Cementoblast associated gene expression during differentiation of various periodontal cell phenotypes

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To my mother.

Thank you for your patience and love.

Abstract

The structures that surround and support the tooth include both hard mineralized tissues (cementum and alveolar bone), and soft tissues (the periodontal ligament and gingiva). These structures collectively form the periodontium, whose major functions include tooth support, proprioception, nutrition, homeostasis, and repair. Of these functions, periodontal tissue homeostasis and repair, in particular, depends upon the control of a wide range of cellular activities such as proliferation, differentiation, collagen turnover, and the synthesis and resorption of soft and hard tissues (Schroeder, 1986). These cellular activities and their regulation are therefore of critical importance in any response to disease or injury.

Most of the common forms of periodontal disease involve chronic inflammatory processes that can lead to the irreversible loss of both soft and hard periodontal tissue which, if left untreated, may ultimately result in tooth loss (Pihlstrom et al., 2005). A recent survey of the Australian population's oral health status has demonstrated that 22.9% of the adult population has some degree of periodontitis (Health, 2009). Clearly, this is a health problem of national significance. Following resolution of the inflammatory pathogenic mechanisms associated with periodontitis, the ultimate goal of periodontal treatment is the regeneration of the lost mineralized and / or soft tissue components of the periodontal attachment apparatus. Hence, it is crucial to understand the mechanism(s) underlining periodontal tissue healing and repair if this goal is to be achievable. Cementogenesis is a key event in periodontal regeneration as it is the cementum layer on the tooth surface into which the fibers of the periodontal ligament

are inserted to provide functional attachment between the tooth and the alveolar bone. For this process to occur on a root surface affected by periodontal disease, cementoblast precursors must first proliferate, migrate, and attach to the denuded root surface and subsequently differentiate into functional cementoblasts (Bosshardt, 2005). Similar to osteoblasts, cementoblasts then express transcription factors such as Runx2, which regulate cellular differentiation and the synthesis of a range of noncollagenous bone matrix proteins, such as alkaline phosphatase (ALP),osteocalcin (OCN), and bone sialoprotein (BSP) (D'Errico et al., 1997, McCulloch, 1987). More recently, a number of other proteins such as cementum attachment protein (CAP), cementum protein-1 (CEMP1) and F-Spondin (SPON1) have been identified as putative makers specifically associated with cementoblast differentiation (Arzate et al., 1992, Alvarez-Perez et al., 2006, Kitagawa et al., 2006).

Undifferentiated mesenchymal cells have been shown to reside in bone and periodontal ligament (Seo et al., 2004, Chen et al., 2006). As these cells can differentiate into the various components of the periodontium, it is important to identify the factors that can induce differentiation to occur. During wound healing and regeneration, several growth factors have been identified as having considerable potential in promoting periodontal regeneration. Platelet derived growth factor (PDGF) and insulin-like growth factor-1 (IGF-1) are two growth factors that have been extensively studied to elucidate their effect on periodontal tissue regeneration, reviewed in (Kao et al., 2009).

Chapter two of this thesis therefore examined the potential of three primary human periodontal cell types to differentiate towards an osteogenic and / or cementogenic phenotype following osteogenic stimulation. Periodontal ligament (PLC), regenerated periodontal defect (RTC) and alveolar bone (OB) cells were cultured *in vitro* and treated with mineralization media up to six weeks. Using a number of histochemical techniques, mineralized matrix formation was observed in these cells after 3 and 6 weeks of culture. Bone and cementum gene expression examined using Real Time-PCR showed BSP and SPON1 were correlated with mineralization formation. Moreover, PLC was the only cell-type that up-regulated expression of the putative cementum markers, CAP and CEMP1.

Chapter three then sought to determine the effect of the growth factors IGF-1 and PDGF on the osteogenic and / or cementogenic potential of these different periodontal cell types. Both growth factors individually up-regulated gene expression to various degrees with OB found to be the most responsive cells, followed by PLC then RTC. The combined treatment of IGF-1 and PDGF together had a synergetic effect on BSP and SPON1 expression in the three cell types, moreover, CAP was also up-regulated in PLC, and OCN and CEMP1 in OB. These results demonstrated IGF-1 and PDGF can differentially regulate osteogenic and / or cementogenic gene expression in these periodontal cells.

Chapter four investigated the heterogeneity in cellular response found among the primary cells examined in chapters 2 and 3. Single cell clones established from these

primary cells showed an array of expression profiles for the bone / cementum markers, which may explain the heterogeneous results shown in the previous chapters.

The final experimental chapter used an animal model to test the ability of PLC cells previously shown to form mineralized matrix and up-regulate osteogenic/cementogenic gene expression following mineralization treatment *in vitro*, to form mineralized tissue *in vivo*. Human PLC cell sheets treated *in vitro* with mineralization media and transplanted into an athymic rat, were found to adhere to the dentine chip and express CEMP1 after six weeks of transplantation.

To date, there is a limited understanding on the periodontal cells differentiation along a mineralized tissue phenotype. To the best of my knowledge, this work presents for the first time, the osteogenic/cementogenic gene expression profile of various periodontal cell phenotypes during osteogenic/cementogenic differentiation for up to six weeks. Not discounting other possible osteoinductive growth factors, this study also illustrates the effect of IGF-1 and PDGF during periodontal cells differentiation. Furthermore, this study demonstrates the importance of cell screening before moving to more complicated cell based tissue regeneration studies *in vivo*. The overall results are consistent with the recent studies proposing the presence of undifferentiated mesenchymal cells in periodontal tissue.

Statement of Originality

I, Haifa Hannawi declare that this work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Declared by

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Haifa Hannawi Date

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Abbreviations

ALP……...Alkaline phosphatase

β-TCP…….....β- tricalcium phosphate

BMP……..Bone morphogenetic protein

BSP……...Bone sialoprotein

cDNA……...complementary deoxyribonucleic acid

CP-23……Cementum protein 23

DF…….Dental follicle

DFDBA……...Demineralised free-dried bone allograft

DFSCs.....Dental follicle stem cells

DMEM……Dulbecco's modification of Eagle's medium

DPSCs.....Dental pulp stem cells

ECM……....Extracellular matrix

EMD…….……Enamel matrix derivatives

FCS……..……Fetal calf serum

FDA…..……Food and drug administration

FGF..……Fibroblast growth factor

GF……....Gingival fibroblasts

GMPC……... Gingival multipotent progenitors cells''

GTR……..…..Guided tissue regeneration

HCEM.....Human cementoblast-like cell line

hESCs.....human Embryonic stem cells

GF……...gingival fibroblasts

hIGF……....human insulin like growth factor

hPDGF……human platelet derived growth factor

INF-λ…..……Interferon-λ

IGF…..……Insulin-like growth factor

iPSCs……..induced pluripotent stem cells

M-CSF.....Macrophage colony stimulating factor

MMP.....Matrix metalloproteinase

mRNA……...messenger ribosomal nucleic acid

MSCs.....Mesenchymal stem cells

RTF……..…..Regenerated tissue fibroblasts

Runx2……Runt related transcription factor 2

OA……....Osteoarthritis

OB……..…Osteoblast

OCN…….Osteocalcin

OPG.....Osteoprotegerin

OPN…….Osteopontin

Osx.....Osterix

PDGF…...…Platelet-derived growth factor

PDGFR...….Platelet-derived growth factor receptor

PDL...……..Periodontal ligament

PDLSCs…..Periodontal ligament stem cells

PLF...……..Periodontal ligament fibroblasts

PLGA…... poly(lactic-co-glycolic acid)

PRP……Platelet rich plasma

Q-PCR…..Quantitative polymerase chain reaction

rADs…....recombinant adenovirus

RANKL.....Receptor Activator for Nuclear Factor κ β Ligand

rhBMP……..recombinant human Bone morphogenetic protein

rhIGF……recombinant human Insulin-like growth factor

rhPDGF…...recombinant human platelet-derived growth factor

SHED……..Stem cells from exfoliating deciduous teeth

TGF-β……..Transforming growth factor-β

TNF-α…....Tumour necrosis factor-α

1.0 Chapter One

Literature Review

1.1 Overview

The research reported in this thesis was undertaken to investigate the gene expression of primary human periodontal cells during differentiation into a bone/cementum phenotype. Cementum formation is a critical step needed to achieve periodontal tissue regeneration because it invests the periodontal fibres and therefore anchor the tooth in the alveolar socket. Cell based periodontal tissue engineering is an emerging technique aimed to achieve more predictable periodontal tissue regeneration, and understanding the molecular processes during periodontal cell differentiation is crucial in order to achieve the ultimate goal of periodontal therapy, i.e. regeneration of the damaged periodontal tissue and restoring periodontal function and architecture.

In the following literature review, I will present a summary of the available research highlighting of our current understanding of the differentiation potential of various periodontal cells, their genetic expression during their differentiation, the effect of growth factors and the heterogeneity among them.

1.2 The Periodontal Attachment Apparatus

The periodontal ligament is a unique connective tissue which lies between root cementum and alveolar bone, and plays an important role in supporting the tooth in the alveolar bone socket (Figure1.1). Additionally, it has sensory, nutritive and homeostatic functions. All of the tissues of the periodontium (i.e. gingiva, periodontal ligament (PDL), cementum and alveolar bone), originate from the ectomesenchyme, a neural crest derived primordium of the developing jaws. In brief, the periodontium originates from dental follicle (DF) cells. During tooth germ development, DF cells differentiate into progenitors of the periodontal lineage and form the three components of the periodontal attachment: periodontal ligament, alveolar bone and cementum. The differentiation of PDL progenitors is initiated in the root forming stage of the tooth germ, and PDL formation is completed when the tooth erupts (Cho and Garant, 2000).

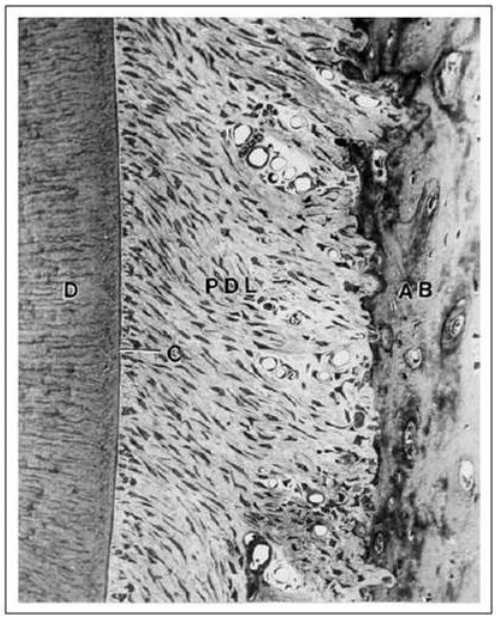

Figure 1.1 Histologic section showing the periodontium structure.
Periodontal ligament (PDL) fibers are inserted into both the alveolar bone (AB) and cementum (C), which lines the root dentine (D). Reprinted with permission from John Wiley and sons, Periodontology 2000 (Cho and Garant, 2000).

Periodontal development commences at the end of the crown stage, when cells of the inner and outer enamel epithelia proliferate from the cervical loop of the enamel organ to form Hertwig's epithelial root sheath. The root sheath initiates the differentiation of dental papilla cells into odontoblasts which lay down root dentine and subsequently secrete a fine matrix of proteins onto the dentine surface, known as the hyaline layer of Hopewell Smith (Lindskog, 1982, Slavkin et al., 1989). It is thought that subsequent fragmentation of the root sheath allows cells of the dental follicle to attach to this protein matrix and differentiate into cementoblasts (MacNeil and Thomas, 1993, Hammarstrom et al., 1996). Subsequently, periodontal ligament fibroblasts derived from the dental follicle form the collagen fibres which become embedded in the newly-formed cementum and are known as Sharpey's fibres (Yamamoto and Hinrichsen, 1993, Yamamoto et al., 1994). Simultaneously, osteoblasts (OB) originating from the dental follicle form bundle bone (the portion of the alveolar process that lines the tooth socket) (Ten Cate, 1975). Insertion of Sharpey's fibres into this newly-forming bone completes the development of the attachment apparatus of the periodontium. Subsequently, the maturation of cementum, periodontal ligament and alveolar bone along with the transformation of reduced enamel epithelium to sulcular and junctional epithelium during tooth eruption, give rise to a complete periodontal attachment apparatus (Ten Cate, 1996).

The PDL is composed of densely packed collagen-rich connective tissue with collagen type I being predominant, but also containing types III, IV, V, VI and XII. Proteoglycans are also present in the PDL extracellular matrix (Huang et al., 1991, MacNeil et al., 1998, Lukinmaa and Waltimo, 1992, Bartold and Narayanan, 2006). The cells residing in the PDL include fibroblasts, osteoblasts, cementoblasts, osteoclasts and

epithelial cells, along with blood vessels, lymphatics and nerve fibers. Indeed, periodontal ligament fibroblasts (PLF) are the dominant resident cell type and a key source of interstitial extracellular matrix. Equally important, PLF play a major role in both homeostasis and healing of the tooth supporting apparatus. In periodontal connective tissue remodeling, fibroblasts are capable of the synthesis and phagocytosis of collagen and other components of the extracellular matrix (Ten Cate et al., 1976). Further, PLF play an important role in the immune system by synthesizing and responding to cytokines and through antigen presentation (Ko et al., 1984, Takashiba et al., 2003). They produce numerous growth factors and cytokines, such as insulin-like growth factor-1 (IGF-1), bone morphogenetic proteins (BMP's), platelet derived growth factor (PDGF), interleukin-1 (IL-1), transforming growth factor β (TGF- β), that mediate tissue destruction and stimulate osteoclastic bone resorption (Genco, 1992, Havemose-Poulsen and Holmstrup, 1997, Graves, 2008). Moreover, PLF have the potential to differentiate into osteoblasts or cementoblasts according to their microenvironment (Gould et al., 1980). They also exhibit osteoblastic properties, such as the expression of runt related transcription factor-2 (Runx2), osterix (Kato et al., 2005), alkaline phosphatise (ALP) (Yamashita et al., 1987), osteopontin (OPN) (Lekic et al., 2001a, Li et al., 2001), osteocalcin (OCN) (Li et al., 2001), periostin (Yamada et al., 2001), Receptor Activator for Nuclear Factor κ β Ligand (RANKL), (Wada et al., 2004), and osteoprotegerin (OPG) (Wada et al., 2004, Wada et al., 2001). On the whole, the functions outlined above underline the central role that fibroblasts play in the normal function of the periodontal ligament during homeostasis and disease.

Traditionally, fibroblasts have been classified as flat elongated cells with an oval nucleus and the ability to synthesize extracellular matrix components. However, PLF are a heterogeneous cell population with diverse PLF subpopulations having different

synthetic products, responses to regulatory molecules, cellular turnover rates and morphological features (McCulloch and Bordin, 1991, Ko et al., 1984, Phipps et al., 1997, Lekic et al., 1997). Electron microscopy studies have revealed a significant morphological heterogeneity among periodontal ligament cells in the periodontal ligament of rats (Roberts and Chamberlain, 1978). In an *in vitro* study of human periodontal ligament cells, Rose et al (1987) proposed that there were at least two subsets of PLF based on morphologic differences: a population containing abundant glycogen pools and one which did not (Rose et al., 1987). Moreover, the PDL also consists of different cell populations in various differentiation stages, sometimes determined by their position in the periodontal ligament. The cell populations with the larger growth potential are generally located in the middle part of the periodontal ligament, while cell populations with higher ALP and mineralization activities are located toward the surface of the root in rat molars' periodontal ligament (Kaneda et al., 2006). Clones derived from human PLF also vary extensively in their expression of cementum and osteogenic markers (Ivanovski, 2001a, Pi et al., 2007). They respond differently to osteogenic induction, as illustrated when a distinct clone from immortalized human periodontal ligament cells was shown to have no mineralized nodule formation and no BMP-2 and OCN expression compared to other clones (Fujita et al., 2005). Porcine PLF clones have also been shown to vary widely in total collagen production and the type of collagen that was synthesized (Limeback et al., 1983).

Cells derived from regenerated periodontal defects have also been studied to understand the biological processes involved in periodontal regeneration. Regenerated periodontal tissue cells (RTC) retrieved from membranes after bone inductive procedures have been shown to form mineralized nodules *in vitro* (Wakabayashi et al., 1996), as well as express different cytokine (Wakabayashi et al., 1997) and protease repertoires

(Wakabayashi et al., 1996). RTC obtained from retrieved membranes of periodontal disease patients treated with GTR and edentulous ridge augmentation were found to produce factors resulting in osteoclasts inhibition (Rowe et al., 1996). RTC cells derived from membranes, as well as regenerated tissue underlying the membrane following GTR, were found to have up-regulated ALP (Kuru et al., 1999), proliferate faster than PLF from uninjured tissue, and exhibit a unique pattern of proteoglycan (Ivanovski, 2001), and bone markers (OCN, BSP and osteopontin) mRNA expression (Ivanovski, 2001a). Granulation tissue fibroblasts derived from both chronically inflamed periodontal lesions and healing wounds were found to behave similarly *in vitro*, and to have distinct morphological features when compared to gingival tissue fibroblasts (Hakkinen and Larjava, 1992). Moreover, RTC were found to express different genes in respect to protein biosynthesis and turnover, structural constituents of the cytoskeleton and extracellular matrix, and signal transduction (Ivanovski et al., 2007).

1.3 Progenitor Capacity of Periodontal Cells

Stem cells have two defining characteristics: (i) the ability for indefinite self-renewal to give rise to more stem cells; and (ii) the ability to differentiate into a number of specialized daughter cells to perform specific function(s) (Smith, 2006). Importantly stem cells can divide asymmetrically, in which case one of the two daughter cells retains the stem cell characteristics while the other is destined for specialization under specific conditions (Morrison and Kimble, 2006). The potential for self-renewal versus differentiation is governed by extracellular signals coupled to intracellular signalling cascades (Watt and Hogan, 2000). There are two main types of stem cells, embryonic stem cells and adult stem cells, which are further classified according to their origin and differentiation potential. Human embryonic stem cells (hESCs), derived from the inner

cell mass of blastocysts, are pluripotent cells capable of differentiating into cells of all three germ layers, ectoderm, mesoderm and endoderm (Thomson et al., 1998). hESCs have two unique properties: (i) virtually unlimited proliferative potential in an undifferentiated state; and (ii) their pluripotentcy, which is the capability of differentiating into cells from all three germ layers mentioned above (Thomson et al., 1998). However, the use of human embryonic stem cells has been hampered by ethical concerns (Klitzman, 2010). Eventually, cells with properties similar to those of embryonic stem cells, termed induced pluripotent stem cells (iPSCs), were generated by the reprogramming of somatic cells into a pluripotent state by the introduction of specific transcription factors, such as Oct4, Sox2, Klf4, and c-myc (Takahashi et al., 2007, Takahashi and Yamanaka, 2006, Lowry et al., 2008, Park et al., 2008, Yu et al., 2007). Besides being good candidates for regenerative medicine, they are also used to study the dynamics of disease, and as systems to screen novel drugs (Fujiwara et al., 2011, Wang et al., 2011, Vitale et al., 2011, Dias et al., 2011).

Adult stem cells are found in the majority of fetal and adult tissues and are thought to function in long-term tissue maintenance and／or repair by replacing cells that are either injured or lost (Leblond, 1964). They are generally multipotent stem cells that can form a limited number of cell types. Adult stem cells are classified depending on their origin and differentiation potential. Two common examples are hematopoietic and mesenchymal stem cells (MSCs). Hematopoietic stem cells have been shown to have therapeutic potential, particularly in the treatment of blood malignancies and immunodeficiency syndromes (Korbling and Estrov, 2003). Mesenchymal stem cells (MSCs) were initially identified in rodent marrow as colony-forming unit fibroblasts capable of forming bone, cartilage and fat, as well as reconstituting the hematopoietic microenvironment (Friedenstein et al., 1974a, Friedenstein et al., 1974b). MSCs can be

derived and propagated *in vitro* from different organs and tissues (da Silva Meirelles et al., 2006). However, they have been found to be heterogeneous, demonstrating functional differences based on their proliferative potentials and developmental capacities *in vitro* and *in vivo* (Pittenger et al., 1999, Gronthos et al., 2003). As the periodontium is mesenchymal in origin, MSCs has been studied in periodontal regeneration research. Both bone marrow and adipose tissue derived MSCs have been shown to form cementum, periodontal ligament and alveolar bone after implantation into periodontal defects *in vivo* (Tobita et al., 2008, Hasegawa et al., 2006, Kawaguchi et al., 2004, Yang et al., 2010).

Traditionally, a small population of periodontal stem cells was believed to be responsible for maintaining homeostasis in the periodontal ligament. It was demonstrated that the human periodontal ligament contains a heterogeneous population of cells (Lekic et al., 2001a, Murakami et al., 2003) capable of differentiating into cementoblasts or osteoblasts *in vitro* (Gould et al., 1980, McCulloch and Bordin, 1991, Isaka et al., 2001) and, under specific conditions, can form mineralized nodules *in vitro* (Pitaru et al., 2002, Lekic et al., 2001c). These cells, which may have their developmental origin in late periodontal mesenchyme of the dental follicle, are thought to be tripotential cell capable of differentiating into cementoblasts, osteoblasts, and periodontal fibroblasts (Bartold et al., 2000b). It has also been suggested that mineralized tissue forming cell lineages and the fibroblast lineage may originate from a common early progenitor cell (Pitaru et al., 2002). Moreover, the progenitor cells in the bone marrow spaces have been found to migrate into the perivascular area of the periodontal ligament by way of vascular channels, and subsequently move to the bone and tooth surface, where they differentiate into osteoblasts or cementoblasts (McCulloch et al., 1987, McCulloch and Melcher, 1983b). Furthermore, the progenitor

cell populations of the periodontal ligament are found to be enriched in locations adjacent to blood vessels and exhibit some of the classical cytological features of stem cells, including small size, responsiveness to stimulating factors, and slow cycle time (Gould et al., 1980, McCulloch and Melcher, 1983a, McCulloch, 1985). More recently, cells derived from human periodontal ligament were found to have osteogenic, adipogenic, chondrogenic, myogenic, angiogenic, vasculogenic and neurogenic differentiation ability *in vitro* (Amin et al., 2011). Indeed, studies of homograft recombinations of enamel organs with papillary, pulpal and follicular mesenchyme (Palmer and Lumsden, 1987) have revealed that the tooth-related part of the periodontal ligament contains cementoblast precursors and the bone-related ligament contains osteoblast precursors. Such findings suggest that the developmental patterns that establish the locations of progenitor cells are preserved in the adult periodontal ligament (Palmer and Lumsden, 1987).

As periodontal ligament cells exhibit relatively slow turnover in normal tissues, wound models have been used to study the location and kinetics of periodontal ligament 'progenitor' cells. During the repopulation response following periodontal ligament extirpation, Gould et al (1980) demonstrated, using ^{3}H-thymidine labeled cells, that there was a fivefold increase in the 'progenitor' cell proportion which was largely due to proliferation of paravascular cells (Gould et al., 1980). These cells exhibited characteristics similar to stem cells in other renewal cell systems. There is also evidence from cell kinetic studies that a second cell population located adjacent to cementum is a separate progenitor population (Gould et al., 1980, McCulloch and Melcher, 1983c). Daughter cells of these progenitors have been shown to repopulate the wounded periodontal ligament (Gould et al., 1980) and the number of new cells generated by mitosis equals the number of cells lost through apoptosis and migration (McCulloch and

Melcher, 1983b). Furthermore, a significant number of periodontal cells do not enter the cell cycle (McCulloch and Melcher, 1983a), suggesting that these cells may act in a similar manner to quiescent, self-renewable and multipotent stem cells. Such cell kinetic studies support the notion of the presence of a progenitor/stem cell population in the normal periodontium.

Studies aimed at determining the cells responsible for eliciting regeneration of the periodontium have suggested that periodontal ligament cells, when appropriately induced, have the capacity to synthesize periodontal ligament, cementum, and alveolar bone (Roberts et al., 1987, Macneil and Somerman, 1999). Indeed, the addition of mineralization media (50ug/ml ascorbic acid, 10mM sodium β-glycerophosphate, and 10^{-7} M dexamethasone) was found to increase the expression of the mineralization tissue markers bone sialoprotein (BSP), bone morphogenetic protein-7 (BMP-7), ALP, OCN, OPN and Runx2, as well as the formation of mineral nodules (Arceo et al., 1991, Cho et al., 1992, Hayami et al., 2007, Khanna-Jain et al., 2010). Furthermore, the addition of low concentrations (5ug/ml) of either cementum or bone extract resulted in increased ALP activity, as well as mineralization in PLF over an observation period of 30 days (Hou et al., 2000). When human periodontal ligament cells isolated from healthy donors were seeded in 3D-static culture on mineralized PLGA scaffolds in a bioreactor and under osteogenic induction, differentiation towards an osteogenic lineage was observed (Inanc et al., 2006).

To date, several different human dental stem/progenitor cell populations have been isolated and characterized. The first dental stem cells were isolated from human pulp tissue and termed ‘postnatal dental pulp stem cells’ (DPSCs) (Gronthos et al., 2000). Subsequently, four more types of dental MSC-like populations were isolated and

characterized: stem cells from exfoliated deciduous teeth (SHED) (Miura et al., 2003), periodontal ligament stem cells (PDLSCs) (Seo et al., 2004), stem cells from apical papilla (SCAP)(Abe et al., 2007, Sonoyama et al., 2006), and those isolated from dental follicle of human third molars, referred to as 'dental follicle precursor cells' (DFPCs) (Morsczeck et al., 2005). More recent studies have identified progenitor cells in gingival tissue (gingival multipotent progenitors cells (GMPC)) (Fournier et al., 2010) and from the regenerated tissue harvested from a 'barrier guided' regeneration site (Lin et al., 2008b).

