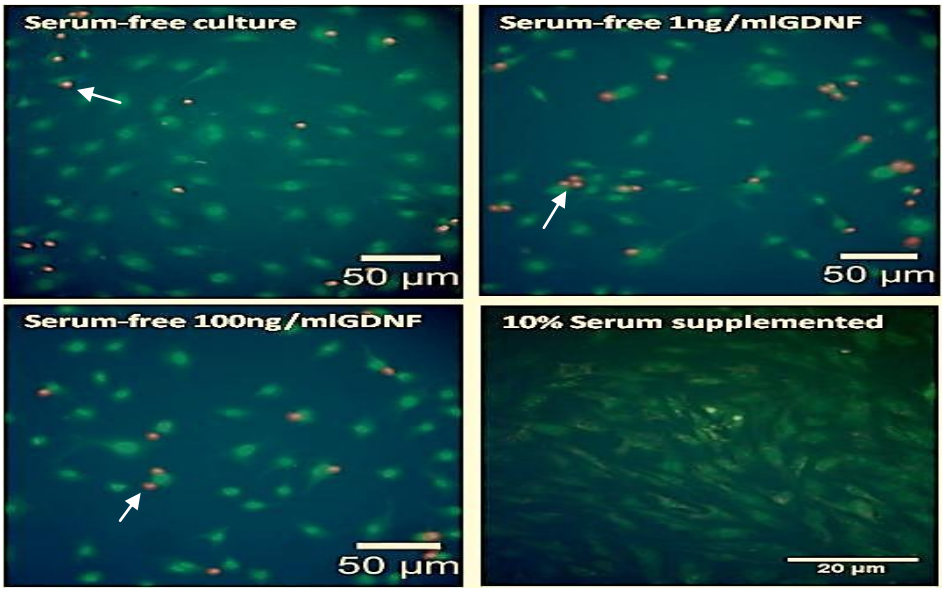
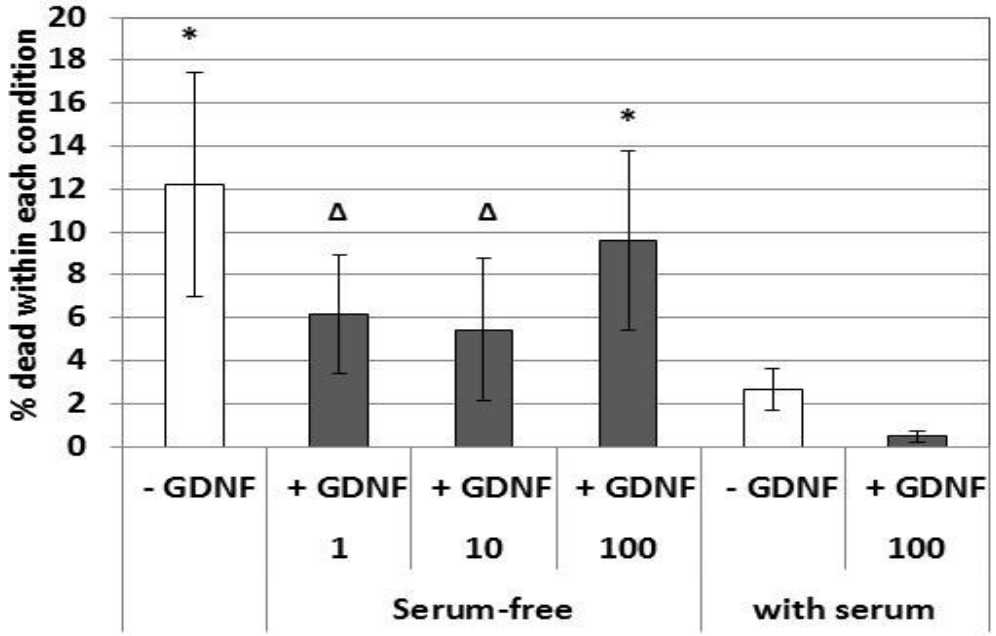


B

Figure 4.3.3 MC3T3-E1 cells under serum-free and serum supplemented conditions at 48 hours. A) The percentage of dead MC3T3-E1 cells within each condition (Serum-free with 0, 1, 10 or 100 ng/ml GDNF and serum supplemented with 0 or 100 ng/ml GDNF). **B**) Representative images of live and dead staining, arrows show non viable cells. Results are expressed as percentage; dead/total MC3T3-E1. (n=3-7, Mean ± SEM; *p =0.0001 *versus* serum supplemented (0 ng/ml GDNF unsupplemented); $^{\Delta}$ p = *versus* serum-free control (0 ng/ml GDNF unsupplemented)).



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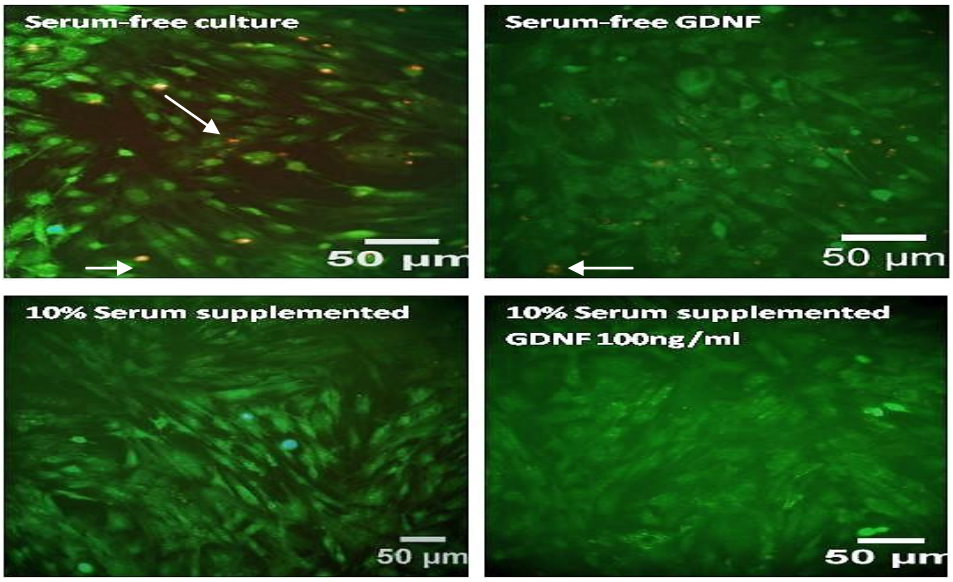
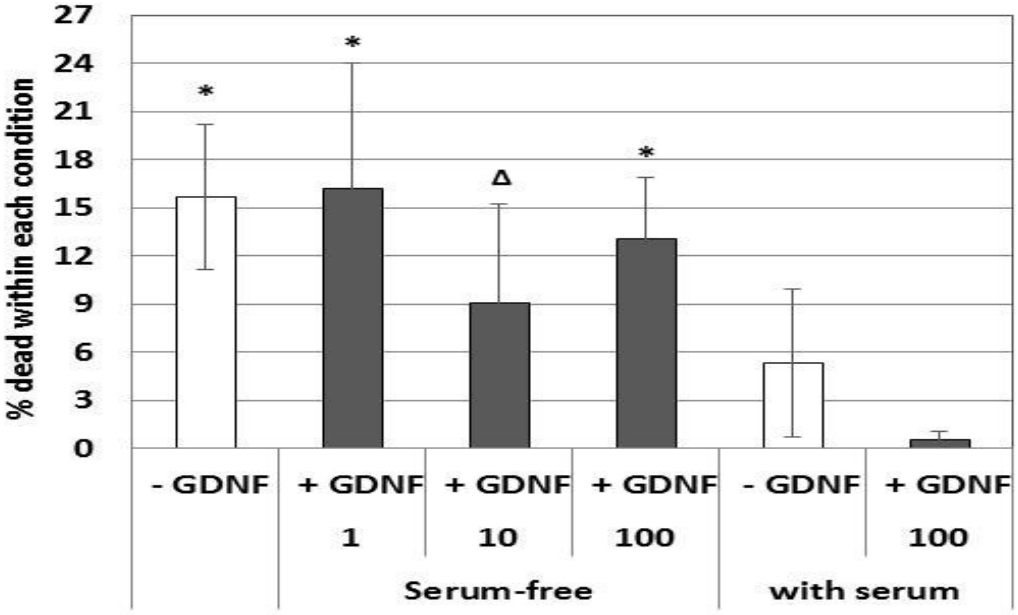


B

Figure 4.3.4 BMSC under serum-free and serum supplemented conditions at 24 hours. A) The percentage of dead BMSC within each condition (Serum-free with 0,1,10 or 100ng/ml GDNF and serum supplemented with 0 or 100 ng/ml GDNF). **B)** Representative images of live and dead staining, arrows show non viable cells. Results are percentage; dead/total BMSC. (n=3-7, Mean ± SEM; *p =0.0001 *versus* serum supplemented (0 ng/ml GDNF unsupplemented); $^{\Delta}$p *versus* serum-free control (0 ng/ml GDNF unsupplemented).



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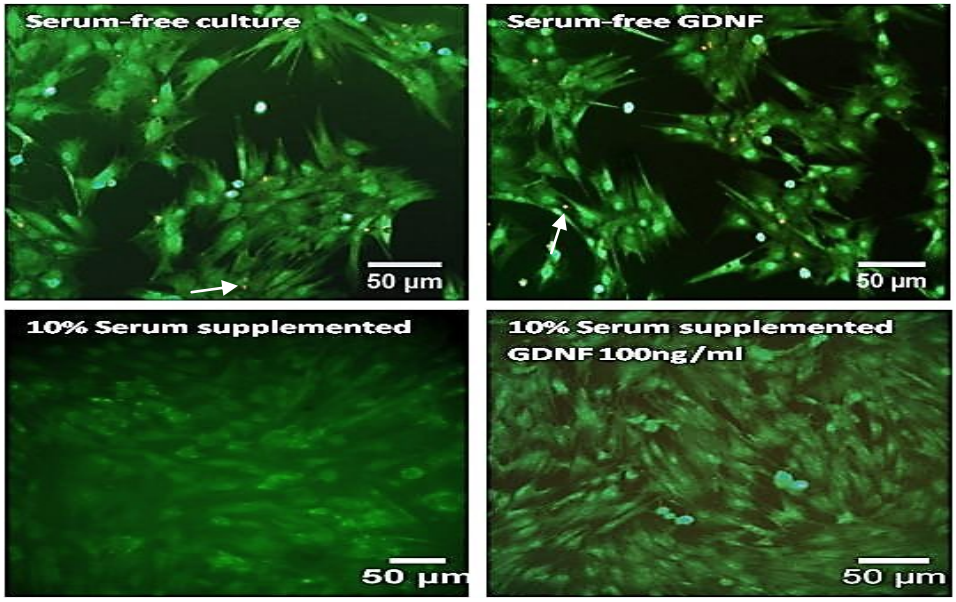


B

Figure 4.3.5 BMSC under serum-free and serum supplemented medium at 48 hours. A) The percentage of dead BMSC within each condition (Serum-free with 0,1,10 or 100 ng/ml GDNF and serum supplemented with 0 or 100 ng/ml GDNF supplementation). **B)** Representative images of live and dead staining, arrows show non viable cells. Results are expressed as percentage; dead/total BMSC. (n=3-7, Mean ± SEM, *p =0.0001 *versus* serum supplemented (0 ng/ml GDNF unsupplemented); $^{\Delta}$ p *versus* serum-free control (0 ng/ml GDNF unsupplemented).

4.2.1 Effect of GDNF on cellular apoptosis and necrosis

To support the live/dead data and detect necrosis and apoptosis within the cultures further biochemical cell death assays were performed under serum-free conditions. The LDH (lactate dehydrogenase) cytotoxicity assay determined the overall levels of cell death from necrosis and/or apoptosis (Xin *et al.* 2001). At 48 hours GDNF addition decreased LDH levels for MC3T3-E1 cells compared to (no GDNF) control or the LDH measurement taken at 24hours. LDH levels for DPC were also reduced at 48 hours compared to 24 hours. However no significant differences to the (no GDNF) control cultures were evident for MC3T3-E1 cells, DPC or BMSC (Figure 4.4).

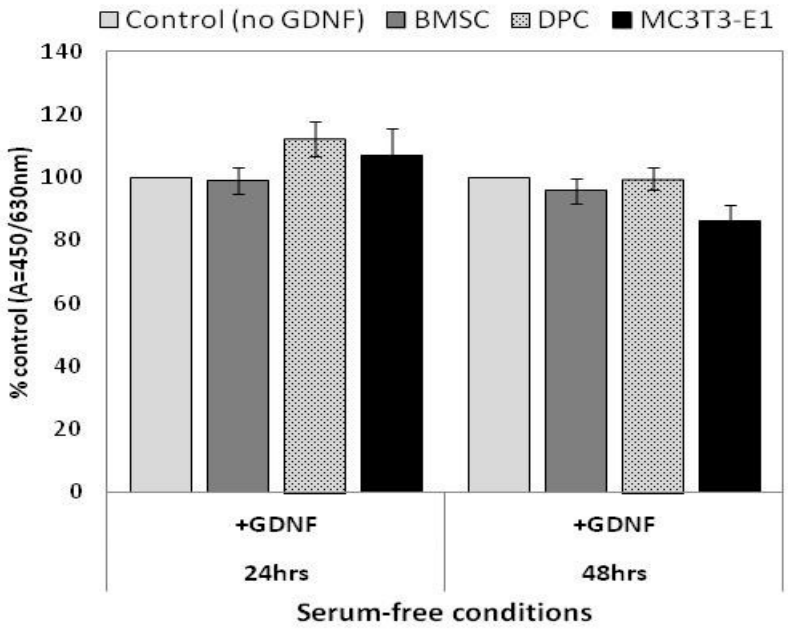


Figure 4.4 Effect of GDNF (100 ng/ml) on LDH levels in supernatants of DPC, MC3T3-E1 and BMSC cultures at 24 and 48 hours under serum-free conditions. Control contained no GDNF. Results are expressed as percentage control (0 ng/ml GDNF unsupplemented (Mean ± SEM, n=3).

At 48 hours the activities of caspases-3/-7 as a marker for apoptosis were investigated within DPC cultures. The activities were significantly increased under serum-free compared to serum supplemented cultures. Moreover, caspase-3/7 levels were significantly reduced on GDNF addition compared to control serum-free cultures containing no GDNF (Figure 4.5). These caspase levels were significantly reduced to levels comparable to that of serum supplemented cultures (1130 RLU and 1065 RLU, respectively) indicating that apoptosis was reduced by GDNF supplementation.

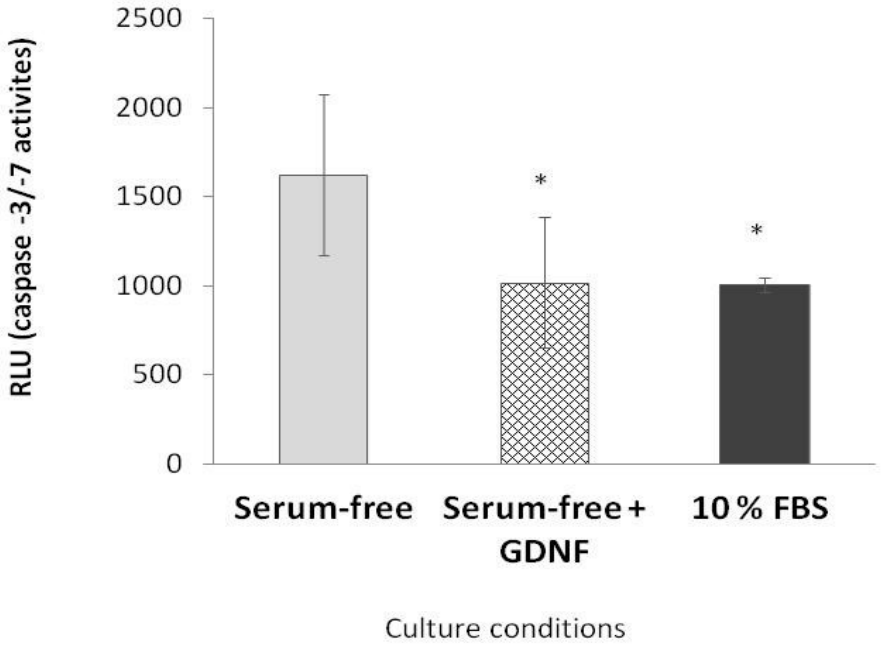


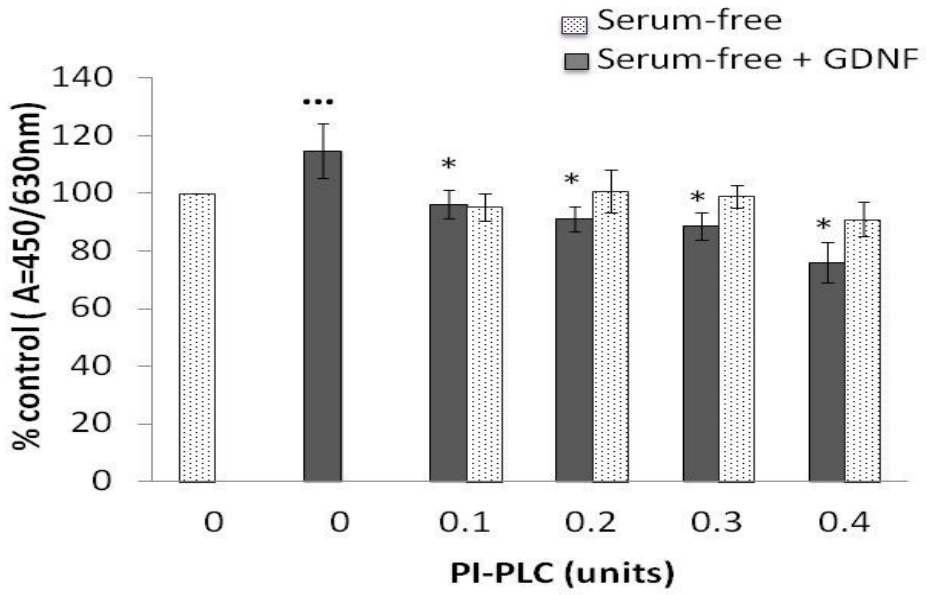
Figure 4.5 Caspase 3/7 activities within serum-free DPC cultures. Caspase levels were significantly reduced after 48 hours culture under serum supplemented conditions and GDNF (100 ng/ml) supplemented serum-free conditions compared to serum-free control (0 ng/ml GDNF unsupplemented). Results are shown as relative light units (RLU) (n=3, Mean ± SEM; T-test *$p<0.05$).

4.3 The effect of attenuating GDNF signalling on DPC and MC3T3-E1 cell viability

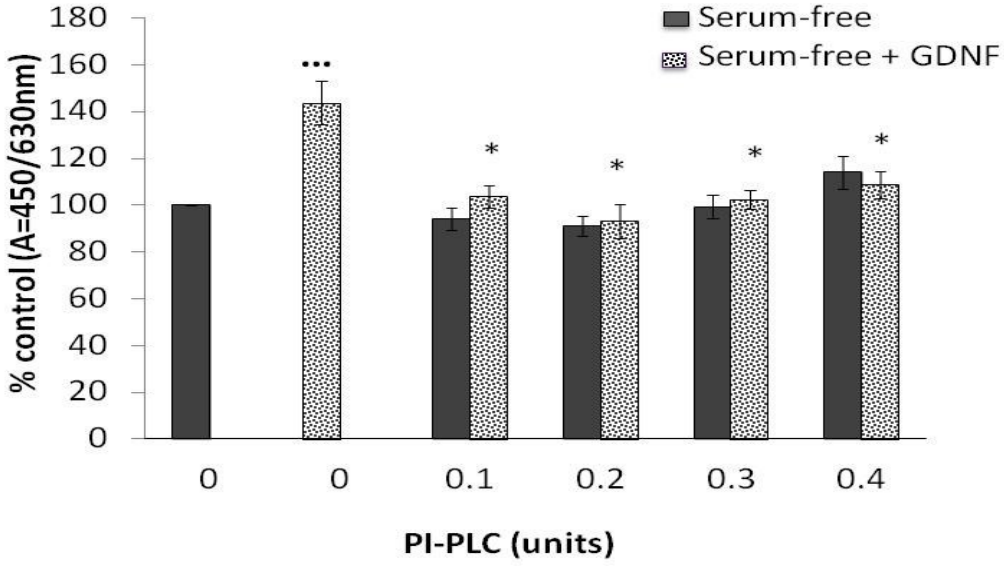
GDNF reportedly binds GFRα1, and can then form dimers with the RET receptors to induce intracellular signalling. (Sariola and Saarma 2003; Kawamoto *et al.* 2004; Bespalov and Saarma 2007; Airaksinen 1999). To confirm that GDNF action was direct signalling via its main receptors RET and GFRα1 was reduced using RPI-1 and PI-PLC inhibitors, respectively (Iwase *et al.* 2005; Cao *et al.* 2006; Cuccuru *et al.* 2004; Lanzi *et al*. 2000, 2003; Mologni *et al.* 2005).

4.3.1 Inhibition of GFRα1 (GPI- linked receptor)

PI-PLC hydrolyses GPI-membrane anchors thus cleaving the GFRα1 binding sites from the membrane. After 48 hours culture with PI-PLC the number of viable cells was assessed using the WST-1 assay. PI-PLC significantly reduced the number of viable DPC and MC3T3-E1 cells under serum-free GDNF supplemented conditions at all concentrations of PI-PLC (from 114 % to 76 % at 0.4 units PI-PLC for DPC and from 144 % to 93 % at 0.2 units PI-PLC for MC3T3-E1 cells), whilst it had no significant effect at any concentration in control serum-free cultures (Figure 4.6 A and B). The number of viable GDNF–stimulated DPC and MC3T3-E1 cells was decreased by the PI-PLC to levels comparable to those of controls at all concentrations of PI-PLC tested. This data indicated that PI-PLC abrogated GDNF activity on DPC and MC3T3-E1 cells (Figure 4.16A and B) suggesting that binding to GFRα1 was needed for GDNF effects on DPC and MC3T3-E1 cells.