Seo et al (2004) demonstrated for the first time that periodontal ligament stem cells (PDLSCs) could be isolated from human periodontal ligament cell culture. Immunohistochemical staining and Western blot analysis showed that cultured PDLSCs expressed an array of cell-surface epitopes associated with mesenchymal progenitors such as STRO-1, CD146, CD29, CD44 and CD106, but did not express CD34, a hematopoietic origin marker, as shown by flow cytometry (Wu et al., 2009). Moreover, PDLSCs were found to exhibit osteogenic, adipogenic, and chondrogenic characteristics under defined culture conditions as well as producing cementum-like and periodontal ligament-like tissues *in vivo* (Seo et al., 2004, Gronthos et al., 2006, Gay et al., 2007, Lindroos et al., 2008, Xu et al., 2009). Recent studies have also shown the ability of PDLSCs to differentiate into neuronal precursors (Techawattanawisal et al., 2007). Human adult PDL tissue contains about 27% STRO-1 positive cells, with 3% being strongly positive (Gay et al., 2007). Flow cytometry revealed that on average 2.6% of periodontal ligament cells were STRO-1(+)/CD146(+), whereas more than 63% were STRO-1(-)/CD146(-) (Xu et al., 2009). Singhatanadgit et al (2009) isolated four homogenous and distinct single cell clones from human periodontal tissue ligament and found that, although they all expressed the stromal cell markers CD29 and CD 44, they

varied in their clonogenicity and multilineage potential, suggesting that isolating a cell clone with clonogenic and multilineage potential may be a critical step before moving to stem cell-based tissue engineering studies *in vivo* (Singhatanadgit et al., 2009).

PDLSCs formed more calcium nodules and had higher ALP activity in mineralized culture medium *in vitro* than DPSCs and PAFSCs, while PAFSCs showed higher clonogenic efficiency and proliferation rates (Jo et al., 2007). Moreover, PDLSCs as well as bone marrow mesenchymal stem cells (BMSCs), were found to have higher mineralization capacity than regenerated tissue derived stem cells (Lin et al., 2008b). PDLSCs were expanded *ex vivo* and then transplanted into immunocompromised mice. A thin layer of cementum-like tissue was formed along with collagen fibers inserted into a newly formed cementum-like layer, suggesting that PDLSCs may contain a subpopulation of cells capable of differentiating into cementoblasts/cementocytes and collagen-forming cells *in vivo*. After transplantation of hPDLSCs into periodontal defects of immunocompromised mice, PDL-like tissue was generated, and these human stem cells were also identified to be closely associated with the trabecular bone next to the regenerated PDL, suggesting their involvement in alveolar bone regeneration (Seo *et al.*, 2004). Park et al., 2011, investigated the ability of three dental stem cells (DSCs) to regenerate severe periodontal defects in beagle dog. PDLSCs, DPSCs and PAFSCs were expanded *ex vivo* and transplanted into the advanced periodontitis defects created by removing alveolar bone using surgical burs. After 8 weeks of transplantation, it was found that both PDLSCs and PAFSCs could regenerate periodontal tissues in an advanced periodontitis model, but PDLSCs provided the best outcome among the three DSCs populations, as evaluated by conventional and immune histology, 3D micro CT and clinical index (Park et al., 2011).

1.4 Periodontal Regeneration

Periodontitis is a disease of the periodontium characterized by irreversible loss of connective tissue attachment and supporting alveolar bone (Pihlstrom et al., 2005). Periodontal defects considered for reconstructive therapy generally exhibit substantial loss of alveolar bone, periodontal ligament and gingival tissues, and the root cementum is usually contaminated due to exposure to the oral environment (Wikesjo and Selvig, 1999). Therefore, understanding the biological mechanisms of periodontal wound healing and homeostasis is very important, not only in relation to periodontal therapy, but also in tooth transplantation and replantation, as well as orthodontic treatment (Shimono et al., 2003). Following conventional periodontal therapy involving debridement of the root surface, the periodontal tissues heal by a repair process characterized by the migration of the epithelium along the previously contaminated root surface (long junctional epithelium), which does not completely restore the architecture or function of the original tissue and prevents connective tissue attachment (Caton et al., 1980).

The American Academy of Periodontology defines regeneration as "a reproduction or reconstitution of a lost or injured part". It is the biologic process by which the architecture and function of lost tissues are completely restored (The American Academy of Periodontology 1996). Therefore, periodontal regeneration entails *de novo* cementogenesis, osteogenesis, and generation of functionally oriented periodontal ligament fibers inserting into both newly formed cementum and alveolar bone (Reddi, 1997, Kassab and Cohen, 2002). A critical step toward achieving periodontal regeneration is the new attachment of connective tissue fibers to the previously contaminated root surface. Cementum plays an important role in this process, as it invests and securely attaches the periodontal ligament fibers to the root surface.

The regeneration of any tissue type requires the interaction of the following three factors: the availability of the appropriate cell type(s), soluble mediators of cell function that activate these cells and an evolving (developing) extracellular matrix (Bartold et al., 2000b) (Figure 1.2). During periodontal regeneration, cells (fibroblasts, cementoblasts and osteoblasts) must be attracted in appropriate numbers, and subsequently induced, at the right time and in the right place, to synthesize their appropriate extracellular matrices and reform the lost periodontal tissues including alveolar bone and cementum. Further, new Sharpey's fibers must form a functional attachment with the new bone and cementum and equally importantly, epithelial down-growth must be excluded from the cementum surface (Bartold et al., 2000a).

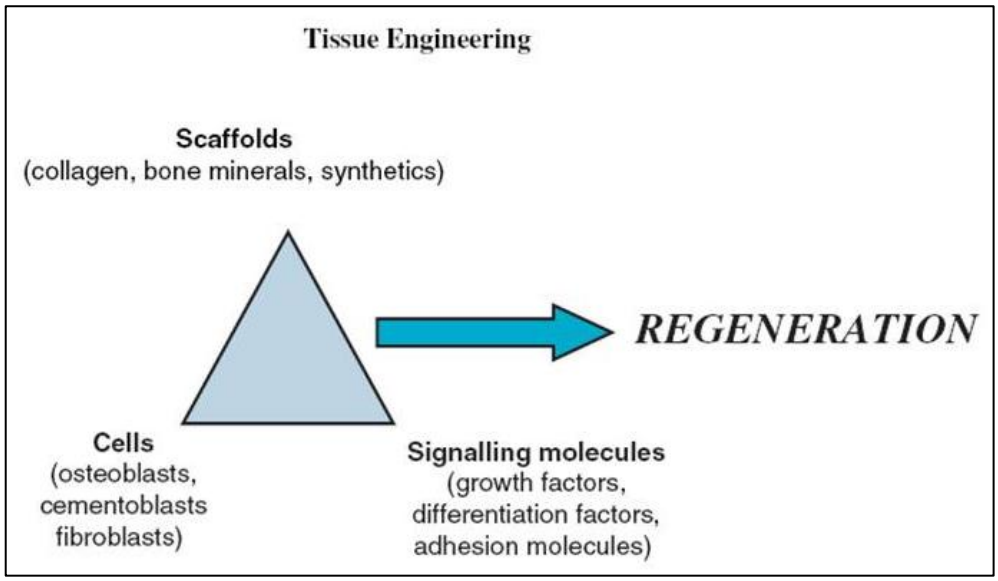

Figure 1.2 Tissue Engineering.
The three major requirements for tissue regeneration, scaffolds, proper cells and signaling molecules. Reprinted with permission from John Wiley and sons, Periodontology 2000 (Bartold et al., 2006b).

Many attempts have been made to develop clinical procedures which might lead to predictable periodontal regeneration. One of the earliest approaches was root surface conditioning using a variety of agents, including demineralization of root surfaces with acids or coating root surfaces with biological attachment agents such as fibronectin, or both. Such treatment was believed to expose collagen fibres on the root surface with which newly-formed Sharpey's fibres could interdigitate, and at the same time discourage the attachment of unwanted epithelial cells. Nevertheless, this procedure did not yield predictable regeneration, and often resulted in both ankylosis and root resorption (Smith et al., 1987). Another common periodontal regeneration procedure involved the use of bone filling materials. Various types of bone grafts have been investigated: (1) alloplastic materials (i.e. synthetic materials); (2) autografts (i.e. grafted tissue from the same individual); (3) allografts (i.e. tissue from the same species but with different genetic composition); and (4) xenografts (i.e. materials from different species). Although utilization of such grafting materials for periodontal defects may result in some gain in clinical attachment levels and produce radiographic evidence of bone fill, histological analysis failed to show signs of periodontal regeneration (Garraway et al., 1998).

Subsequently, synthetic barrier membranes have been used to encourage appropriate progenitor cell populations from the periodontal ligament to re-populate the defect site using the clinical procedure of guided tissue regeneration (GTR). This approach was based on the observation that the periodontal ligament, but not gingival connective tissue or bone, contains cells capable of establishing new attachment between cementum and bone (Nyman et al., 1980, Karring et al., 1980, Nyman et al., 1982a). GTR procedures facilitate periodontal regeneration by using barrier membranes to selectively

promote the repopulation of a periodontal defect by periodontal ligament and bone cells at the expense of epithelial and gingival connective tissue cells (Nyman et al., 1982b).

Although tissue regeneration of periodontal defects using the GTR procedure is possible, it does not always have a predictable outcome. Clinical efficacy of GTR procedures depends on different patient factors such as smoking, plaque control, residual pocket depths, and local factors including the anatomy and morphology of the defect (Villar and Cochran, 2010). It has been shown that GTR results in significantly greater probing depth reductions and clinical attachment gains in narrow and deep periodontal intrabony defects and in class II mandibular furcation involvement (Pontoriero and Lindhe, 1995, Tonetti et al., 1996, Tonetti et al., 1993). To increase the predictability and clinical success of GTR, growth factors and bone filling substitutes have been combined with GTR. A recent systematic review came to the conclusion that most preclinical studies have histologically demonstrated periodontal regeneration when grafting materials are combined with barrier membranes (Sculean et al., 2008).

Cell-based regenerative periodontal therapy has recently gained increasing attention, especially since the identification of stem cells within the periodontal ligament with the ability to achieve new attachment formation *in vivo* (Seo et al., 2004). Cultured PDL cells applied in various scaffolds or in suspension have been shown to be able to induce the formation of a new periodontal tissue apparatus on root dentine surfaces and dental implants (Lang et al., 1998b, Choi, 2000, Lekic et al., 2001c, Dogan et al., 2002, Nakahara et al., 2004, Hasegawa et al., 2005, Seo et al., 2004). Regeneration of a new cementum / periodontal ligament-like structure was recently achieved using *ex vivo* expanded PDLSCs (Seo et al., 2005, Sonoyama et al., 2006, Liu et al., 2008), and MSCs (Kawaguchi et al., 2004, Yamada et al., 2006, Yang et al., 2010). Chen et al (2008) used

autologous MSCs, cultured *ex vivo* and engineered to express the BMP-2 gene by replication-defective adenovirus, to treat periodontal defects in New Zealand white rabbits and found that this approach regenerated all components of the periodontal attachment apparatus (Chen et al., 2008). Moreover, recent studies have focused on the effectiveness of periodontal ligament cell sheets to regenerate periodontal defects *in vivo* (Akizuki et al., 2005, Hasegawa et al., 2005, Flores et al., 2008) and currently this approach is being trialed in humans using autologous cells derived from nonfunctioning wisdom teeth (Ishikawa et al., 2009).

As discussed earlier, enamel matrix proteins, produced by Hertwig's epithelial sheath, are known to play an important role in cementogenesis, as well as in the development of the periodontal attachment apparatus. It is thought that fragmentation of the root sheath allows cells of the dental follicle to attach to this protein matrix and subsequently differentiate into cementoblasts (MacNeil and Thomas, 1993, Hammarstrom et al., 1996). *In vitro* studies have demonstrated that the addition of enamel matrix derivative (EMD) proteins to cultures of periodontal fibroblasts results in enhanced proliferation, protein and collagen production, as well as enhanced mineralization (Gestrelius et al., 1997, Van der Pauw et al., 2000). Enamel matrix derivative (EMD) has been approved by the U.S. Food and Drug Administration for use in achieving periodontal regeneration in angular bony defects (Heijl et al., 1997, Heden, 2000, Rasperini et al., 1999, Zetterstrom et al., 1997). Commercially available enamel matrix derivative (Emdogain®) is now in widespread clinical use. It consists of porcine enamel matrix proteins within a propylene glycol alginate gel vehicle. Whilst histologic evidence of periodontal regeneration has been shown in a human dehiscence model after application of enamel matrix derivative (Zetterstrom et al., 1997), further human case reports have reported inconsistent histologic evidence of regeneration (Yukna and Mellonig, 2000,

Sculean et al., 2004). A subsequent Cochrane systematic review which assessed the use of enamel matrix derivative for periodontal tissue regeneration in the treatment of intrabony defects concluded that statistically significant improvements in probing attachment levels and probing pocket reduction were achieved in comparison with open flap surgery (Esposito et al., 2004).

Although the aforementioned procedures have demonstrated a potential for regeneration of root surface cementum, alveolar bone and periodontal ligament, the clinical results vary greatly and are often unpredictable (Bartold et al., 2000b, Wang et al., 2005, Zohar and Tenenbaum, 2005) and a complete and predictable reconstruction of periodontal tissues is still difficult to obtain. As a result, the use of polypeptide growth factors to promote the wound healing process has been proposed as a way to enhance the regenerative process by promoting the proliferation and/or differentiation of cells capable of facilitating periodontal regeneration rather than repair.

1.5 Growth Factors

Growth factors are proteins that may act locally or systemically to affect the growth and function of cells in various ways. They may act in an autocrine or paracrine fashion to control cell growth and hence the number of cells available to produce a specific tissue (Shimono et al., 2003). Moreover, growth factors regulate key cellular events during tissue repair, including cell proliferation, chemotaxis, differentiation, and matrix synthesis by binding to specific cell-surface receptors. The sequence of events necessary for periodontal regeneration relies on the above processes for osteogenesis, cementogenesis, and connective tissue formation (Cochran and Wozney, 1999). Therefore, another approach to induce periodontal regeneration is using polypeptide

growth factors in order to influence cell migration, proliferation and differentiation, and hence facilitate the cascade of wound healing events that lead to the formation of new cementum and periodontal attachment.

Soluble mediators of importance in regeneration include growth factors such as platelet-derived growth factor (PDGF), Insulin-like growth factor (IGF), transforming growth factor- β (TGF-β), and fibroblast growth factor (FGF), cytokines including interleukin-1 (IL-1), tumor necrosis factor-α (TNF- α), and interleukin-4, lymphokines such as interferon-γ (INF-γ), as well as fibronectin and other adhesion molecules (Bartold et al., 2000b). Two of these growth factors, IGF-1 and PDGF, have been classified as anabolic cytokines able to promote the synthesis and inhibit the degradation of proteins (Nietfeld, 1993). They have been extensively studied and found to enhance periodontal regeneration in animal and human periodontal defects (Giannobile et al., 1996, Jin et al., 2004, Cho et al., 1995, Lynch et al., 1989, Nevins et al., 2003, Nevins et al., 2005, Nevins et al., 2007, Mellonig et al., 2009, Camelo et al., 2003, Howell et al., 1997). Another promising group of polypeptide growth factors are the bone morphogenetic proteins (BMP) which have also been shown to have good potential for stimulating bone and cementum regeneration (Chen et al., 2008, Chen et al., 2007). Platelet-rich plasma (PRP) has also been shown to improve periodontal healing and promote bone regeneration, and autologous preparations of PRP have been utilized in periodontal practice (Tozum and Demiralp, 2003).

Growth factors cannot diffuse across the cell membrane and thus must exert their activity by first binding to high-affinity cell membrane receptors. These receptors are transmembrane antigens which, on binding of the respective growth factors, produce a cascade of intracellular signals that stimulate different cell activities including,

chemotaxis, cell growth, differentiation and the production of extracellular matrix (Dereka et al., 2006, Shimono et al., 2003, Lee et al., 2010). The capacity of a cell to respond to a given factor is therefore dependent on the presence of these receptors and they are also likely to be of fundamental importance in human periodontal growth and regeneration. PDGF and IGF receptors have been identified in periodontal tissues and have been implicated in the growth and differentiation of cells from these tissues (Blom et al., 1992, Parker et al., 2001). Flow cytometry has been used to show that cells derived from healthy human gingiva and periodontal ligament expressed PDGF receptor-β (PDGF-Rβ), whereas IGF-R was detected in a proportion of the total cell population (Parker et al., 2001). Both PDGF and IGF receptors were detected in cells from regenerating periodontal defects. However, it has been demonstrated that PDGF-Rβ was up-regulated in regenerated tissue fibroblasts (RTF) compared to gingival fibroblasts (GF) and periodontal ligament fibroblasts (PLF) (Parker et al., 2001). Furthermore, periodontal fibroblasts and cementoblasts associated with deciduous human teeth were found to express IGF-1 receptor (Götz et al., 2001).

1.5.1 Insulin like growth factor

Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) are peptides with biochemical similarities to insulin. They are produced by the liver in response to growth hormone and considered potent mitogenic growth factors that mediate the growth-promoting activities of growth hormone postnatally (Daughaday and Rotwein, 1989). Both IGF-1 and IGF-2 are expressed in many tissues and cell types and may have autocrine, paracrine and endocrine functions. More importantly, IGFs are responsible for the growth and development of somatic tissues, such as skeletal muscle and bone (Ebberink

et al., 1989) and promote differentiation of myoblastic and osteoblastic tissues into muscle and bone (Schmid et al., 1983, Pfeilschifter et al., 1990, Tanaka et al., 2002).

IGF's have been shown to stimulate both proliferation of preosteoblasts and differentiation of osteoblasts (OB) *in vitro* and *in vivo* by acting through specific membrane receptors (Conover and Kiefer, 1993, Centrella et al., 1990, Jonsson et al., 1993, Rosen and Donahue, 1998). For example IGF-1 was shown to be essential for matrix mineralization in osteoblast-specific IGF-R knockout animals (Zhang et al., 2002). Similarly, both IGF-1 and IGF-2 knockout mice, while viable, are approximately 40% smaller than littermates (Liu et al., 1993). IGF-1 infusion was found to restore the level of osteoblast markers in old rats (Tanaka et al., 1994, Liang et al., 1992, Tanaka et al., 2002) and stimulated both cortical bone formation and trabecular bone apposition which was age dependent, being more effective in older rats (Spencer et al., 1991). Moreover, IGF-1 is considered to be a link between bone formation and bone resorption (Rosen, 2004) and IGF-1 administration was shown to increase bone mass in osteoporotic women (Ebeling et al., 1993), IGF-1 also increase collagen production by osteoblasts and inhibit collagen degradation by suppressing collagenase synthesis (Kream et al., 1990).

Studies investigating the effect of IGF-1 on undifferentiated progenitor cells showed that its effect on differentiation were restricted to more mature cells of the osteoblastic lineage (Walsh et al., 2003). Thomas et al., (1999) found that IGF-1 and -2 did not modulate the commitment of hMSCs to differentiate into OBs, and it did not affect the expression of the transcription factor Runx2, nor collagen I, OCN and ALP activity (Thomas et al., 1999). However, IGF-1 induced Type I collagen gene expression in mature osteoblasts and a bipotential hMSC line, when used together with

preconditioned osteogenic differentiation media containing Dexamethasone (Dex), 1,25-dihydroxyvitamin D3, L-ascorbic acid phosphate, and β-glycerophosphate (Thomas et al., 1999). Another study found that primary hMSC, when exposed to rhIGF-1, did not differentiate into osteoblasts as determined by ALP activity. However, the addition of Dex did not alter the outcome of IGF-1 treatment on primary hMSCs in this study (Walsh et al., 2003). Hence, it has been postulated that the effect of IGF-1 and 2 on OB differentiation is dependent on the stage of cell maturation (Thomas et al., 1999).

More recently, the effect of IGF-1 on the osteoblastic differentiation of mesenchymal stem cells showed different results (Koch et al., 2005, Celil et al., 2005, Ogata, 2008). After adenoviral delivery of hIGF-1 to hMSCs, it was found that IGF-1 participates in the commitment of progenitor cells to the osteoblastic lineage via up-regulation of the early marker genes Runx2, Type I collagen, and ALP. Moreover, in the presence of BMP-2, hMSC transduced with Ad-IGF-1 achieved a state of matrix mineralization without the requirement of Dex treatment (Koch et al., 2005). IGF-1 also up-regulated Osterix (Osx), a zinc finger-containing transcription factor that plays an important role in the osteoblast differentiation of hMSCs (Celil et al., 2005). Recently, IGF-1 was shown to increase bone sialoprotein (BSP) mRNA levels *in vitro* in human osteoblast-like cells as well as rat stromal bone marrow (RBMC-D8) cells (Ogata, 2008).

In fibroblast systems, IGF-1 is a proliferation progression factor and a strong mitogenic factor for periodontal ligament fibroblasts (Ivanovski, 2001, Matsuda et al., 1992, Palioto et al., 2004), gingival fibroblasts (Ivanovski, 2001), and fibroblasts obtained from regenerated periodontal defects in humans (Ivanovski, 2001). IGF-1 enhances the chemotaxis of PDL cells (Matsuda et al., 1992), PLF protein synthesis (Matsuda et al.,

1992), and proteoglycan synthesis in cultured articular cartilage (McQuillan et al., 1986), as well as periodontal gingival and regenerated periodontal defect cells (Ivanovski, 2001). Furthermore, IGF-1 has been shown to reduce apoptosis in cultured periodontal ligament fibroblasts compared to gingival fibroblasts and enhance the survival of PLF by up-regulation of anti-apoptotic molecules and down-regulation of pro-apoptotic molecules (Han and Amar, 2003). IGF-1 has also been shown to induce BMP-2 and BMP-4 expression (Li et al., 2001).

1.5.2 Platelet derived growth factor

Platelet-derived growth factor (PDGF) is a dimeric molecule, existing in several subtypes that consist of homodimers and heterodimers of PDGF-A and PDGF-B gene products. Lately, two other subtypes, PDGF-C and PDGF-D, has been identified (Bergsten et al., 2001, Uutela et al., 2001, Fredriksson et al., 2004). PDGF is produced by platelets, fibroblasts, macrophages, vascular endothelium, keratinocytes and osteoblasts (Antoniades et al., 1991, Uutela et al., 2004, Niessen et al., 2001) and is recognised as having an important role in wound healing (Barrientos et al., 2008). Upon injury, PDGF is released from degranulating platelets and stimulates chemotaxis and proliferation of neutrophils, macrophages, fibroblasts, and smooth muscle cells at the wound site (Heldin and Westermark, 1999). PDGF is particularly important in angiogenesis and blood vessel maturation. It also plays a role in re-epithelialisation by up-regulating the production of IGF-1 and thrombospondin-1 (Rabhi-Sabile et al., 1996). IGF-1 has been shown to increase keratinocyte motility, and thrombospondin-1 delays proteolytic degradation and promotes a proliferative response in *in vitro* wound models (Ando and Jensen, 1993, Krishnaswami et al., 2002). PDGF also enhances the proliferation of fibroblasts and the production of extracellular matrix (Lin et al., 2006),

and enhances fibroblast motility and consequently cutaneous wound healing (Rajkumar et al., 2006). In addition, it stimulates fibroblasts to contract collagen matrices and induces a myofibroblast phenotype (Rhee and Grinnell, 2006). During tissue remodelling, PDGF helps to break down collagen by up-regulating matrix metalloproteinases (Jinnin et al., 2005).

At a cellular level, PDGF is a potent mitogen for cells of mesenchymal origin including human periodontal ligament (Oates et al., 1993b, Boyan et al., 1994, Matsuda et al., 1992, Bartold and Raben, 1996a, Marcopoulou et al., 2003, Haase et al., 1998, Ivanovski, 2001, Dennison et al., 1994, Cesari et al., 2006, Mailhot et al., 1996, Wang et al., 1994), gingival fibroblasts (Haase et al., 1998, Ivanovski, 2001, Dennison et al., 1994, Marcopoulou et al., 2003, Bartold et al., 1992), regenerated tissue fibroblasts (Ivanovski, 2001), cementoblasts (Saygin et al., 2000) and osteoblasts (Strayhorn et al., 1999, Hsieh and Graves, 1998, Wildemann et al., 2007, Canalis et al., 1989). It also promotes PDL cell proliferation when applied to EDTA demineralized dentin surfaces (Zaman et al., 1999). Moreover, PDGF induces PLF chemotaxis (Matsuda et al., 1992), migration (Bartold and Raben, 1996a), collagen synthesis (Matsuda et al., 1992, Ojima et al., 2003), and extracellular matrix synthesis (Bartold and Raben, 1996a, Ivanovski, 2001). It also promotes protein synthesis by gingival fibroblasts (Bartold, 1993, Ivanovski, 2001) and regenerated tissue fibroblasts (Ivanovski, 2001).