A



B

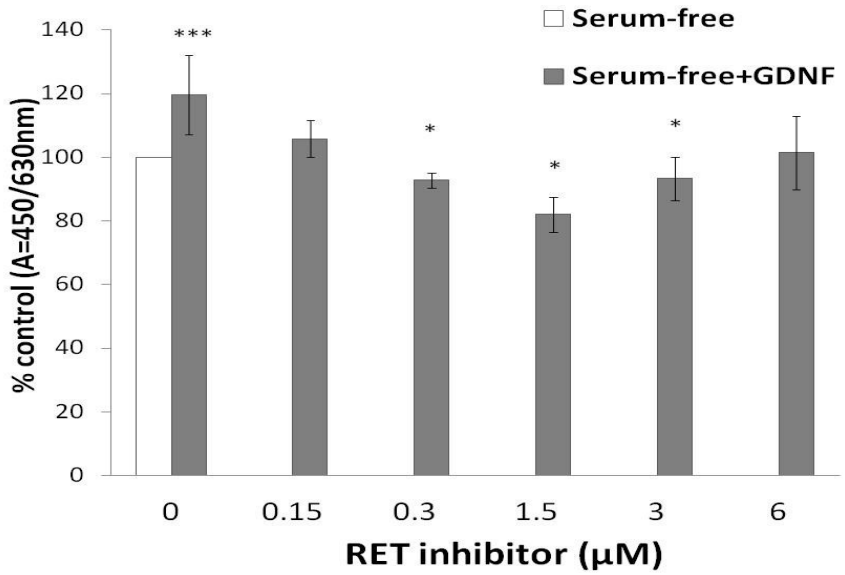
Figure 4.6 PI-PLC effect on A) GDNF-stimulated DPC and B) GDNF-stimulated MC3T3-E1 cells at 48 hours under serum-free conditions. GDNF was added to cultures at 100 ng/ml. Results are expressed as percentage controls (n=6, Mean ± SD; *p =0.0001 *versus* serum-free + GDNF control; ⋯p=0.01 *versus* serum-free control (0 ng/ml GDNF unsupplemented).

4.3.2 The effect of inhibition of RET receptor DPC and MC3T3-E1 cell viability

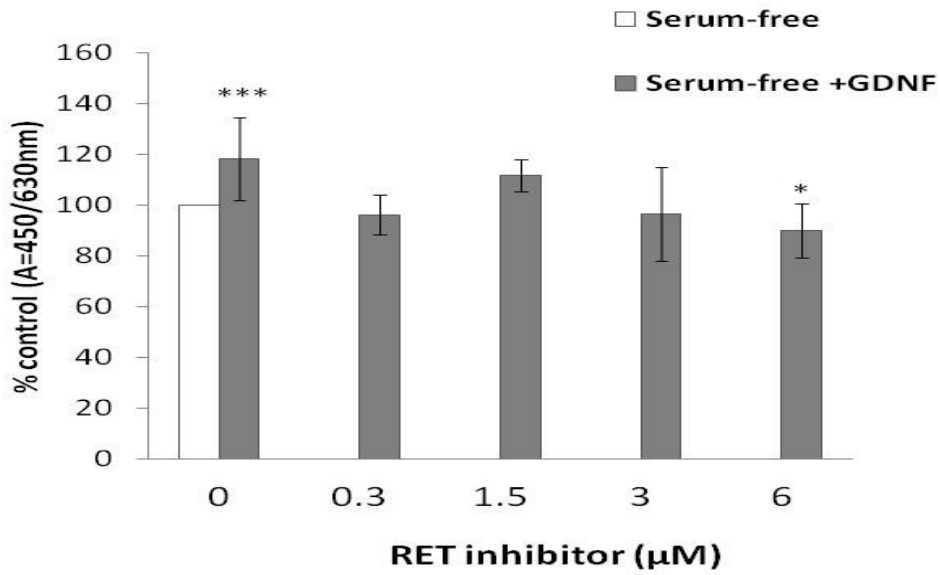
RPI-1 competitively inhibits RET receptors by binding at an active site of the receptor and thereby blocking auto-phosphorylation and subsequent activation of intracellular signalling pathways (Figure 1.1, pg 4) (Mologni *et al.* 2005, 2006; Kawamoto *et al.* 2004). In serum-free controls, high RPI-1 concentrations (>30 μM) significantly decreased viable DPC numbers compared to the control cultures indicating RPI-1 was toxic at these concentrations. Such toxicity has previously been recorded for this compound (> 30 μM) (Lanzi *et al.* 2000). At 30-60 μM the RET inhibitor had a similar cytotoxic effect on MC3T3-E1 cells.

RPI-1 (0.3-3 μM) significantly decreased GDNF-stimulated viable DPC cell number under serum-free conditions suggesting that GDNF effect was dependent on signalling through the RET receptor (Figure 4.6.1A). Addition of GDNF with RPI-1 (6 μM) significantly decreased viable MC3T3-E1 cell numbers to levels comparable to the levels of the serum-free controls (Figure 4.6.1B). The addition of 0.1 ng/ml of GDNF significantly increased viable glioma cell number (Figure 4). Due to the relatively high "sensitivity" of this cell line to GDNF, the inhibitor was added at higher concentrations within the range that showed effects on DPC and MC3T3-E1 cells, to ensure adequate attenuation of the receptors. [increased sensitivity may indicate increased receptor numbers (Davidson *et al.* 2011)]. The addition of the inhibitor at 3 μM and 6 μM decreased the numbers of glioma viable cells (Figure 4.6.2). This data demonstrated that RET inhibition reduced the GDNF stimulated increase in the number of viable

DPC, MC3T3-E1 and C6 glioma cells under serum-free conditions. These data indicated that GDNF effects were mediated by the RET receptor for all cell types tested.



A



B

Figure 4.6.1 RPI-1 effect on A) GDNF-stimulated DPC viable cell number and B) GDNF-stimulated MC3T3-E1 viable cell number at 48 hours under serum-free conditions. GDNF was added to cultures at 100 ng/ml. Results are expressed as percentage controls. (n=2, Mean ± SEM, *p =0.0001 v*ersus* serum-free + GDNF; *** p= *versus* serum-free control (0 ng/ml GDNF unsupplemented)).

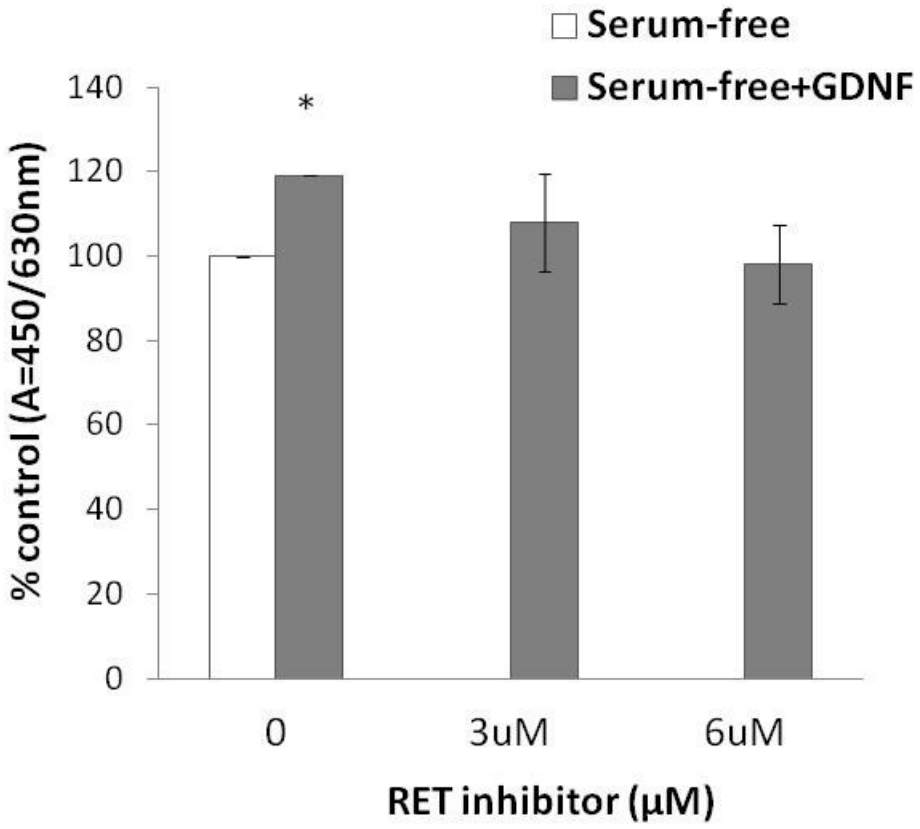


Figure 4.6.2 RPI-1 effect on GDNF-stimulated glioma cell number at 48 hours under serum-free conditions. GDNF was added to cultures at 0.1 ng/ml. Results are expressed as percentage controls. (n=7, Mean ± SD; *p= 0.0001 *versus* serum-free control (0 ng/ml GDNF unsupplemented).

CHAPTER 5 RESULTS

GDNF effects on cell differentiation and *in vitro* mineralisation

5 Short-term *in vitro* differentiation

The stimulation of DPC, BMSC and MC3T3-E1 cell differentiation during disease may limit the effects of infection and repair damage by generating cells (i) to replace lost/damaged cells and (ii) reform the mineralised tissues of the tooth and bone. In this study alkaline phosphatase (ALP) was used as a marker of early differentiation of DPC, BMSC and MC3T3-E1 cells in response to GDNF. ALP levels can reportedly be used as an early indicator of differentiation (Matsui *et al.* 1992; Porter *et al.* 2003; Beck *et al.* 1998).

ALP activities were decreased for all cell types studied under serum-free compared to serum-supplemented culture conditions. Under serum-free conditions, GDNF (100 ng/ml) significantly decreased BMSC and MC3T3-E1 cell ALP activities (Figure 5B and 5C). Under serum-supplemented conditions DPC and BMSC displayed non-significantly reduced ALP activities in response to increasing GDNF concentration. However changes in MC3T3-E1 cell ALP activities under serum supplemented conditions were only apparent at 100 ng/ml GDNF where ALP activities were significantly increased (Figure 5B). This data suggested that GDNF may inhibit early differentiation of DPC, BMSC and MC3T3-E1cells under serum-free conditions yet stimulate differentiation of MC3T3-E1 cells under serum supplemented conditions at 100ng/ml (Figure 5).

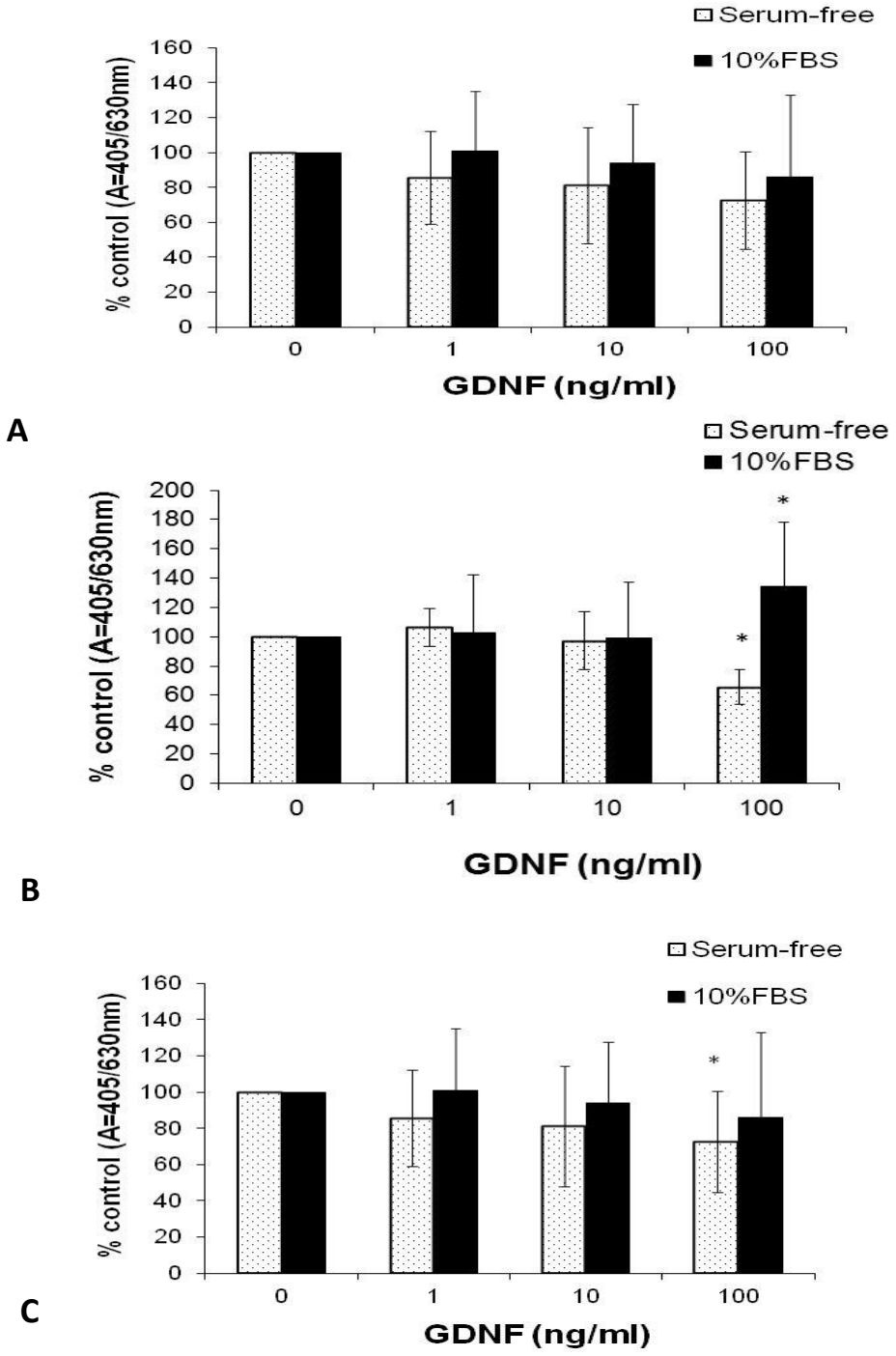


Figure 5 GDNF effect on (A) DPC (B) MC3T3-E1 cells and (C) BMSC ALP activities under serum-free and serum supplemented conditions. Results are expressed as percentage of control (0 ng/ml GDNF unsupplemented conditions) (Mean ± SEM; n=4, BMSC and MC3T3-E1 cells, Mean ± SEM; n=3, *p<0.05 *versus* control).

5.1 Long-term *in vitro* mineralisation

GDNF induced proliferation of DPC and MC3T3-E1 cells at day 2 (see Chapter 4) along with changes in ALP levels shown above (Figure 5). These data suggested that GDNF may be stimulating early differentiation/expansion within short term cultures under serum-free conditions and may therefore also affect late differentiation over the longer term. Notably ECM mineralisation has been reported to be preceded by transiently increased ALP levels (Hoemann *et al.* 2009; Wei *et al*. 2007; Lui *et al.* 2007).

Staining of mineralised ECM under osteogenic conditions [(supplementation with 10mM β-glycerophosphate (β-gp), 50 μg/ml ascorbic acid (AA) and 10^{-7} M dexamethasone (DEX)] demonstrates that cultures contain fully differentiated functionally active secretory cells, therefore Alizarin Red Staining (ARS) was used to visualise and semi-quantify mineralised areas of the cell cultures.

Preliminary mineralisation studies were performed in the absence of GDNF to determine the capacity of the cell cultures to produce mineralised matrix in standard serum supplemented cultures. MDPC-23 cells were used as a positive control to represent odontoblast-like cells known to produce mineralised ECM in response to osteogenic additives (Hanks *et al.* 1998; Sun *et al.* 1998; Pang *et al.* 2006).

Under serum-supplemented osteogenic conditions all cultures showed significant mineralisation compared to standard serum supplemented conditions that had minimal mineral deposition (Figure 5.1). MDPC-23 and MC3T3-E1 cells displayed relatively early mineralised ECM formation and generally stained more intensely compared to the DPC cultures. Staining of mineralised ECM within MDPC-23 and MC3T3-E1 cell cultures was evident at day 3, while DPC staining remained comparatively weak even after 24 days of culture (Figure 5.1). Subsequently the effect of GDNF was investigated under osteogenic serum-free culture conditions at 2 and 3 week intervals compared to serum supplemented osteogenic conditions to assess GDNF effects on mineralisation of the cultures.

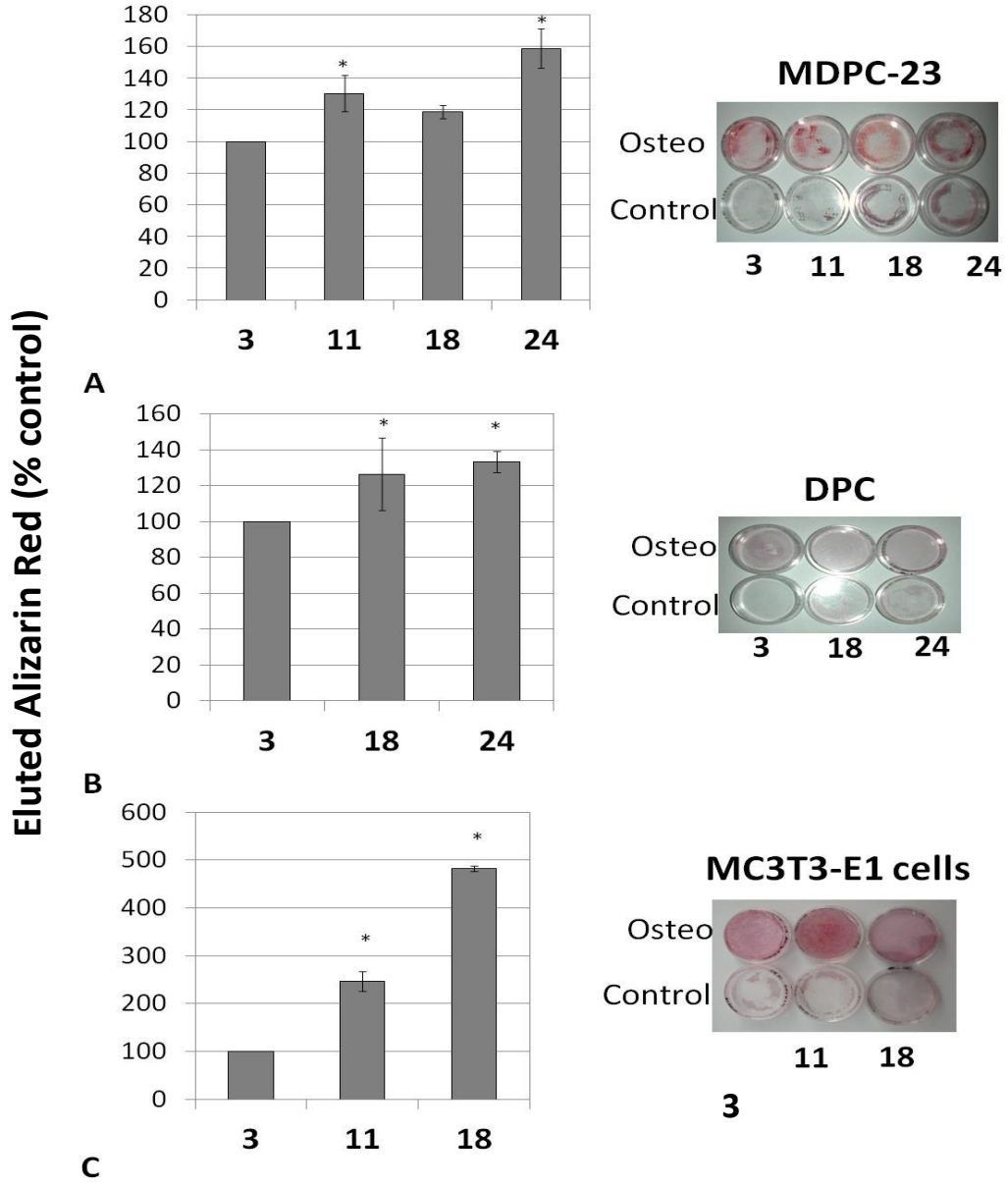


Figure 5.1 Effect of osteogenic culture conditions on *in vitro* mineralised matrix formation. Results are shown as percentage of control (day 3), for (**A**) MDPC-23 cells (**B**) DPC and (**C**) MC3T3-E1 cells. Corresponding images of Alizarin red staining (ARS) under osteogenic (OSTEO) or control (standard serum supplemented) conditions are shown on the right. Results are expressed as percentage of day 3 osteogenic absorbance value (450/630nm; Mean ± SD; n=3, *p <0.005 versus day 3 osteogenic conditions).

5.1.1 *In vitro* mineralisation under serum-free conditions

DPC were cultured under serum-free osteogenic supplemented conditions in order to study the longer term effects of GDNF. The addition of osteogenic supplements and GDNF to serum-free conditions significantly increased ARS staining, compared to serum-free osteogenic cultures and also compared to serum-free cultures without osteogenic supplements (Figure 5.2). The subsequent mineralisation of the ECM indicated that GDNF was potentially stimulating osteo/odontogenic differentiation.

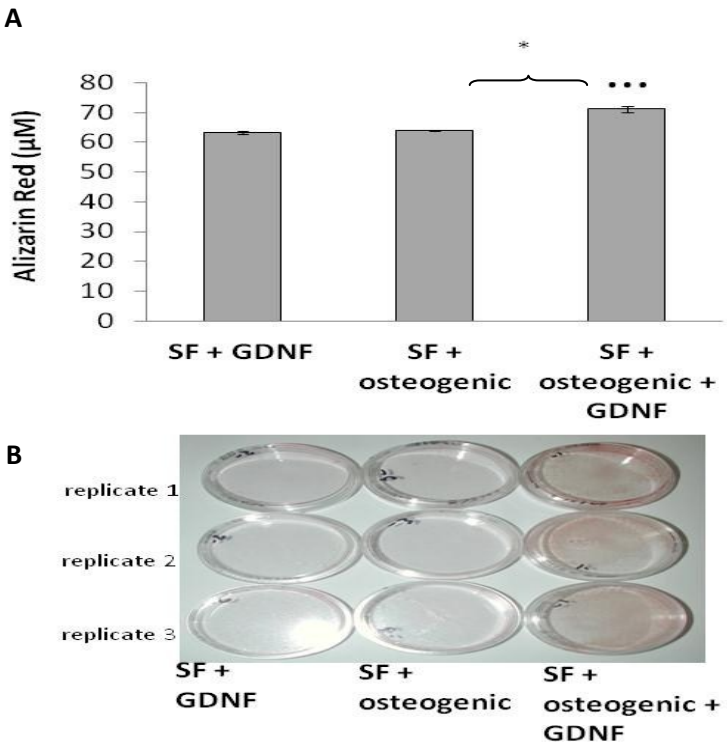
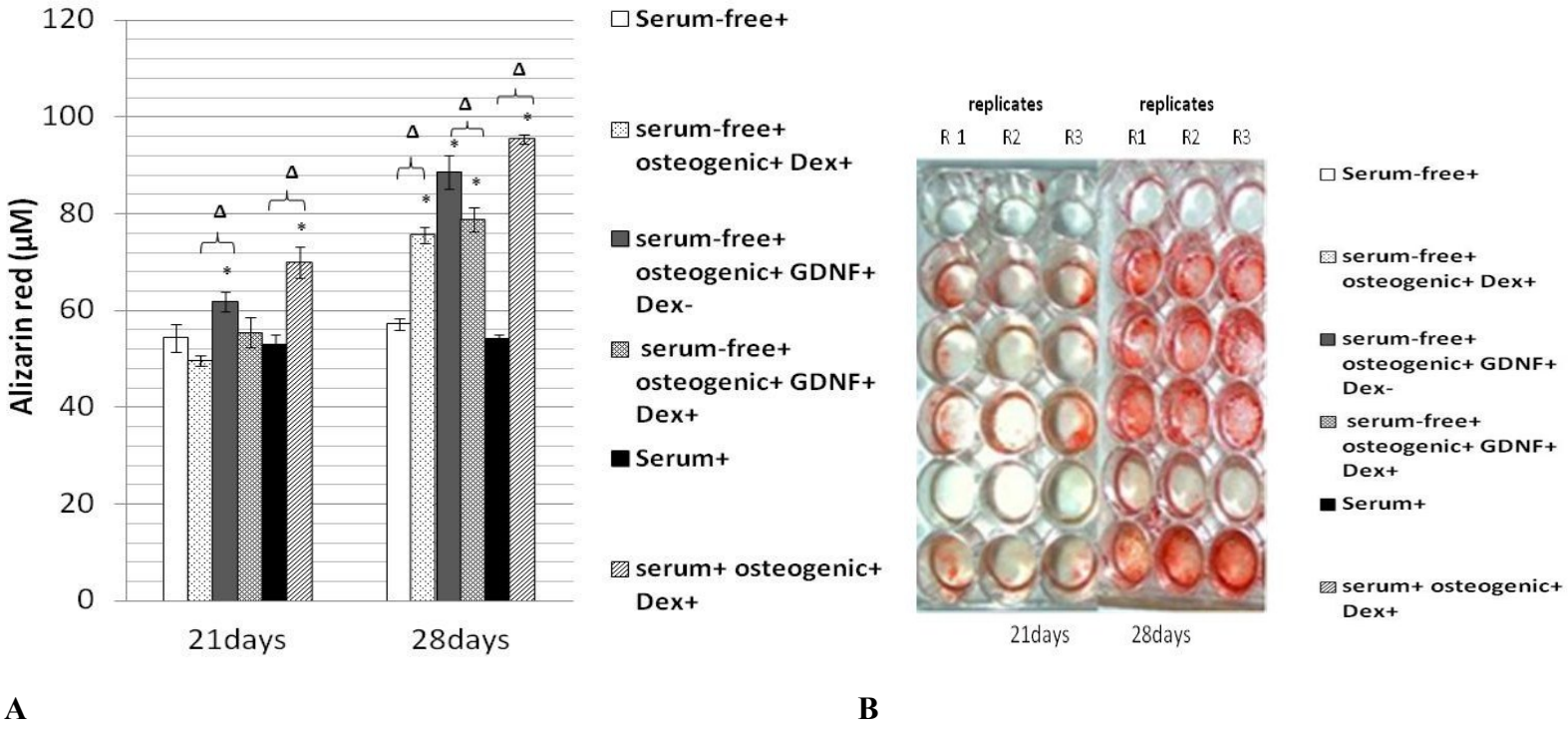


Figure 5.2 Effect of GDNF on mineralised matrix formation for DPC cultures at 7 day. (A) Concentration of eluted Alizarin red (μM) after culture under osteogenic or serum-free (SF) conditions and **(B)** corresponding images of Alizarin red staining. Results are expressed as Alizarin red concentration (μM) (Mean ± SD; n=3, *p<0.001 significantly different groups; p$^{...}$ *versus* serum-free unsupplemented controls).

The effects of GDNF on *in vitro* mineralisation were studied using DPC and MC3T3-E1 cells cultured for 21 and 28 days under serum-free osteogenic conditions with GDNF, using serum supplemented osteogenic culture conditions as a positive control for mineralisation. Areas of mineralised ECM were detected within DPC and MC3T3-E1 cell cultures and the addition of osteogenic supplements to serum-free conditions increased ARS staining 28 and 21 days of culture respectively. ARS was significantly increased within GDNF-stimulated cultures under serum-free conditions (Figure 5.3).

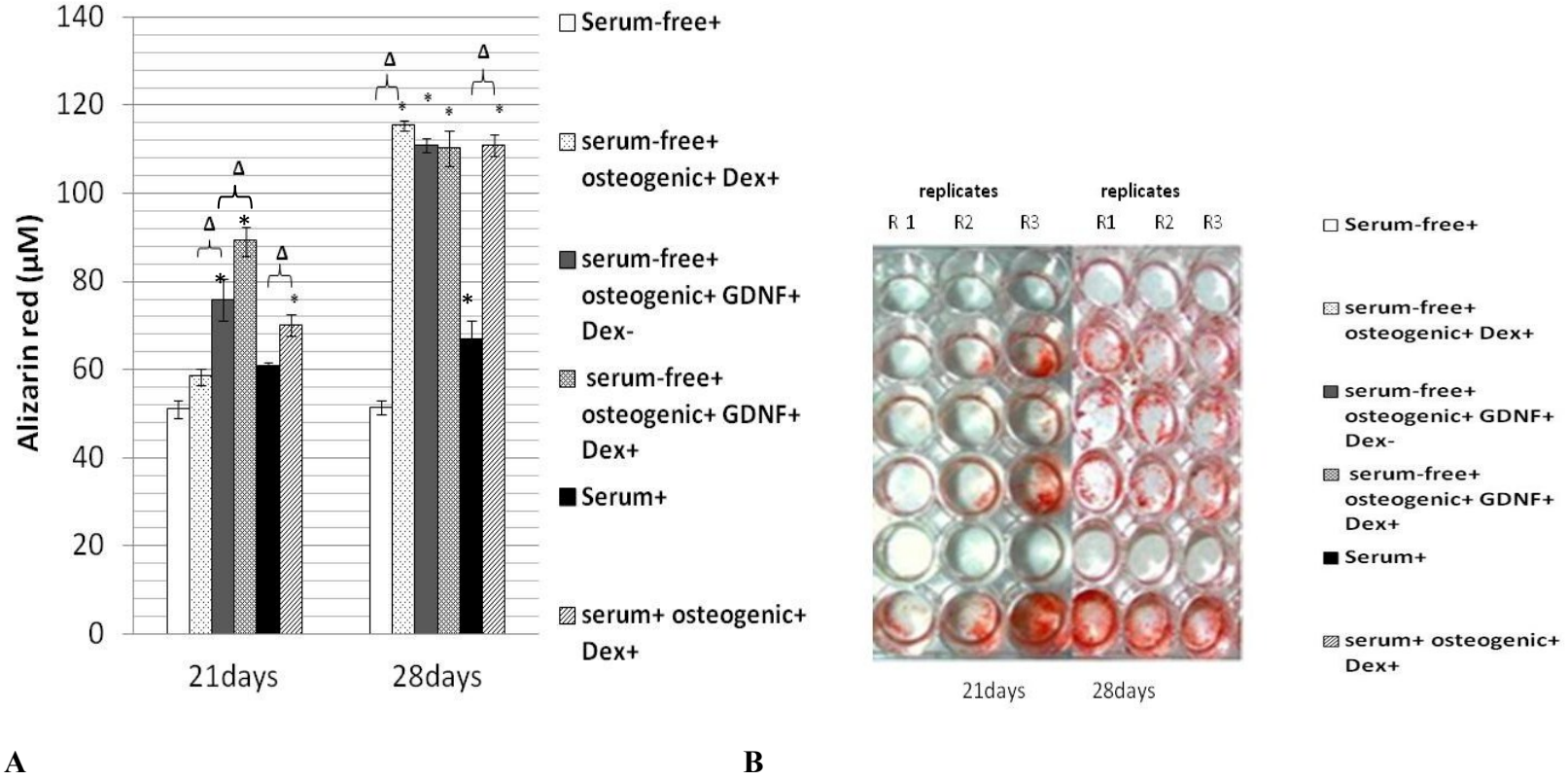
Interestingly when DEX was not used for supplementation the GDNF-stimulated serum-free DPC osteogenic cultures displayed increased ARS staining indicating that DEX addition decreased GDNF effects on differentiation. The highest level of staining was observed for serum supplemented cultures with osteogenic supplements, furthermore GDNF treated DPC serum-free cultures without DEX showed comparable staining levels to serum supplemented osteogenic conditions (Figures 5.3).

A similar pattern of staining was evident for MC3T3-E1 cells, albeit at day 21 osteogenic supplements increased ARS staining under serum-free conditions compared to controls that contained no osteogenic supplementation. The addition of GDNF to the osteogenic supplements further increased the intensity of staining detected. In contrast to the results for DPC, the addition of DEX and GDNF to MC3T3-E1 cell cultures resulted in staining more intensely than all other conditions at day 21 (Figure 5.4). These data indicated that DEX and GDNF may act synergistically to increase MC3T3-E1 cell differentiation. The DPC cultures again stained more weakly under all conditions compared with MC3T3-E1 cell cultures. At day 28, all osteogenic supplemented conditions for both DPC and MC3T3-E1 cultures were significantly increased compared to the controls (Figure 5.4).



A **B**

Figure 5.3 Effect of GDNF on *in vitro* DPC cultures mineralised matrix formation (**A**) Concentration of eluted Alizarin red (µM) after culture under osteogenic or control conditions at 21 or 28 days of culture within 48 well plates. Results are expressed as Alizarin red concentration (µM) **(**Mean ± SD; n=3, *$p<0.05$ versus respective control (serum-free or serum supplemented) at time point 21 days or 28 days; $^{\Delta}p<0.05$ significantly different groups at 21 days or 28 days). Results from the 28 day group were not compared to 21 day group (**B**) corresponding images of Alizarin red staining in triplicate for each condition.



A **B**

Figure 5.4 Effect of GDNF on *in vitro* MC3T3-E1 cell culture mineralised matrix formation. (A) Concentration of eluted Alizarin red (µM) after culture under osteogenic or control conditions at 21 or 28 days of culture within 48 well plates. Results are expressed as Alizarin red concentration (µM) **(**Mean ± SD; n=3, *$p<0.000$ *versus* respective control (serum-free or serum supplemented) at time point 21 days or 28 days; $^{\Delta}p<0.05$ significantly different groups at 21 days or 28 days). Results from the 28 day group were not compared to 21 day group. **(B)** Corresponding images of Alizarin red staining in triplicate for each condition.

CHAPTER 6 RESULTS

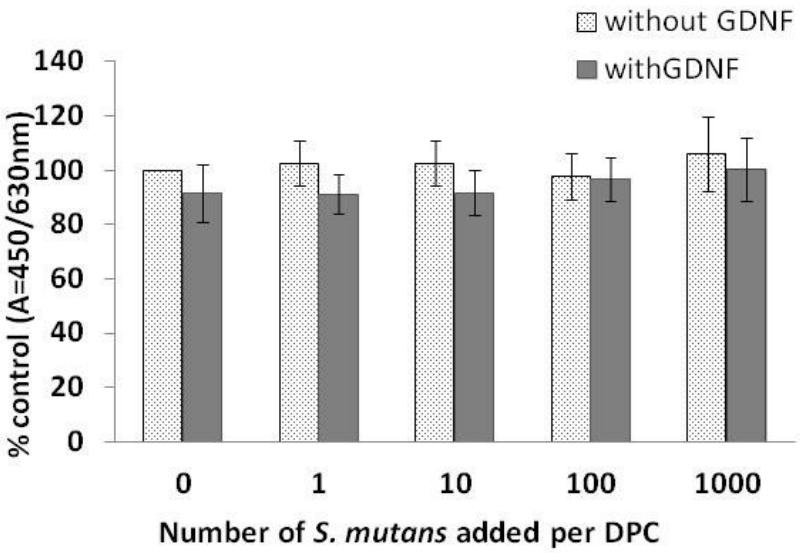
Potential role of GDNF during postnatal repair and infection

6 GDNF effects on DPC and/or MC3T3-E1 cell behaviour in response to;

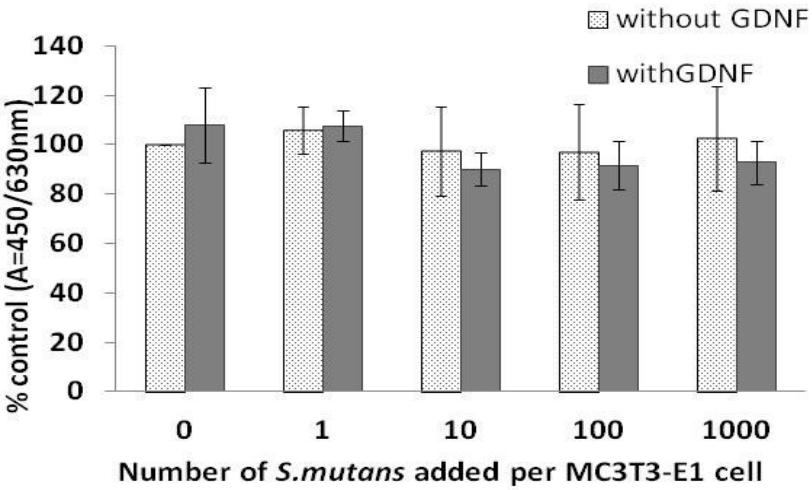
6.1 *Streptococcus mutans (S.mutans)*

DPC and MC3T3-E1 cell survival and proliferation may be limited following exposure to bacteria and *in vivo* such infections, therefore limit tissue repair and regeneration. *S.mutans* is a gram positive bacteria present within the plaque biofilm on teeth (Michelich *et al.* 1980; Marsh 2006; Verstraeten *et al.* 2008) and is associated with dental caries progression (Rosengren and Winblad 1975; Meikle *et al.* 1982; Jackson *et al.* 1997). *S.mutans* is therefore regarded as an instigator of tooth attrition and can contact dental pulp and periodontal bone during aggressive infections. However, the direct effect of *S.mutans* on dental pulp cells and bone cells still warrants further investigation (Hahn and Liewehr 2007; Law *et al.* 2008; Wayman *et al*. 1992; Duany *et al.* 1971).

Initially DPC and MC3T3-E1 viable cell number in response to heat inactivated *S. mutans* with and without GDNF supplementation were investigated. DPC and MC3T3-E1 cells were cultured with *S. mutans* (at 0, 1, 10 and 100 cells per DPC or MC3T3-E1 cell), with and without 100 ng/ml GDNF and under serum supplemented conditions. The addition of *S. mutans* did not significantly affect the numbers of viable DPC or MC3T3-E1 cells (Figure 6A and B); furthermore GDNF addition had no significant effect on DPC or MC3T3-E1 cell viability in the presence of heat inactivated *S.mutans* (Figure 6A and B).



A



B

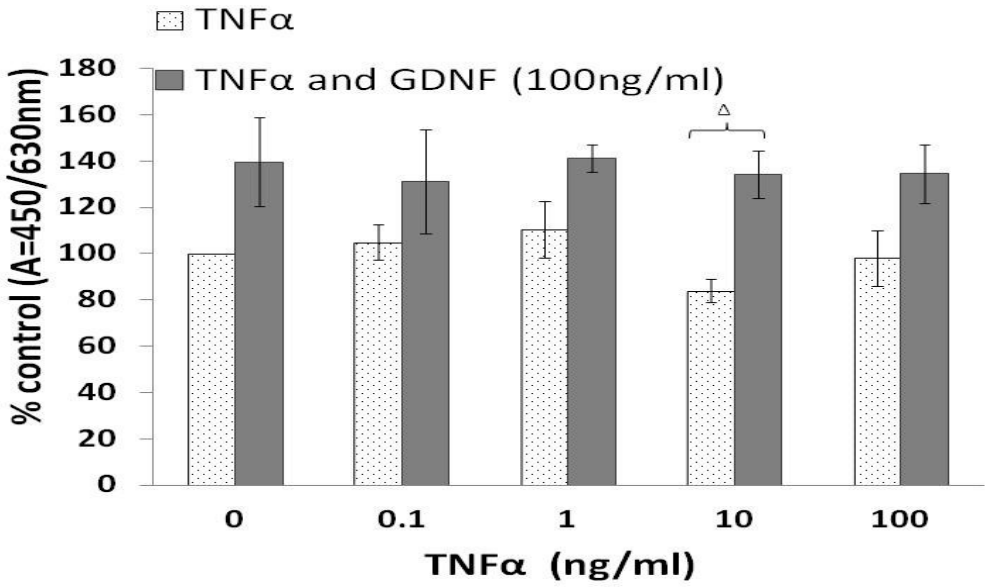
Figure 6 GDNF (100 ng/ml) effect on viable cell number with or without *S. mutans* addition for (A) DPC and **(B)** MC3T3-E1 cell cultures after 48 under serum supplemented conditions. Bacterial cell concentrations were 0, 1, 10 and 100 cells per DPC or MC3T3-E1 cell. Viability was determined by WST-1 assay. Results are expressed as percentage control (0 ng/ml GDNF unsupplemented conditions) (Mean ± SD; n=2).

6.1.1 TNFα

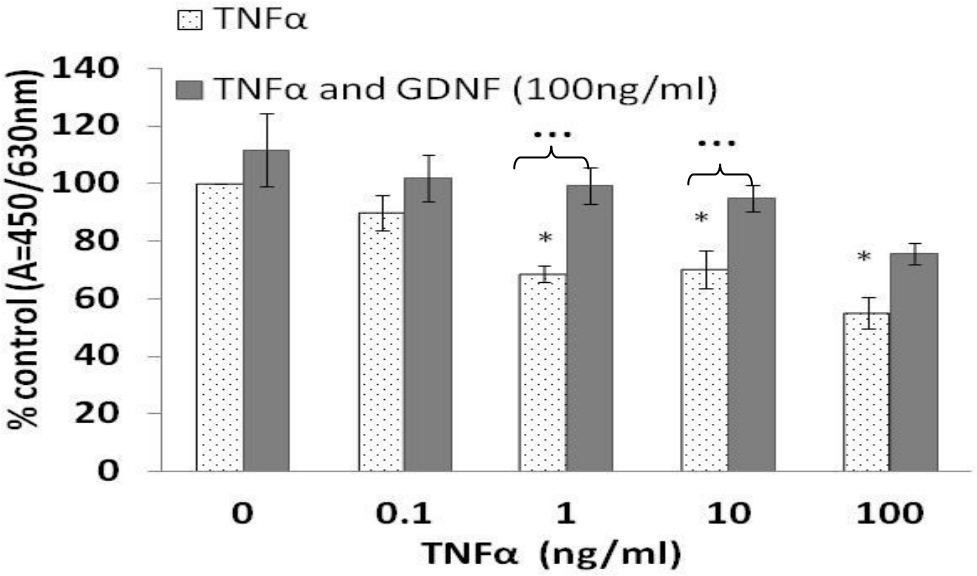
There is potential for the use of the anti-apoptotic properties of GDNF (Steinkamp *et al.* 2003; Botchkareva *et al.* 2000; Dalkara *et al.* 2011; Ng *et al.* 2009) to aid repair of dental pulp and bone *in vivo* during inflammatory conditions. GDNF activity under *in vitro* conditions representative of some aspects of disease may improve our understanding of GDNF function during dental and bone disease. TNFα is a pro-inflammatory cytokine, present during infections of the dental pulp and during inflammatory bone and skeletal joint diseases such as rheumatoid arthritis (Kokkas *et al.* 2007; Cooper *et al.* 2010; Scott and Kingsley 2006). In this study TNFα supplemented cultures provided a simple *in vitro* model of aspects of inflammation to investigate the action of GDNF on cells-derived from mineralised tissue during injury and inflammation.