PDGF has been found to be expressed at early and late phases during osteoblast differentiation (Huang et al., 2007, Smith et al., 2011). However, the role of PDGF in osteoblast differentiation is controversial. Earlier studies have shown that PDGF enhances DNA and collagen synthesis in rat osteoblast cultures (Centrella et al., 1989), increases bone matrix deposition in cultured calvaria (Pfeilschifter et al., 1990) and

increases bone formation and promotes complete bony reunion of calvarial defects (Park et al., 1998). In an *in vivo* study, PDGF was also found to enhance bone formation in a subcutaneously implanted scaffold (Howes et al., 1988). PDGF stimulated OPN mRNA expression in a dose-dependent manner when applied for 48hr to mesenchymal cells from adult and old rats (Tanaka and Liang, 1995). In an injured growth plate model, PDGF was found to be important in bone healing and repair (Chung et al., 2009), and has been shown to be up regulated during successful long bone fracture healing in humans (Weiss et al., 2009).

On the other hand, PDGF was also found to have a modest inhibitory effect on the differentiation of osteoblasts (Canalis et al., 1989, Tanaka and Liang, 1995, Hock and Canalis, 1994). Hesieh and Davis found that multiple brief (1 day) PDGF treatments of OB's increased mineralized nodule formation, but long term PDGF treatment decreased mineralized nodule formation by inhibiting OB differentiation as measured by ALP activity (Hsieh and Graves, 1998). In undifferentiated mesenchymal cell lines and in osteoblasts, treatment with PDGF for 15 days was found to have no effect on osteoblastic differentiation (Wildemann et al., 2007). However, the combined use of PDGF and dexamethasone induced mesenchymal stromal cells to reduce ALP, type I collagen and OCN secretion after 48hrs of treatment (Tanaka and Liang, 1995). PDGF-BB was also shown to promote *in vivo* mineralization when treated cementoblasts were transplanted into immunodeficient mice while *in vitro,* it inhibited biomineralization, decreased BSP and OCN expression and increased OPN expression after 24hrs (Saygin et al., 2000). Strayhorn et al (1999) also examined the effect of PDGF and IGF-1 on osteoprogenitor cells for up to 8 days. PDGF was found to reduce the expression of OPN and OCN, while IGF-1 promoted the expression of BSP and OPN. PDGF was able to block the effect of IGF on bone marker gene expression when the cells were treated

with a combination of the two growth factors. Further, PDGF inhibited the expression of Runx2 (Strayhorn et al., 1999).

More recently, the role of PDGF in osteogenic differentiation and bone formation was investigated using a PDGF receptor (PDGFR) inhibitor. Imatinib (Gleevec, Novartis, Basel, Switzerland) is an inhibitor of specific protein tyrosine kinases associated with the platelet-derived growth factor (PDGF) receptor (Savage and Antman, 2002). At therapeutic concentrations, it may also inhibit the macrophage-colony stimulating factor (M-CSF) receptor, c-fms (Taylor et al., 2006). Therefore Imatinib is used as a first line therapy for diseases characterized by increased PDGFR signaling, including patients with bcr-abl–positive chronic myeloid leukemia (CML) (O'Brien et al., 2003) and gastrointestinal stromal cells tumors (GISTs) (Verweij et al., 2003). It has been shown that PDGFR inhibition stimulates differentiation and inhibits proliferation and survival of osteoblasts (Fitter et al., 2008, Tibullo et al., 2009, Fierro et al., 2007, O'Sullivan et al., 2007) as well as inhibiting osteoclastogenesis (O'Sullivan et al., 2007, Dewar et al., 2006, Gallet et al., 2006, El Hajj Dib et al., 2006). On the other hand, PDGFR inhibition was found to decrease the expression of OCN in rat mesenchymal cells and impair bone healing (Chung et al., 2009).

Studies in humans treated with Imatinib suggest a biphasic effect of the drug on bone turnover. The early phase of treatment showed an increase in biochemical markers of bone formation such as OCN and procollagen type I N-terminal propeptide [PINP] , mean values of which doubled within 3 months, but subsequently tended to decline toward baseline values between 3 and 6 months (Grey et al., 2006). In a prospective study, bone mineral density (BMD), assessed by dual-energy X-ray absorptiometry (DXA) was either slightly increased or stable during 2 years of Imatinib therapy

(O'Sullivan et al., 2009). In a retrospective analysis of trephine iliac spine biopsies, trabecular bone volume was substantially increased in patients treated with Imatinib (Fitter et al., 2008). A cross-sectional analysis comparing patients with CML treated with Imatinib to healthy controls found higher BMD (by DXA) and higher cortical, but not trabecular, volumetric BMD by peripheral quantitative computed tomography (pQCT) in the Imatinib-treated group (Jonsson et al., 2008). However, the markers of bone turnover, OCN and bone specific ALP were significantly lower in treated patients compared with controls (Jonsson et al., 2008). More recently, Imatinib treatment of healthy rats for 5 weeks showed no increase in bone volume (O'Sullivan et al., 2011). The inconsistent findings and the complex effect of Imatinib on skeletal tissue might be the result of differences in study design and methods of skeletal assessment.

1.5.3 The Combination of PDGF and IGF-1

The combination of PDGF and IGF-1 was found to enhance the mitogenic and differentiation abilities of these two growth factors. Both PDGF and IGF-1 have been shown to regulate the proliferation and differentiation of osteoblasts during the bone formation process, and the combination of PDGF with IGF-1 has a synergistic effect on osteoblasts activity resulting in enhanced cell proliferation greater than the combined effect of the individual growth factors (Giannobile et al., 1997, Mott et al., 2002). The combination of IGF-1 and PDGF had an additive effect on DNA synthesis in rat calvaria cells (Canalis et al., 1989), stimulated bone matrix apposition *in vitro* (Pfeilschifter et al., 1990), and stimulated cell proliferation in an additive manner in bone marrow stromal cells (Tanaka and Liang, 1995). Recently, it was shown that the combination of PDGF and IGF-1 enhanced the proliferation of undifferentiated mesenchymal cell lines and osteoblasts but were not able to enhance cell differentiation

(Wildemann et al., 2007). Further, the combination of PDGF and IGF-1 has been shown to enhance the growth of PLF, GF and RTF (Giannobile et al., 1996, Giannobile et al., 1994, Lynch et al., 1991b, Lynch et al., 1989, Rutherford et al., 1992b, Ivanovski, 2001, Matsuda et al., 1992). However, Cesari *et al* showed that PDGF had a greater mitogenic effect on PLF than IGF-1 or the combination of PDGF and IGF-1 (Cesari et al., 2006). The combination led to an increase in the expression of OCN in the femurs of adult and old rats following marrow ablation which suggests that this combination stimulates bone maturation and mineralization (Tanaka et al., 2002). The synergistic action of PDGF and IGF-1 has been demonstrated in wound healing (Lynch et al., 1991b), periodontal bone formation (Lynch et al., 1989) and bone repair in a cortical defect model (Lynch et al., 1994). A previous report found that the combination of PDGF and IGF-1 enhanced the density of callus mineralization *in vivo*, which was not affected by either PDGF or IGF-1 alone (Lynch et al., 1994). Furthermore, the combination of both factors was found to be chemotactic for cells derived from the periodontal ligament (Matsuda et al., 1992, Bartold and Raben, 1996b, Haase et al., 1998).

1.5.4 PDGF and IGF-1 in Periodontal Regeneration

Experimental studies have shown that the local application of rhPDGF alone or together with IGF-1 may enhance regeneration of bone (Vikjaer et al., 1997, Lynch et al., 1994), periodontal attachment (Wang et al., 1994, Cho et al., 1995, Howell et al., 1997, Park et al., 1995, Nevins et al., 2003, Nevins et al., 2005, Nevins et al., 2007), and peri-implant bone defects (Becker et al., 1992, Lynch et al., 1991a). Rutherford et al (1992), found that the combination of PDGF and IGF promoted periodontal regeneration and attachment gain in periodontal defects in monkeys (Rutherford et al., 1992a). New attachment and bone fill was also found when these two growth factors were applied in

monkey periodontal osseous defects (Giannobile et al., 1996). In a randomized control trial, the local application of the same combination delivered in a methylcellulose gel to intraosseous defects resulted in statistically significant new bone formation and defect fill compared to controls after 6–9 months of healing (Howell et al., 1997). A regenerative study in beagle dogs examined the effects of PDGF / IGF-1 individually and in combination. Histological evidence of new attachment was reported after 3 months of the combined treatment, however, PDGF alone was found to be as effective as the combination of PDGF and IGF-1. No significant effect was found when IGF-1 was used alone (Lynch et al., 1989).

In two case reports/series of a limited number of patients (Nevins et al., 2003, Camelo et al., 2003) the local application of rhPDGF-BB on previously conditioned root surfaces and with demineralized freeze-dried bone allograft (DFDBA) in intra-bony or Class II furcation defects, resulted in substantial periodontal and bone regeneration which was confirmed clinically and histologically. The rhPDGF-BB/DFDBA treatment without GTR led to significantly more bone formation than DFDBAGTR therapy (Nevins et al., 2003). Moreover, treatment with rhPDGF-BB stimulated a significant increase in the rate of clinical attachment level gain, reduced gingival recession at 3 months post-surgery, and improved bone fill at 6 months (Nevins et al., 2003). With regards to the 9-month defect associated clinical parameters, the rhPDGF-BB/DFDBA treatment resulted in a robust improvement compared to pre-surgery values. Moreover, the histological analysis of biopsy specimen showed that rhPDGF-BB/DFDBA was capable of regenerating a complete functional periodontal apparatus, including new bone, cementum and PDL (Camelo et al., 2003).

A multicenter randomized controlled trial evaluated the clinical and radiographic outcomes of two different doses of rhPDGF-BB (0.3 and 1.0 mg/ml) combined with β-tricalcium phosphate (β-TCP) in the treatment of deep intraosseous defects (Nevins et al., 2005). After 6 months of healing, increased gain in clinical attachment level (CAL) was shown in the 0.3 mg/ml rhPDGF-BB/β-TCP group compared to the control group (significantly different at 3 months). Moreover, both test groups were significantly more effective than the control group in terms of radiographically determined linear bone growth and percentage of bone defect fill. Furthermore, a statistically significant difference was detected between the two test groups (0.3mg/ml and 1mg/ml rhPDGF-BB) for both linear bone growth and percentage of bone defect fill, favoring the 0.3mg/ml rhPDGF-BB dose (Nevins et al., 2005). Recently, the clinical and radiographic effect of the combination of rhPDGF-BB with a freeze-dried bone allograft (FDBA) in the treatment of periodontal intraosseous defects has been investigated in a human case series. Surgical re-entries were performed up to 11 months post-surgery, revealing complete bone fill and an improvement in clinical and radiographic parameters (Nevins et al., 2007).

Several studies have investigated the potential of PDGF gene therapy for periodontal engineering. Recombinant adenovirus (rAds) encoding PDGF-A was found to enhance the mitogenic and proliferative response in cells derived from the periodontium (osteoblasts, PLF's and GF's) (Zhu et al., 2001). Furthermore, it was demonstrated that gene transfer of PDGF-B stimulates alveolar bone and cementum regeneration in rat periodontal defects (Jin et al., 2004). A novel gene-activated matrix consisting of embedded chitosan/plasmid DNA nanoparticles encoding PDGF and a porous chitosan/collagen composite scaffold has recently been used to study periodontal tissue engineering. The plasmid DNA entrapped in the scaffolds showed a sustained and

steady PDGF release over 6 weeks. The results of co-culture with PDLCs revealed that PDLCs maintained their fibroblast shape and their proliferation increased (Peng et al., 2009).

On the basis of the compelling evidence for a role of PDGF in promoting wound healing and regeneration of bone in periodontal and periimplant defects, a new product named growth-factor enhanced matrix GEM 21S® was recently released and approved by the FDA for treatment of periodontal defects. It is a combination of recombinant human platelet derived growth factor (rhPDGF-BB) and β-Tricalcium phosphate (β-TCP) (Lynch et al., 2006, Hollinger et al., 2008).

In summary, the primary effect of PDGF is that of a mitogen, initiating cell division and modulating proliferation. The combination of PDGF and IGF-1 further increases mitogenesis (Ivanovski et al., 2001), migration (Giannobile, 1996), promotes collagen and total protein synthesis (Matsuda et al., 1992), and results in a significant increase in new periodontal attachment formation and osseous defect fill (Giannobile, 1996).

1.6 Periodontal Tissue Engineering Using Cell Sheets

Tissue engineering is an emerging field of science aimed at developing techniques for the fabrication of new tissues to replace damaged tissues and is based on the principles of cell biology, developmental biology and biomaterial science (Vacanti et al., 1991, Narem and Sambanis, 1995, Reddi, 1997, Reddi, 1998, Bartold et al., 2000b). It involves combining biomaterial scaffolds, containing appropriately selected and primed cells, together with an appropriate mix of regulatory factors that facilitates growth and maturation of the cells and matrix, which are subsequently implanted into a defect site.

Tissue engineering techniques may be utilized to facilitate periodontal regeneration (Bartold et al., 2000a). This strategy eliminates some of the limitations associated with conventional regenerative procedures because the direct delivery of growth factors and progenitor cells overcomes the normal lag phase of progenitor cell recruitment to the site.

Cell sheet engineering without scaffolds is a new approach to tissue engineering. This technique involves the use of a temperature-responsive cell culture surface developed in 1990 by Yamada and co-workers (Yamada et al., 1990) which responds reversibly to temperature changes. This temperature-responsive surface is coated with poly N-isopropylacrylamide which is hydrophilic below 32°C and hyodrophobic above this temperature. Thus intact cell sheets together with their extracellular matrix cultured on this material may be harvested by simply lowering the temperature (Hirose et al., 2000, Nishida et al., 2004a, Okano et al., 1993).

The use of such a non-enzymatic cell harvesting system has been shown to be non-invasive, gentle, and harmless to cells (Yamato et al., 1998, Kushida et al., 1999, Yamato et al., 1999). Periodontal ligament fibroblasts cultured on the temperature-responsive culture surface can be harvested from culture dishes as a contiguous cell sheet, with an abundant extracellular matrix and retained intact integrins (Hasegawa et al., 2005). The safety and efficacy of human PDL (hPDL) cell sheets for use in clinical trials was examined both in vitro and *in vivo*, with the results showing that harvested hPDL cell sheets are safe products. Moreover, their efficacy for periodontal regeneration and their safety are maintained after implantation in vivo (Washio et al., 2010).

The cell sheet method has been used to promote periodontal regeneration (Akizuki et al., 2005, Hasegawa et al., 2005, Gomez Flores et al., 2008). Gomez Flores et al (2008) showed the formation of periodontal ligament fibres with a perpendicular orientation inserted into a newly deposited cementum like tissue when PLF cell sheets were transplanted together with dentine slices in athymic mice. Similar findings were found when PLF cell sheets were transplanted into periodontal bony defects (Hasegawa et al., 2005) and buccal bone dehiscences (Akizuki et al., 2005), suggesting that this technique can be useful for periodontal regeneration.

1.7 The Extracellular Matrix and Periodontal Regeneration

The extracellular matrix within each periodontal component comprises both fibrous and non-fibrous elements including collagens, elastin, fibronectin, laminin, glycoproteins, a variety of growth factors and other noncollagenous proteins, proteoglycans, lipids, minerals and water. Aside from the role of the extracellular matrix as a structural scaffold, isolation and characterization of the matrix components associated with wound healing has revealed a multifunctional role that includes regulation of cellular function and activity. The extracellular matrix components that appear to be actively involved in the healing events include collagenous proteins, proteoglycans (decorin, biglycan) (Young et al., 1992b), and glycoproteins (OPN and BSP) (Chen et al., 1992a).

It has been postulated that elevated expression of bone and cementum markers may be used to identify cells with the potential to differentiate and facilitate hard tissue formation and hence periodontal regeneration (Bartold et al., 2000a). For the purposes of the series of studies described in this thesis, three generic mineralized tissue associated markers (BSP, OCN, Runx2) and three putative cementum specific markers

(CAP, CEMP1, F-Spondin) were chosen to assess the differentiation of periodontal cells.

1.7.1 Bone Sialoprotein

Bone sialoprotein (BSP) is a phosphorylated and sulfated 34-kDa protein that represents one of the major noncollagenous extracellular matrix (ECM) proteins associated with mineralized tissues. Constituting between 8 and 12% of the total noncollagenous proteins component in bone and cementum (Fisher et al., 1983, Fisher et al., 1987, Fisher et al., 1990b). This protein is selectively associated with clusters of "needle-like" crystals of hydroxyapatite. It contains polyacidic amino acid segments through which it can bind to hydroxyapatite, and an RGD sequence which can mediate cell attachment through the $\alpha_v\beta_3$ integrin (Oldberg et al., 1988). It has been found to be effective in promoting the nucleation of hydroxyapatite and facilitating mineral growth (Hunter and Goldberg, 1993). In accordance with its nucleating activity, BSP has a high binding affinity for calcium and hydroxyapatite. In the presence of 60 mM KCI, BSP binds 83 Ca^{2+} ions/molecule, with binding constants in the 0.5-1.0-mM range (Chen et al., 1992b). Moreover, BSP can also be an effective inhibitor of hydroxyapatite crystal growth. Based on comparisons of native BSP with fragments of recombinant BSP, this inhibitory activity, unlike the nucleating activity, is strongly dependent upon post-translational modifications (Stubbs et al., 1997). Moreover, IGF-1, which is known to promotes bone formation by stimulating the proliferation and differentiation of osteoblasts, has been shown to stimulate BSP transcription by targeting the fibroblast growth factor 2 response element and homeodomain protein-binding site in the proximal promoter of the BSP gene through the tyrosine kinase, Ras/MAPK and phosphatidylinositol 3-kinase/Akt pathways (Nakayama et al., 2006).

Expression of BSP is highly specific for mineralizing tissues, including bone, mineralizing cartilage, dentin, cementum and ameloblasts (Oldberg et al., 1988, Fisher et al., 1990b, Chen et al., 1991, Chen et al., 1993, MacNeil et al., 1995, MacNeil et al., 1996a, Chen et al., 1998, Bosshardt et al., 1998, McKee, 1996), with the exceptions being osteoclasts and trophoblasts (Fisher et al., 1990a). BSP is first expressed at the onset of bone, cementum and dentin formation (Chen et al., 1992a) and the levels of expression in mineralized cartilage, dentin, and enamel are much lower than in newly forming bone and cementum (Chen et al., 1992a, Shen et al., 1995). In developing rat calvaria, BSP mRNA expression is detected in osteoblasts along with type I collagen, OCN, osteonectin, and ALP, and is also found in hypertrophic chondrocytes (Cowles et al., 1998). Similar to OCN, BSP is up-regulated during osteoblast differentiation *in vitro* as the extracellular matrix mineralizes. However, BSP reaches peak levels slightly earlier than OCN and is down regulated in mature osteocytes (Malaval et al., 1999). Northern hybridization analysis of the temporal expression of BSP in rat calvariae and tibiae has revealed that the induction of the BSP gene coincides with the initial formation of a mineralized matrix in both membranous and endochondral bones, and that maximal levels are attained during embryonic bone formation (Chen et al., 1992a). *In situ* hybridization has further demonstrated that BSP mRNA is highly expressed by newly formed cuboidal osteoblastic cells actively forming bone matrix on the ectocranial surface of the 21- day embryonic rat cranium (Chen et al., 1992a). Therefore, BSP appears to be associated with the late stage of osteoblast differentiation and the early stages of matrix mineralization.

BSP has also been specifically localized to the mineralized tissues of normal and healing rat periodontium (Lekic et al., 1996a, Lekic et al., 1996b). Immunolocalization

of BSP has been described in dental root formation and early cementogenesis in the rat (Bronkers et al., 1994, Bosshardt et al., 1998). A comprehensive analysis of cementum formation has shown that the BSP gene is activated concurrently with cementoblast differentiation, and that high levels of expression are associated with the early and rapid stages of the formation of mineralized cementum matrix (MacNeil et al., 1994, MacNeil et al., 1996a, Yamamoto et al., 2007). *In vitro*, BSP mRNA expression was noted in the PLF and RTF, more so than in GF (Ivanovski et al., 2001a). Subsequently, as BSP has been shown to be expressed almost exclusively by mineralized tissue-forming cells (Chen et al., 1991). It is commonly used as a marker for osteogenic differentiation and mineralized tissue formation, including PLF differentiation and cementogenesis (Pi et al., 2007, Titorencu et al., 2007, Kitagawa et al., 2006a, Inanc et al., 2006, Inanc et al., 2007, Ogata, 2008).

1.7.2 Osteocalcin

Osteocalcin (OCN), a small protein with a molecular mass of approximately 6kDa and containing 49 amino acids, is a major noncollagenous protein (~15%) of bone (Hauschka and Wians, 1989). It is characterized by the presence of three residues of vitamin K dependently synthesized Gla-proteins (Hauschka and Wians, 1989). The Gla residues interact with the mineral phase of bone and lead to its incorporation into the bone matrix (Atkinson et al., 1995). OCN binds to hydroxyapatite and regulates crystal growth (Hauschka, 1985). Moreover, OCN has been found to be expressed by osteoblasts, odontoblasts, and hypertrophic chondrocytes at the onset of tissue mineralization, and accumulates in the bone extracellular matrix (Hauschka and Wians, 1989).

Osteocalcin is involved in the formation and remodelling of bone. It is produced by mature osteoblasts during the mineralization phase and is only marginally detectable during the early phases of proliferation and matrix maturation. During osteoblast differentiation, strong suppression of OCN transcription signals predominate in early osteoprogenitors, while enhancer factors are found in mature postproliferative osteoblasts (Owen et al., 1990, Towler et al., 1994, Banerjee et al., 1996, Newberry et al., 1998). Studies in warfarin-treated rats which exhibit greater than 90% depletion of OCN from bone (Price, 1988), and in OCN null mutant mice, have elucidated the function of OCN (Ducy et al., 1996). In both studies, postnatal bone formation and mineral deposition appear normal. However, although no defects in cell and tissue organization were observed in the knockout mice at birth, increased bone density is observed several months postnatally. Furthermore, analysis of the mineral phase of bone suggests a crystal maturational defect in the absence of OCN (Boskey et al., 1998). The increase in bone mass implicates OCN as a regulator of bone formation, although a role for OCN in bone resorption is strongly supported by a number of *in vitro* and *in vivo* studies showing a role for OCN in osteoclast recruitment (Lian et al., 1984, Glowacki et al., 1991).

Immunolocalization of OCN has been described during dental root formation and early cementogenesis in rats (Bronkers et al., 1994, Bosshardt et al., 1998). In a recent proteomic study of PLF differentiation, OCN was not found to be up-regulated during the early stages of differentiation. However, OCN was significantly up-regulated in the late stages of osteogenic differentiation of periodontal ligament cells, which was confirmed by quantitative polymerase chain reaction (Wu et al., 2009). However, some studies found that OCN gene expression can be induced in hMSCs progenitor cells only

after additional treatment with calcitriol (1α,25-dihydroxyvitamin D3) or 9-RA retinoic acid (Titorencu et al., 2007, Jaiswal et al., 1997, Jadlowiec et al., 2004).

1.7.3 Runx2

The process of cellular differentiation that gives rise to bone is regulated by lineage-specific gene expression and is ultimately under the control of transcription factors that act as key switching mechanisms to induce and regulate specific gene expression (Baek and Kim, 2011). Many transcription factors including Runx2 and Ostrix involved in bone development have been identified through studies in human patients and genetically modified mice (Katagiri and Takahashi, 2002). Some of these are expressed predominantly in the skeleton during development and have a function restricted to controlling skeletal cell proliferation and differentiation (Javed et al., 2010). Runx2 is the most widely studied transcription factor associated with osteogenesis.

The Runt domain (Runx) is a highly conserved 128 amino acid sequence that defines a metazoan family of sequence specific DNA binding proteins important for the developmental control of cell fate (Coffman, 2003). Three mammalian Runx genes encode transcription factors that share a conserved region termed the runt domain (RD), reviewed in (Wheeler et al., 2000)); Runx1 (alternatively designated as Aml1/Cbfa2/Pebp2aB), Runx2 (Aml3/Cbfa1/Pebp2aA), and Runx3 (Aml2/Cbfa3/Pebp2aC). On the whole, the Runx family of transcription factors are critical for cellular differentiation and organ development (Ito, 1999) and have been associated particularly with the development of hematopoietic and skeletal tissues, reviewed in (Ducy, 2000).

Ablation of the Runx2 gene in mice results in a complete absence of intramembranous and endochondral bone that is attributed to the maturational arrest of hypertrophic chondrocytes and osteoblasts (Komori et al., 1997). Animals in which this gene is deleted have a cartilaginous skeleton, do not develop bone and die at birth because their soft rib cage does not support respiration. The human genetic disease called cleidocranial dysplasia, a dominantly inherited developmental disorder of bone, is the result of dysfunction of one allele of the Runx2 gene (Otto et al., 1997, Tsai et al., 2000). It is characterized by a delay in osteoblast differentiation in bones forming through intramembranous ossification and also results in dental defects that include supernumerary teeth and delayed eruption of the permanent dentition (D'Alessandro et al., 2010).