Under serum-free conditions no significant effects of TNFα on viable DPC cell numbers were detected (Figure 6.1A). The addition of GDNF (100 ng/ml) to the TNFα-containing cultures increased viable DPC numbers to levels seen for previous experiments where GDNF alone was added to DPC (Chapter 4). This data indicated that TNFα displayed no evident interaction with GDNF-induced cell survival and proliferation under serum-free conditions.

Under serum-supplemented conditions, TNFα (1-100 ng/ml) significantly decreased viable DPC numbers. Further supplementation with GDNF (100 ng/ml) abolished TNFα-induced decrease in viable DPC numbers (Figure 6.1B). Conversely, under both serum-free and serum supplemented conditions, MC3T3-E1 cell numbers were significantly increased with 10 and 100 ng/ml TNFα supplementation. Moreover TNFα and GDNF elicited a synergistic effect significantly increasing MC3T3-E1 viable cell numbers above those obtained from either TNFα or GDNF addition alone (Figure 6.1.1). These data indicate that GDNF may negate the deleterious effects of TNFα on DPC, whereas for MC3T3-E1 cells GDNF enhances the effects of TNFα.

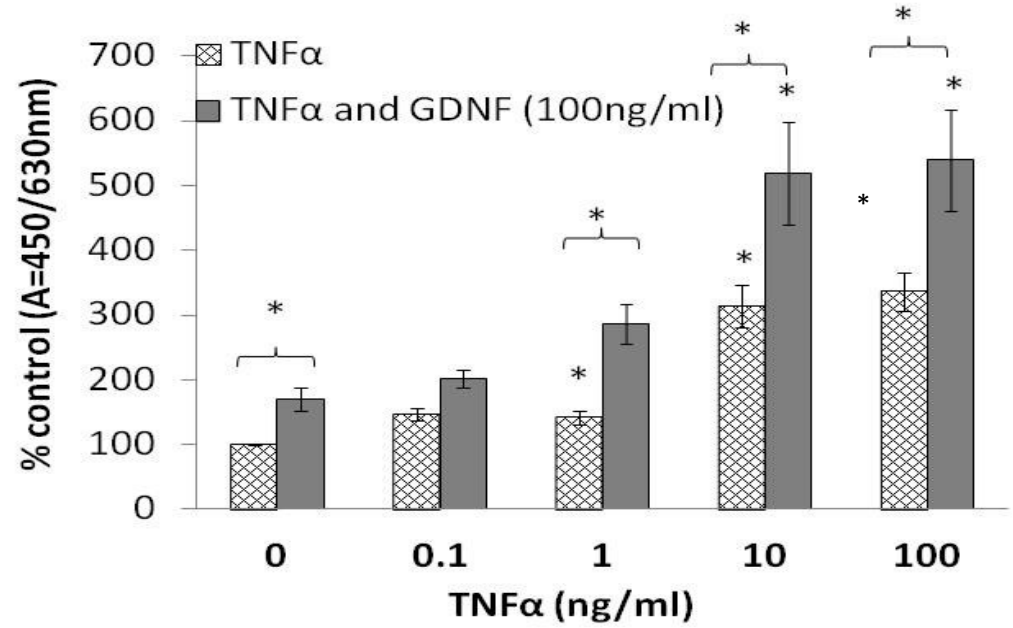


A

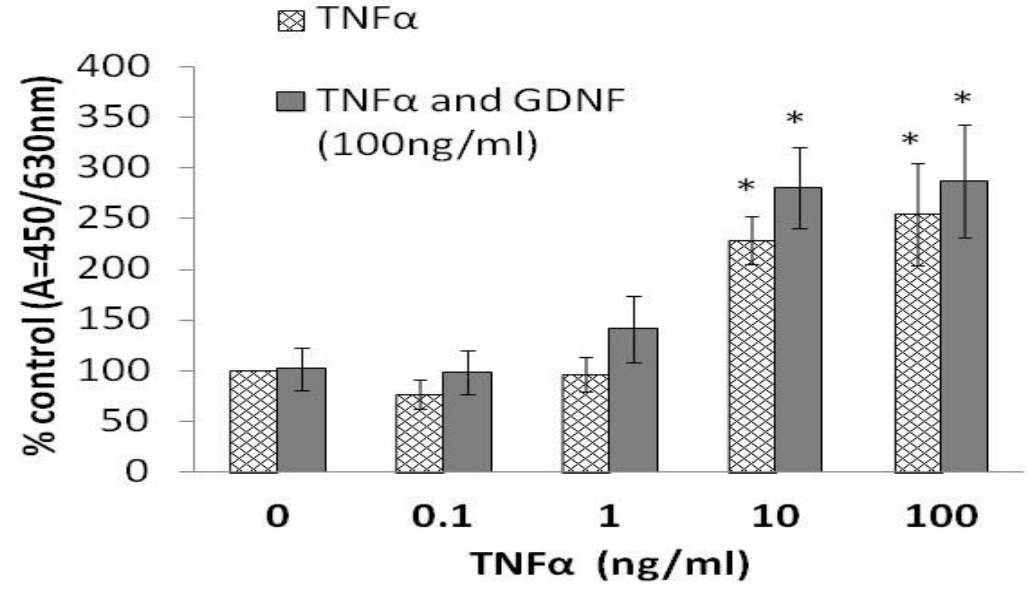


B

Figure 6.1 TNFα effect on viable DPC number with or without GDNF (100 ng/ml) supplementation (**A**) DPC cultured under serum-free conditions and (**B**) under serum supplemented conditions for 48hrs. Results are expressed as percentage of controls (0 ng/ml GDNF unsupplemented conditions) (Mean ± SEM; n=3, *p= 0.0001 *versus* serum supplemented control; $^{\cdots}$p<0.0001 significantly different groups; $^{\Delta}$p= 0.032 significantly different groups).



A



B

Figure 6.1.1 TNFα effect on viable MC3T3-E1 cell number with or without GDNF (100 ng/ml) supplementation (**A**) MC3T3-E1 cells cultured under serum-free conditions and (**B**) under serum supplemented conditions for 48h. Results are expressed as percentage controls (0 ng/ml GDNF unsupplemented conditions) (Mean ± SEM; n=3, *p= 0.0001 *versus* serum-free control (*p= 0.023 *versus* serum supplemented control).

6.1.2 NGF (nerve growth factor)

The neurotrophin, NGF (nerve growth factor) may activate RET, up regulate GDNF expression and has been detected with GDNF during infection (Dechant *et al.* 2002; Appel *et al.* 1997; Martinelli *et al.* 2006). NGF is also involved in dentine generation during development and reportedly present during dental pulp injury where it may promote dentine repair (Mitsiadis and Luukko 1995; Byers *et al.* 1990; Woodnutt *et al.* 2000; Shiomi *et al.* 2000; O'hara *et al.* 2009; Arany *et al.* 2009; Kurihara 2007; Mizuno 2007). NGF may also be involved in osteoblast survival and bone repair (Mizumo *et al.* 2007; Yada *et al.* 1994; Mogi *et al*. 2000; Asaumi *et al*. 2000; Aiga *et al.* 2006).

Subsequently, initial experiments were performed to assess the effect of NGF on DPC viability. NGF significantly reduced viable DPC number under serum-free conditions at all concentrations analysed and significantly increased viable DPC number under serum supplemented conditions (Figure 6.2).

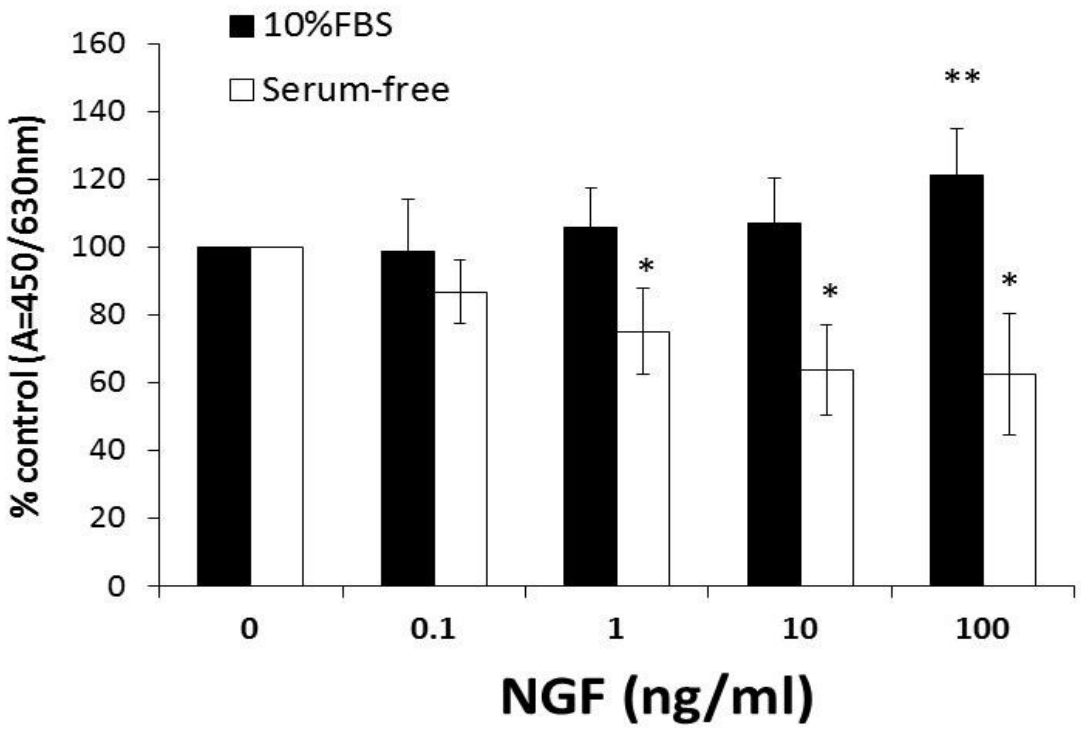


Figure 6.2 NGF effect on viable DPC number under serum-free and 10% serum supplemented conditions as assessed by WST-1 assay. Results are expressed as percentage of controls (0 ng/ml NGF, unsupplemented cultures) (Mean± SD; n=6, *p<0.000 *versus* control;**p< 0.05 *versus* control).

In addition, NGF (100 ng/ml) supplementation significantly increased DPC ALP levels under serum-free conditions; however, no effect was evident under serum supplemented conditions (Figure 6.2.1). These results indicated that NGF may stimulate differentiation under serum-free conditions and early differentiation/proliferation under serum supplemented conditions.

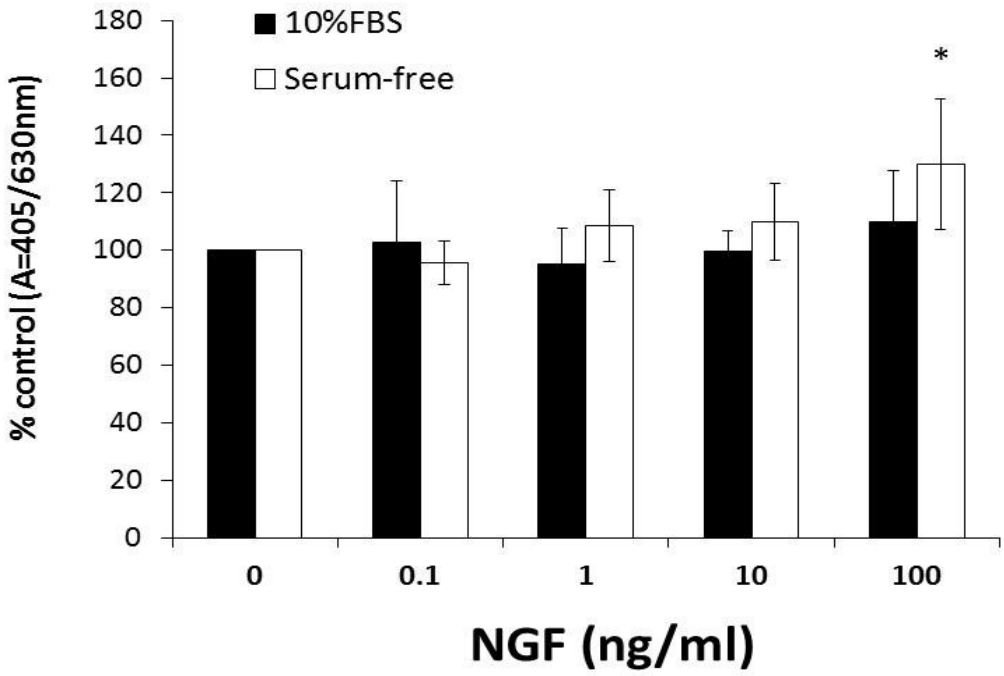


Figure 6.2.1 NGF effect on ALP activity under serum-free and 10% serum supplemented conditions. Results are expressed as percentage of controls (0 ng/ml NGF unsupplemented cultures) (Mean ± SD; n=6, *p<0.007 *versus* control).

6.2 Effect of GDNF on short term gene expression in DPC and MC3T3-E1 cells

This study thus far demonstrated that GDNF has effects on viability and proliferation in both DPC and MC3T3-E1 cell cultures. These effects may be mediated by several intracellular signalling cascades induced by GDNF-RET-GFRα1 binding (Airaksinen *et al.* 1999; Sariola and Saarma 2003). To further investigate the cellular effects of GDNF, the expression of a panel of genes were analysed where the gene expression related to: (i) cell proliferation (PCNA, p27kip1), (ii) cell survival/apoptosis (GDNF, IL-6, p27kip1, TNFα) and (iii) differentiation (Cfbα1, BSP, IL-6). In addition, the expression of GDNF and associated receptors (GFRα1, RET) were investigated under the differing culture conditions and in response to GDNF (Figure 6.3. and 6.3.1).

Results from the sqRT-PCR analyses demonstrated clear differences in IL-6 gene expression for DPC following GDNF treatment. In response to GDNF, DPC IL-6 gene expression was increased under serum-free conditions, whereas under serum supplemented conditions IL-6 expression was significantly decreased. BSP expression in DPC was also significantly decreased under serum-free conditions compared to serum supplemented conditions (Figure 6.3). Cbfα1 gene expression was increased for DPC and MC3T3-E1 cells by GDNF (Figure 6.3 and 6.3.1). In MC3T3-E1 cells BSP expression exhibited a similar profile to that exhibited by DPC and with BSP decreased relative levels under serum-free conditions were seen compared to serum supplemented conditions.

GDNF gene expression was relatively (albeit non-significantly) increased for both DPC and MC3T3-E1 cells under serum-free conditions in response to GDNF stimulation suggesting a possible positive autocrine feedback mechanism was in effect

(Figure 6.3 and 6.3.1). GFRα1 expression demonstrated the same pattern of expression as GDNF for both DPC and MC3T3-E1 cells. Unique to DPC cells, GFRα1 appeared differentially regulated under serum-free compared to serum supplemented conditions in response to GDNF addition (Figure 6.3). For both DPC and MC3T3-E1 cells RET receptor expression demonstrated the same profile and was increased under serum-free conditions compared to serum supplemented conditions. RET gene expression further decreased in response to GDNF supplementation under both serum-free and serum supplemented conditions (Figure 6.3 and 6.3.1).

For DPC, TNFα expression appeared increased under serum-free compared with serum supplemented conditions and GDNF supplementation had a minimal effect on TNFα expression. $p27^{kip1}$ expression decreased with GDNF addition in both DPC and MC3T3-E1 cells under serum supplemented conditions. Under serum-free conditions GDNF had opposing effects on $p27^{kip1}$ expression in DPC and MC3T3-E1 cells, decreasing and increasing in relative levels respectively. PCNA expression demonstrated a similar pattern of expression in response to GDNF although this was less evident for MC3T3-E1 cells, where PCNA expression was increased under serum supplemented conditions compared to serum-free conditions. GDNF addition decreased PCNA gene expression under serum conditions for both DPC and MC3T3-E1 cells. Under serum-free conditions relatively small increases in PCNA expression were detected (Figure 6.3 and 6.3.1).

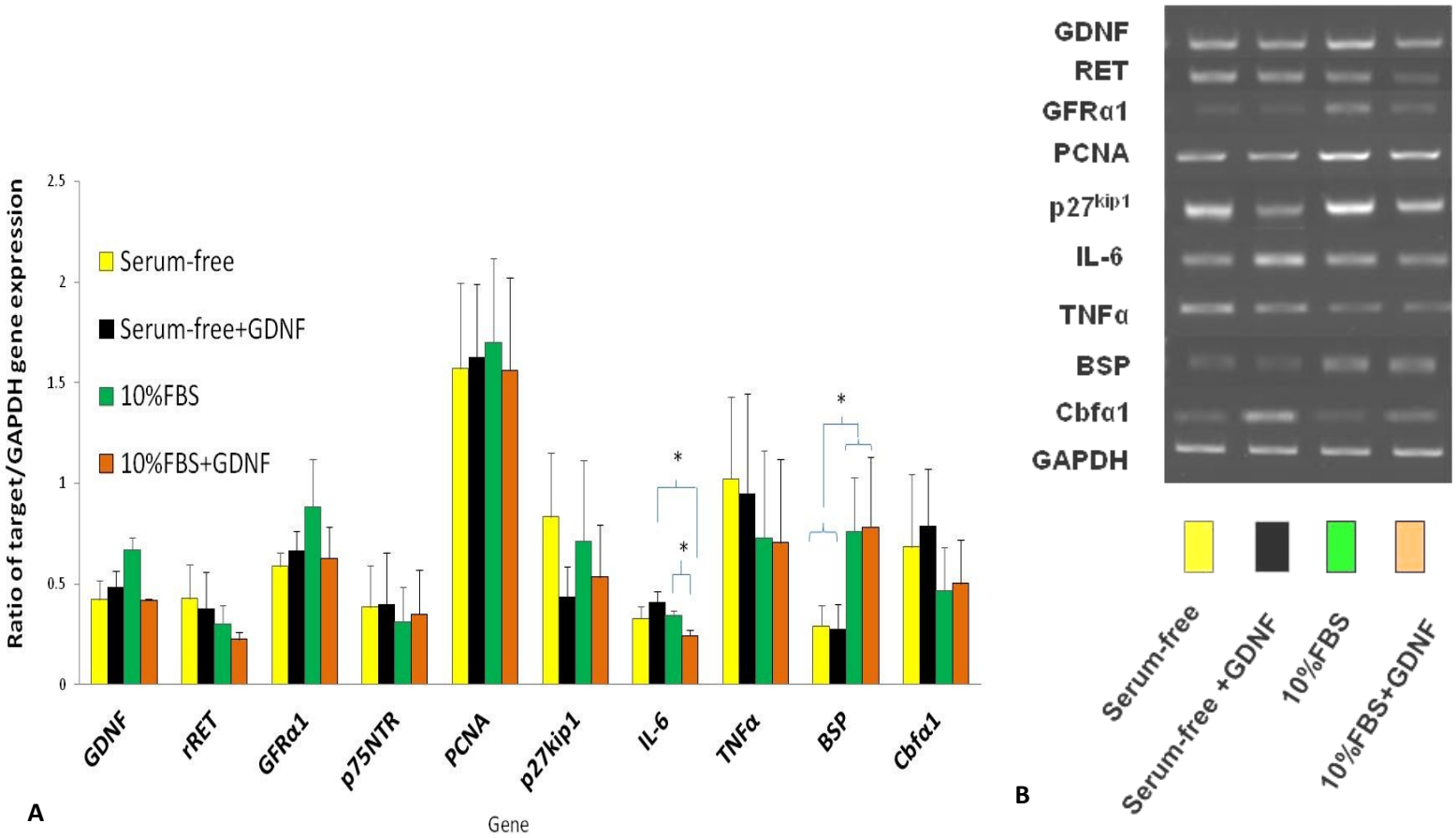


Figure 6.3 The effect of GDNF (100 ng/ml) supplementation on relative gene expression in DPC under serum-free and serum supplemented (10%FBS) conditions at 48 hours. A) Graphical representation of relative gene expression levels and **B**) representative electrophoresis gel images of transcripts detected for each gene amplified. GAPDH levels were used as a normalising control. (Mean ± SEM; n=3, T-test *p<0.05).

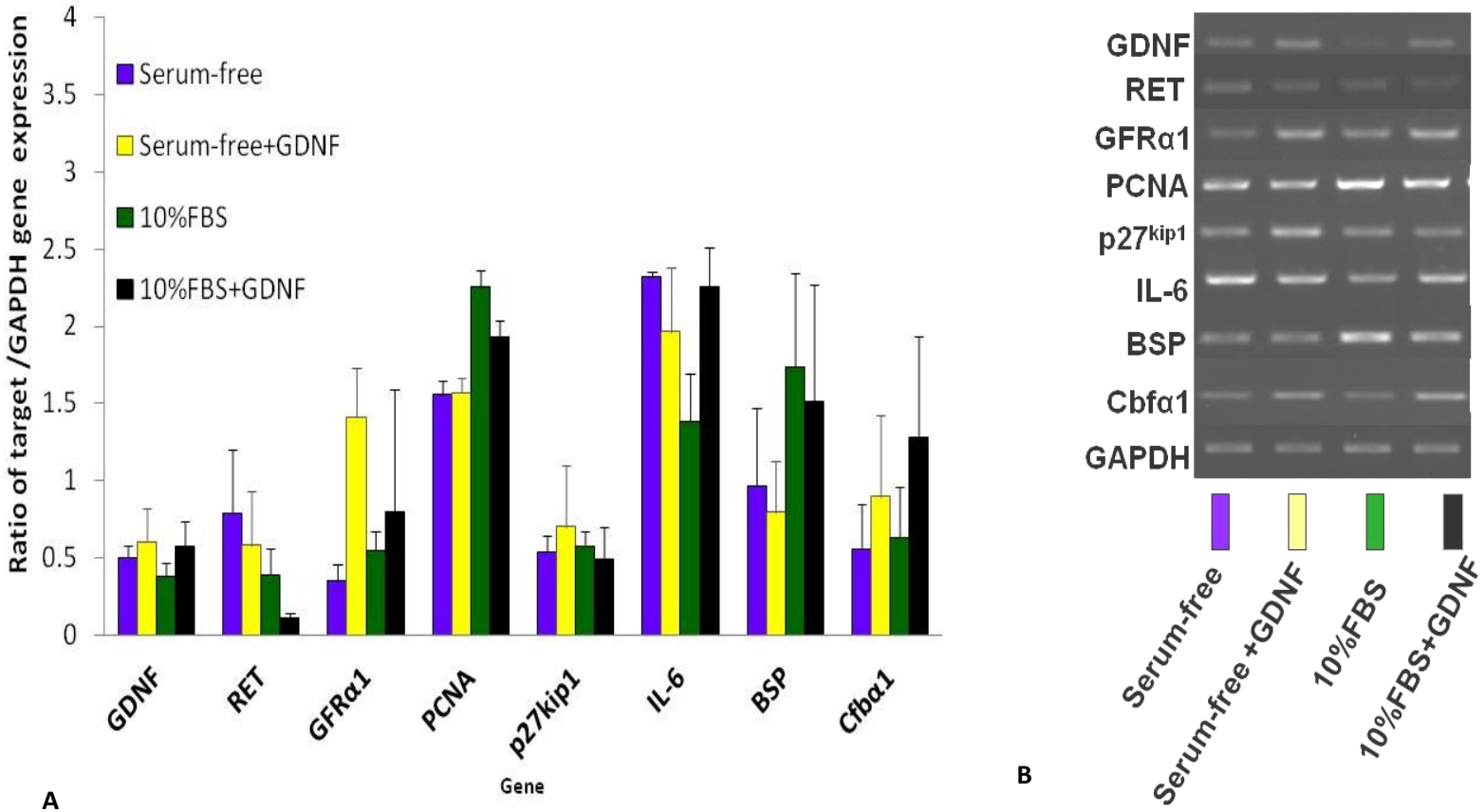


Figure 6.3.1. The effect of GDNF (100 ng/ml) supplementation on relative gene expression of MC3T3-E1 under serum-free and serum (10 %FBS) supplemented conditions at 48 hours. **A**) Graphical representation of relative gene expression levels and **B**) representative electrophoresis gel images of transcripts detected for each gene amplified. GAPDH levels were used as a normalising control. (Mean ± SEM; n=2).

CHAPTER 7 DISCUSSION

7 DPC culture model characterisation

The enzymatically-derived DPC culture model has previously been characterised in terms of odontoblast differentiation, effect of cryopreservation, pellet culture techniques, multi-potentiality and "stemness" (Huang *et al.* 2006; Zhang *et al.* 2006; Woods *et al.* 2009; Yu *et al.* 2010; Iohara *et al.* 2004, 2009; Alge *et al.* 2010; Miura *et al.* 2003; Paino *et al.* 2010). Key papers produced by Gronthos and co-workers furthered interest in the regenerative capacity of the dental pulp by identifying putative stem cells within it. Gronthos *et al.* (2000, 2001, 2002, 2006) found that enzymatically-derived DPCs could be expanded for up to 20-30 population doublings and were capable of differentiating along various cell lineages as well as forming a dentine-pulp complex *in vivo* when these cells were transplanted into immune-compromised mice. In these and more recent studies, DPC have been isolated by digestion using trypsin for up to 30 mins (Patel *et al.* 2009). This approach, effective for deriving many different cell types from the ECM, released cells from the dental pulp by cleavage of cell surface peptide bonds on the C-side of Arginine and Lysine residues (Gilbert *et al.* 2006). Enzymatic digestion may therefore significantly disrupt cellular membranes and affect viability, reducing cell yields (Kirkpatrick 1985). Indeed yield per isolation is a critical factor when potentially only a proportion of clonogenic DPC (possibly < 20 %) are capable of more than 20 doublings (Gronthos *et al.* 2002; Mao *et al.* 2006).

Couble *et al.* (2000) applied an explant approach to obtain DPC, identifying differentiated odontoblast-like cells at four weeks within confluent cultures following addition of ascorbic acid and β-glycerophosphate to the media. This method has since

been used by several groups to study the interaction of these cells with bacteria, dental materials, pulpal innervation and ECM (Staquet *et al.* 2008; Farges *et al.* 2011; Durand *et al.* 2006; Maurin *et al.* 2004; Seux *et al.* 1991; About *et al.* 2000; Carrouel *et al.* 2008).

The use of bone explants to obtain human osteoblast cultures emerged in the late 1970's and these osteoblast-like cells have since been extensively characterised (Jones and Boyde 1977; Ecarot-Charrier *et al.*. 1983; Gallagher *et al.* 2003; Lian and Stein 1992, 2003, 2004, 2006). Other techniques for isolating cells from mineralised tissues have also been developed (Iohara *et al.* 2004; Sasaki *et al.* 2008; Tjäderhane *et al.* 1998; Téclès *et al.* 2008). Cao *et al.* (2006) found a short period of enzymatic digestion followed by the explanting technique produced cells more quickly compared with the enzymatic culturing technique when used for isolating osteoblasts. Technically the explant approach comprises fewer steps, is less time consuming and requires minimal manipulation of the tissue compared to the enzymatic digestion method and in the study presented here this approach was found to produce consistent results. Although enzymatic digestion approaches may result in cell damage or derivation of less robust cells, a more heterogeneous cell population containing DPC at different levels of differentiation may be obtained which may be more representative of the native pulpal tissue (Patel *et al.*. 2009; Balic and Mina 2010; Balic *et al.* 2010).

In this study, explant outgrowth resulted in cells of homogenous polygonal morphology although this approach took several hours longer to obtain cells compared with the enzymatically released DPC approach (Figure 2.1). The explant approach proved to be more efficient, where the reuse of explants was possible thereby producing further DPC outgrowths (Couble *et al.* 2000). Therefore the explant-derived model,

albeit a more "selective" approach, also appears an appropriate and suitable method for obtaining DPC.

7.1 GDNF and GDNF receptor expression in DPC cultures

To further characterise the explant and enzymatically-derived cells used in this study relative gene expression levels were compared. Data presented here demonstrate that explant-derived DPC displayed a different profile of GDNF, GFRα1 and RET mRNA expression compared with enzyme-digested DPC, however both were suitable models to study GDNF affects on DPC *in vitro*. Indeed explant-derived DPC expressed relatively higher expression levels of both RET and GFRα1 receptors compared with enzymatically derived DPC. This differential gene expression suggested that different cell types may have been selected according to the isolation procedure. However further qPCR is needed for precise quantification of transcript levels involved. Indeed differential gene and protein expression according to the isolation procedure used has previously been reported for neuronal, smooth muscle and dental pulp cell cultures (Iohara *et al.* 2004; Arthur *et al.* 2008; Zimmerman 2006; Kirschenlohr *et al*. 1995; Huang *et al.* 2006b). Zimmermann *et al.* (2006) found that osteopontin levels were significantly increased within explanted compared to enzymatically digested smooth muscle cell cultures. In addition DPC gene expression level for collagen type I was increased while DSP levels decreased, in enzyme-digested compared with explant-derived DPC cultures (Huang *et al.* 2006). Furthermore Zhang *et al.* (2005) found 5 % of the DPC population expressed the STRO-1 antigen (a putative mesenchymal stem cell marker) within explanted rat cultures whereas Laino *et al.* (2006) found only 9.98 % STRO-1 antigen positive cells within enzymatically isolated DPC.

Interestingly while RET is reported to have extremely low postnatal expression, *in vitro* expression was detectable within DPC cultures. RET expression can represent the degree of differentiation of some cell types, for example during neuronal differentiation RET expression increases as cells become more differentiated (Ibanez and Ernfors 2007; Hoffman *et al*. 2005; Hoffman 2008). It is conceivable that the relative difference in gene expression of GDNF and the GDNF receptors between explanted and enzymatically-derived cell cultures may represent the presence of cell populations exhibiting varying degrees of differentiation.

7.1.1 Confirmation of the mesenchymal nature of DPC culture models

RT-PCR analysis indicated that the dental pulp and DPC cultures consisted of cells of mesenchymal nature as evidenced by the expression of the putative MSC markers $p75^{NTR}$ (CD271), CD44 (hyaluronan receptor) and CD105 (endoglin is also a TGF-β co-receptor) (Pittenger *et al.* 1992, 2008; Jones *et al.* 2002; Bühring *et al.* 2007; MoscatelliI *et al.* 2009). $p75^{NTR}$ expression in DPC was relatively low compared with dental pulp tissue and this may have reflected the lack of neuronal cells present within these cultures. $p75^{NTR}$ is regarded as a putative stem cell marker (Kendirci *et al.* 2010; Stemple and Anderson 1992; Moscatelli *et al.* 2009) and its expression may also indicate the presence of multipotent cells within cultures (Gronthos *et al.* 2000). Notably the MSC markers (CD44, CD105 and p75NTR) have been used to isolate multipotent MSC from pulpal tissue using FACS and these enriched sub-populations can subsequently form mineralised ECM *in vitro* (Huang *et al.* .2009, Bianco *et al.* 2001).

GFL (Glial Family Ligands) are known survival factors for neural crest and neural crest-derived cells (Enomoto *et al.* 2005; Sariola and Saarma 2003; Chalazonitis *et al.* 1998 Bunone *et al.* 2000; Insua *et al.* 2003; Hofmann *et al.* 2005). The dental papilla and hence DPC are of neural crest origin and the expression of GDNF, RET and GFRα1 may reflect this neural crest lineage (Saavedraa 2008; Paratcha and Ledda 2008). GDNF gene expression as detected in this study concurs with the demonstration of GDNF in rat tooth by Western blot analysis (Kvinnsland *et al.* 2004) and GDNF mRNA localisation within the inner dental epithelium (IDE), enamel knots, sub-odontoblast and odontoblast layers (Nosrat *et al.* 1998; 2002). GFRα1 and RET were also expressed in the epithelial and mesenchymal dental papilla, respectively, during tooth morphogenesis (Luukko *et al.* 1997; Nosrat *et al.* 1997) suggesting a role in the reciprocal epithelial-mesenchymal interactions which occur during early tooth development. However this study now demonstrates for the first time the GDNF receptor expression occurring within *in vitro* DPC cultures.

7.1.2 Characterisation of bone cell cultures for GDNF and GDNF receptor expression

The co-expression of RET and GFRα1 in BMSC and osteoblast-like cells as shown here is also a novel observation. GDNF mRNA and protein expression have previously been detected in BMSC cultures (Ye *et al.* 2005; Chen *et al.* 2007; Garcia *et al.* 2004) and this study confirmed the expression of GDNF within MC3T3-E1 osteoblast-like cells suggesting a role for GDNF in osteoblast regulation. GDNF transcript expression in BMSC was shown to be relatively weak, although immunocytochemical staining was clearly apparent. Differences between the level of gene and surface protein expression may occur and could be due to post-transcriptional

and post-translational modifications/processing. Indeed GDNF receptor protein expression is influenced by post-translational modification, as 30% of translated RET may misfold in the endoplasmic reticulum and subsequently be degraded (Abrescia *et al.* 2005, Kjaer and Ibàñez 2003). Further work based on these studies is now warranted to analyse the *in situ* distribution of GDNF and GDNF receptors in whole tooth and bone tissue. It should also be noted that the expression of the GDNF receptors Gfrα1 and RET does not necessarily denote responsiveness to GDNF. Indeed intracellular cross-talk with other growth factor receptor systems occurs whereby TGF-β is required for RET receptor activation (Tsui-Pierchala *et al.* 2002; Dechant 2002; Airaksien 1999; Sariola and Saarma 2003). Nonetheless, the data from the studies presented here demonstrated that GDNF exerted direct effects mediated by Gfrα1 and RET on non-neuronal cell types from the dental pulp, calvarial bone and to a lesser extent the bone marrow (see below).

7.1.3 *In vitro* culture under serum-free conditions

Serum-free cultures facilitate the study of the direct effects on cells without interference from serum factors, but also present a model mimicking pathological conditions involving cellular insult and injury due to growth factor deprivation (Goyeneche *et al.* 2006). In this study DPC and MC3T3-E1 cell cultures displayed reduced cell viability when cultured under serum-free conditions in agreement with other reports (Onisha *et al.* 1999; Tarle *et al.* 2010; Tumber *et al.* 2000). Interestingly, BMSC cultures retained relatively good viability which corresponded with previous studies showing that serum-free conditions did not affect viable BMSC numbers during a 7 day experimental period (Sauerzweig *et al.* 2009; Pochampally *et al.* 2004). Interestingly Sauerzweig *et al.* (2009) identified those BMSC proliferating under

standard serum-free conditions as differentiating towards a primitive neural lineage. It would therefore be interesting to investigate whether BMSC can respond to subsequent GDNF addition by differentiating along a neural cell lineage.

Serum deprivation is known to arrest mitosis and serum starved cultures have been shown to exhibit differential gene expressions compared with serum supplemented cell cultures (Raff *et al.* 1992; Ye and Lotan 2008). Interestingly Onisha *et al.* (1999) have previously reported that canine DPC could not be cultured under serum-free conditions without additional supplements. Reportedly cell-cell and cell-ECM interactions are likely important in regulating survival under stressful conditions. Indeed fibronectin (FN) coating of culture surfaces decreased the number of floating, apoptotic SHED (Stem cells from human exfoliated deciduous teeth) under serum-free conditions (Tarle *et al.* 2010) indicating that the loss of cell attachment under these conditions could be rescued by cell-ECM contact. In addition, differentiated osteoblasts secrete FN (Stephansson 2002; Garcia and Reyes 2005; Globus *et al.* 1998; Moursi *et al.* 1996) and apoptosis was induced in MC3T3-E1 cells by neutralising FN binding (Globus *et al.* 1998). BMSC also synthesise FN constitutively *in vitro* and FN is important in BMSC cell-cell adhesion *in vivo;* however, additional FN coating of BMSC culture surfaces had no effect on BMSC adherence (Jo *et al.* 2011; Lerat *et al.* 1993; Van der Velde-Zimmermann *et al.* 1997). Combined this may suggest that variations in endogenously secreted ECM such as FN may be responsible for differences in cell viability of DPC and BMSCs under serum-free culture conditions.

In future, investigations utilising sub-optimal conditions for BMSC culture may be useful to study the positive effects of mitogens or survival factors such as GDNF on BMSC. Sub-optimal conditions may be established by reducing seeding density or

perhaps blocking FN adherence using antibodies against integrin β1 binding sites in serum-free culture conditions. Data presented here indicated that although DPC and MC3T3-E1 cell viability was reduced at day 1 and 2 sufficient cell numbers remained after 10 days of culture, indicating that serum-free conditions can be used to support long-term studies.

7.2. Short term effect of GDNF on cell proliferation under serum-free conditions

This study demonstrated that GDNF increased DPC and MC3T3-E1 viable cell numbers and cell proliferation under serum-free conditions. The increase in DPC viable cell numbers by GDNF corresponded with a decrease in $p27^{kip1}$ expression confirming that cell replication/cycle progression was evident and GDNF regulation of $p27^{kip1}$ expression was in line with other reports (Kalechman *et al.* 2003; Baldassarre *et al.* 2002). GDNF did not however affect $p27^{kip1}$ expression in the osteoblast-like cells suggesting that this may represent a cell type-specific effect. PCNA (proliferating cell nuclear factor) is also a marker and regulator of cell cycle progression and cell proliferation (Maga and Hubscher *et al.* 2003; Coltrera and Gown 1991; Sasaki *et al.* 1992; Golberg *et al.* 2008). In this study PCNA transcript expression was detected as being both up- and down-regulated in accordance with increasing and decreasing proliferation as assessed by BrdU incorporation, thus confirming the GDNF-induced changes in proliferation under serum-free conditions.

7.2.1 GDNF and cell survival

GDNF is a well characterised cell survival factor (Enomoto *et al.* 2005; Sariola and Saarma 2003; Chalazonitis *et al.* 1998 Bunone *et al.* 2000; Insua *et al.* 2003; Hofmann *et al.* 2005). It is known that the cellular repertoire involved in cell-cycle

control and hence proliferation is closely associated with pro-survival signalling (Maddika *et al.* 2007). To assess whether the increased viable cell numbers evident in response to GDNF were also due to increased survival of DPC and MC3T3-E1 cells, live/dead, LDH and caspase assays were performed to assess the levels of cell death and apoptosis within all cultures. GDNF decreased numbers of dead DPC that were observed under serum-free conditions. Moreover, caspase levels indicated that apoptosis occurred within serum-free DPC cultures and that this was reduced in response to GDNF addition. This finding is in agreement with other studies demonstrating that serum withdrawal induces cell death in neuronal cell cultures and this is abrogated by GDNF supplementation (Kobori *et al.* 2006). Further work is needed to confirm and visualise apoptosis, e.g. using the TUNEL assay. In addition, the anti-apoptotic effect elicited by GDNF remains to be confirmed for the MC3T3-E1 cell cultures. Interestingly, up-regulation of NTGF receptors under serum-free conditions in the absence of ligand resulted in apoptosis in several cell types (Ahn *et al.* 2005; Fauchais *et al.* 2008; Yuan and Yanker 2000). Within this study RET and GFRα1 expression were detected under all culture conditions tested and the inhibitor studies indicated that the functional effects were mediated by GDNF receptor binding.

7.2.2 Osteogenic differentiation and *in vitro* mineralisation

Cbfα1/runx2 gene expression in DPC and MC3T3-E1 cells was up-regulated by GDNF under serum-free conditions; moreover relative expression levels of Cbfα1 were increased in DPCs under serum-free compared with serum-supplemented conditions. Cbfα1 is considered a pivotal osteoblast transcription factor, although it may not be essential for odontoblast differentiation (Narayanan *et al.* 2001; Fen *et al.* 2002; Qin *et al.* 2007). Cbfα1 is used as a marker of the osteoblast lineage and may reflect the

osteogenic potentiality of cells in culture (Galindo *et al.* 2005; Wang *et al.* 1999). Moreover, ALP activities in the DPC, BMSC and MC3T3-E1 cell cultures were decreased by GDNF supplementation under serum-free conditions, suggesting an inverse relationship between cell proliferation and differentiation (Yokose *et al.* 2000; Hoemann *et al.* 2009; Maddika *et al*. 2007).

An *in vitro* mineralisation assay was conducted as a measure of functional osteogenic differentiation. Cultures were grown for up to 28 days under *in vitro* osteo-inductive conditions and stained with alizarin red (ARS) to detect mineralised ECM under serum-free conditions. Initial experiments indicated that mineralisation occurred at a later time point for DPC compared to MC3T3-E1 cells suggesting that DPC cultures were less differentiated or less inductive under these study conditions compared with the osteoblast-like cell line. The relatively long-term mineralisation experiments indicated that GDNF stimulated osteogenic differentiation of both DPC and MC3T3-E1 cell cultures. It may be possible that cell death occurring under serum-free conditions related to the formation of mineralised ECM. Lynch *et al.* (1998) found apoptosis was co-regulated with osteoblast differentiation and Fratzl-Zelman *et al.* (1998) found rapid mineralisation occurred within cultures containing dead cells. However, other studies have reported mineralisation within osteoblast and BMSC cultures under serum-free conditions (Gronowicz *et al.* 1989; Sakamoto *et al.* 1989; Huffman *et al.* 2007; Solmesky *et al.* 2010). In this study the mineralisation observed under serum-free conditions with osteogenic induction became significant only after 28 days; thus it is improbable that this calcification was associated with dead cells which normally occurs relatively rapidly within the early stages of culture (Fratzl-Zelman *et al.* 1998).

The level of ARS under serum-free osteogenic conditions was changed with the addition of GDNF and/or removal of DEX. GDNF without DEX supplementation in MC3T3-E1 cell cultures decreased ARS staining, whereas ARS staining was increased in DPC cultures, indicating cell specific effects of DEX in combination with GDNF. The mechanism of action for GDNF increased ECM calcification remains unclear. GDNF may stimulate early osteogenic/odontogenic differentiation of mesenchymal cells and/or expansion of existing progenitor cells; moreover, GDNF may exert late effects on the synthesis of ECM proteins (Del Rio *et al.* 2011; Lin *al.* 2001). GDNF may also have promoted endogenous production of growth factors such as BMPs that subsequently enhanced cell differentiation during the *in vitro* mineralisation experiments. Indeed GDNF may act synergistically with BMP-4 and -7 during neuronal development and proliferation (Chalazonitis *et al.* 2004; Harvey *et al.* 2005).