Runx2 acts as an inducer of osteoblast differentiation and it has all the characteristics of a differentiation regulator in the osteoblast lineage (Ducy et al., 1997, Komori et al., 1997). Key studies have established that Runx2 is required for osteoblast differentiation and *in vivo* bone formation (Ducy et al., 1997, Otto et al., 1997, Komori et al., 1997, Ivanovski et al., 2011, Smith et al., 2011). As bone formation is not solely controlled at the level of osteoblast differentiation, Runx2 is also required for osteoblast function (Ducy et al., 1999). Runx2 has been found to be able to induce both early and late markers for osteoblast differentiation, including ALP, type I collagen, OPN, BSP, and OCN in several cell lines (Harada et al., 1999). *In vivo*, ectopic expression of Runx2 in transgenic animals leads to ectopic endochondral ossification. *In vitro,* over-expression of Runx2 in non-osteoblast cells induces osteoblast-specific expression of all bone marker genes, including OCN and BSP (Yang and Karsenty, 2002). Moreover, Runx2 over-expression in BMSCs accelerated healing of critical-sized defects (Wojtowicz et al., 2010). Runx2 expression decreases with age, which might explain the impaired

osteoblast function and reduced bone formation shown with increasing age (Christiansen et al., 2000). However, genes involved in the bone resorption process, such as RANKL, OPG, and matrix metalloproteinase-13 (MMP-13) are also regulated by Runx2 (Geoffroy et al., 2002b). During bone development, Runx2 induces osteoblast differentiation and increases the number of immature osteoblasts, which form an immature bone matrix. However, it has been shown that Runx2 expression has to be subsequently down-regulated for differentiation of mature osteoblasts, which form mature bone (Komori, 2010). Moreover, Liu *et al* found that over expression of Runx2 increases osteoblast numbers but inhibits their terminal maturation, resulting in accumulation of less mature osteoblasts and consequent osteopenia (Liu et al., 2001). Osteoclastogenesis is increased, possibly by the increased production of RANKL and MMP-13 (two factors involved in the bone formation–resorption coupling) by immature osteoblasts (Geoffroy et al., 2002a). Thus Runx2 has been found to control the differentiation of osteoblasts and the expression of extracellular matrix protein genes, as well as maintaining osteoblasts in an immature stage.

Runx2 is essential for tooth development up to the bell stage, being necessary for the formation of the enamel knot which controls growth and folding of the enamel organ epithelium, and its expression is largely restricted to osteoblasts, cartilage, teeth and areas of mesenchymal condensation during bone formation (D'Souza et al., 1999). Whether Runx2 is essential for the later stages of tooth development is still unknown, as Runx2 knockout mice do not survive beyond birth (Camilleri and McDonald, 2006). Enamel matrix derivatives have also been found to increase the expression of Runx2 (Narukawa et al., 2007). Runx2 is also expressed in periodontal ligament (PDL) fibroblasts (Jiang et al., 1999) and in human cementoblasts cell lines (Kitagawa et al., 2006a).

1.8 Cementum

Cementum is a highly specialized mineralized tissue produced by cementoblasts which covers the root surfaces and is crucial for the anchoring of teeth to the surrounding alveolar bone via the insertion of PDL fibers. Therefore, cementum is critical for achieving periodontal regeneration, because it invests and securely attaches periodontal ligament fibres to the root surface. The origin of cementoblasts during cementum formation is not fully elucidated. It has been suggested that during root formation, Hertwig's epithelial root sheath (HERS) disintegrates and undifferentiated mesenchymal cells from the dental follicle migrate and attach to the root surface. These undifferentiated cells subsequently differentiate into cementoblasts on the root surface and deposit the initial cementum matrix (Cho and Grant, 1988).

Collagen fibers are the major organic cementum component. Immunohistochemical and biochemical methods have been used to identify the noncollagenous matrix molecules within cementum, including noncollagenous glycoproteins, proteoglycans, plasma derived macromolecules, and several growth factors (Bosshardt, 2005). Cementoblasts are thought to share many molecular properties with osteoblasts including the expression of type I collagen and noncollagenous proteins such as OCN, OPN and BSP (D'Errico et al., 1997, Bosshardt, 2005). Nevertheless, the characterization of cementoblast-specific properties is hampered by the difficulty in isolating 'pure' colonies of cementoblasts. This is a result of anatomical constraints, and due to the fact that the cementum type that is crucial for periodontal attachment formation and regeneration (acellular extrinsic fibre cementum) is, by definition, acellular. This means that the precursor cells reside within the periodontal ligament, which is a heterogeneous

mix of mesenchymal cells with a variety of functions. Nevertheless, several groups have tried to isolate 'putative' cementoblast cell lines.

Kitagawa *et al,* (2006) established human cementoblast-like cell lines (HCEM) and human PDL cell lines via hTERT transfection, which were used to examine the molecular events associated with cementoblast differentiation (Kitagawa et al., 2006a). HCEM cell lines showed high alkaline phosphatase (ALP) activity, calcified nodule formation, and the expression of type I collagen, ALP, Runx2, OCN, BSP, and CP-23. On the other hand, hPDL showed low ALP and mineralization activity, and did not express OCN and BSP (Kitagawa et al., 2006a). PDL subpopulations obtained from the cementum half of the PDL by sequential enzymatic digestion showed that the PDL subpopulation on the root surface had high levels of proliferation and mineralization (Kaneda et al., 2006).

The formation of new cementum with newly inserted Sharpey's fibers on the root surface is a prerequisite and a crucial step in the regeneration of periodontal tissues (Saygin et al., 2000). It is clear that cementum formation is critical for appropriate maturation of the periodontium, both during development and regeneration (Pitaru et al., 1995). However, one major impediment to investigating cementum formation and understanding the molecular mechanisms that regulate periodontal regeneration is a lack of specific cementum markers (Alvarez-Perez et al., 2006, Bosshardt, 2005). Several cementum related proteins have been suggested as cementum markers, such as collagen type I and III, fibronectin, OPN, BSP, OCN, vitronectin and growth factors such as TGFß and BMP-2. However, these molecules are not cementum-specific (Bosshardt, 2005) and are shared by other mineralized tissue forming cells such as osteoblasts.

Recent studies have identified 'putative' new makers that have been suggested to be uniquely localized to cementum, such as cementum-derived attachment protein (CAP) (Arzate et al., 1992) and cementum- derived protein (CP-23) (Alvarez-Perez et al., 2006). F-Spondin has also been recently identified as a gene demonstrating high expression in human cementoblast-like cells (Kitagawa et al., 2006).

1.8.1 Cementum Attachment Protein

Cementum Attachment Protein (CAP) is the most widely studied putative cementum-specific protein. It is a 56kDA molecule and has been characterized as a collagen-like protein. A putative sequence for CAP was established from a cDNA clone isolated from a human cementifying fibroma cDNA library. CAP has high affinity to hydroxyapatite and fibronectin and low affinity for collagen (Pitaru et al., 1992). Furthermore, it does not cross react with fibronectin, OPN or sialoprotein II (Arzate et al., 1992). It has been found to enhance the attachment and migration of osteoblasts and PLF, and to a lesser extent gingival fibroblasts, but not gingival epithelium (Pitaru et al., 1993, Pitaru et al., 1995, McAllister et al., 1990). CAP expression was found to be restricted to cementum and periodontal derived cell lineages with the potential of forming mineralized tissues (Arzate et al., 1992, Bar-Kana et al., 1998, Liu et al., 1997) as well as human root derived cells (Nunez et al., 2010). During cementogenesis, CAP was found to be limited to the cementum matrix and cementoblasts. Isolated cells from the dental follicle have been shown to adhere to surfaces containing CAP (Saito et al., 2001). These findings suggest that CAP may have a function in cementogenesis (Saito et al., 2001). Furthermore, when bone morphogenetic protein-2 was added to periodontal fibroblasts clones and mineralized tissue forming cell lineages (osteoblasts and cementoblasts), CAP expression was induced (Pitaru et al., 2002).

1.8.2 Cementum Protein-1

Cementum Protein-1 (CEMP1), also known as Cementum Protein-23 (CP-23), was derived from human cementoblastoma and has been found to be highly expressed in cementoblasts, as well as in progenitor cells located in the paravascular zone of the periodontal ligament and endosteal spaces of bone (Arzate et al., 2002), and human root derived cells (Nunez et al., 2010). Human PDL cell lines were shown to express CEMP1 mRNA using RT-PCR (Kitagawa et al., 2006a). CEMP-1 has also been found to be highly expressed in cementoblast-like cells and to a lesser extent in periodontal ligament cells cultured *in vitro* (Alvarez-Perez et al., 2006), but not in osteoblasts (Kitagawa et al., 2006). Moreover, in a severe periodontitis dog model, periodontal regeneration was achieved using PDSCs. The rhCEMP1 was detected only in the newly formed cementum and PDL but not in dentin or alveolar bone (Park et al., 2011). Therefore CEMP1 has been proposed as a candidate for a periodontal ligament/cementum marker (Alvarez-Perez et al., 2006).

Transfection of CEMP1 into human gingival fibroblasts (GF) was shown to induce mineralization (Carmona-Rodríguez et al., 2007). The transfected GF cells had higher alkaline phosphatase activity and proliferation rates and they expressed genes for ALP, BSP, OCN, OPN, the transcription factor Runx2, and CAP (Carmona-Rodríguez et al., 2007). These findings suggest that CEMP1 may have a potential role in cementum and bone formation.

1.8.3 F-Spondin

F-Spondin (SPON1) is an extracellular matrix, heparin-binding glycoprotein of the thrombospondin family that regulates neuronal outgrowth in the embryonic floor plate (Klar et al., 1992). It is important in development, modulating the migration of commissural axons (Burstyn-Cohen et al., 1999), promotes neurite outgrowth of dorsal root ganglion cells (Wang et al., 1999, Somerman et al., 1988) and spinal cord neurons in cell culture (Burstyn-Cohen et al., 1999) and promotes the differentiation of neural precursor cells (Cheng et al., 1994). F-Spondin is also a marker of hypertrophic and mineralized cartilage. Functional studies indicate that F-Spondin regulates endochondral bone growth in organ cultures, and promotes chondrocyte terminal differentiation and mineralization in cell cultures (Palmer et al., 2010). Moreover, F-Spondin was found to be up-regulated in osteoarthritic (OA) cartilage (Attur et al., 2009). In OA cartilage explant cultures, F-Spondin treatment was found to increase both type II collagen degradation and MMP-13 secretion, suggesting its ability to regulate articular chondrocyte metabolism.

Recently, F-Spondin has been found to be expressed specifically in dental follicle cells during tooth germ development (Nishida et al., 2007). Using temporal and spatial expression analysis, F-Spondin has been suggested to play a role in the early stages of PDL formation (Nishida et al., 2007). Kitagawa *et al* (2006) identified F-Spondin as a promoter for cementoblastic differentiation. Compared to PDL cell lines, F-Spondin was found to be highly expressed in human cementoblast-like cell lines (HCEM) using microarray analysis and the results were confirmed with RT-PCR. F-Spondin protein expression was observed to specifically localise to root lining cells by immunohistochemistry. Furthermore, no F-Spondin expression was found in osteoblast cell lines, suggesting that F-Spondin expression is specific to cementoblasts (Kitagawa et al., 2006). Moreover, Human periodontal ligament cells transfected with F-Spondin

changed their morphology and exhibited increased expression of ALP, OCN, BSP, CP-23 mRNA and ALP (Kitagawa et al., 2006). In summary, F-Spondin was found to be unique to cementoblasts, and shown to promote the differentiation of PDL cells towards mineralized tissue forming cells. Recently, Oka *et al* (2011), found that F-Spondin is essential to protect the root surface from resorption, by down-regulating the recruitment and differentiation of osteoclastic precursors (Oka et al., 2011).

Hypothesis

Previous studies have demonstrated that periodontal tissue contains mesenchymal progenitor cells able to express bone and cementum associated markers, and this expression can be influenced by osteogenic/mineralization media. Additionally, growth factors such as IGF-1 and PDGF can influence cellular differentiation as assessed by the expression of bone and cementum markers. Furthermore, it is proposed that periodontal cells are a heterogeneous population with respect to the expression of mineralized tissue-associated proteins, and this may reflect the cells' potential for cementogenesis *in vivo*. However, the potential of primary periodontal cells to differentiate into bone/cementum forming cells is not clear. Our hypothesis is that periodontal cells are a heterogeneous cell population containing undifferentiated cell sub-populations able to differentiate and form mineralized tissue in response to a variety of external stimuli including growth factors treatment/s.

Aims

- To describe the temporal expression of bone and putative cementum marker genes during periodontal cells differentiation over a six week period.
- To identify the effect of IGF-1 and PDGF on differentiation of periodontal cells.
- To identify periodontal cell clones best able to differentiate into a bone or cementum forming phenotype.
- To assess the ability of periodontal cell derived clones to form cementum *in vivo.*

2.0 Chapter Two

The Differentiation of Primary Cultures of Human Periodontal Cells.

2.1 Abstract

Background: The formation of bone and cementum is important for periodontal regeneration and the differentiation of progenitor cells along a mineralization forming phenotype is a critical step for osteogenesis and cementogenesis.

Objective: To examine the osteogenic/cementogenic differentiation of primary cultures of cells derived from the periodontal ligament (PLC), regenerating periodontal tissue (RTC) and alveolar bone (OB).

Method: Three human primary cell types (PLC, RTC, OB) were cultured in duplicate and subsequently exposed to osteogenic media containing 50ug/ml ascorbic acid, 10^{-7} M Dexamethasone, and 10 mM β-Glycerophosphate for 72hrs, one, three and six weeks. To examine the mineralization of the cell cultures, alizarin red S staining, von Kossa staining, calcium release and hydroxyapatite formation were assessed. Real time PCR (RT-PCR) was utilized to quantify the expression of the mineralized tissue associated markers, bone sialoprotein (BSP), Osteocalcin (OCN), and Runx2; and the putative cementum markers: cementum attachment protein (CAP), cementum protein-1 (CEMP1), and F-Spondin (SPON1).

Results: Histochemical analysis showed that the three primary cell types tested (PLC, RTC and OB) were all able to differentiate into mineralized tissue forming cells after treatment with mineralization media, however, both PLC and OB showed more mineralization than RTC. RT-PCR showed that mineralization media up-regulated the cementum markers CAP and CEMP in the PLC cultures but not in the OB and RTC

cultures. Moreover, BSP and SPON1 expression was more consistently associated with mineralization.

Conclusion: Periodontal ligament, regenerating periodontal tissue and alveolar bone cells, all contained cells able to differentiate into a bone/cementum forming phenotype. PLC however, appeared to be the most responsive in terms of the up-regulation of cementum marker expression and hence appears to be the most suitable candidate for use in periodontal regeneration.

2.2 Introduction

The formation of new cementum with inserted Sharpey's fibres on a root surface previously affected by periodontal disease is a key requirement for periodontal regeneration. For this process to occur, cementoblast precursors must proliferate, migrate, attach to the denuded root surface and differentiate into functional cementoblasts (Bosshardt, 2005).

Recently, undifferentiated mesenchymal stem cells have been shown to reside in the periodontal ligament (Seo et al., 2004, Chen et al., 2006, Somerman et al., 1988, Cheng et al., 1994). These cells expressed the mesenchymal stem-cell markers STRO-1 and CD146/MUC18 and under defined culture conditions, they differentiated into cementoblast-like cells, adipocytes, and collagen-forming cells. When transplanted into immunocompromised rodents, they showed a capacity to form a cementum/periodontal ligament-like structure and contribute to periodontal tissue repair by forming collagen fibres, similar to Sharpey's fibres (Seo et al., 2004). Other possible sources of osteoblast and cementoblast precursors are the endosteal spaces of alveolar bone, and these cells have been observed to migrate to paravascular locations in the periodontal ligament of mice (McCulloch, 1987).

The periodontal ligament has been shown to be a critical source of cells required for periodontal regeneration (Nyman et al., 1982a). This is in contrast to gingiva and bone, which do not appear to have the potential to facilitate periodontal regeneration, reviewed in (Bosshardt and Sculean, 2009a). Based on these observations, the surgical technique of 'Guided tissue regeneration' (GTR) was developed to allow a selective

repopulation of periodontal defects with cells from the periodontal ligament, which have the potential to facilitate periodontal regeneration. Fibroblasts derived from regenerated periodontal defects (regenerated tissue 'fibroblast-like' cells (RTC) derived from defects treated with GTR) have been cultured and studied to understand the cellular and molecular mechanisms involved in periodontal regeneration (Rowe et al., 1996, Wakabayashi et al., 1996, Wakabayashi et al., 1997, Kuru et al., 1999, Parker et al., 2001, Ivanovski, 2001a, Ivanovski, 2001, Ivanovski et al., 2007, Lin et al., 2008b).

Although cementum differs from bone in its histological profile which is characterized by a lack of innervation and vascularization, as well as limited remodelling potential, it also shares many properties with bone, most notably a remarkable similarity in biochemical composition (Saygin et al., 2000, Bosshardt, 2005). Cementoblasts express a similar profile of mineralized tissue markers as do osteoblasts, including proteins like alkaline phosphatase (ALP), runt-related gene 2, type I collagen, and noncollagenous proteins, such as bone sialoprotein (BSP) and osteocalcin (OCN) (D'Errico et al., 1997, D'Errico et al., 2000, Kitagawa et al., 2006a).

OCN and BSP are well established extracellular macromolecules that are associated with mineralization (Young et al., 1992a), while the transcription factor *Runx2* has been implicated in the commitment of pluripotent mesenchymal cells into osteoblastic lineage and in the regulation of mineralization (Ducy et al., 1997, Komori, 2010). Although BSP, OCN and Runx2 has been identified in cementum (Ivanovski, 2001b, Ivanovski et al., 2000, Kitagawa et al., 2006a), they are not necessarily cementum-specific, and have also been identified in bone. Recently, considerable efforts have been put into identifying cementum-specific markers. CEMP1 (also known as CP-23), CAP

and F-Spondin (SPON1) have subsequently been suggested to be unique to cementum (Arzate et al., 1992, Alvarez-Perez et al., 2006, Kitagawa et al., 2006).

CAP has been proposed as a marker for putative cementoblast progenitors in the adult human periodontal ligament (Bar-Kana et al., 1998, Liu et al., 1997). Its expression was found to be restricted to cementum and periodontal derived cell lineages with the potential of forming mineralized tissues (Arzate et al., 1992, Bar-Kana et al., 1998).

Cementum Protein 1 (CEMP1, CP23) is derived from human cementoblastoma and has been found to be highly expressed in cementoblasts, as well as in progenitor cells located in the paravascular zone of the periodontal ligament, the endosteal spaces of bone (Arzate et al., 2002), in established cementoblast-like cell lines from human cementum-lining cells (Kitagawa et al., 2006a) and in periodontal ligament cells cultured in vitro (Alvarez-Perez et al., 2006). Therefore, it has been proposed as a cementum marker candidate (Alvarez-Perez et al., 2006). Indeed transfection of CEMP-1 into human gingival fibroblasts increased the alkaline phosphatase activity and the expression of BSP, OPN, Runx2 and CAP, supporting a possible role for CEMP1 in cementoblast differentiation (Carmona-Rodríguez et al., 2007). Recently, hrCEMP1 was shown to play a role during the mineralization process by promoting octacalcium phosphate crystal growth (Villarreal-Ramirez et al., 2009).

Kitagawa et al (2006) identified F-Spondin (SPON1) as a promoter for cementoblastic differentiation. F-Spondin was shown to be highly expressed in human cementoblast-like cells (Kitagawa et al., 2006). Furthermore, human periodontal ligament cells

transfected with the F-Spondin gene showed a similar phenotype to cementoblasts, such as up-regulation of mineralized tissue related genes, ALP, OCN and BSP (Kitagawa et al., 2006).

The purpose of this study therefore was to assess the gene expression of bone and cementum markers from cells derived from the human periodontal ligament (PLC), regenerated periodontal defect (RTC) and alveolar bone (OB) and to examine cellular differentiation along osteogenic/cementogenic lineages following exposure to osteogenic culture conditions.

2.3 Materials and Methods

2.3.1 Cell Culture

Periodontal ligament cells (PLC) and osteoblasts (OB) were isolated and cultured as previously described (Ivanovski, 2001, Haase et al., 2003). Briefly, teeth were collected from patients requiring extraction. The selected teeth had no periodontal disease or carious cavity. Ethical approval for the use of this redundant tissue was attained through the Griffith University Human Research Ethics Committee (DOH/17/7/HREC).

For establishment of the PLC cultures, the extracted teeth were collected into explant medium containing Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco) supplemented with 10% Fetal Calf Serum (FCS), 2.5 μg/ml fungizone, 100 units/ml penicillin, 100 μg/ml streptomycin and 1% non-essential amino acids. As soon as the samples reached the research laboratory, PLC explants were curetted from the middle

third of the periodontal ligament still adherent to the root. The tissues were then cut into small pieces (less than 3mm) and placed in tissue-culture dishes to allow the establishment of explant cultures. Subsequent subcultures were maintained in DMEM containing 10% FCS, 50 units/ml penicillin, 50 ug/ml streptomycin and 1% non-essential amino acids at 37°C in a 5% CO_2 incubator.

For generation of OB cells, alveolar bone collected during third molar extraction was treated with 0.05% trypsin (37°C, 30 min) followed by 0.2% collagenase (37°C, 30 min), then washed three times with phosphate-buffered saline before the bone was chipped into small segments, covered with explant media and placed into a 5% CO_2 incubator at 37°C (Haase et al., 2003). The explant cultures were subsequently subcultured as described above for the periodontal ligament cells.

The regenerating tissue derived cells were obtained from frozen low passage number stock (3^{rd} passage), prepared as previously described (Ivanovski et al., 2001). Briefly, following attainment of institutional ethics approval and informed consent from three subjects, full-thickness buccal flaps were raised around periodontal defects associated with non-carious molar teeth destined for extraction in preparation for immediate denture construction. Granulation tissue was removed from the defects and the tooth surface was thoroughly debrided before placement of an ePTFE membrane (Gore Tex Periodontal Material-single tooth wide configuration, W. L. Gore & Associates, Flagstaff, Arizona). Wound closure included flap repositioning coronal to the cementoenamel junction and suturing (Figure 2.1). After a six week healing period, the membrane was removed, the regenerated tissue excised, and the tooth extracted. The

cells obtained from the regenerated area were termed regenerated tissue associated cells (RTC).

Ultimately, five cell lines of each type were established. For the following experiments two cell lines from each type was selected on the basis of strong cell growth following retrieval from liquid nitrogen. Duplicate experiments (biological replicates) were subsequently performed and measurements for each assay were made in triplicate (technical replicates).

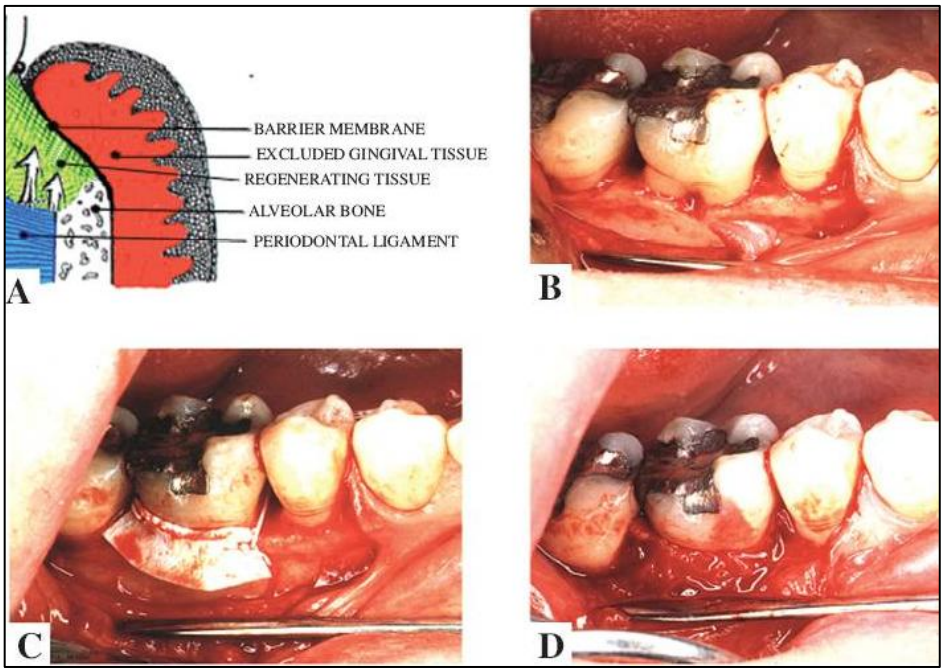

Figure 2.1 Guided tissue regeneration surgical procedure.

A. Diagrammatic representation of GTR. A. barrier membrane is used to prevent the downgrowth of gingival and epithelial cells and allows the repopulation of the periodontal defect site with periodontal ligament and alveolar bone cells. B-D. Surgical procedure. B. Surgical flap elevation and tooth root debridement. C. Barrier membrane secured in place. D. Six weeks re-entry into the surgical site.

Reprinted with permission from Mary Anne Liebert, Inc, Tissue Engineering (Ivanovski et al., 2007).

2.3.2 Mineralization Media

Cells were cultured in DMEM supplemented with 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin and non-essential amino acids. When the cells became confluent, they were detached using 0.2% trypsin and plated in six well plates at a density of $1x10^5$ cells/well. After the cells reached 50% confluence (after 48 hrs), the cells were left in the existing culture medium or were treated with mineralization medium (MM). In the control group, the cells remained in the culture medium of DMEM containing 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin and non-essential amino acids (control media, CM). The experimental group (MM) was cultured in control media supplemented with 50ug/ml ascorbic acid, 10^{-7} M Dexamethasone, and 10mM β-Glycerophosphate. This supplemented medium has been shown to induce osteogenic differentiation (Arceo et al., 1991, Cho et al., 1992, Hayami et al., 2007, Khanna-Jain et al., 2010). The media were changed every 2-3 days.