7.3 Short-term effect of GDNF on cell proliferation and differentiation under serum-supplemented conditions

GDNF decreased viable MC3T3-E1 cell number by inhibiting proliferation under serum supplemented conditions as confirmed using a BrdU assay. This study also demonstrated that relative gene expression of the osteogenic-associated transcription factor Cbfα1 was up-regulated in MC3T3-E1 cell cultures under serum supplemented conditions by GDNF. GDNF is known to induce differentiation not only for neuronal cell types, but also for cells of the testes and kidney (Peterson *et al.* 2004; Toshifumi *et al.* 2005; Baldassarre *et al.* 2002; Meng *et al.* 2000; Insua *et al.* 2003; Park *et al.* 2005). In this study relative gene expression of BSP in the osteoblast cultures was enhanced under serum supplemented conditions. Furthermore the decrease in proliferation in GDNF supplemented cultures may also be indicative of

cytodifferentiation (Aubin and Heersche 2000; Blagosklonny 2003). Interestingly, GDNF/RET signaling has been shown to be responsible for decreased proliferation of an embryonic neural precursor carcinoma cell line (Baldassarre *et al.* 2002). This effect, mediated by $p27^{kip1}$, was suggested to be a mechanism by which GDNF regulates cell growth to initiate terminal differentiation (Baldassarre *et al.* 2002). Moreover the short-term experiments performed in this study demonstrated that ALP levels were increased concomitantly with decreasing proliferation. Further long-term studies will be required to investigate in detail the role of GDNF in osteogenic proliferation, differentiation and bone formation.

7.4 *In vitro* culture under "challenging" conditions that reflect some aspects of infection and inflammation

In vivo bacterial infection or injury to bone and dentine can lead to the proteolysis of the ECM and elicit an inflammatory reaction by the host immune system. 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). Previous studies have demonstrated that LPS (lipopolysaccharide) and NFκB (a TNFα regulated transcription factor) signalling up-regulated NGF expression during dental inflammation (Freund-Michel and Fossard 2008; Heese *et al.* 1998; Magloire *et al.* 2001). The cytokine TNFα is well known for its ability to stimulate recruitment and activation of immune cells to the sites of infection (Zganiacz *et al.* 2004; Abe *et al.* 2010; Stashenko *et al.* 1991; Stashenko *et al*. 1987; Li and Stashenko 1992), however it is also reportedly able to stimulate osteogenic migration, proliferation and differentiation (Gowen *et al.* 1988; Frost *et al.* 1997; Glass *et al.* 2011; Lencel *et al.* 2011; Modrowski *et al.* 1995). TNFα also directs

the differentiation of osteoclasts that resorb bone, although the exact mechanisms by which this occurs are not yet fully ascertained (Robinson *et al.* 2007; Nanes 2003). Given GDNF effects on osteoblast-like and odontoblast-like cell differentiation and survival under serum-starved conditions, the effect of GDNF under challenged conditions recapitulating some aspects of infection and inflammation were investigated.

7.4.1 *Streptococcus mutans* (*S.mutans)* exposure

S.mutans is a gram positive bacterium associated with dental caries and Rosengren and Winblad (1975) demonstrated that *S.mutans* infections are also involved in deeper destruction of bone during dental infections. However, the effects of *S.mutans* on the viability of the dental pulp or osteoblasts are not elucidated. Meikle *et al.* (1982) found *S.mutans* antigens contributed to inhibition of MC3T3-E1 cell protein synthesis and that this may contribute to bone loss. In accordance, bacterial wall components of *S.mutans* and LTAs (Lipoteichoic acid) have been found to induce bone resorption (Nair *et al.* 1996).

Elson *et al.* (2007) studied the direct effect of heat inactivated gram positive and negative bacteria compared with purified LPS on TLR activation and concluded that heat inactivation was a valid method for studying TLR responses of the innate immune system. Previously, Paterson *et al.* (1982, 1987) have indicated that live *S.mutans* decreased dental pulp cell numbers *in vivo*; however decreasing cell viability was evident from day 7 only suggesting that this was not a direct cytotoxic effect of the bacteria. More recently Abe *et al.* (2010) reported heat inactivated *S.mutans* stimulated DPC osteogenic and dentinogenic differentiation. Interestingly the literature suggests that GFL expression during bacterial infection may increase cell survival, whereby

infecting gram positive bacteria express outer protein structures that mimic NTGF structure and subsequent exposure to the host cells has been reported to increase neuronal cell differentiation and survival during bacterial infection (Lu and PereiraPerrin 2008). Moreover, Durand *et al.* (2006) found that LTA exposed odontoblasts up-regulated mRNA for hypoxia-inducible factor 1α (HIF1α), a transcription factor regulating GDNF expression (Foti *et al.* 2010). Within this study no effect of heat inactivated *S.mutans* on viable DPC or MC3T3-E1 cell number was evident. Likewise *S.mutans* had no evident effect on GDNF stimulated DPC and MC3T3-E1 viable cell number. Further work is needed to assess *S.mutans* effect in isolation from serum factors and to determine whether bacterial components modulate GDNF effects under these conditions.

7.4.2 GDNF interaction with TNFα

Interestingly the NFκB transcription factor has a binding site on the human GDNF promoter, suggesting a potential regulatory feedback loop involving GDNF in response to inflammatory signalling (Appel *et al.* 1997; Woodbury *et al.* 1998). Under serum-free conditions TNFα exerted no effect on viable DPC numbers and GDNF addition increased viable cell numbers to levels previously seen without TNFα addition. Thus *in vitro* experiments were conducted using serum to mimic the multitude of proteins present under physiological conditions. This study demonstrated that under serum supplemented conditions, TNFα decreased DPC viable cell numbers, and this effect was abated by GDNF addition. These data suggested that GDNF may protect DPC against TNFα induced apoptosis/necrosis in agreement with previous reports (Talley *et al.* 1995; Yang *et al.* 2002; Sheng *et al.* 2005; Peralta-Solter *et al.* 1996).

Interestingly, TNFα promoted osteoblast proliferation which was further enhanced in the presence of GDNF. The mechanisms by which GDNF interacted with TNFα to stimulate osteoblast proliferation are as yet unknown, but plausibly the effect may have been due to the stimulation of converging pathways that may include CREB (cAMP response element), IKK and NFκB signalling (Ono *et al.* 2004; Kalechman *et al.* 2003; Encinas *et al.* 2008). Furthermore TNFα also is proposed to regulate GDNF expression and secretion. (Tanaka *et al.* 2000; Woodbury *et al.* 1998; Kim *et al.* 2009; Simon *et al.* 2007; Esseghir *et al.* 2007; Kuno *et al.*. 2006; Appel *et al.* 1997). Thus, it is possible that increased GDNF synthesis by TNFα could lead to autocrine positive feedback mechanisms.

7.4.3 Cell specific effects of GDNF

This study demonstrated that whilst GDNF had direct effects on DPC and MC3T3-E1 cell viability, GDNF only decreased ALP levels in BMSC cultures under serum supplemented conditions but had no obvious comparable effect on BMSC proliferation or viability. These data corresponds with previous findings by Kramer *et al.* (2006). These apparent cell-specific effects of GDNF may reflect cell-specific receptor expression and intracellular signalling (Encinas *et al.* 2008; Tsui-Pierchala *et al.* 2002; Encinas *et al.* 2001; Focke *et al.* 2001; Mason *et al.* 2006) and/or may be due to the differential expression of tissue specific genes such as PAX and SOX genes in long bones as opposed to neural crest derived tissues (Fernandas *et al.* 2004; Betters *et al.* 2010; Dezawa *et al.* 2005; Otto *et al* . 2009; Basch *et al.* 2006; Wang *et al* . 2009). Indeed DPC and MC3T3-E1 cells displayed similar proliferative responses to GDNF. Furthermore it should be noted that MC3T3-E1 cells are of foetal origin whereas BMSC are postnatally derived cells. Gattei *et al.* (1997) hypothesised that

GDNF has a role within the bone marrow, activating signalling between BMSC expressing GFRα1 and RET expressing hematopoietic cells to stimulate monocytes differentiation.

This study highlighted another novel action of GDNF, that the neurotrophic factor modified cytokine IL-6 gene expression in DPC and MC3T3-E1 cell cultures. These findings are consistent with previous studies indicating a role for IL-6 in late stage differentiation and survival of osteoblasts and fracture healing (Jüttler *et al.* 2002; Li *et al.* 2010; Jilka *et al.* 1998; Iwasaki *et al* . 2008; Tsiridis and Giannoudis 2006). IL-6 is also a known neurokine able to sustain neuronal cell survival (Hirano *et al.* 2000; Bromberg and Wang 2009) and it may therefore be speculated that IL-6 may play a role downstream of GDNF as described in the current study.

7.4.4 NGF stimulation of DPC

It was previously proposed that the neurotrophin, NGF, may be involved in odontoblast differentiation and dentine formation during tertiary dentinogenesis (Arany *et al.* 2009; Kurihara 2007; Woodnutt *et al.* 2000; Shiomi *et al.* 2000; O'hara *et al.* 2009). Neurotrophins have been detected during fracture repair and NGF may also be involved in the repair of bone (Asaumi *et al.* 2000; Aiga *et al.* 2006). NGF was reported to stimulate differentiation and survival of osteoblasts (Mizumo *et al.* 2007; Yada *et al.* 1994; Mogi *et al.* 2000). NGF can also up-regulate GDNF expression (Appel *et al.*1997) and may modulate its action *in vivo* during infection and inflammation (Martinelli *et al.* 2006). This study demonstrated NGF increased DPC viable cell numbers under serum supplemented conditions. Conversely, NGF decreased viable DPC numbers and increased ALP activity under serum-free conditions suggesting NGF stimulated cell

differentiation. Further studies are required to investigate the inter-relationship between GDNF and NGF in the regulation of DPC and osteoblast activity.

7.5. Summary

In summary, this PhD study demonstrated that BMSC and MC3T3-E1 cells which are well characterised models for osteoblast differentiation expressed both GDNF receptors (GFRα1 and RET). Dental pulp and dental pulp-derived cell cultures also express the GDNF receptors. Furthermore, direct cell-specific, receptor-mediated effects of GDNF were demonstrated on the proliferation and cell survival of DPC cultures. However, GDNF did not affect BMSC proliferation or viability nor did GDNF influence MC3T3-E1 cell survival under serum-starved conditions. GDNF effects on DPC and osteoblast cell proliferation were inversely related to ALP levels. Nonetheless, GDNF stimulated *in vitro* mineralisation in long-term DPC and MC3T3-E1 cultures. Further work is warranted to investigate the precise effects of GDNF on osteoblast and odontoblast differentiation, the mechanisms involved in GDNF signalling in these mesenchymal/NCC-derived cells and whether GDNF may be used as a bioactive therapeutic agent in bone or dental repair.

CHAPTER 8 CONCLUSION

In conclusion the findings from this research demonstrate that GDNF is able to directly stimulate odontoblast and osteoblast-like cell proliferation and differentiation. From these findings, it is proposed that GDNF may play an important role in the regulation of dental pulp homeostasis and bone metabolism. Moreover GDNF promoted DPC survival under "challenged conditions" indicating that GDNF may be involved in the survival and differentiation of DPC and possibly odontoblasts during the repair of mineralised tissues.

8.1 Clinical and therapeutic applications

The putative role for GDNF during development and homeostasis, regulating cell differentiation, survival and proliferation may be exploited for clinical regenerative or therapeutic applications.

8.1.2. GDNF as a bioactive in tissue repair and regeneration

GDNF has already been used within clinical trials for neuronal diseases and GDNF regulation may be involved in rare disease such as Waardenburg-Hirschsprung diseases (Pelet *et al.* 1998; Angrist *et al.* 1996; Chan *et al.* 2003). GDNF receptor signalling may be also involved in neuro-endocrine cancers, breast cancers, leukaemias and drug dependence (Morandi *et al.* 2011; Plaza-Menacho et al. 2010; Lanzi *et al.* 2003, 2009; Gattei *et al.* 1999; Niwa *et al.* 2007; Petrangolini *et al.* 2006). Indeed RPI-1 and other indoline blockers that inhibit RET have been proposed as clinical treatments for such diseases (Morandi *et al.* 2011; Phay and Shah 2010).

Non-steroidal anti-inflammatory drugs (NSAIDS) are commonly used to treat bone diseases such as osteoarthritis, rheumatoid arthritis, ankylosing spondylitis and osteomyelitis, however their effects are limited (Donnelly and Hawkley 1997; Harder and An 2003). Anti-TNFα therapy is a promising new treatment however this has many reported serious side effects (Bongartz *et al.* 2006; Williams *et al.* 1992; Vaz *et al.* 2009). GDNF appears to modify TNFα effects and may represent a target for inhibition in cases where anti-TNFα treatment is used. However for this application GDNF regulation and signalling requires further elucidation. Moreover GDNF interactions with TNFα, highlight a GDNF/TNFα synergy in stimulating osteoblast differentiation which could possibly be harnessed to promote bone formation. MSC have previously been genetically engineered and used to deliver growth factors as a part of gene therapy and similar investigations regarding the dental pulp have been proposed to stimulate natural reparative events *in situ* (Moutsatsos *et al.* 2001; Nakashima *et al.* 2002, Kimelman *et al.* 2007). BMSC and DPSC have also gained much interest for use in neuronal regeneration due to DPC release of NTGF and DPC have been implanted in *vivo* to deliver NTGF or differentiate along neuronal lineages (Bolliet *et al.* 2008; Huang *et al.* 2009; Nosrat *et al.* 2001; Lillesaar *et al.* 2001, 2003; Apel *et al.* 2009). There is now scope within the field of dentistry for the addition of bioactives such as GDNF. Bioactives may be used within scaffolds, dental sealants/fillings and via implantation of bioactive secreting MSC to encourage regeneration of the pulp by increasing adherence (to biomaterials such as implants/scaffolds), survival, proliferation, differentiation and migration of dental pulp cells along with promoting angiogenesis (Brydone *et al.* 2010; Hing 2004; Zuk *et al.* 2008; Sun *et al.* 2010). This

study raises the possibility that GDNF may play a role in MSC survival, recruitment and proliferation during dentine repair (Shi *et al.* 2008).

8.1.3 Role of GDNF in tooth inflammation and analgesia

GDNF may protect against TNFα induced cytotoxicity during painful pulpal inflammation where high levels of TNFα occur such as during irreversible pulpitis (Kokkas *et al.* 2007; Donaldson 2006). Current treatment for pain relief during irreversible pulpitis involves tooth extraction or pulp removal (pulpectomy/extirpation) (Komabayashi and Zhu 2010). GDNF has recently been reported to have analgesic effects on pain induced by damaged nerves (Takasu *et al.* 2011; Adler *et al.* 2009). Therefore novel dental pulp clinical therapies using GDNF can be envisaged which maintain and promote tooth vitality (Komabayashi and Zhu 2010; Wang *et al.* 2010, 2011).

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APPENDIX

PUBLISHED WORK

Gale Z, Cooper P.R and Scheven B.A.A **(2011)** Effects of GDNF on dental pulp cells. *Journal of Dental Research*. 90(10):1240-5

Gale Z, Cooper PR, Scheven BA **(2012)** Glial cell line-derived neurotrophic factor influences proliferation of osteoblastic cells. *Cytokine*. 57(2):276-81

ABSTRACTS/CONFERENCE PAPER

Gale Z, Cooper P.R and Scheven B.A.A Glial Cell Line-Derived Neurotrophic Factor Influences Osteoblast Proliferation. British Society of Oral and Dental Research Conference Sheffield University. 12-15th September **2011**

Gale Z, Cooper P.R and Scheven B.A.A. Survival of dental pulp stromal/stem cells in response to Glial Cell Line-Derived Neurotrophic Factor (GDNF) Festival of Research and Enterprise, University of Birmingham 4-5th April **2011**

Gale Z, Cooper P.R and Scheven B.A.A. Effects of GDNF on dental pulp cells. International Association for Dental Research, Barcelona July 14-17th **2010**

Gale Z, Cooper P.R and Scheven B.A.A. Regulation of dental pulp cells by GDNF. College of Medical and Dental Sciences Research and Enterprise Gala 19-20th April. University of Birmingham **2010**

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PRIZES

Highly commended **poster prize** (**2011)** College of Medical and Dental Sciences Research and Enterprise Gala. University of Birmingham. 4-5th April

British Society of Oral and Dental Research **MINTIG prize** (Mineralised Tissue Group) (**2010)** IADR. Barcelona. July 14-17th

Medici Prize for best poster in the category enterprise and innovation **(2008)** Festival of Research and Enterprise. University of Birmingham. December

AUTHORS PERSONAL COPIES OF PUBLISHED WORK

PAPER 1

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, GFRα1 and RET. Addition of recombinant GDNF to cultures in serum-containing medium did not significantly affect DPC growth; however, GNDF 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 GFRα1 and RET suggesting receptor-mediated events. This study also demonstrated that GDNF counteracted TNFα-induced DPC cytotoxicity, suggesting that GDNF may be cytoptotective 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 TGFβ 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 (GFRα1) 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 GFRα1 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 (~5mm^3) samples and cultured in tissue culture flasks containing αMEM supplemented with 20% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin and 2.5 μg/ ml Amphotericin B (Sigma Aldrich, UK) in a humidified 5% CO_2 incubator at 37 ^{O}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 αMEM/10% FBS at 5,000 cells/well. After 24 hours, the cultures were replenished with either serum (10% FBS) or serum-free αMEM 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 (TNFα; Peprotech, UK) which was co-incubated with 100 ng/ml GDNF in αMEM/10%FBS for 2 days before analysis. For the receptor inhibitor experiments, DPC cultures were treated for 1 hour with different concentrations of phosphoinositide phopsholipase C (PI-PLC; Sigma) which blocks signalling *via* GFRα1 (Krieglstein *et a*l., 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 (4μM) (stains nuclei of live cells) and ethidium bromide (4μM) (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 caspsase-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 μM 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 ^{0}C in a humidified 5% CO_2 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% H_2O_2 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 ^{0}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 GFRα1 and RET transcripts were present in postnatal dental pulp and DPC cultures (Fig. 1A). Immunocytochemical staining of DPC using specific antibodies against GFRα1 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 GFRα1 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 TNFα-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 TNFα, a pro-inflammatory cytotoxic cytokine up-regulated in pulpitis (McLachlan *et al.,* 2004). The results demonstrated that TNFα dose-dependently decreased DPC numbers; however, cultures supplemented with GDNF showed significantly increased DPC viability as compared with TNFα controls, indicating that GDNF counteracted TNFα-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 GFRα1 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, GFRα1 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 GFRα1/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 TNFα, highlighting a possible protective role of this neurotrophic factor in pulpitis. TNFα is able to elicit various cellular responses, depending on cell types and conditions; in particular, TNFα'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 TNFα 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 (TNFα-related apoptosis-inducing ligand)-induced cell death (Murata et al., 2006). Interestingly, TNFα induced production of GDNF by astrocytes and glioma cells possibly *via* the NFkβ 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 TGFβ which induces the translocation of GFRα1 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 TGFβ1 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 mutli-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.

ACKNOWLEDGEMENTS

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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
GFRα1	F-AATGCAATTCAAGCCTTTGG R-TGTGTGCTACCCGACACATT	U59486	218bp	60

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

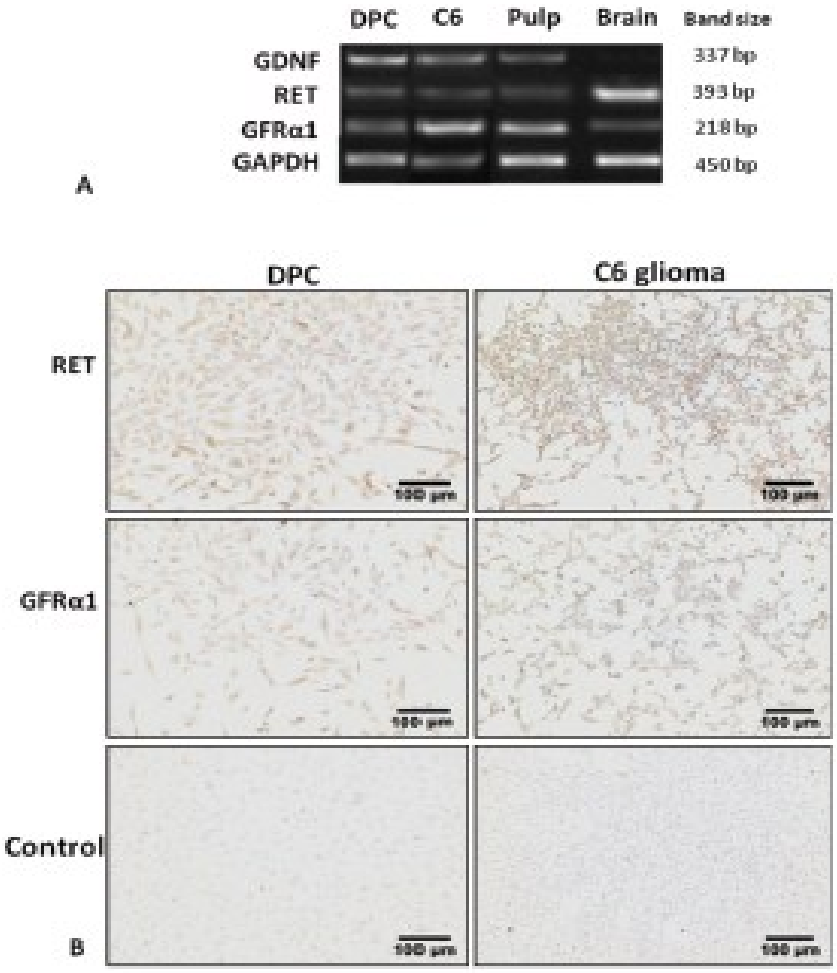

Figure 1: A. RT-PCR gel images showing transcripts of GDNF and its receptors GFRα1 and RET in dental pulp mesenchymal cells (DPCs), dental pulp and brain. **B**. Immunocytochemical staining of DPSC for GFRα1 and RET. Non-immune rabbit serum without specific primary antibody was used as control.

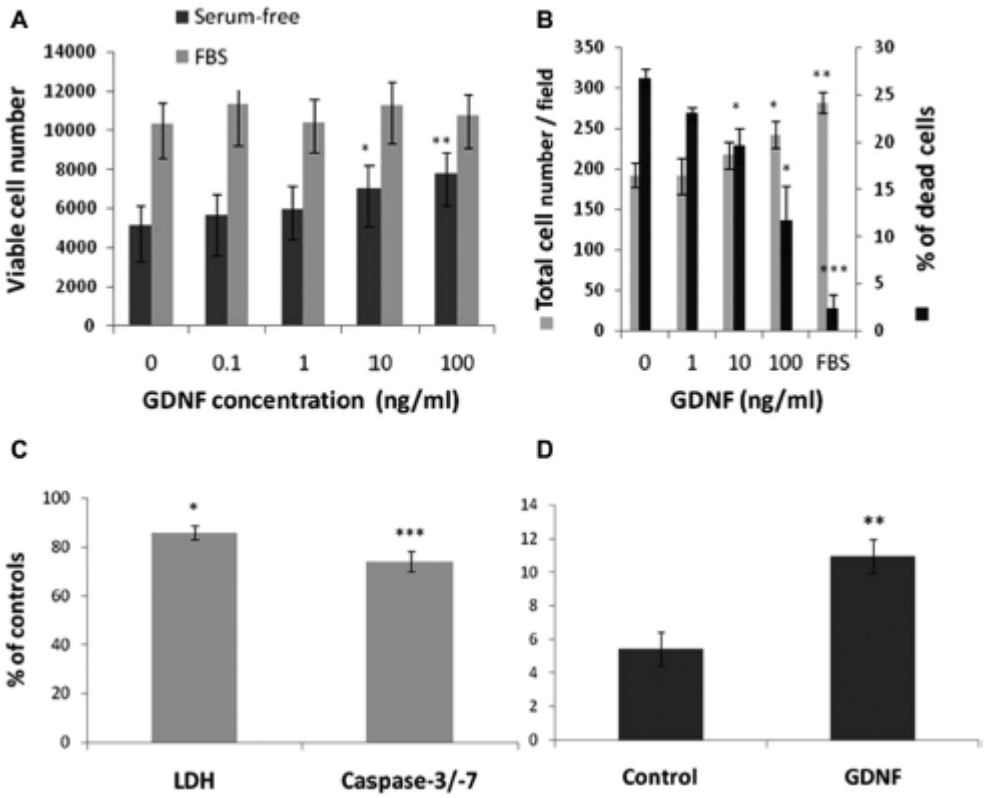

Figure 2. **A.** Effect of GDNF on viable cell number in serum-supplemented or serum-free cultures as assessed by WST-1. Results are expressed as percentage of controls (mean ± SD; n=4). **B.** Effect of GDNF on the number of dead cells as determined by the live/dead assay. Results show percentage of dead cells (mean ± 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 ± 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: * $p < 0.05$, **$P<0.01$, *** $p<0.001$.

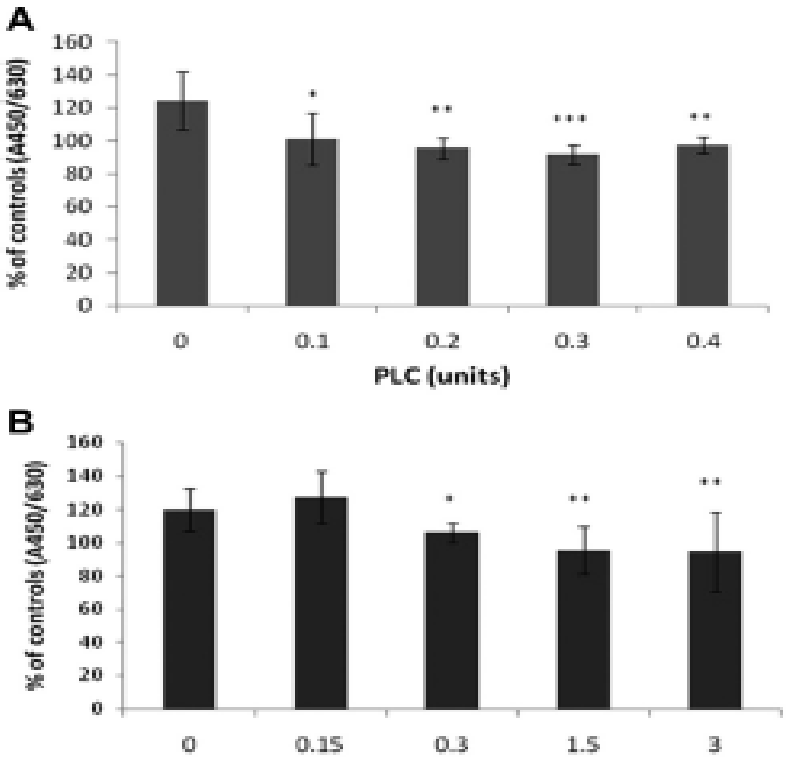

Figure 3: Effects of GDNF receptor inhibitors on GDNF-stimulated DPC cultures. A. Effect of phosphatidylinositol-specific phospholipase C (PI-PLC) on viable cell number as assessed by WST-1 in cultures supplemented with 100 ng/ml GDNF. B. Effects of the RET kinase inhibitor, RPI-1, on viable cell number 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.

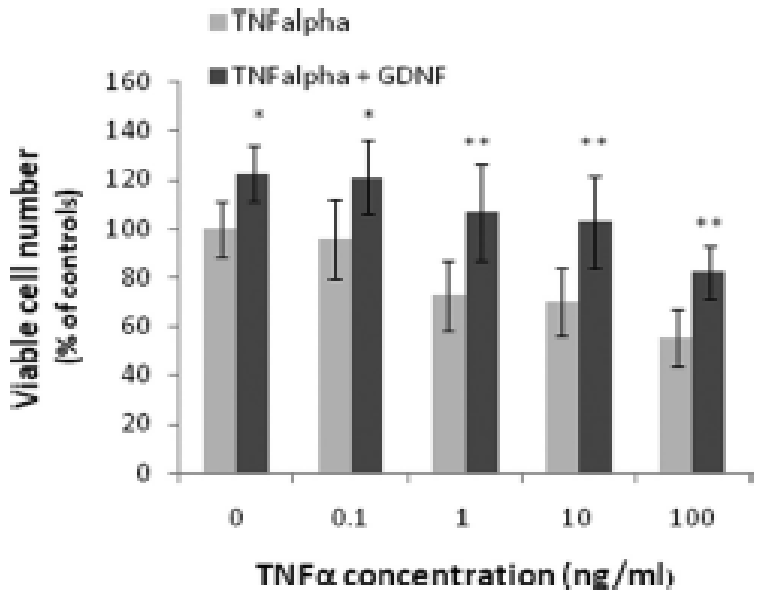

Figure 4: Effects of 100 ng/ml GDNF on 2-day DPC cultures in the presence of increasing concentrations of TNFα. Results are percentage of control values determined by the WST1 assay (mean $\pm$ SD; n=5-6). *P< 0.05, **P<0.01 *versus* corresponding TNFα cultures.

PAPER 2

Glial cell line-derived neurotrophic factor induces proliferation of osteoblastic cells

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ABSTRACT

Little is known about the role of neurotrophic growth factors in bone metabolism. This study investigated the short-term effects of glial cell line-derived neurotrophic factor (GDNF) on calvarial-derived MC3T3-E1 osteoblasts. MC3T3-E1 expressed GDNF as well as its canonical receptors, GFRα1 and RET. Addition of recombinant GDNF to cultures in serum-containing medium modestly inhibited cell growth at high concentrations; however, under serum-free culture conditions GDNF dose-dependently increased cell proliferation. GDNF effects on cell growth were inversely correlated with its effect on alkaline phosphatase (ALP) activity showing a significant dose-dependent inhibition of relative ALP activity with increasing concentrations of GDNF in serum-free culture medium. Live/dead and lactate dehydrogenase assays demonstrated GDNF did not significantly affect cell death or survival under serum-containing and serum-free conditions. The effect of GDNF on cell growth was abolished in the presence of inhibitors to GFRα1 and RET indicating that GDNF stimulated calvarial osteoblasts via

its canonical receptors. Finally, this study found that GDNF synergistically increased tumor necrosis factor-α (TNF-α)-stimulated MC3T3-E1 cell growth suggesting that GDNF interacted with TNF-α-induced signaling in osteoblastic cells. In conclusion, this study provides evidence for a direct, receptor-mediated effect of GDNF on osteoblasts highlighting a novel role for GDNF in bone physiology.

Key words: GDNF, neurotrophic factor, TNF-alpha, osteoblast, bone, calvarial, cell proliferation

1. Introduction

Glial cell line-derived neurotrophic factor (GDNF) is a pleiotropic signaling molecule playing a pivotal role in the development and regulation of the nervous system (1-2). GDNF has been recognized as a potent survival factor for neuronal cells in addition to its essential roles in neural migration and differentiation (1-3). GDNF is also widely expressed outside neuronal tissues and has been suggested to be involved in epithelial-mesenchymal interactions during development of urogenital and dental tissues (1, 4-6). GDNF is able to elicit various intracellular signalling cascades via multiple receptor systems, primarily through the glycosyl-phosphatidylinositol-anchored, GDNF family receptor α (GFRα1) and the tyrosine kinase transmembrane co-receptor RET (2-3).

Neurotrophic growth factors and cytokines including GDNF have been shown to be expressed in bone marrow stromal cells prompting an emerging interest in therapeutic regenerative application of bone marrow-derived mesenchymal stem cells in neurological disorders (7-11). Interestingly, whilst GFRα1 expression was detected

along with GDNF in bone marrow stromal cells, RET proved to be absent in these cells (12-13). However, GDNF/GFRα1 complexes cleaved from the stromal cells were shown to elicit functional signaling through RET expressed on hematopoietic and leukemic cells suggesting a signaling pathway involving cell-cell interactions within the bone marrow environment (13-14). In this study we addressed the question whether GDNF may be involved in bone metabolism. In particular, this study focused on the short term effects of GDNF on the proliferation and survival of osteoblastic cells using a non-transformed calvarial-derived cell line as model system for osteoblasts. In addition, the research investigated a possible interaction between GDNF and the multifunctional, pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α).

2. Materials and methods

2.1 Cell cultures

The MC3T3-E1 cell line is a non-transformed, clonal osteoblast-like cell line established from mouse calvaria and has extensively been used as a physiologically relevant *in vitro* model for calvarial osteoblasts, osteogenic differentiation and bone formation (15). MC3T3-E1 were acquired from the European Collection of Cell Cultures (ECACC) and were cultured in αMEM containing 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin, 200 mM glutamine and 2.5 μg/ ml Amphotericin B (Sigma Aldrich, UK) in a humidified 5% CO_2 incubator at 37^o C. Subconfluent cell cultures were trypsinised using Trypsin/EDTA (Gibco, UK) and plated into 96-multiwell plates in αMEM/10% FBS at 5,000 cells/well. After 24 hours, the cultures

were replenished with either serum (10% FBS) or serum-free αMEM supplemented with 0.1% bovine serum albumin (BSA). Recombinant human GDNF (rhGDNF, provided by Amgen, Thousand Oaks, USA) or TNF-α (PeproTech, UK) was added to the cultures for a further two days. For the receptor inhibitor experiments, cultures were treated for 1 hour with different concentrations of phosphoinositide phopsholipase C (PI-PLC; Sigma) which blocks signalling via GFRα1 (16), or RPI-1 (Merck/Calbiochem), a specific RET receptor tyrosine kinase inhibitor (17), followed by further culture with the respective inhibitors in media with or without GDNF.

2.2 Cell number and viability assays

The WST1 assay (Roche Applied Biosciences) was used to assess the number of viable cells (18); the absorbance of the reduced compound was measured at a wavelength of 450 with a reference filter at 630nm using a Biotek plate reader. The "live/dead" assay used 4μM acridine orange to stain nuclei of live cells and 4μM ethidium bromide to label nuclei of dead cells. The number of live and dead cells per microscopic field was counted under a Nikon Eclipse fluorescent microscope using 480 and 520nm filters, respectively. 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-days' culture for the presence of LDH. Absorbance was determined at 490/630nm using the Biotek plate reader.

2.3 BrdU cell proliferation assay

Cell proliferation was assessed using a 5-bromo-2-deoxy-uridine (BrdU) labeling and detection kit (Roche Applied Sciences). In brief, cells were labeled with 10 μM BrdU for the final hour of the 48 hours' 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 labeled and non-labeled nuclei were counted in 50 independent microscope fields.

2.4 Biochemical alkaline phosphate (AlP) assay

Cells were lysed in 0.1% Triton X-100 and incubated for 10 min in 1 M diethanolamine buffer (pH 9.8) containing 1mg/ml p-nitrophenyl phosphate (pNPP) at 37° C. Production of PNP (p-nitrophenol) was quantified spectrophotometrically at an absorbance of 405 nm using an automatic plate reader.

2.5 Semi-quantitative RT-PCR (sqRT-PCR) analysis

Cells were lysed in RLT buffer containing β-mercaptoethanol followed by RNA isolation using the RNeasy minikit (Qiagen, UK). Subsequently, 1μg 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).

2.6 Immunocytochemistry

MC3T3-E1 were seeded onto multispot microscope slides and incubated for 24h at 37 ^{0}C in a humidified 5% CO_2 incubator (15,000 cells/well). The adherent cells were fixed with ice-cold acetone for 5 min followed by rinsing in phosphate-buffered saline (PBS) containing 1% BSA. Following incubation in 3% H_2O_2 for 30 min (to block endogenous peroxidase), the slides were washed in PBS 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^o C. Both antibodies have been shown to specifically recognise the respective protein receptors as determined by immunoblotting, and have been validated for use in immunocytochemical staining of cell membrane receptors (see manufacturer's datasheets). To demonstrate that the immunocytochemical staining was specific for the primary antibody, the primary antibody was substituted with 20% normal rabbit serum. The slides were rinsed in PBS/1% BSA and labeled and stained with biotin-streptavidin-HRP using a Biogenex detection kit (LP000-UL). The slides were counterstained with haematoxylin before examination under a Zeiss microscope.

2.7 Data and statistical analysis

Data obtained from the WST-1, LDH and ALP assays were corrected for background values and expressed as percentage of controls. Data were analysed using ANOVA with Tukey's posthoc test.

3. Results

3.1 Expression of GDNF, GFRα1 and RET in calvarial osteoblasts

sqRT-PCR analysis revealed that GDNF and its receptors GFRα1 and RET were expressed in the osteoblast cell line MC3T3-E1 (Fig. 1A). No obvious changes in gene expression were evident in serum-free cultures as compared to cultures maintained in serum supplemented media (Fig. 1A). This observation was supported by gel image analysis (unpublished observations). Immunocytochemical staining of MC3T3-E1 using specific antibodies against GFRα1 and RET confirmed the presence of these GDNF receptors (Fig. 1B).

3.2 GDNF stimulates MC3T3-E1 cell proliferation

Addition of GDNF to the osteoblast-like cells did not elicit major changes in the number of viable cells over a 2-day culture period in medium supplemented with FBS; however, at 100 ng/ml GDNF a significant, albeit modest decrease in cell number was evident (82.9% of controls). Conversely, GDNF dose-dependently increased viable MC3T3-E1 numbers in serum-free cultures (Fig. 2A).

Biochemical analysis of alkaline phosphate (ALP), a non-specific marker for early osteoblast differentiation (19-20) demonstrated that GDNF had no significant effect on overall ALP activity (data not shown), but following correction for cell numbers a dose-dependent decrease in ALP levels at increasing GDNF concentrations in serum-free cultures was evident (Fig. 2B). These data indicated that the reduced relative ALP activity in GDNF-treated serum-free cultures corresponded with increased cell growth.

To corroborate the WST-1 data, cell proliferation was further analyzed using the BrdU-incorporation assay. The BrdU data demonstrated that the mitotic activity in serum-free cultures was greatly reduced compared to the serum-supplemented cultures (Fig. 3). GDNF had a modest, albeit non-significant, effect on BrdU labeling in serum-containing cultures (23% reduction compared to controls). However, GDNF significantly increased the number of BrdU-labeled cells in serum-free cultures by 103.7% (i.e. two-fold increase) demonstrating that GDNF stimulated cell replication under these conditions (Fig. 3).

3.3 GDNF does not affect osteoblast cell survival

To further investigate whether GDNF influenced cell survival, the live-dead assay was applied. Results demonstrated that significant cell death occurred in control, serum-free cultures as compared to serum-supplemented cultures (Fig. 4A). GDNF did not significantly affect the number of dead cells in either serum-containing or serum-free cultures suggesting that GDNF did not affect cell death or survival under either condition (Fig. 4A).

These data were corroborated by the biochemical cytotoxicity LDH assay demonstrating that GDNF did not influence the level of cell death under serum-free conditions (Fig. 4B).

3.4 Receptor-mediated effects of GDNF

To determine whether GDNF affected the cells through its canonical receptors GFRα1 and RET, cultures were treated with specific compounds known to block GDNF signalling. PI-PLC which hydrolyses the GFRα1 subunits from their glycosylphosphatidylinositol (GPI)-anchored membrane proteins thereby negating GDNF signalling via this receptor, abrogated GDNF effects on viable cell numbers in serum-free cultures (Fig. 5). These data underline an essential role for GFRα1 in the GDNF effects on osteoblast viability. RPI-1, a competitive ATP-dependent RET kinase inhibitor, dose-dependently blocked GDNF action indicating that activation of the RET co-receptor was necessary to elicit GDNF signalling in these cells (Fig. 5).

3.4 Interaction of GDNF with TNFα

Finally this study investigated the effects of GDNF in the presence of the pro-inflammatory cytokine TNF-α, which is known to have profound effects on bone cells including MC3T3-E1 osteoblastic cells (21-23). TNF-α dose-dependently increased viable cell numbers in both serum-containing and serum-free MC3T3-E1 cultures (Fig. 6). Addition of GDNF to the cultures supplemented with TNF-α further promoted osteoblastic cell growth in these cultures: The stimulating effects of GDNF appeared

relatively modest and non-significant in the 10% FBS cultures (Fig. 6A); however, GDNF synergistically increased cell numbers in the presence of TNF-α in serum-free cultures (Fig. 6B).

4 Discussion

The current study provides evidence of a direct effect of GDNF on calvarial-derived osteoblasts suggesting a potential role for this neurotrophic factor in the regulation of craniofacial bone metabolism. GDNF as well as both of its canonical receptors GFRα1 and RET were shown to be expressed in MC3T3-E1 osteoblastic cells and signaling through both receptors was needed for GDNF effects on osteoblast cell growth. This is a novel and interesting finding, as previous studies reported that only GDNF and GFRα1were present in two human osteosarcoma cell lines (Saos-2, MG63) and primary bone marrow stromal cells, but not RET (12). Thus this latter work had led to the conclusion that GDNF signaling in the bone marrow environment involved interaction with RET-expressing hematopoietic cells (13-14). Previous studies have suggested that isolated cells from calvarial bones may behave differently than osteoblasts derived from long bones ((21-23)), which may reflect the mechanistically different processes by which the different structures in the skeleton develop (i.e. flat bones via intramembranous bone formation, whereas long bones through the process of endochondral bone formation; (27)). Moreover, it is worth noting that a significant part of the craniofacial skeleton originates from neuronal crest “ecto-mesenchymal” progenitor cells and therefore the calvarial osteoblasts may therefore exhibit a different molecular repertoire and cell behavior than mesodermal/mesenchymal-derived bone-forming cells present in long bones (24-26). RET is considered important for

development and differentiation of neural crest-derived tissues, including cranial tissues (28-30). GDNF was shown to be co-expressed with GFRα1/2 and RET in dental epithelial and mesenchymal cells during tooth development (31,32). Our recent studies indicated that mesenchymal/stromal cell cultures derived from dental pulp also displayed co-expression of GDNF and GFRα1/RET (33). RT-PCR analysis suggested that the calvarial osteoblasts expressed GDNF and the receptors GFRα1 and RET in serum-free cultures to a similar degree as cells maintained in serum-supplemented media.Previous studies demonstrated gene expression of both GDNF receptor components GFRα1 and RET in adrenal medullary cells and glial cells cultured in serum-free medium (34, 35).Interestingly, addition of serum upregulated the transcription levels of GFRα1 and RET in these cells (34, 35). Further quantitative RT-PCR is recommended to evaluate and substantiate the effects of culture conditions on gene expression in osteoblasts; notwithstanding our observations indicate that the calvarial osteoblastic cell line expressing the primary GDNF receptors represents a suitable model to investigate GDNF signaling in either serum-containing or serum-free culture media.

This study demonstrates that GDNF stimulated MC3T3-E1 cell proliferation in serum-free conditions, which corresponded with a concomitant inhibitory effect on relative ALP activity, a marker of early osteogenic differentiation. Moreover, our data indicate that GDNF at high concentrations exerted a cell growth curbing effect on osteoblasts maintained in serum-supplemented culture medium; an effect that appeared to be associated with a slight increase in ALP activity (Fig. 2). It is well established that cell differentiation is often inversely related with mitotic activity and our observations indicate that GDNF effect on calvarial osteoblasts mainly involves an action on cellular

proliferation with a concomitant inverse effect on immediate differentiation. Interestingly, GDNF/RET signaling has been shown to be responsible for the anti-mitotic action in an embryonic neural precursor carcinoma cell line (36). This effect mediated by $p27^{kip1}$ was suggested to be a mechanism by which GDNF regulates cell growth to initiate terminal differentiation (36). Long-term culture experiments will be required to investigate in further detail the role of GDNF in osteogenic proliferation, differentiation and bone formation.