Mineralization media, also described as osteogenic or osteodifferentiation media, is a mixture of ascorbic acid, β-glycerophosphate, and Dexamethasone and is commonly used to examine the differentiation and mineralization ability of different cell types. Ascorbic acid is used to permit collagen type I fibril assembly while the β-glycerophosphate provides the mineral content for mineralization of collagen fibrils (Quarles et al., 1992, Bellows et al., 1992). Dexamethasone is a glucocorticoid receptor agonist that is used to stimulate osteoblast differentiation (Chang et al., 2006).

The three cell types (PLC, RTC and OB) were cultured in ether standard or mineralization media for 72hrs, 7 days, 3 weeks or 6 weeks for each of the assays described below.

2.3.3 Alizarin Red S Staining

Alizarin Red S staining was used to assess the calcium content of the cells in culture, Alizarin red S, an anthraquinone derivative, has been shown to react with the calcium portion to form an Alizarin red S-calcium complex in a chelation process. Twenty thousand cells per well of PLC, RTC and OB were cultured with and without mineralization media (MM) treatment in 24 well plates. The staining was carried out using a modification of the method described by Reinholz et al. (2000). After 72hrs, one week, three weeks and six weeks, the cell monolayer was first washed in PBS then fixed using 10% formalin for 10 minutes. The cells then washed with PBS and 1ml of 2% Alizarin red S was added for 20 minutes. Subsequently the wells were washed four times with PBS. Staining was assessed visually and via optometric analysis (Reinholz et al., 2000).

Quantification of staining was carried out using the methodology described by Gregory et al, (2004). Briefly, 400 µL 10% (v/v) acetic acid was added to each well, and the plates were incubated on a shaker for 30min. The monolayer was then scraped with a cell scraper (Cell Scraper, 23cm, Nunc) and transferred to a 1.5-mL microcentrifuge tube with a wide-mouth pipette. After vortexing for 30 s, the slurry was overlaid with mineral oil (Sigma–Aldrich), heated to 85 °C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000*g* for 15 min and 250 uL of the

supernatant was removed to a new 1.5-mL microcentrifuge tube. Subsequently, 100 uL of 10% (v/v) ammonium hydroxide was added to neutralize the acid. The pH was measured at this point to ensure that it was between 4.1 and 4.5. Aliquots of 100uL were read in triplicate at 405 nm in 96-well plates (Gregory et al., 2004).

2.3.4 von Kossa Staining

Von Kossa staining was carried out to assess the phosphate content of the fixed cell cultures. The silver nitrate in the von Kossa staining solution stains calcium phosphate and calcium carbonate, by binding the positive silver ions with the negative phosphate or carbonate portion. Twelve thousand cells per well of PLC, RTC and OB were cultured and treated with and without mineralization media (MM) in 48 well plates. After 72hrs, one week, three weeks and six weeks the cell monolayer was fixed using 10% formalin for 10 minutes, the cells were then washed with PBS and 500ul of 2% silver nitrate solution was added and subjected to ultra-violet light for 1hr. Subsequently, the wells were washed by three changes of distilled water, and then 500uL 5% sodium thiosulfate was added for 2min and washed again in three changes of distilled water. Staining was assessed visually and photographed.

2.3.5 Calcium Release

A QuantiChrom^{TM} Calcium Assay Kit (DICA-500 by BioAssay Systems) was used to measure the concentration of calcium ions released into the media by the various primary cell cultures (PLC, RTC and OB). A phenolsulphonephthalein dye in the kit forms a blue coloured complex specifically with free calcium. The intensity of the

colour, measured spectophotometrically at 612 nm, is directly proportional to the calcium concentration in the sample.

Culture media was collected from the plates of cultured PLC, RTC and OB cells after 72hrs, one week, three weeks and 6 weeks of tissue culture with and without mineralization treatment (MM). The assay was carried out according to the manufactures instructions (DICA-500 by BioAssay Systems). Briefly, 5 µL of media samples, along with diluted standards ranging from 0-20mg/dL, were transferred into the wells of a clear bottom 96-well plate. Subsequently, 200 µL of the supplied working reagent was added to the plate and incubated for 3 min at room temperature. Optical density at 570- 650nm was read (peak absorbance at 612nm) in 96-well plates using the POLARstar Omega Plate reader (BMG LABTECH, Germany).

The blank optical density (OD) (0mg Ca^{2+}) reading was subtracted from the standard OD values and the OD plotted against Ca^{2+} standard concentrations. The slope was determined using linear regression and the calcium concentration of the sample was calculated as $OD_{(SAMPLE)} - OD_{(BLANK)}$ / Slope (mg/dL).

2.3.6 Hydroxyapatite Formation

To quantify the *in vitro* mineralization of the primary cell cultures of PLC, RTC and OB, the OsteoImage™ Mineralization Assay (Lonza) was used. This assay is a fluorescent *in vitro* assay for assessing bone cell mineralization based on specific binding of the fluorescent OsteoImage™ Staining Reagent to the hydroxyapatite (HA)

portion of bone-like nodules deposited by cells (OsteoImage™ Mineralization Assay, Lonza).

In 48 well plates, 12,000 cells per well of PLC, RTC and OB were cultured with and without mineralization media (MM) treatment. After 72hrs, one week, three weeks and six weeks the cell monolayer was fixed using 10% formalin for 10 minutes. After fixation, the wells were rinsed with OsteoImage™ Wash Buffer (X10) and 200ul of OsteoImage™ Staining Reagent (X100) was added to each well. The plates were then incubated at room temperature, protected from light, for 30 minutes. After the incubation step, the wells were washed 3 times with 400ul OsteoImage™ Wash Buffer (X10), leaving wash buffer in the wells for ~5 minutes per wash. After the final wash, the wash buffer was added and the solution was transferred to the plate reader for analysis. The excitation and emission wavelengths used were 492nm and 520nm respectively.

2.3.7 Real Time-PCR

Total RNA was harvested from cultured PLC, RTC and OB using TRIzol® Reagent (Invitrogen™ life technologies) at baseline (BL) and after 72hrs, one, three and six weeks of cell culture. The quality and quantity of RNA was measured using a spectrophotometer (NanoDrop® ND-1000). cDNA was prepared from the total RNA samples by reverse transcription using the iScript™ cDNA synthesis kit (BIO-RAD). Real time PCR was performed using an ABI PRISM 7900 (Applied Biosystems) thermocycler with TaqMan® Universal PCR Master Mix and Taqman® 'Assay-on-Demand' primer/probe sets (Applied Biosystems).

The cycling conditions consisted of an initial uracil-N-glycosylase (UNG) activation step at 50ºC for 2 min (UNG) to prevent the reamplification of carryover-PCR products by removing any uracil incorporated into single- or double-stranded DNA (Longo et al., 1990). This was followed by 10 min at 95ºC to activate the AmpliTaq Gold® DNA polymerase, followed by 45 cycles of denaturation at 95°C for 15 sec and primer/probe annealing and extension at 60ºC for 1 min.

Primer/probe preparations for each of the target bone markers (BSP, OCN, Runx2), putative cementum markers (CAP, CEMP1, and F-Spondin) and reference genes (B2MG, Rps13 and GAPDH) were obtained from a commercial source (Assays-on-Demand Gene Expression, Applied Biosystems, CA, USA).

The geNorm^{TM} software analysis package was used to determine the most stable reference gene (housekeeping gene) and to calculate the expression of normalized genes of interest (Vandesompele, 2002). The geNorm VBA applet determines the most stable reference genes from a set of tested reference genes in a given cDNA sample panel, and subsequently, calculates a gene expression normalization factor for each tissue sample based on the geometric mean of a user-defined number of reference genes (http://medgen.ugent.be/genorm/).

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2.4 Statistical analysis

For each cell type (PLC, RTC and OB) two cell lines from different patients were used for each functional analysis. These experiments were carried out in duplicate for each of the two cell lines (biological replicates). Assay measurements were performed in triplicate. The student's *t*-test was used to compare samples with and without treatment. One Way ANOVA with Bonferroni's post test correction and Two Way ANOVA were used to compare differences between the three cell types at each time point, and changes for each cell type over time. Results were considered statistically significant at $p<0.05$. GraphPad Prism5 software was used for the statistical analysis.

2.5 Results

2.5.1 Mineralization Analysis of Cells in Control Media

When cells were cultured in control media (10% FCS only), Alizarin red S staining was significantly increased at week six in all three cell types compared to the earlier time points, and was more intense in OB compared to both PLC and RTC (Figure 2.2A). Calcium release into the media was significantly higher from OB cells compared to both PLC and RTC 72hr only. After this time there was no significant difference in calcium release between cell types although the concentration of released Ca^{+2} ions from all cell types increased at later time points (Figure 2.2B). Hydroxyapatite formation was greatest in PLC at all time points and significantly higher than OB at week three. Hydroxyapatite formation was also significantly higher in OB at week 6 compared to week 1 and 72hrs (Figure 2.2C).

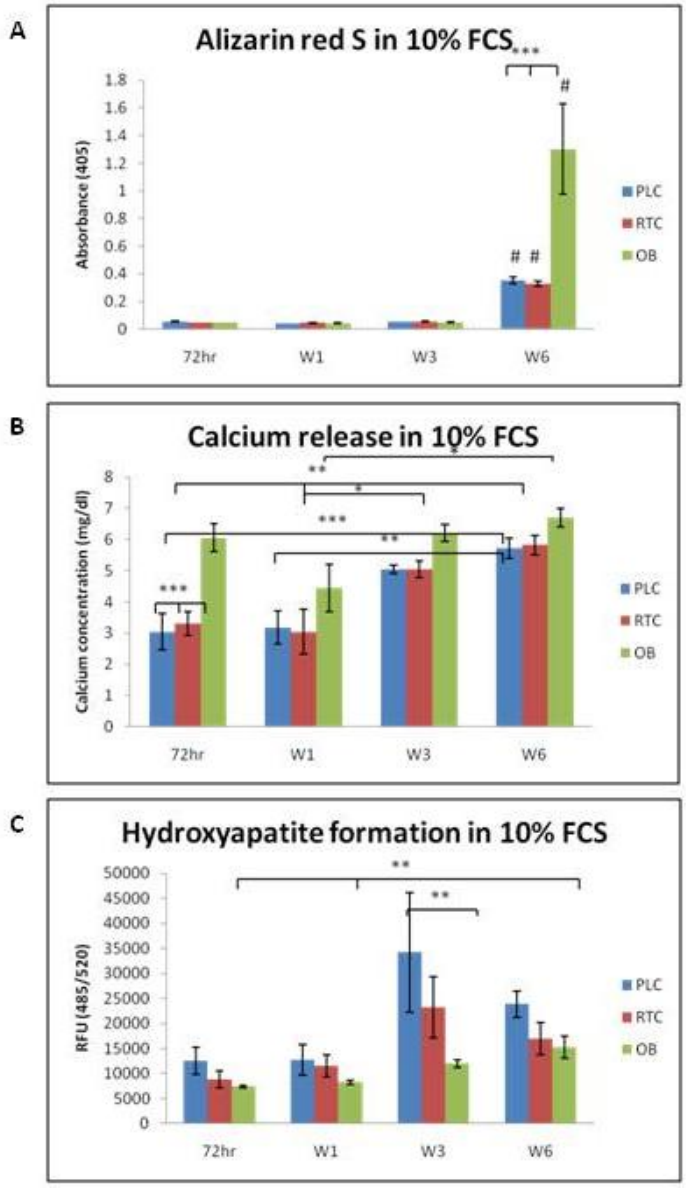


Figure 2.2 Histochemical analysis in control media.

Alizarin red S, Ca^{+2} release and Hydroxyapatite formation in PLC, RTC and OB cells cultured in control media. (#) Statistically significant compared to earlier time points ($p < 0.001$). (*) $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Values represent mean of quadruplicate measurements ± SEM.

2.5.2 von Kossa Staining

Von Kossa staining of all cell types was weak after 72hr and one week of treatment with mineralization media (data not shown). After three weeks of osteogenic treatment, all of the cells exhibited more intense staining than the negative controls (Figure 2.3A). Both PLC and OB displayed more staining than the RTC. After six weeks, the staining had increased further with the osteogenic treated wells darker than the osteogenic treated wells at week three for all cell types. Furthermore, the staining was most intense in OB followed by PLC and RTC (Figure 2.3B).

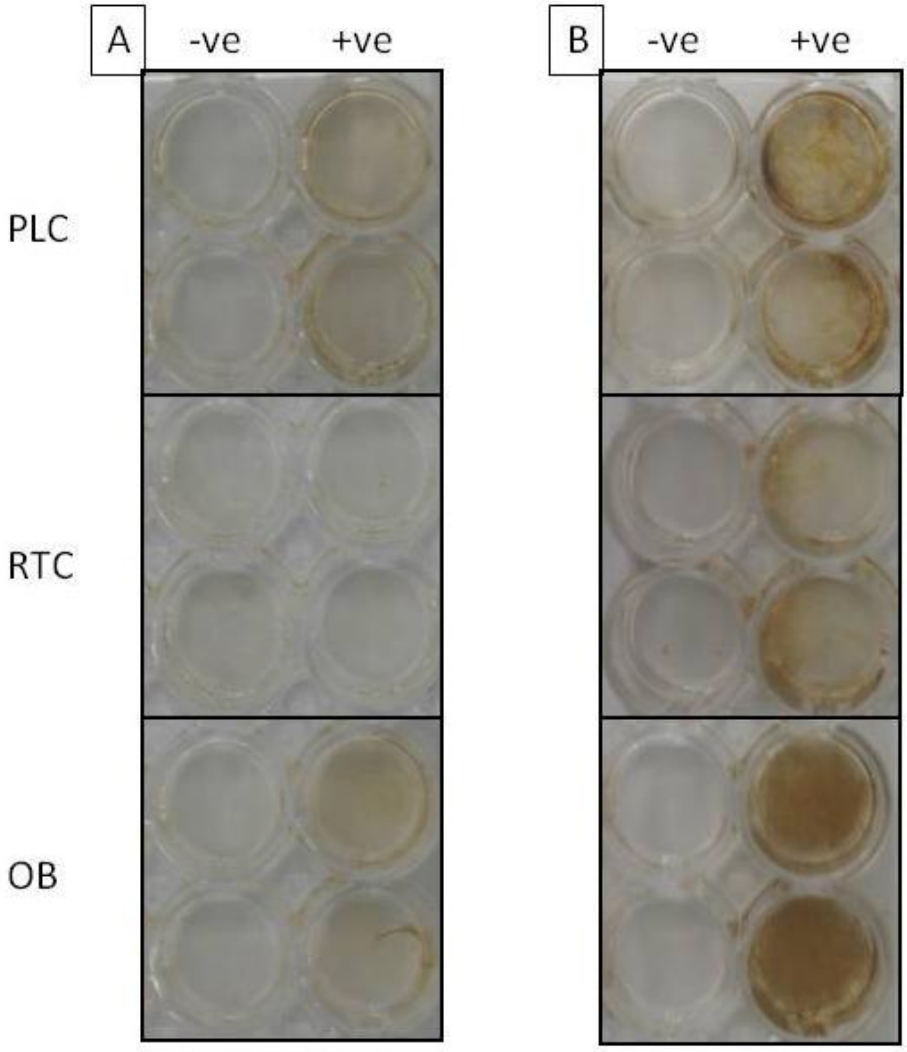


Figure 2.3 Von Kossa staining.

PLC, RTC and OB cells cultured in mineralization media (+ve) and a negative control (-ve) for A. 3 weeks and B. 6 weeks.

2.5.3 Alizarin red S Staining of Cells in Control and Mineralization Media

In general all cell types demonstrated more intense staining after mineralization treatment at three and six weeks, however the staining was more intense in both PLC and OB compared with RTC. The microscopic images taken from the middle of the wells illustrate the relative intensity of staining between the cells and show the presence of mineralization "crystals" and "nodules" (Figure 2.4 and 2.5).

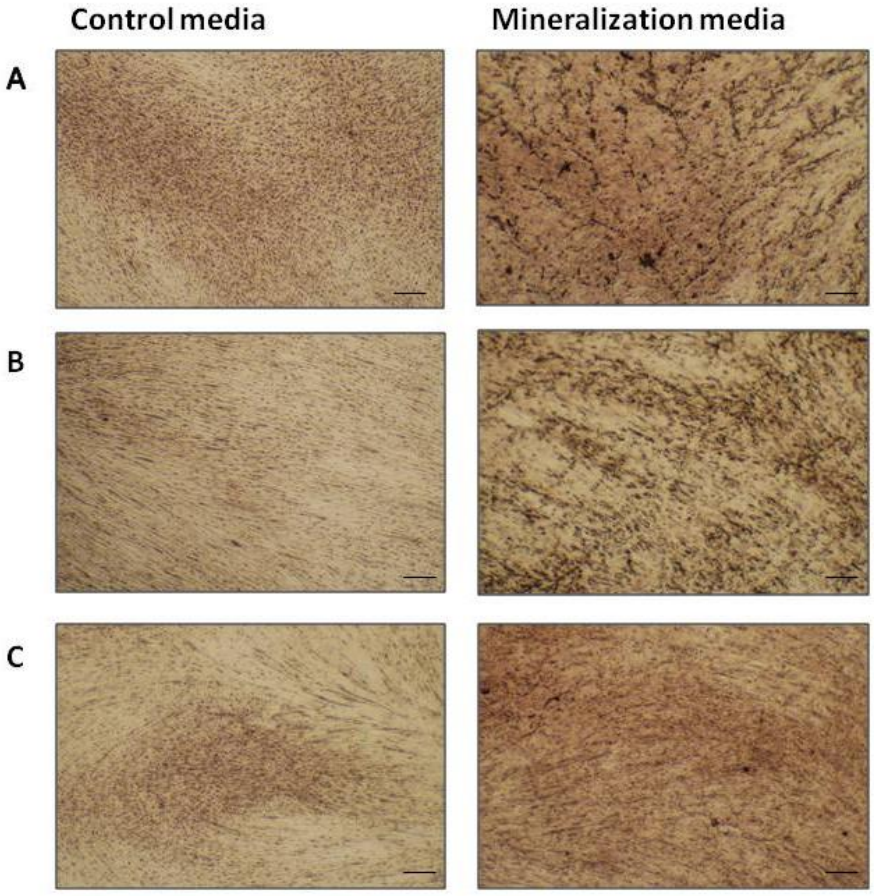


Figure 2.4 Qualitative alizarin red S staining at week 3.
Rrepresentative culture well of each cell type at week three. A. is PLC, B. is RTC and C. is OB with and without mineralization treatment. Scale bar is 100µm. Note the feathery appearance of the crystals

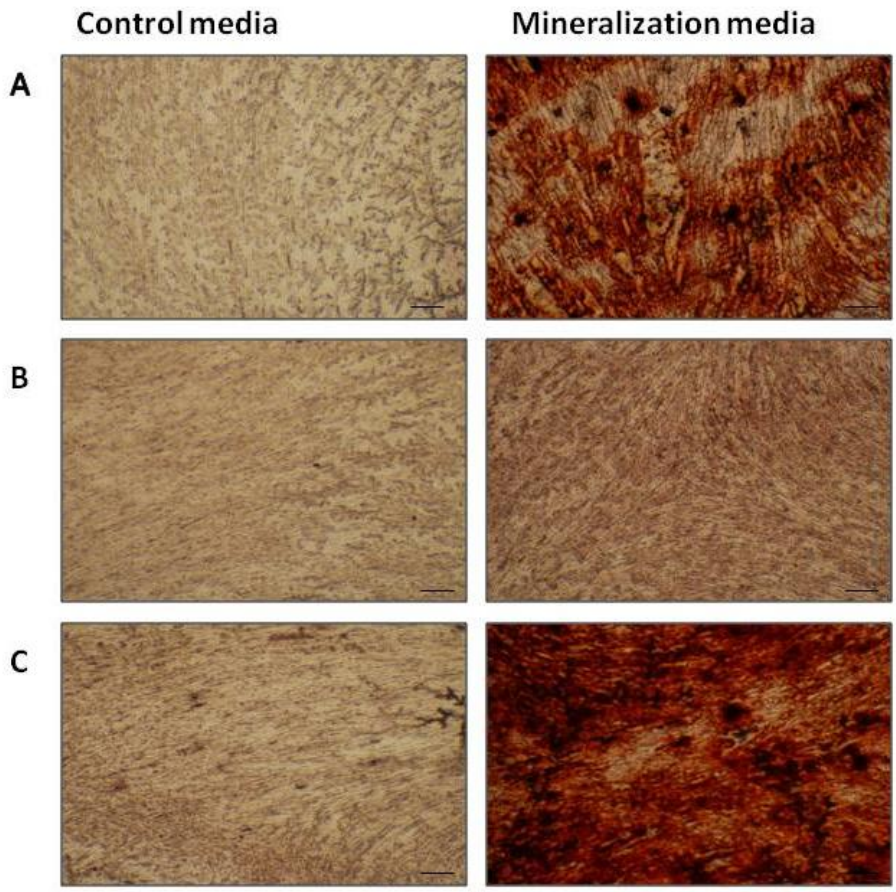


Figure 2.5 Qualitative alizarin red S staining at week 6.
Representative culture well of each cell type at week six. A. is PLC, B. is RTC and C. is OB with and without mineralization treatment. Scale bar is 100µm. Note the feathery appearance of the crystals

The results are also presented as the relative increase in staining at each time point in the mineralization media (MM) compared with the control media (CM) (Figure 2.6). All three cell types showed greater relative increases in absorbance at weeks three and six compared to 72hr and week one. No statistically significant differences in relative absorbance readings were found between week three and six except for PLC.

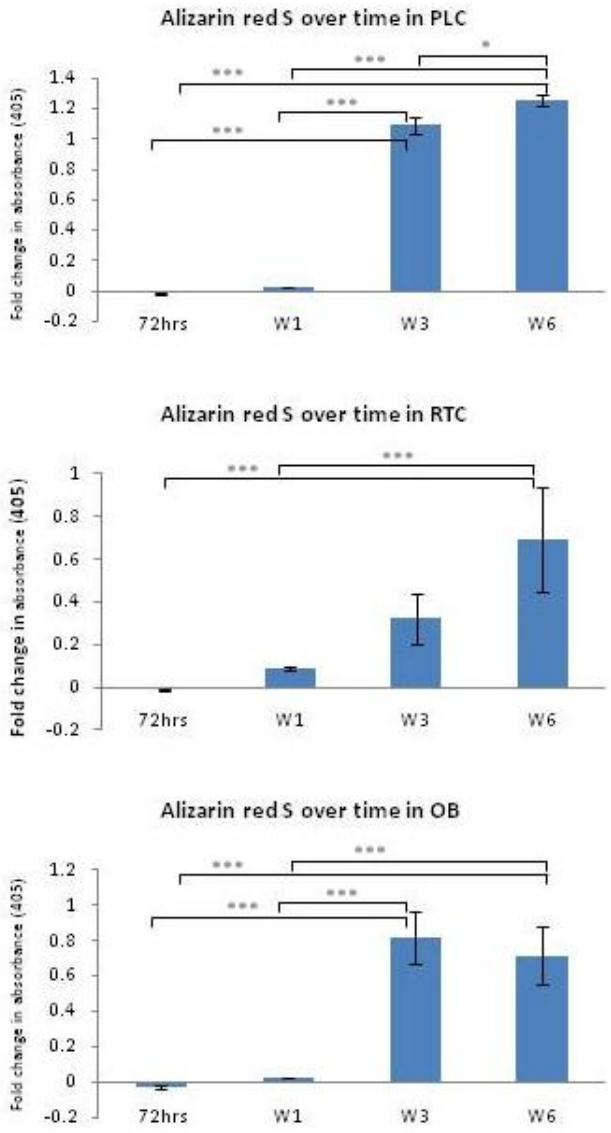


Figure 2.6 Quantitative alizarin red S staining over time.

PLC, RTC and OB were cultured in mineralization media for six weeks. The results are presented as log fold change compared to control media. (*) represents significance between cells. $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Values represent mean of quadruplicate measurements ±SEM.

Statistically significant differences between mineralization media treated wells and their corresponding negative control wells in all three cell types after three and six weeks of culture were found (Figure, 2.7). After three weeks of osteogenic treatment, both PLC and OB had significantly greater relative absorbance readings than RTC. After six weeks, the relative increase in PLC staining was significantly greater than both RTC and OB.

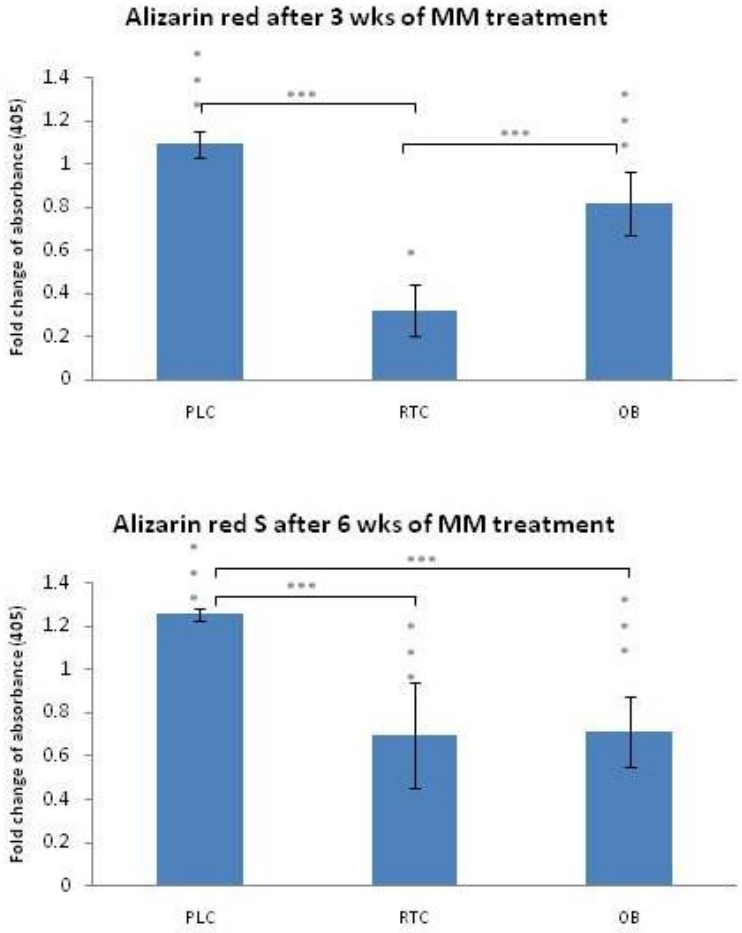


Figure 2.7 Quantitative alizarin red S staining between cell types

PLC, RTC and OB cultured in mineralization media (MM). The results are presented as log fold changes compared to control media at week 3 and 6. (*) at the top of each bar represents significance compared to control, (*) on the arch represents significance between cells. (*) $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Values represent mean of quadruplicate measurements ±SEM.

2.5.4 Calcium Release

No difference was found in the relative changes (MM/CM) in calcium release concentrations for any of the cell types between 72hr and week one, and also between week three and week six. However, relative calcium concentrations decreased in both OB and PLC cell lines between both 72hr and week one compared to both weeks three and six. No difference was found over time in the RTC cells (Figure 2.8).

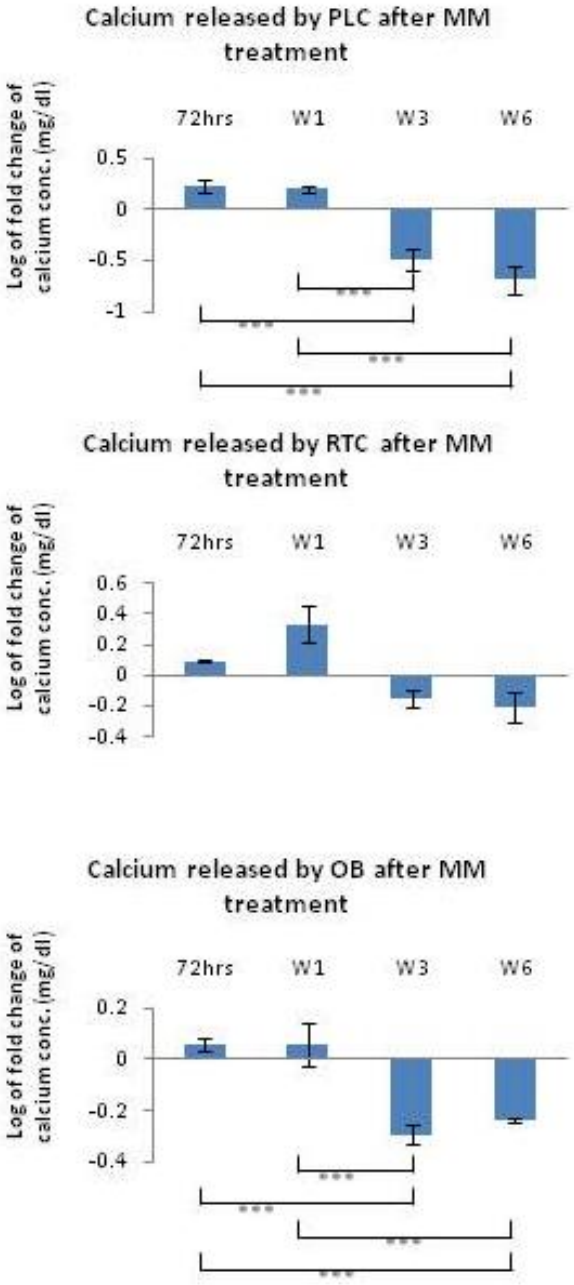


Figure 2.8 Calcium released over time.

PLC, RTC and OB were cultured in mineralization media for six weeks. The results are presented as log of the fold changes compared to negative control (control media). (*) represents significance between time points. $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Bars represent mean of quadruplicate measurements ±SEM.

No differences in relative calcium release were noted between the different cell types at 72hr and week one. Relative to control media, treatment with mineralization media decreased calcium levels in the media in all three cell types after three weeks, and also at week six in the OB and PLC but not the RTC cultures. The relative decrease in released calcium concentrations was statistically significantly greater in PLC than RTC at week three and greater in PLC compared to both RTC and OB at week three (Figure 2.9).

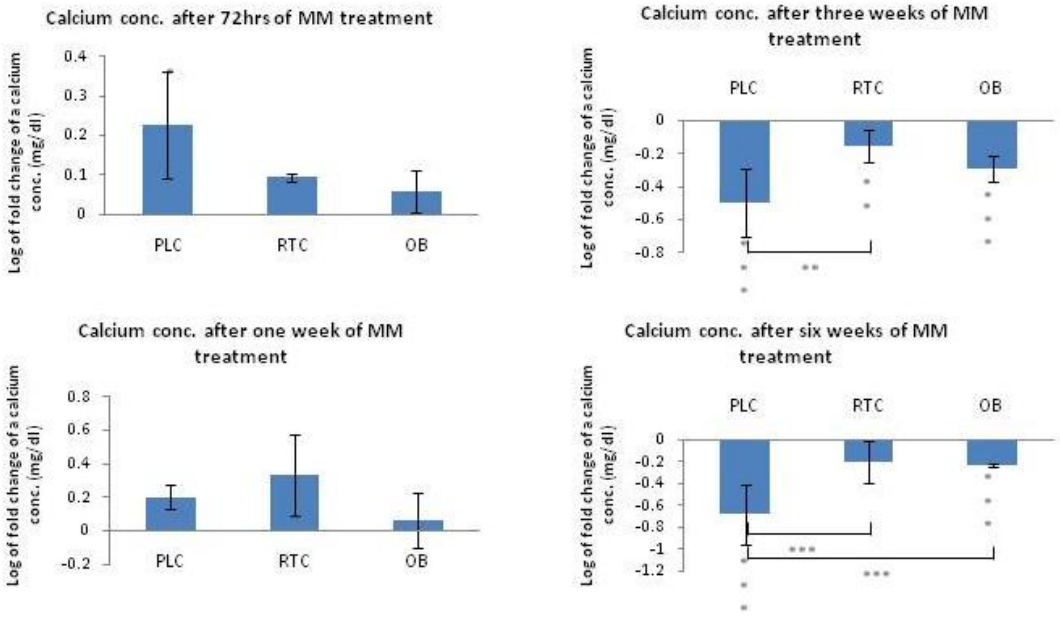


Figure 2.9 Calcium release between cell types.

The relative changes in released calcium ion concentrations for the three cell types at each time point. The results are presented as log fold changes of mineralization treatment (MM) compared to control media. (*) at the top of each bar represents significance comparing to control. (*) on the arch represents significance between cells. $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Bars represent mean of quadruplicate measurements ±SEM.

2.5.5 Hydroxyapatite nodule formation

In general there was a statistically significant relative (MM/CM) increase in fluorescence at week three and six compared to 72hr and week one for all of the three cell types. There was no difference between 72hr and week one. Furthermore, only the RTC cells exhibited a statistically significant increase in relative hydroxyapatite formation between week 3 and 6 (Figure 2.10).

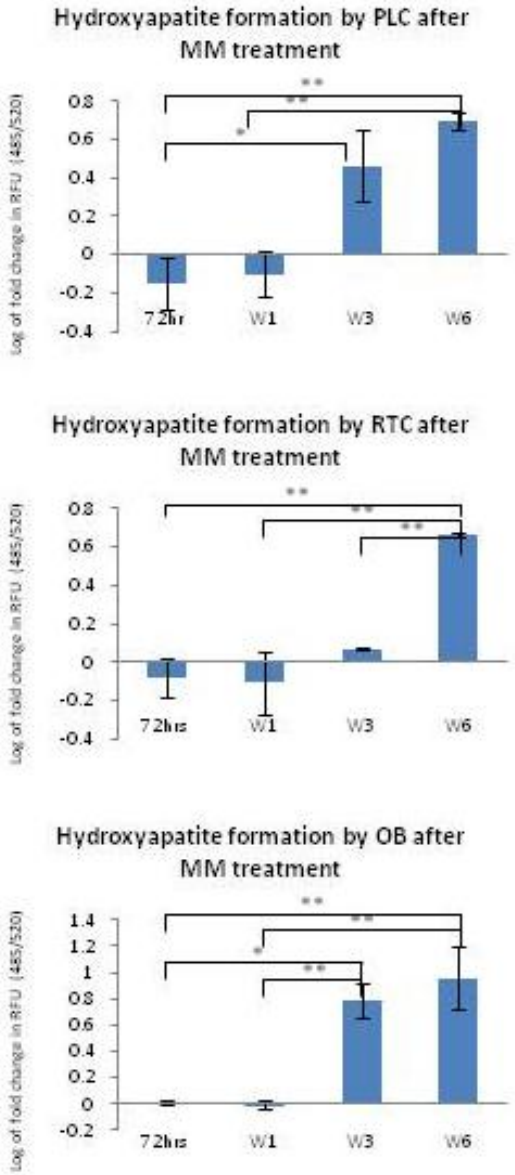


Figure 2.10 Relative hydroxyapatite formation for each cell line over time.

The results presented as log of the fold changes comparing to control media. (*) at the top of each bar represents significance comparing to control. (*) on the arch represents significance between cells. $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Bars represent mean of quadruplicate measurements ±SEM.

At the 72hrs and week one time points there were no statistical differences in relative hydroxyapatite (HA) levels between cells (data not shown). However, at week 3 a statistically significant difference in fluorescence between mineralised media and control was found in PLC and OB but not RTC. Furthermore, OB showed a statistically higher relative increase in fluorescence compared with RTC. At week six, a statistically significant increase in fluorescence was found in all three cell types in the mineralization media compared to the control media (Figure 2.11) .

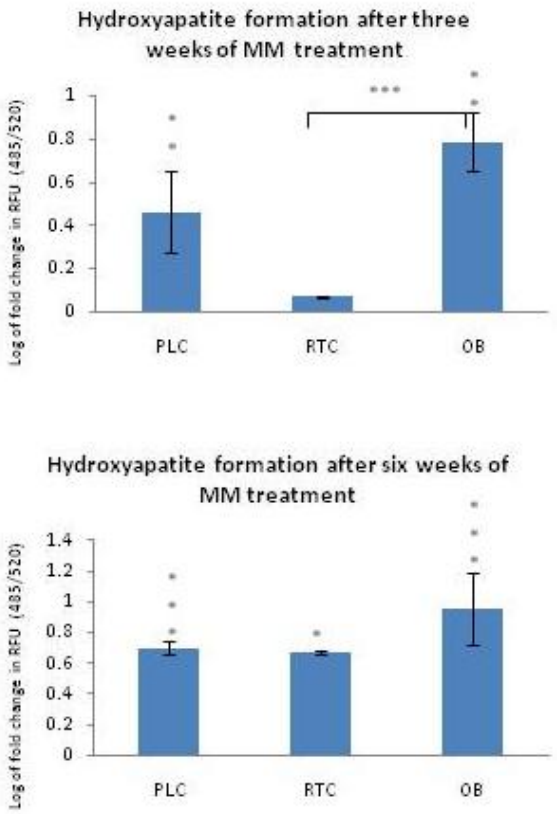


Figure 2.11 Relative hydroxyapatite formation for each cell type at 3 and 6 weeks.
The results are presented as log fold changes compared to control media. (*) on the arch represents significance between time points. $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Bars represent mean of quadruplicate measurements ±SEM.

2.5.6 Real Time-PCR

PLC, RTC and OB were cultured for 48hours in standard culture media containing DMEM supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and non-essential amino acids. RT-PCR results showed a trend towards greater BSP, SPON1 and Runx2 expression in OB and PLF cells compared with RTC. However, only the difference between PLC and RTC's SPON1 expression reached statistical significance. No significant difference in the expression of OCN, CEMP1 or CAP was noted between the cells (Figure 2.12).

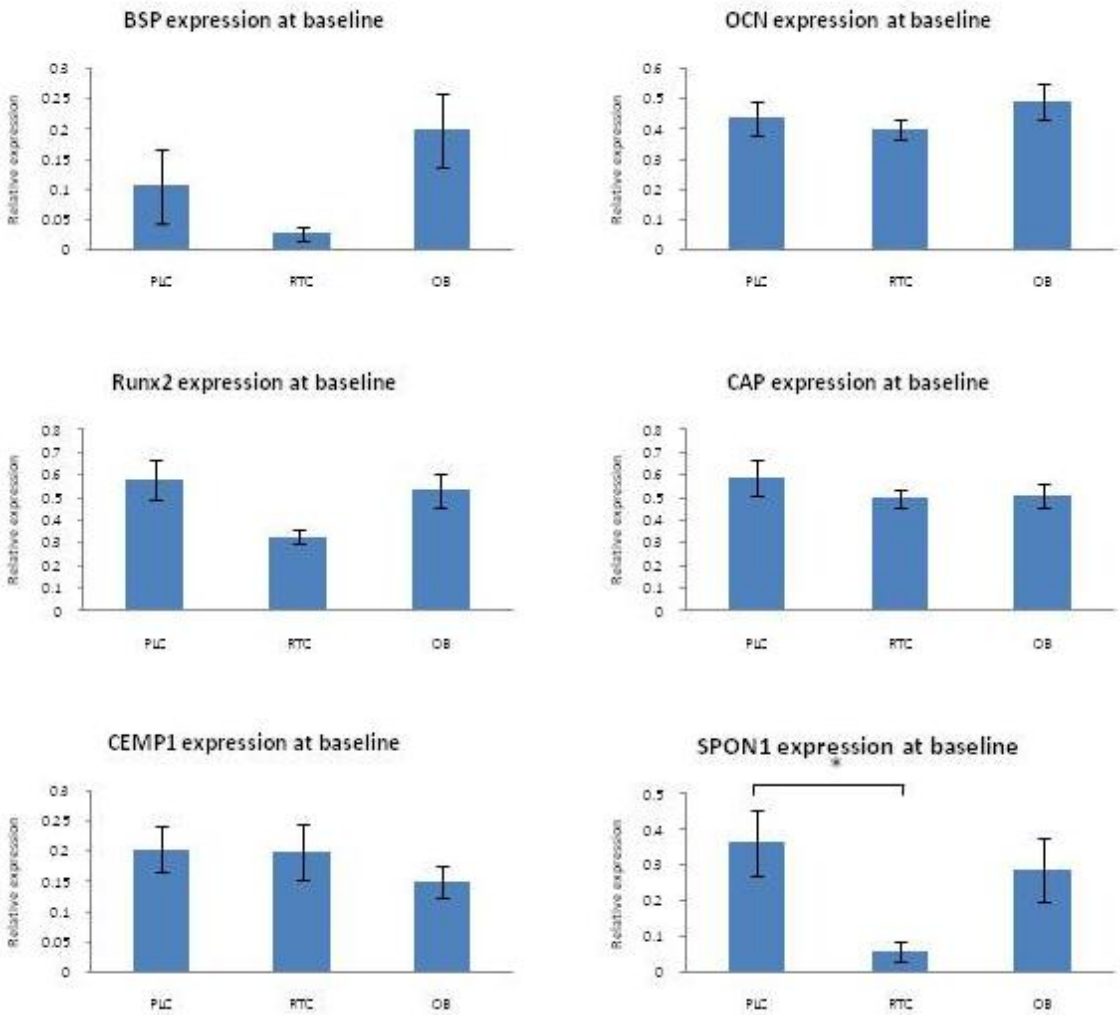


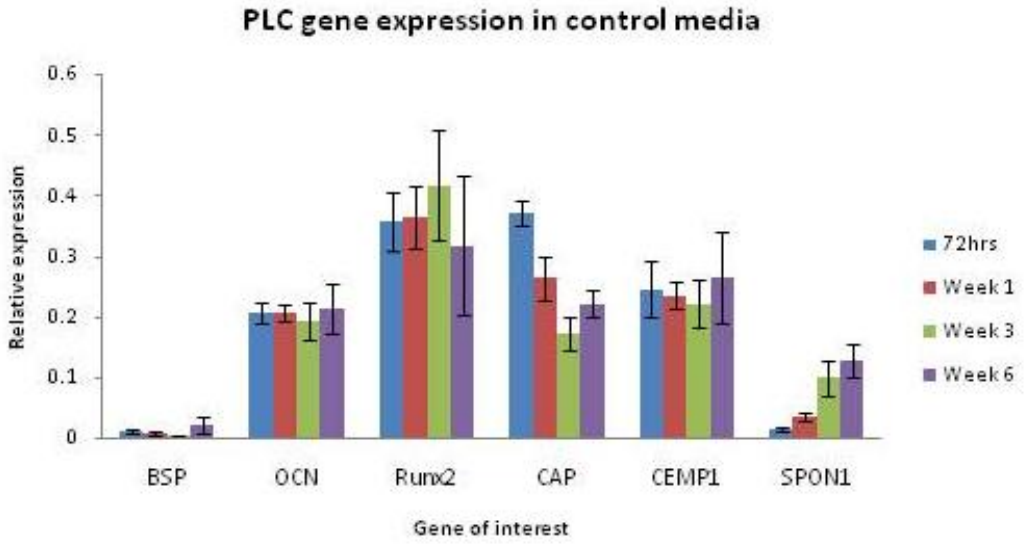
Figure 2.12 RT-PCR results at baseline.

BSP, OCN, Runx2, CAP, CEMP1 and SPON1 expression at baseline in the three cell types examined (PLC, RTC and OB).

(*) significant difference between the cell types, $p<0.05$. Values represent mean of quadruplicate ± SEM.

Culture of PLC in 10% FCS only (control media) displayed expression of all of the six markers tested at all time points. Further, culture in control media was shown to progressively increase the expression of SPON1 and decrease CAP expression over the 6 week period (Figure 2.13, graph).

Treating PLC with mineralization media resulted in a large up-regulation of BSP expression at week 6. Furthermore, modest increases in OCN, CAP and CEMP1 were noted at later time points of week 3 and week 6, as well as a modest decrease in Runx2 at week 6. The expression of SPON1 was not influenced by mineralization media (Figure 2.13, table).

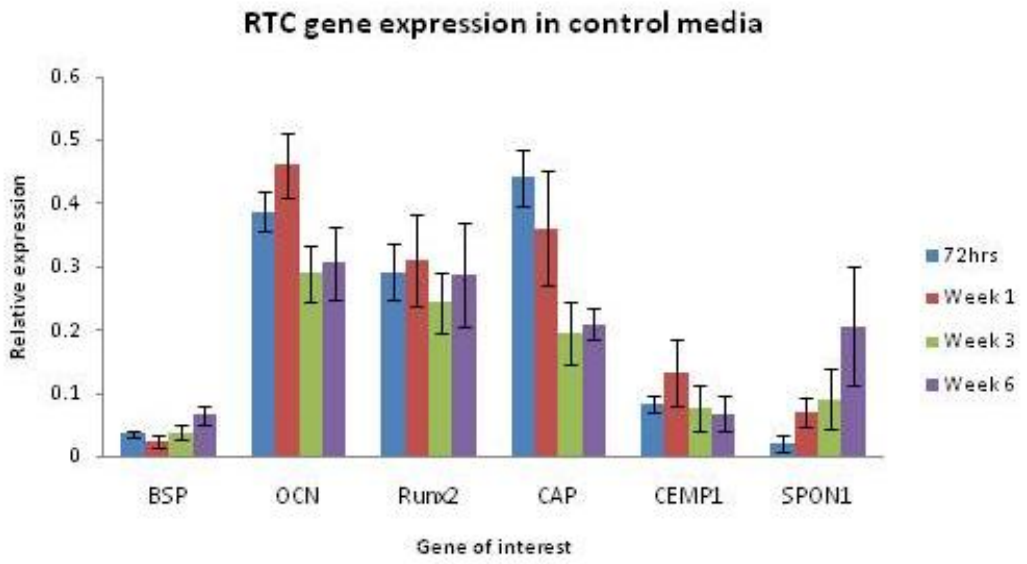


Cell type	Time points	Fold change in gene expression after MM treatment					
		BSP	OCN	Runx2	CAP	CEMP1	SPON1
PLC	72hr	–	–	–	–	–	–
	W1	2.7±1.5	–	–	–	–	–
	W3	–	2.0±0.8	–	2.0±0.6	–	–
	W6	28.0±9.5	–	2.1±0.4	2.8±1.2	3.4±2.4	–

Figure 2.13 PLC gene expression.

The graph shows the gene expression in the control media and the table shows the ratio of the up-regulated (red) or down-regulated (blue) genes (threshold of two fold change). Values in the graph represent mean of quadruplicate ± SEM and in the table represent the ratio of the change in the expression ± SEM (MM/CM).

RTC cultured in control media showed expression of all markers tested, and a gradual up-regulation of BSP and SPON was noted over the 6 week period. However CAP and OCN were down-regulated over time, while little difference was found in the expression of CEMP1 and Runx2 over the duration of the study (Figure 2.14, graph). Treating RTC with mineralization media was able to up-regulate the expression of BSP and F-SPON only (Figure 2.14, table).

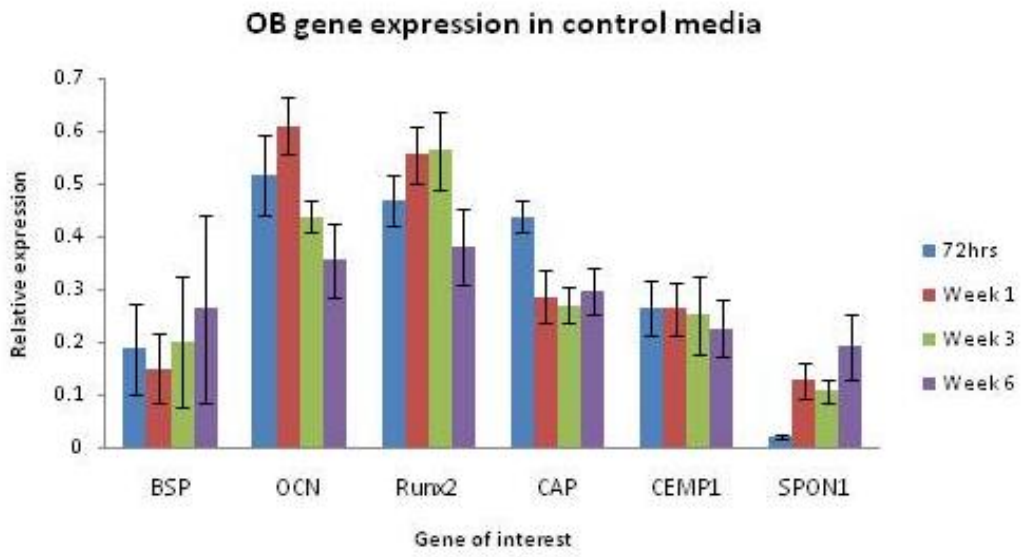


Cell type	Time points	Fold change in gene expression after MM treatment					
		BSP	OCN	Runx2	CAP	CEMP1	SPON1
RTC	72hrs	–	–	–	–	–	4.1±1.6
	W1	2.0±1.0	–	–	–	–	–
	W3	–	–	–	–	–	4.1±2.2
	W6	–	–	–	–	–	6.2±4.1

Figure 2.14 RTC gene expression.

The graph shows the gene expression in the control media and the table shows the ratio of the up-regulated (red) genes (threshold of two fold change). Values in the graph represent mean of quadruplicate ± SEM and in the table represent the ratio of the change in the expression ± SEM (MM/CM).

OB expression of OCN, Runx2 and CAP showed a trend towards decreased expression over time, while no difference was noted for CEMP-1. However, BSP and SPON1 both showed a tendency to increase expression over time with culture in control media (Figure 2.15, graph). Treatment of OB with mineralization media was shown to up-regulate both BSP and SPON1 expression at the latter time points (weeks 3 and 6) (Figure 2.15, table).



Cell type	Time points	Fold change in gene expression after MM treatment					
		BSP	OCN	Runx2	CAP	CEMP1	SPON1
OB	72hrs	–	–	–	–	–	–
	W1	2.8±0.7	–	–	–	–	–
	W3	4.0±1.9	–	–	–	–	7.0±4.6
	W6	3.3±2.6	–	–	–	–	6.9±3.9

Figure 2.15 OB gene expression.

The graph shows the gene expression in the control media and the table shows the ratio of the up-regulated (red) genes (threshold of two fold change). Values in the graph represent mean of quadruplicate ± SEM and in the table represent the ratio of the change in the expression ± SEM (MM/CM).