The mitotic and cell survival actions of GDNF through GFRα1/RET signaling are well documented in the literature underscoring the multifunctional role of GDNF in tissue maintenance, repair and regeneration (1-3). Shi et al (37) described that GDNF promoted mesenchymal stem cell migration and survival; a mechanism by which GDNF may deliver renoprotection and kidney repair. The physiological implications of our findings that GDNF stimulated calvarial osteoblast proliferation under serum-deprived conditions are as yet unclear, but may allude to a novel role of this neurotrophic factor in bone remodeling and repair. GDNF is considered a member of the TGFβ superfamily as it has a partial amino-acid sequence homology and similar structural confirmation to TGFβ (38), which comprise growth factors including bone morphogenetic proteins pivotal in regulation of bone development, metabolism and repair. TGFβ1 can have diverse and multiple effects in different cell systems; notably this signaling molecule has been ascribed a central role in bone cell recruitment, proliferation and differentiation (39). Interestingly, TGFβ1 induces translocation of GFRα1 to the plasma membrane thereby enabling GDNF signaling through this receptor (40). It is also worth noting that GDNF is a potent inducer of the nuclear transcription factor, murine GDNF

inducible factor (mGIF) which is homologous to the human TGFβ inducible early gene (TIEG) (41). TIEG expression which has been suggested to play a pivotal role in the regulation of osteoblast differentiation (42) is highly induced in human osteosarcoma cells as well as immortalized human fetal osteoblasts following treatment with TGFβ (43). It would be fascinating to explore whether GDNF signaling in calvarial osteoblasts is related to induction of TIEG/mGIF.

Considering that GDNF is produced by bone marrow stromal cells as well as osteoblasts (9, 44, 45), it is tempting to speculate that GDNF in conjunction with other auto- and paracrine factors may be involved in the regulation of osteoblast recruitment in bone growth and remodelling. Indeed, this study demonstrated that GDNF cooperated with the cytokine TNF-α to stimulate osteoblastic cell growth suggesting an interaction between GDNF and TNF-α signalling pathways in osteoblasts. TNF-α has been ascribed a multifunctional role in bone metabolism (22); TNF-α effects may involve a pro-resorptive (osteoclastic bone degradation) action during inflammatory conditions (21), but TNF-α has also been recognised as an anabolic cytokine stimulating osteogenic migration, proliferation and differentiation (46-50). Interestingly, TNF-α has been shown to have neuroprotective capabilities which in part may be dependent on induction of cytoprotective neutrotrophic growth factors such as GDNF (51-53). Moreover TNF-α was reported to induce GDNF in chondrocytes underscoring a potential role for GDNF in skeletal cells under pro-inflammatory conditions (54). Further research is warranted to explore the precise role and mechanistic interaction of these pleiotrophic signaling molecules in bone remodeling and their potential therapeutic use in bone regeneration and repair.

In conclusion, this is the first study to report that the neurotrophic factor GDNF is able to influence the proliferation of calvarial osteoblasts via its canonical receptors GFRα/RET expressed on these cells highlighting a novel regulatory pathway in craniofacial bone physiology.

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Tables and figures

Table 1. PCR primer sequences and annealing temperature (Tm)

Gene symbol	**Primer sequence (5' to 3')**	**Genbank Accession no.**	**Product size**	**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
GFRα1	F-AATGCAATTCAAGCCTTTGG R-TGTGTGCTACCCGACACATT	U59486	218bp	60

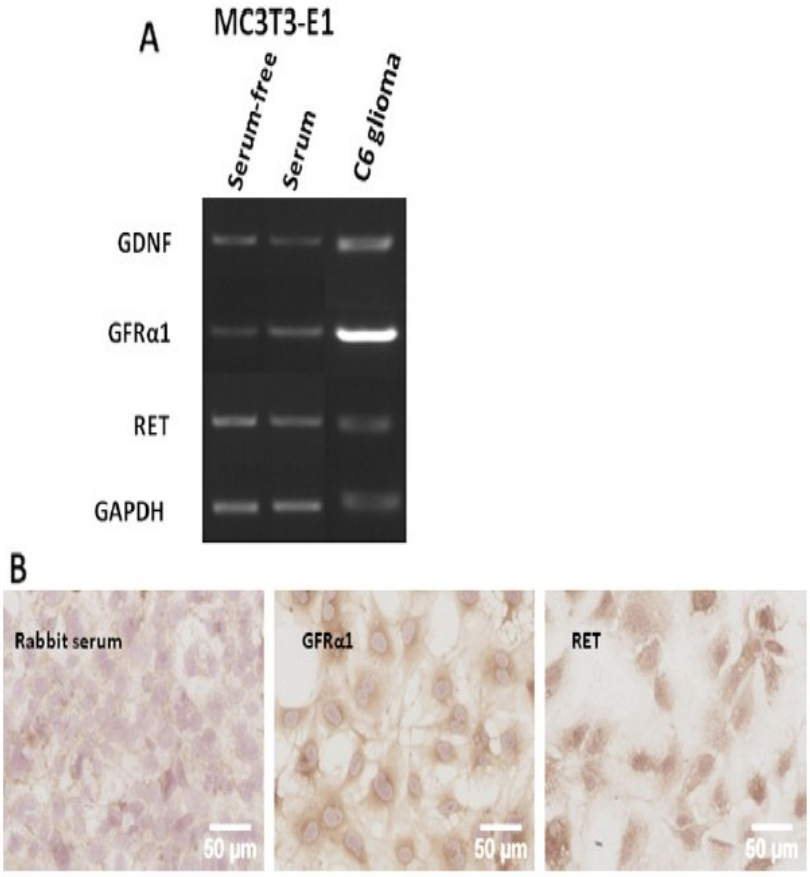

Fig. 1. (A) Representative RT-PCR gel images demonstrating the presence of transcripts for GDNF and its receptors GFRa1 and RET in MC3T3-E1 calvarial osteoblasts cultured
in serum-supplemented (10% FBS) or serum-free culture medium for 2 days. C6 glioma cells were used as positive control, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control, housekeeping gene. Respective cycle number used for GDNF, GFRa1, RET and GAPDH were 35, 45, 45 and 25. **(B)** Immunocytochemical staining of MC3T3-E1 for GFRa1 and RET. Positive staining (brown) for both receptors was clearly evident in the MC3T3-E1 cells, whilst immunostaining was absent in controls (specific
primary antibody was substituted with non-immune rabbit serum). (For interpretation of the references to colour in this figure legend, the reader is referred to the web
version of this article.)

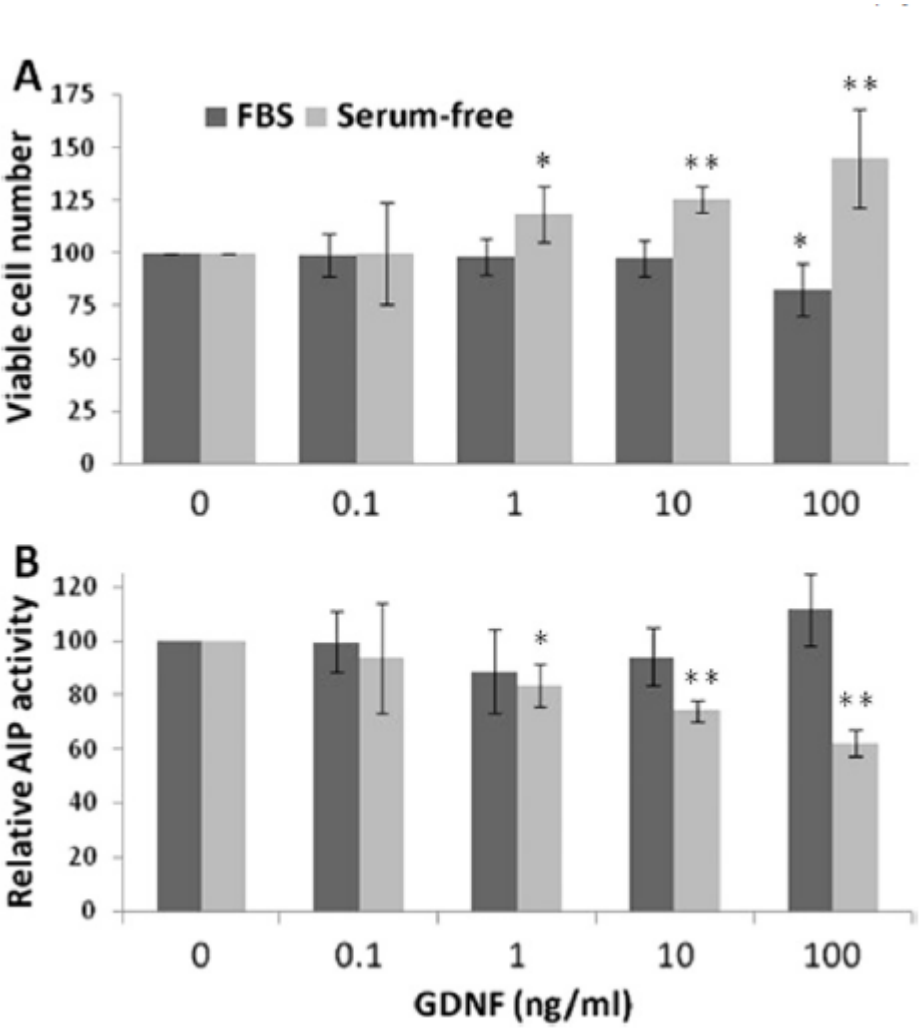

Fig. 2. (A) Effect of GDNF on viable MC3T3-E1 cell number in 2-day serum supplemented
(10% FBS) or serum-free (0.1% BSA) cultures as assessed by WST-1.
Results are expressed as percentage of controls (mean ± SD; n = 4). **(B)** Effect of GDNF on alkaline phosphatase (AlP) activity in osteoblast cultures after 2 days of culture. Results represent relative AlP activity corrected for cell number (percentage of controls; mean ± SD; n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control values.

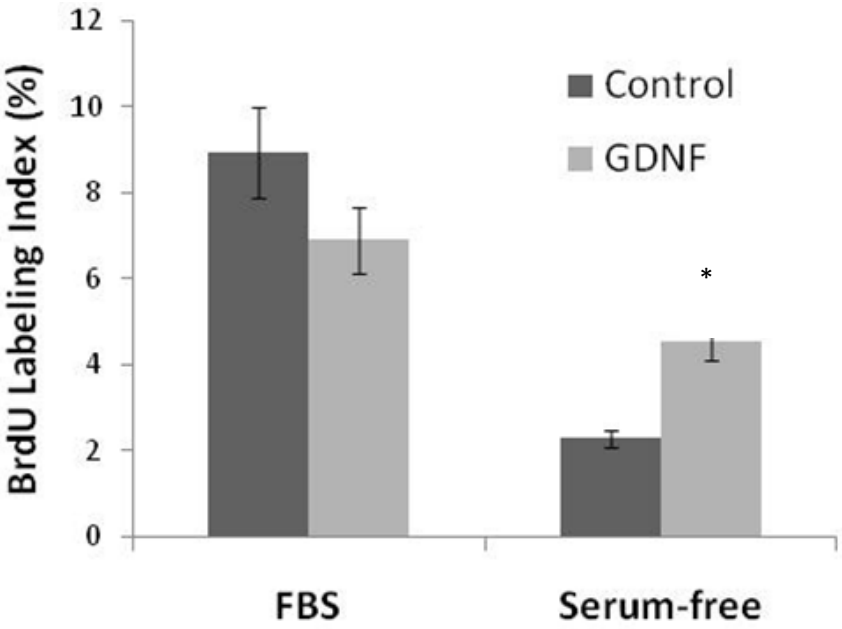

Fig. 3. BrdU incorporation in 2-day MC3T3-E1 cultures in serum-supplemented (10% FBS) or serum-free (0.1% BSA) cultures. The results show the proportion of cells labeled positively for BrdU (Li: labeling index). Significantly different from controls: $P < 0.05$.

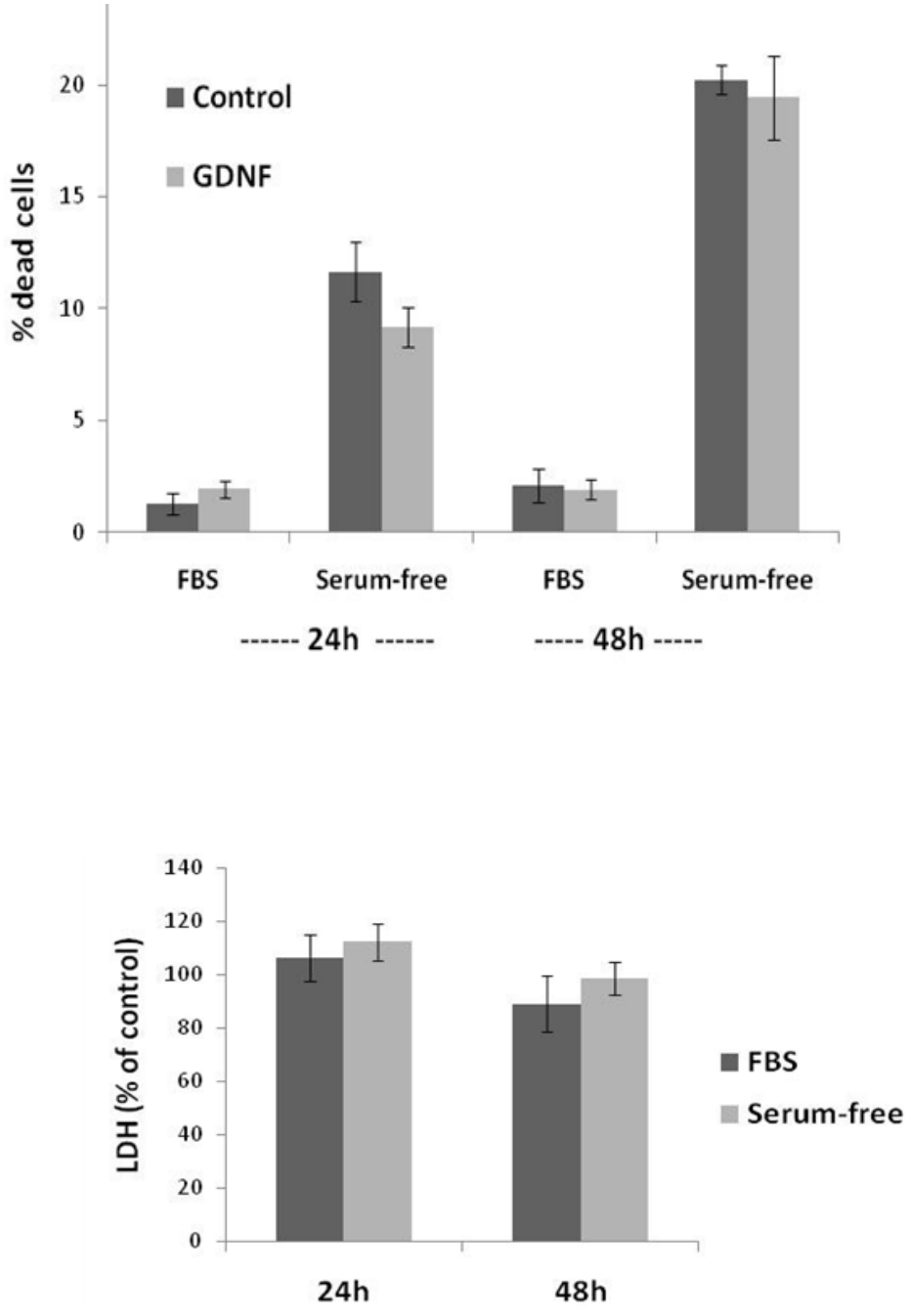

Fig. 4. Effects of GDNF on cell death in MC3T3-E1 cultures**. (A)** The number of dead cells as determined by the live/dead assay in cultures treated with 100 ng/ml GDNF. Results show percentage of dead cells in 1- and 2-day cultures (mean ± SD; n = 3). **(B)** Relative LDH levels after 2-days culture in serum-containing or serum-free medium cultures supplemented with 100 ng/ml GDNF (mean ± SD; n = 3). Results are mean ± SD (n = 3–4).

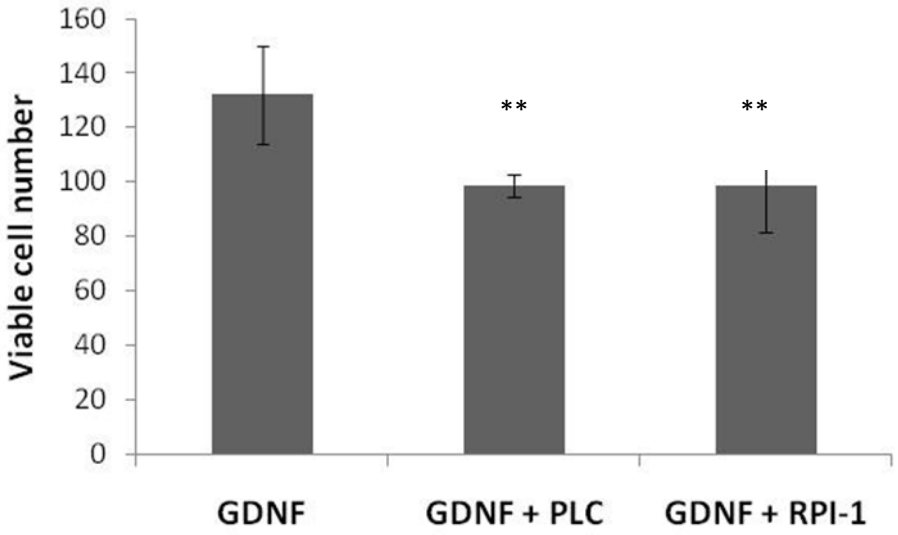

Fig. 5. Effects of GDNF receptor inhibitors on GDNF-stimulated MC3T3-E1 cultures. **(A)** Effect of phosphatidylinositol-specific phospholipase C (PLC) on viable cell number in cultures supplemented with 100 ng/ml GDNF. **(B)** Effects of the RET kinase inhibitor, RPI-1, on viable cell number in 2-day GDNF-treated cell cultures. Results are percentage of control values as determined by the WST1 assay (mean} SD of 6.8 replicates). Data from GDNF control cultures (without inhibitor) were significantly different from controls without GDNF ($P < 0.01$). ..$P < 0.01$ versus GDNF cultures.

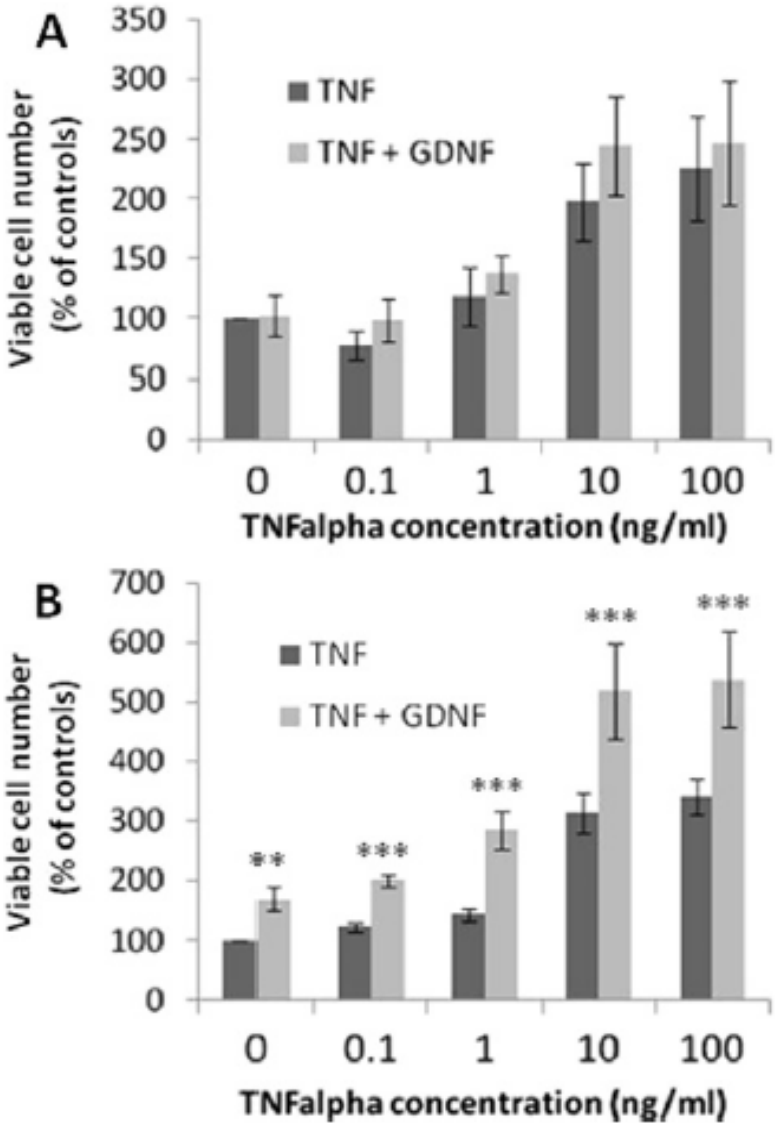

Fig. 6. Osteoblastic MC3T3-E1 cell numbers after 2-days' culture in the presence of increasing concentrations of TNF-a with or without 100 ng/ml GDNF in media supplemented with 10% serum (A) or in serum-free cultures (B).Viable cell numbers were assessed using the WST-1 assay; results are expressed as percentage of controls (mean ± SEM; n = 3). /P < 0.05, //P < 0.001 GDNF-supplemented cultures versus corresponding TNF-a controls.