2.6 Discussion

The repair of the lost mineralized and soft tissue components of the periodontal attachment apparatus in periodontitis affected patients remains a significant clinical problem. The ultimate goal of periodontal treatment is full regeneration of the original architecture and function of the periodontium. This requires the formation of new cementum on the diseased root surface, regrowth of bone and formation of newly oriented periodontal fibres inserting into new cementum and bone (Bartold et al., 2000a). For successful repair and remodelling of periodontal defects, a cell population capable of producing and remodelling the periodontal tissue must be present. These cells can be either recruited from the host tissue or delivered to the defect site.

Cementum is critical for achieving periodontal regeneration because it invests and securely attaches periodontal ligament fibers to the root surface. The precise origin and the molecular factors that regulate cementoblast recruitment and differentiation however are not fully understood (Bosshardt, 2005). It has been suggested that during root formation, Hertwig's epithelial root sheath (HERS) disintegrates and undifferentiated mesenchymal cells from the dental follicle migrate and attach to the root surface. These undifferentiated cells subsequently differentiate into cementoblasts at the root surface and deposit the initial cementum extracellular matrix (Cho and Grant, 1988).

To determine the possible source of cementoblast precursors, this study examined the behaviour of three different cell types in response to mineralization (osteogenic differentiation) treatment. The cells used were periodontal ligament derived cells (PLC), fibroblast-like cells derived from regenerated periodontal defects (RTC) and alveolar bone derived cells (OB). In order to investigate the mechanisms involved in

osteoblastic/cementoblastic differentiation, we compared the gene expression profiles of three traditionally used 'mineralization tissue' associated proteins, OCN, BSP and Runx2, and three putative cementum markers, CAP, CEMP1 and SPON1 in the three cell types (PLC, RTC and OB). This gene expression pattern could be correlated with a series of *in vitro* histochemical assays commonly used to identify and quantify mineralization.

The mineralization process has been suggested to occur in two phases. The first is the initiation phase (1–2-week), during which cells proliferate, express ALP activity and bone specific genes, and produce a collagen matrix. This is followed by a maturation phase occurring in the second (Wang et al., 1999, Somerman et al., 1988) or third (Quarles et al., 1992, Bellows et al., 1992, Cheng et al., 1994) week when matrix mineralization is observed. In this study, the von Kossa assays indeed showed more staining after mineralization treatment for three and six weeks in the three cell types examined. Similarly the Alizarin red S optometric readings were higher after three and six weeks of treatment. Calcium release into the media showed the opposite pattern, suggesting that calcium is incorporated into mineralized matrix resulting in significantly fewer calcium ions being released into the media at three and six weeks. Moreover, hydroxyapatite formation was also significantly increased at weeks three and six.

Elevated levels of extracellular Ca^{2+} ions have been implicated in osteogenesis where they, stimulate the proliferation and differentiation of osteoblasts (Yamauchi et al., 2005, Yamaguchi et al., 1998, Dvorak et al., 2004, Godwin and Soltoff, 2002, Pi et al., 2000). Recently, Kanaya *et al* investigated the Ca^{2+}-sensing mechanism in cementoblasts by testing the activation of fibroblast growth factor-2 (FGF-2), which

plays a pivotal role in bone and cementum formation (Marie, 2003, Sato et al., 2004, Murakami et al., 1999, Takayama et al., 2001), and found that elevated extracellular Ca^{2+} ion concentration increases FGF-2 gene and protein expression levels via a cAMP/PKA dependent pathway (Kanaya et al., 2010). In our study the level of calcium deposited in the matrix was measured by Alizarin red S staining and the level of extracellular calcium released into the media was also measured. Those cells with a high concentration of Ca^{2+} ions in the matrix as measured by Alizarin red S staining showed less calcium release. Moreover, we found that PLC had more alizarin red S staining and less calcium released into the media after mineralization treatment compared to both RTC and OB.

Similarly, studies have shown that extracellular inorganic phosphate (Pi) is an important regulator of gene expression during bone mineralization and cementoblast function (Fatherazi et al., 2009, Foster et al., 2006, Rutherford et al., 2006). The Von Kossa staining principle is a precipitation reaction in which silver ions react with phosphate (Meloan and Puchtler, 1985). In our study, OB appeared to have more phosphate deposition than PLC. This was in contrast to the calcium based assays which showed that PLC had both more calcium deposition and less release into the media.

Both Alizarin red and von Kossa staining are commonly used to detect mineralized bone nodule formation, however, mineral deposits in osteoblast cultures are mostly composed of calcium-phosphate substituted hydroxyapatite similar to that seen in bone, cartilage and teeth. In the process of mineralization, Ca^{2+} ion concentration is raised in the matrix through binding to OCN and other glycoproteins, Moreover, OB also release membrane-enclosed vesicles rich in ALP and other enzymes which increase the PO_4^-

ions locally. With high concentrations of both ions, these matrix vesicles serve as foci for the formation of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] crystals, the first visible step in calcification. A commercially available assay kit (OsteoImage, Lonza) has been developed to detect the levels of hydroxyapatite in mineralized tissue. In our study, the results obtained with this assay were consistent with the other mineralization assays. Hydroxyapatite formation was increased in the PLC and OB cell cultures at weeks three and six, and HA levels were increased in the RTC cultures by week 6. The pattern of HA formation was similar to that seen in the von Kossa staining, with OB forming more mineralized tissue than PLC and RTC.

Interestingly, when cells were cultured in control media, without any supplemented mineralization treatment, all the cells types showed staining with Alizarin red S at week 6, which was strongest in the OB cell culture. Moreover, HA formation was also increased after 3 and 6 weeks of tissue culture, suggesting that these cell types were able to differentiate into a mineralized forming phenotype regardless of mineralization treatment, albeit to a lesser degree. This is not entirely surprising as the cells will 'mature' over time in culture.

Real-time PCR was utilized to investigate changes in gene expression during the process of mineralization. Six genes identified in the literature as markers of bone/cementum expression were assessed. BSP is one of the major noncollagenous, extracellular matrix (ECM) proteins associated with mineralized tissues (Oldberg et al., 1988), and is believed to be associated with clusters of needle-like crystals of hydroxyapatite (Uutela et al., 2001). BSP mRNA expression was also noted previously in both PLC and RTC (Ivanovski, 2001a).

In this study, BSP showed a tendency to increase gradually over time even in the control media, however, treatment with mineralization media up-regulated the expression of BSP in all cell types. Moreover, the up-regulation (fold change) was greater in treated PLC than treated OB, which may be explained by the higher BSP expression by OB cells at baseline and in control media. This suggests that these OB cells may be further differentiated along a mineralized tissue forming lineage, and the lower fold change in BSP expression is consistent with findings that show BSP is down-regulated in mature osteocytes (Bergsten et al., 2001).

OCN is known to act as a bone formation regulator, with strong suppression signals for OCN predominant in early osteoprogenitors, while enhancer factors for OCN transcription are found in mature post-proliferative osteoblasts (Owen et al., 1990, Towler et al., 1994, Banerjee et al., 1996, Newberry et al., 1998). Furthermore, a role for OCN in bone resorption is strongly supported by a number of *in vitro* and *in vivo* studies showing an association of OCN with osteoclast recruitment activity (Lian et al., 1984, Glowacki et al., 1991). In this study, OCN was shown to be expressed in all cells at baseline, however, there was a tendency for a decrease in expression over time in RTC and OB. PLC was the only cell type in which expression was maintained over the time of the experiment. Further, following mineralization treatment, PLC was again the only cell type to increase OCN expression at a later time point (3 weeks). OCN however has been shown to be expressed at a later stage compared to BSP expression (Bergsten et al., 2001), and hence OCN expression may have been demonstrated later if the experiment was extended for a longer time period.

Runx2 is a transcription factor that has been shown to control the differentiation of osteoblasts and the expression of extracellular matrix protein genes. Runx2 has been implicated in maintaining osteoblasts in an immature stage of the differentiation cascade, and hence, Runx2 expression has to be down regulated for differentiation into mature osteoblasts (reviewed in (Komori, 2010). In this study, Runx2 expression was shown in all cells at BL, and tended to decrease by week 6 in both PLC and OB when grown in the control media. In response to the mineralization media, down-regulation of RunX2 expression was found only in PLC after 6 weeks.

Cementum attachment protein (CAP) has been proposed to be a cementum specific protein (Arzate et al., 1992, Bar-Kana et al., 1998), which has a key role during cementogenesis (Saito et al., 2001). CAP has also been suggested to be a marker for putative cementum progenitors in adult human periodontal ligament cells (Bar-Kana et al., 1998, Liu et al., 1997). In this study, CAP was found to be expressed in all cells similarly at BL, and decreased over time in control media. Mineralization media up-regulated the expression of CAP only in PLC cells at later stages of treatment. Therefore, this study showed that cells residing in the periodontal ligament may be able to differentiate along a cementum lineage and express CAP, but not the cells from the regenerated tissue or alveolar bone.

Cementum protein 1 (CEMP1), also known as cementum protein 23 (CP-23), is another putative cementum specific marker which was originally derived from human cementoblastoma (Arzate et al., 1998). It has been shown to be highly expressed in cementoblasts, progenitor cells within the periodontal ligament and endosteal spaces of bone (Arzate et al., 2002), periodontal ligament fibroblasts (Alvarez-Perez et al., 2006),

but not in osteoblasts (Kitagawa et al., 2006a). In the present study, RT-PCR identified expression of CEMP1 in all cells at base line, while culturing in mineralization media up regulated the expression in PLC cells at week 6. Similar to CAP expression, PLC was the only cell type able to express CEMP1 after 6 weeks of mineralization treatment suggesting that cells reside in the periodontal ligament may be able to differentiate along a cementogenic lineage.

In 2006, Kitagawa *et al* was the first to identify F-Spondin (SPON1) as a promoter for cementoblastic differentiation, and to show that it is highly expressed in human cementoblasts, but not in osteoblasts (Kitagawa et al., 2006). F-Spondin has also been found to be expressed specifically in dental follicle cells during tooth germ development (Nishida et al., 2007). In this study, F-Spondin was found to have a higher expression in PLC compared with RTC, and OB at baseline. After 6 weeks culture in control media, a slight increase was found over time in all three cell types. However, mineralization media up-regulated the expression of F-Spondin in OB and RTC, but not PLC, suggesting that SPON might not be specific to cementum and cementoblasts.

The polymerase chain reaction (PCR) technique allows the massive amplification of DNA which can be visualized at the end of the reaction. Real-time-PCR is able to more precisely quantify DNA than traditional PCR, because it measures the accumulation of PCR product during amplification, rather than a prespecified endpoint (Kim, 2001). However, most of the available literature evaluating gene expression during periodontal cell differentiation uses PCR, which is less accurate in quantifying gene expression. Using ‘standard’ PCR, both PLC and RTC in culture were found to express OCN and BSP, with no significant difference in the expression between the two cell types

(Ivanovski, 2001a). In the current study, real-time-PCR was used to allow the more accurate quantification of osteogenic markers expression, which generally occurs at relatively low levels *in vitro*. It was found that the increase in BSP expression was higher in PLC than RTC, but this may be attributed to the long treatment with osteogenic treatment as compared to the study conducted by Ivanovski et al (2001a).

Moreover, the findings of this thesis were consistent with other studies demonstrating that PDL cells are osteogenic in nature and may differentiate into either osteoblasts or cementoblasts (Mukai et al., 1993, Bartold et al., 2000a, Cho et al., 1992, Arceo et al., 1991). The results also show that alveolar bone derived osteoblasts can differentiate and express bone markers. Furthermore, the RTC results suggest that this population is heterogeneous, and contains cells derived from the periodontal ligament or bone that are able to differentiate along a bone forming phenotype, albeit at a reduced rate than primary PLC or OB cultures.

Indeed, cells derived from regenerated periodontal defects have been shown to demonstrate a heterogeneous range of genomic and phenotypic characteristics including: formation of mineralized nodules *in vitro* (Wakabayashi et al., 1996), expression of specific cytokines (Wakabayashi et al., 1997) and protease repertoires (Wakabayashi et al., 1996), production of factors resulting in osteoclast inhibition (Rowe et al., 1996), up-regulation of alkaline phosphatase activity (Kuru et al., 1999), faster proliferation than periodontal ligament fibroblasts and a unique pattern of proteoglycan mRNA expression (Ivanovski, 2001), expression of bone markers (OCN, BSP and osteopontin) (Ivanovski, 2001a) and expression of a unique gene profile in respect to protein biosynthesis and

turnover, structural constitutes of the cytoskeleton and extracellular matrix, and signal transduction (Ivanovski et al., 2007).

In summary, this chapter has demonstrated that cells derived from the periodontal ligament, regenerated periodontal defects and alveolar bone contain undifferentiated cells able to differentiate in response to mineralization treatment into an osteogenic/cementogenic phenotype. This was demonstrated by the deposition of mineralized tissue after 3 weeks of treatment, which was significantly increased after six weeks. Moreover, PLC showed up-regulation of the putative cementum markers, CAP and CEMP1 after 6 weeks of mineralization treatment, which may suggest that cells derived from the periodontal ligament might contain cells capable of differentiating along a cementogenic phonotype. It's well known that gene expression is dependent not only on the cell phenotype but also on the stage of cell differentiation. In this study, it was difficult to correlate the expression of the tested genes with mineralization formation. Therefore, further studies, including the use of a larger number of cells, and more frequent analysis of gene expression over time coupled with histochemical analysis, are needed to understand the appropriate cell candidate(s) for periodontal regeneration as well as the cascade of gene expression associated with mineralization.

2.7 Conclusion

The data using von Kossa and alizarin red S staining, Ca^{2+} release, hydroxyapatite formation and the expression of bone/cementum markers commonly used to test the osteogenic/cementogenic differentiation potential of a cell, showed that mineralization

treatment promotes and induces differentiation of PLC, RTC and OB into bone/ cementum forming phenotypes. Moreover, PLC may contain cells able to differentiate into cementoblast forming cells more than both RTC and OB. BSP and SPON1 gene expression appeared to be most consistently associated with mineralization.

3.0 Chapter Three

Growth Factor Effects on the Expression of Bone and Cementum Markers

3.1 Abstract

Background: Periodontal regeneration is the reconstitution of lost periodontal tissue including bone, periodontal ligament and cementum. Growth factors have been used to induce cellular differentiation of periodontal cells along a mineralization forming phenotype in order to facilitate the formation of bone and cementum.

Objective: This study was undertaken to determine the effect of insulin-like growth factor-1 (IGF-1), Platelet-derived growth factor (PDGF), and the combination of PDGF and IGF-1 on the expression of bone and cementum markers by periodontal ligament (PLC), periodontal regenerated tissue (RTC) and alveolar bone cells (OB).

Method: Three primary cell types (PLC, RTC and OB), derived from three different patients, were exposed to various growth factors (IGF-1 100ng/ml, PDGF-BB 10ng/ml and IGF-1/PDGF-BB 100/10ng/ml) for six weeks. Real time PCR was utilized to quantify the expression of the bone markers bone sialoprotein (BSP), osteocalcin (OCN) and Runx2, and the putative cementum specific markers: cementum attachment protein (CAP), cementum protein-1 (CEMP1), and F-Spondin (SPON1).

Results: A similar pattern of gene expression was note in both 'control' media (1% FCS and 10% FCS), however, 1% FCS induced higher expression of most of the genes tested compared to 10% FCS. Both IGF-1 and PDGF up-regulated the expression of BSP and OCN by osteoblasts. PLC responded to IGF-1 and the combination of IGF-1/PDGF with up-regulated expression of BSP. CAP and CEMP1 expression by the three cell types was not particularly responsive to the growth factors, although the combination of IGF-1/PDGF mildly increased CAP expression in PLC and CEMP1 in OB. The RTC were

less responsive to growth factor treatment than both OB and PLC. Moreover, F-Spondin expression was up-regulated by the growth factors at most time points in all of the cell types.

Conclusion: IGF-1 and/or PDGF may be able to differentiate periodontal cells along mineralized tissue-forming phenotypes as evidenced by their up-regulation of bone and cementum markers. The most responsive cells in regards to bone/cementum marker expression were PLC and OB.

3.2 Introduction

Periodontitis is a globally prevalent inflammatory condition that leads to a progressive destruction of periodontal tissues and it is a major cause of tooth loss in adults (Pihlstrom et al., 2005). Regenerative periodontal therapy aims to restore the periodontal apparatus that is damaged as a consequence of periodontal disease (Bosshardt and Sculean, 2009b).

Several coordinated biological processes are required for periodontal regeneration and reconstitution of the lost hard and soft tissues. Firstly, appropriate cells must be attracted to the wound site. Furthermore, an appropriate matrix must be secreted by these cells in order to provide an environment conductive to cell proliferation and differentiation (Bartold et al., 2000a). If critical factors capable of attracting periodontal tissue precursors to the periodontal defect can be identified, it may be possible to treat periodontal defects with such factors for the purpose of regenerating periodontal tissues *in vivo* (Kao et al., 2009).

Growth factors are natural biological molecules that mediate and regulate key cellular events during tissue repair, including cell proliferation, chemotaxis, differentiation, and matrix synthesis by binding to specific cell-surface receptors. The sequence of events necessary for periodontal regeneration relies on the above processes for osteogenesis, cementogenesis, and connective tissue formation (Cochran and Wosney, 1999, Shimono et al., 2003). Soluble mediators of importance in periodontal regeneration include growth factors such as platelet-derived growth factor (PDGF), transforming growth factor- β (TGF-β), fibroblast growth factor (FGF) and insulin like growth factor-1, reviewed in (Lee et al., 2010).

Both IGF-1 and PDGF have been studied extensively either separately or together in many tissue regeneration applications including periodontal regeneration (Bartold et al., 2000a). PDGF's primary effect is mitogenic, initiating cell division (Cochran and Wosney, 1999). PDGF has been found to increase the mitosis of periodontal ligament cells (PLC) (Ivanovski, 2001, Marcopoulou et al., 2003, Matsuda et al., 1992, Bartold and Raben, 1996b, Haase et al., 1998, Cesari et al., 2006), gingival cells (GC) (Ivanovski, 2001, Marcopoulou et al., 2003, Haase et al., 1998), regenerated tissue 'fibroblast-like' cells (RTC) (Ivanovski, 2001), alveolar bone osteoblasts (OB) (Strayhorn et al., 1999) and cementoblasts (Saygin et al., 2000). Furthermore, it stimulates periodontal cell migration (Bartold and Raben, 1996a), chemotaxis (Matsuda et al., 1992), and protein synthesis (Bartold and Raben, 1996a, Ivanovski, 2001).

IGF-1 is also a potent mitogenic growth factor that mediates the growth-promoting activities of growth hormone postnatally. In fibroblast systems, it is a proliferation progression factor, exerting a strong mitogenic influence on PLC (Matsuda et al., 1992, Ivanovski et al., 2001, Palioto et al., 2004). gingival fibroblasts (Ivanovski et al., 2001, Bartold and Raben, 1996a), and RTC (Ivanovski, 2001). In bone cell systems, IGF-1 stimulates both proliferation of preosteoblasts and differentiation of osteoblasts, including the induction of type I collagen synthesis (Wikesjo and Selvig, 1999, Shimono et al., 2003, Olney, 2003). IGF-1 enhances the chemotaxis of PDL cells (Matsuda et al., 1992) and promotes PDL protein synthesis (Matsuda et al., 1992). Conversely, Palioto et al (2004) found that IGF-1 does not affect PDL cell adhesion, migration and expression of type I collagen (Palioto et al., 2004).

Experimental studies have shown that the local application of recombinant human platelet-derived growth factor (rhPDGF) alone or together with IGF-1 may enhance

regeneration of cranial- (Vikjaer et al., 1997), periodontal-(Giannobile et al., 1996, Jin et al., 2004, Cho et al., 1995, Lynch et al., 1989, Nevins et al., 2003, Nevins et al., 2005, Nevins et al., 2007, Mellonig et al., 2009, Camelo et al., 2003, Howell et al., 1997), and peri-implant bone defects (Lynch et al., 1991a, Becker et al., 1992), as well as promote vertical and horizontal bone augmentation (Simion et al., 2006, Schwarz et al., 2009).

In light of the aforementioned reports concerning the role of growth factors in periodontal regeneration, the aim of this study was to determine the effect of PDGF-BB, IGF-1 and the combination of both on periodontal cell differentiation, as determined by the expression of the bone markers, BSP, OCN and Runx2, as well as the putative cementum markers, CAP, CAMP1 and SPON1.

3.3 Materials and Methods

3.3.1 Cell culture

The establishment of the PLC, RTC and OB primary cell lines was carried out as described in the previous chapter. However, for this experiment, three cell lines of each of the three cell types (PLC, RTC and OB) were examined.

3.3.2 Growth Factor Treatment

Cells were cultured in DMEM supplemented with 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin and non-essential amino acids. When the cells became confluent, they were detached using 0.2% trypsin and plated in six well plates at a density of $1x10^5$cells/well. After 48hours, the cells had reached 50% confluency. Subsequently, the cells were treated with one of the following six treatments: 1) ‘control’ media containing

DMEM, 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin and non-essential amino acids; 2) 'negative control' media for the growth factor treatment containing DMEM, 1% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin and non-essential amino acids; 3) IGF-1 group consisting of negative control media supplemented with 100ng/ml of IGF-1 (R&D Systems, Inc, catalog no. 291-G1); 4) PDGF group consisting of the negative control media supplemented with 10ng/ml of PDGF-BB (R&D Systems, Inc, catalog no. 220-BB).; and5) IGF-1/PDGF-BB group consisting of the negative control media supplemented with 100ng/ml of IGF-1 and 10ng/ml of PDGF. The media was changed every 2-3 days for the duration of the in vitro study period of up to 6 weeks.

The growth factor dosage was selected based on previous studies by our group as well as other reports in the literature (MacNeil et al., 1996b, Matsuda et al., 1992, Strayhorn et al., 1999, Bartold and Raben, 1996a, Bartold PM, 1992, Bartold, 1993, Oates et al., 1993a, Saygin et al., 2000, Marcopoulou et al., 2003, Ivanovski, 2001a, Ivanovski, 2001, Haase et al., 1998).

3.3.3 Real Time-PCR

Total RNA was collected from the cells following 72hrs, 1, 3 and 6 weeks of culture as described above. Real Time-PCR was subsequently carried out as described in chapter 2 to determine the relative expression of the bone and cementum markers.

3.4 Results

A real time-PCR system was used to compare gene expression based on data acquired during the exponential phase of PCR, rather than relying on the comparison of products obtained after a pre-determined endpoint, as is the case of 'standard' PCR. In the cell populations used in this study, the bone/cementum marker gene expression was variable (heterogeneous) between the triplicates of the same cell type. Furthermore, inherent expression varied significantly between different cell lines from the same cell type. This made statistically significant changes difficult to evaluate. Notwithstanding this, differences in gene expression were evident and in order to present these findings, we defined any 2 fold or more increase in gene expression compared to its negative control as up-regulation, while a 2 fold or more decrease in gene expression was considered as down-regulation.

3.4.1 Periodontal Ligament Cells Gene Expression

Periodontal ligament cells (PLC) grown in high (10% FCS) and low (1% FCS) concentrations of FCS showed similar patterns of gene expression (Figure 3.1). However, OCN and CEMP1 expression are consistently higher in 1% FCS compared with 10% FCS. Furthermore, Runx2 expression was higher in response to 1% FCS compare to 10% FCS at 3 weeks. BSP expression was higher in response to 10% FCS at the early time points (72hrs and week 1) and subsequently, the pattern reversed and the expression was higher in the 1% FCS media at week 6. SPON1 expression was higher in the 10% FCS media at week 6.

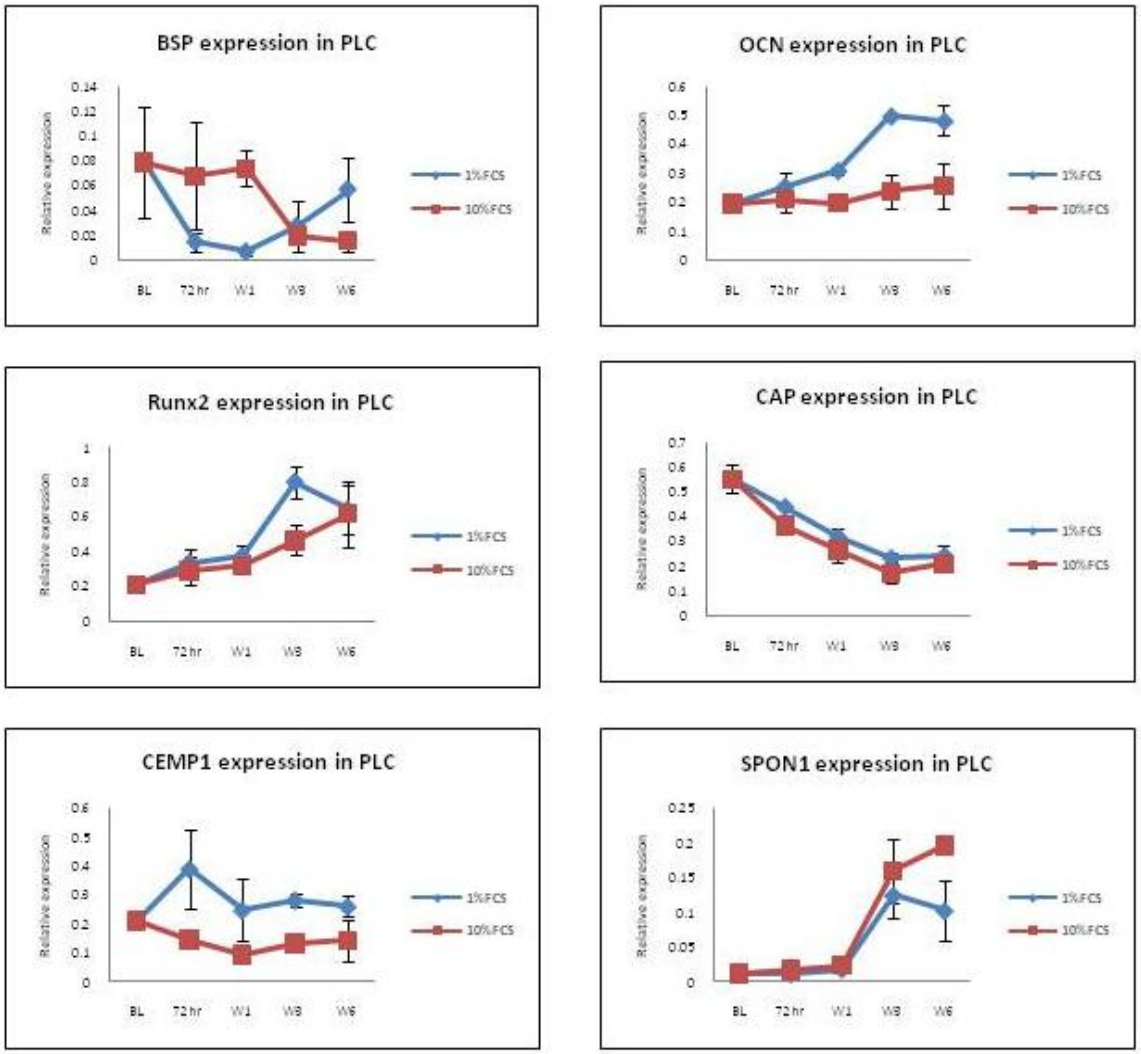


Figure 3.1 Real-time PCR results of PLC cultured in 10% FCS and 1% FCS. Values in the graph represent mean of triplicate ± SEM.

IGF-1 treatment alone up regulated the expression of BSP at week 1 and 6, and also SPON1 at 72hr (Figure 3.2). PDGF alone up-regulated only SPON1 at week 6, while the combination of IGF-1 and PDGF up-regulated BSP at all time points and also CAP expression by week 6. In summary, BSP was the most responsive gene and in particular the combination of IGF-1/PDGF treatment resulted in the most consistent increases in gene expression.

Treatment	Time points	Fold change in gene expression in PLC					
		BSP	OCN	Runx2	CAP	CEMP1	SPON1
IGF-1	72hr	–	–	–	–	–	3.4±2.2
	W1	4.0±1.7	–	–	–	–	–
	W3	–	–	–	–	–	–
	W6	3.2±1.7	–	–	–	–	–
PDGF	72hr	–	–	–	–	–	–
	W1	–	–	–	–	–	–
	W3	–	–	–	–	–	–
	W6	–	–	–	–	–	3.2±1.0
IGF-1/PDGF	72hr	3.3±1.3	–	–	–	–	–
	W1	4.0±2.2	–	–	–	–	–
	W3	7.1±2.7	–	–	–	–	–
	W6	2.4±1.5	–	–	2.4±1.4	–	–

Figure 3.2 The effect of growth factors on PLC gene expression.
Values represent the ratio of the change in the gene expression ± SEM (growth factor treatment/1% FCS negative control).

3.4.2 Regenerated Tissue Cells Gene Expression

Regenerated tissue cells (RTC) grown in 1%FCS and 10% FCS also had a similar gene expression profile at most time points as seen for PLC. However, notably, all of the markers (except CAP) displayed an increased expression at week 6 in response to 1% FCS (Figure 3.3).

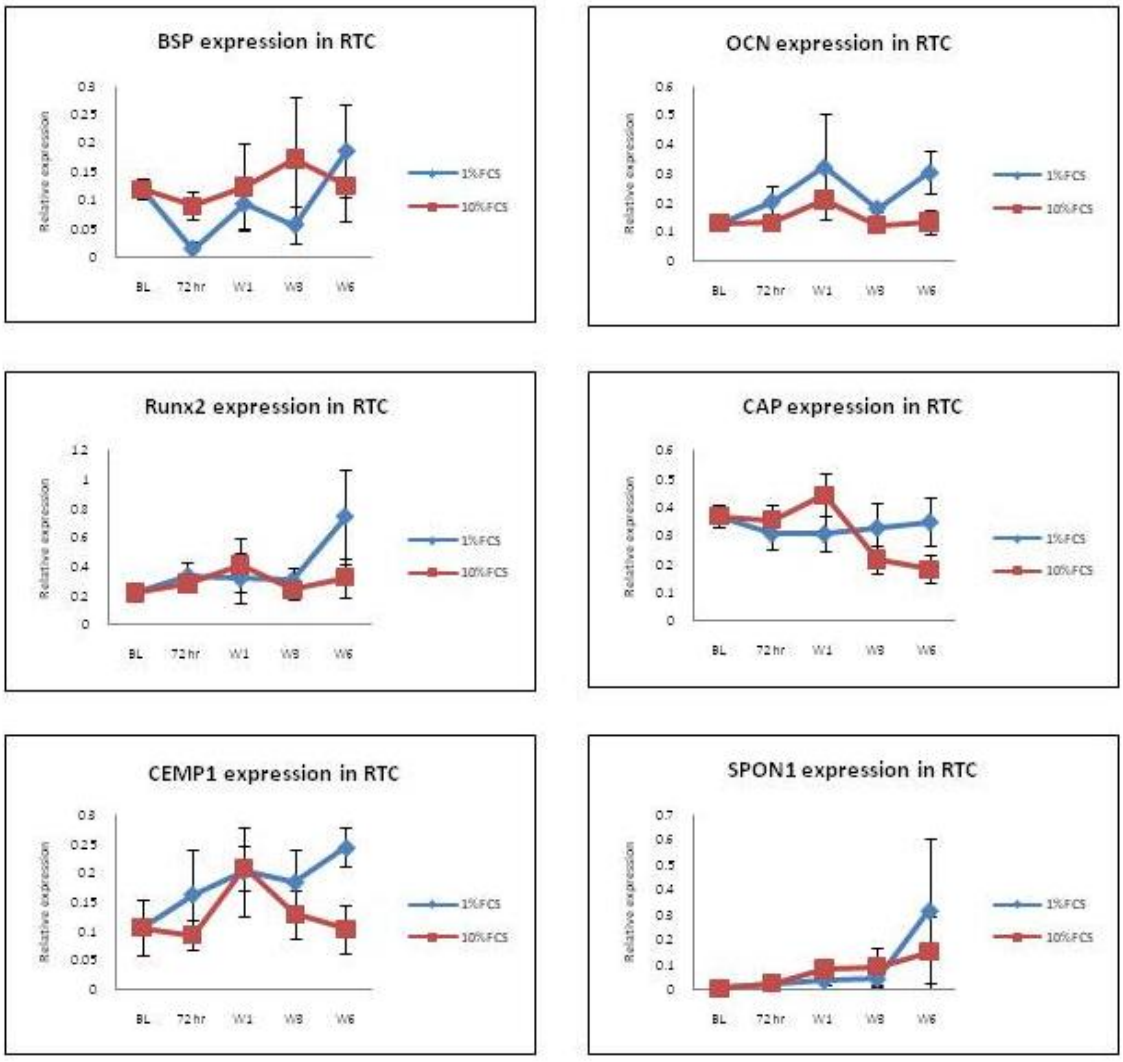


Figure 3.3 RT-PCR results of RTC cultured in 10% FCS and 1% FCS.
Values in the graph represent mean of triplicate ± SEM.

Treatment of RTC with the growth factors had only a minor effect with both IGF-1 and the IGF-1/PDGF combination treatment up-regulating BSP expression at week 1 (Figure 3.4). All growth factor treatments however up-regulated SPON1 expression at week 3.

Treatment	Time points	Fold change in gene expression in RTC					
		BSP	OCN	Runx2	CAP	CEMP1	SPON1
IGF-1	72hr	–	–	–	–	–	–
	W1	2.8±1.3	–	–	–	–	–
	W3	–	–	–	–	–	2.9±0.6
	W6	–	–	–	–	–	–
PDGF	72hr	–	–	–	–	–	–
	W1	–	–	–	–	–	–
	W3	–	–	–	–	–	2.5±0.3
	W6	–	–	–	–	–	–
IGF-1/PDGF	72hr	–	–	–	–	–	–
	W1	2.1±0.9	–	–	–	–	–
	W3	–	–	–	–	–	4.3±0.6
	W6	–	–	–	–	–	–

Figure 3.4 The effect of growth factors on RTC gene expression.
Values represent the ratio of the change in the gene expression ± SEM (growth factor treatment/1% FCS negative control).

3.4.3 Osteoblast Gene Expression

Osteoblasts grown in 1% FCS and 10% FCS showed similar pattern of gene expression at most time points with the exception of week 6 when 1% FCS induced higher gene expression for BSP, OCN, CEMP1 and Runx2 than 10%FCS (Figure 3.5). Interestingly, the expression pattern for OCN was almost identical to that seen for PLC. Treatment with IGF-1 up-regulated the expression of BSP, OCN and SPON1 at weeks 3 and 6 (Figure 3.6). PDGF also up-regulated BSP and OCN at weeks 3 and 6. However BSP down-regulation was detected at 72hr. Further, Runx2 was up-regulated at week 6. The combined growth factor treatment up-regulated BSP, OCN and SPON1 at the later time points (weeks 3 and 6). No changes in CAP expression were found, but PDGF modestly up-regulated Runx2 at week 6 and IGF-1/PDGF up-regulated CEMP1 at week 3.

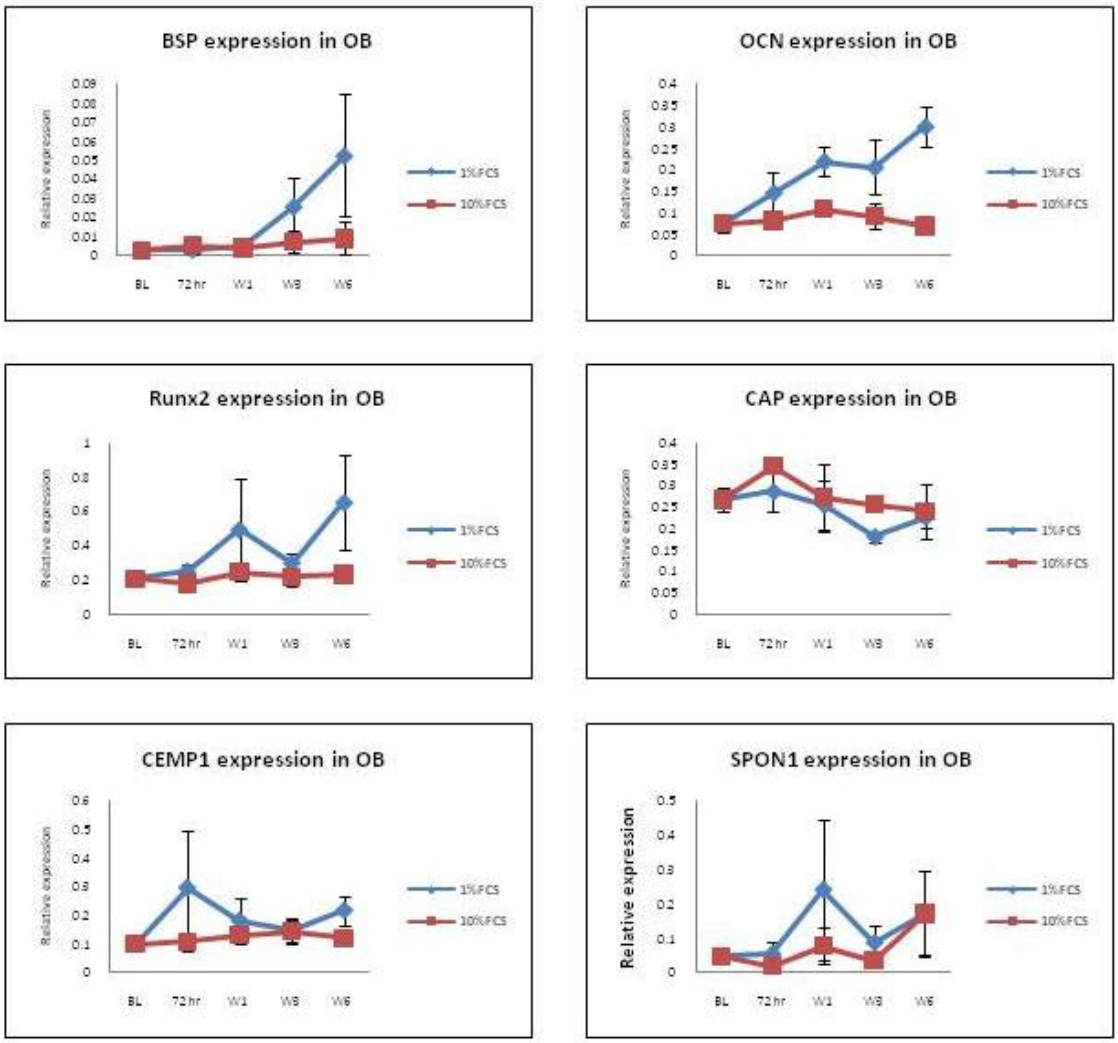


Figure 3.5 RT-PCR results of OB cultured in 10% FCS and 1% FCS.
Values in the graph represent mean of triplicate ± SEM.

Treatment	Time points	Fold change in gene expression in OB					
		BSP	OCN	Runx2	CAP	CEMP1	SPON1
IGF-1	72hr	–	–	–	–	–	–
	W1	–	–	–	–	–	–
	W3	2.9±0.2	2.2±0.4	–	–	–	3.3±1.5
	W6	3.6±1.2	3.3±1.6	–	–	–	2.9±0.7
PDGF	72hr	2.1±0.4	–	–	–	–	–
	W1	–	–	–	–	–	–
	W3	3.0±0.9	2.2±0.9	–	–	–	2.7±0.9
	W6	3.0±1.2	2.4±0.5	2.2±0.8	–	–	3.4±0.5
IGF-1/PDGF	72hr	–	–	–	–	–	–
	W1	–	–	–	–	–	2.2±0.4
	W3	3.3±0.2	2.5±1.1	–	–	2.4±0.5	5.1±2.1
	W6	–	–	–	–	–	3.2±1.3

Figure 3.6 The effect of growth factors on OB gene expression

Values represent the ratio of the change in the gene expression ± SEM (growth factor treatment/1% FCS negative control).

3.5 Discussion

Periodontal regeneration involves the reconstitution of lost periodontal tissues destroyed by periodontal disease. This requires the *de novo* formation of both alveolar bone and cementum, and the insertion of periodontal ligament fibers into the newly formed cementum and bone (Bartold et al., 2000a). It is generally accepted that regeneration depends on the availability of cells within the wound that have the capacity for growth and differentiation into new tissue (Melcher, 1976). In this respect, the chemotaxis, migration, attachment, differentiation and synthetic activity of the undifferentiated progenitor cells residing in the periodontal defect can be modulated by biologically active molecules that reside in the extracellular matrix. Several growth factors have been studied for their ability to promote periodontal regeneration. Both PDGF and IGF-1, either alone or in combination, have been extensively tested *in vitro*, *in vivo* and in clinical human studies, and have been shown to have the potential to promote periodontal wound healing and regeneration, reviewed in (Lee et al., 2010, Dereka et al., 2006).

This first objective of this study was to determine the effect of cell culture over time in 'standard' culture conditions. This is important, as the potential effect of any growth factor treatment is likely to be significantly influenced by the basal or endogenous level of expression of the genes of interest in the study. The effect of IGF-1 and PDGF, alone or in combination, on the expression of bone and cementum markers by cells derived from the periodontal ligament (PLC), regenerating periodontal tissue (RTC) and osteoblasts (OB) was then determined.

In the current study, the cells were grown in medium containing either 10% or 1% FCS concentration to elucidate the role of FCS concentration on cell differentiation. Generally, both concentrations appeared to have a similar effect on bone and cementum marker

expression over time. However, the lower level of nutrients present in 1% FCS was found to increase gene expression more than the 10%FCS. This is in agreement with similar published effects shown in relation to Runx2 expression in osteoblasts, whereby cells exposed to 10%FCS exhibited lower levels of Runx2 compared to a 0.2% negative control (Strayhorn et al., 1999).

In the previous chapter, it was shown that PLC, RTC and OB were a heterogeneous population containing cells able to differentiate and produce a mineralized matrix after three and six weeks of osteogenic treatment, as determined by histochemical assays and RT-PCR. In this chapter, real-time PCR was also carried out to investigate the effect of growth factor (IGF-1, PDGF and combination of IGF-1/PDGF) treatment on osteogenic and cementogenic marker expression over a six week period in the same three periodontal cell types (PLC, OB and RTC) that were investigated in the previous chapter.

The differentiation effect of IGF-1 and PDGF alone or in combination on periodontal cells has been investigated previously, and both growth factors were found to have a differentiation effect on PLC and RTC in short term (24hrs) studies using standard PCR (Ivanovski, 2001a). However, conventional PCR does not have good quantitative sensitivity. Therefore, in the current study, we utilized real-time PCR. Furthermore, the study was carried out for a longer (six week) period.

The results showed that the growth factors were able to induce OBs to express higher levels of bone/cementum markers, suggesting a differentiation into a mineralized tissue forming cell phenotype. The three growth factors treatments increased the expression of BSP, OCN and SPON1 at the late stages of treatment. BSP expression has been associated with the late stages of osteoblast differentiation and the early stage of matrix

mineralization (Chen et al., 1992a), and OCN is produced by mature osteoblasts during the mineralization phase and is only marginally detectable during the early phases of proliferation and matrix maturation. It is found to bind to hydroxyapatite and regulate crystal growth (Hauschka, 1985). IGF-1 has been found to induce the differentiation of undifferentiated mesenchymal cells (Koch et al., 2005) as well as the differentiation of mature cells of the osteoblast lineages (Canalis, 1980, Conover and Kiefer, 1993, Jonsson et al., 1993, Hock et al., 1988). The role of IGF-1 in OB differentiation and increased expression of BSP has been shown in several *in vitro* studies (Strayhorn et al., 1999, Nakayama et al., 2006, Ogata, 2008). IGF-1 stimulates BSP transcription by targeting the fibroblast growth factor 2 response element and homeodomain protein-binding site in the proximal promoter of the BSP gene through the tyrosine kinase, Ras/MAPK and phosphatidylinositol 3-kinase/Akt pathways (Nakayama et al., 2006). Since OCN is known to be produced by mature OB and to regulate the calcification of bone tissue, it's possible that both IGF-1 and PDGF may stimulate OB's maturation. The results of the present study are consistent with Jonsson et al who investigated the effect of IGF-1 on human OB's (Jonsson et al., 1993). Tanaka et al found that the administration of the combination of IGF and PDGF in old rats enhanced OCN expression, but not when each growth factor was administered alone (Tanaka et al., 2002). Furthermore, Strayhorn *et al* (1999) found that PDGF-BB at the concentration of 10 and 20 ng/ml had no effect on the differentiation of a mouse osteoprogenitor cell line (Strayhorn et al., 1999). In the current study, human alveolar bone derived primary cells were used, which are likely to represent a more heterogeneous cell population than commercially available cell lines.

It is generally accepted that progenitor cells are located in the periodontal ligament space, and have the ability to differentiate and replace lost periodontal tissues (Bartold et al., 2006a). As mentioned earlier, IGF-1 has been shown to have a differentiation effect on

undifferentiated mesenchymal cells. Moreover, BSP mRNA expression was noted in PLC and RTC, but not in gingival fibroblasts (Ivanovski, 2001a). In our study, IGF-1 but not PDGF, was able to up-regulate the expression of BSP in PLC. Previous studies have shown that PDGF treatment for 8 days blocked the gene expression of OCN and OPN in mesenchymal cells (Strayhorn et al., 1999). Furthermore, the addition of 10ng/ml of PDGF and 10ng/ml of IGF-1 to mesenchymal cells for 8 days blocked the differentiation effect noted when cells were treated with IGF-1 alone (Strayhorn et al., 1999). In the present study, the addition of PDGF did not block the effect of IGF-1, and PLC's were still able to highly express BSP in the presence of the combination of PDGF and IGF-1. These results may be explained by the use of primary cell cultures containing a diversity of cell phenotypes at different stages of their differentiation, rather than a commercial cell line as used by Strayhorn et al. It should be noted that the use of primary cell lines holds greater clinical relevance as periodontal regeneration strategies generally rely on locally available progenitors for the reconstitution of the lost attachment apparatus.

Runx2 has been identified as an osteoblast-specific transcription factor and more specifically, controls the differentiation of osteoblasts and the expression of extracellular matrix protein genes. It is also thought to maintain osteoblasts in an immature stage of differentiation. Subsequently, it has been suggested that Runx2 expression has to be down-regulated for differentiation into mature osteoblasts to occur (Komori, 2010). In the current study, Runx2 was up regulated in OB when treated with PDGF for six weeks, suggesting a role for PDGF in maintaining cells in the early stages of OB differentiation. This finding is in contrast with the previous findings by Strayhorn et al, who found that PDGF blocked the expression of Runx2; however, they used a commercially available cell line and the cells were treated for only 8 days (Strayhorn et al., 1999).

As mentioned above, cementum formation is critical for appropriate maturation of the periodontium because it invests and securely attaches the periodontal ligament fibers to the root surface. However, one major obstacle for identifying cementoblasts required for this new cementum formation is the lack of a suitable cementum marker (Bosshardt, 2005). Cementum attachment protein (CAP) and cementum protein (CP-23 or CEMP1) have been identified as putative cementum specific proteins, and have therefore been proposed as markers for both cementum producing cells and progenitors of mineralized tissue forming cells within the periodontal ligament. CAP and CEMP1 antibodies have been localized in cementum, cementoblasts, cells lining alveolar bone endosteal spaces, dental follicle derived cells, and perivascular cells in the PDL (Arzate et al., 2002, Arzate et al., 1992, Bar-Kana et al., 1998, Liu et al., 1997, Nyman et al., 1982a, Saito et al., 2001). In the present study, the combination of IGF-1/PDGF was the only treatment able to up-regulate CAP and CEMP1 in PLC and OB, suggesting the presence of a progenitor cells within these primary cell cultures with the ability to differentiate into cementoblasts. PDGF and IGF-1 have been shown to act synergistically in promoting periodontal bone formation (Giannobile et al., 1996, Lynch et al., 1989) and bone repair in a cortical defect model (Lynch et al., 1994).

Recently, F-Spondin (SPON1) was reported to be specific to cementoblasts and promote cementoblastic differentiation (Kitagawa et al., 2006). Moreover, F-Spondin was found to be expressed specifically in dental follicle cells during tooth germ development (Nishida et al., 2007). Temporal and spatial expression analysis during tooth germ development suggested that F-Spondin plays a role in the early stages of PDL formation (Nishida et al., 2007). In the present study, SPON1 was found to be up-regulated by all of the treatments under investigation. The pattern of expression and the effect of the treatment were different from one cell to another, but generally up-regulated at later time points. Further

studies are needed to elucidate the localization and role of F-Spondin in order to determine more clearly whether SPON1 is unique to cementum and cementogenesis.

Regenerated tissue derived cells have been studied to further understand the cellular and molecular processes within the regenerating periodontal defect. *In vivo* immunohistochemical studies have shown that osteogenic and cementogenic proteins, including BSP and OCN, as well as CAP, are localized within regenerated periodontal defects (Amar et al., 1997, Ivanovski et al., 2000). In fact, fibroblasts derived from regenerated periodontal defects were found to be a heterogenous population which may contain cells with characteristics consistent with their ability to facilitate periodontal regeneration (Rowe et al. 1996; Wakabayashi et al. 1996; Wakabayashi et al. 1997; Kuru et al. 1999; Ivanovski et al. 2000; Ivanovski et al. 2001; Parker et al. 2001; Ivanovski et al. 2001a). More recently, Lin et al found cells with characteristics of putative mesenchymal stem cells in regenerating periodontal tissues, and that the level of mineralization of RTC was lower than that of periodontal ligament stem cells and bone marrow stromal stem cells (Lin et al., 2008b). In the current study, RTC also responded to the treatments with the growth factors to a lesser degree compared to that seen for PLC and OB. However, some up-regulation of BSP and SPON1 was found, which suggests that this cell population may contains undifferentiated mesenchymal cells with osteogenic and possibly cementogenic potential.

3.6 Conclusion

In summary, the results from this study show that IGF-1 and PDGF promoted the differentiation of periodontal cells by increasing the expression of both BSP and OCN. The most responsive cells were OB followed by PLC, while the RTC cells were least

responsive. IGF-1 treatment, but not PDGF, also up-regulated BSP expression in PLC cells. Furthermore, CAP and CEMP1 expression was up-regulated in PLC and OB respectively after treatment with IGF-1/PDGF, while SPON1 expression was up-regulated in almost all cells and all treatments.

In conclusion, the use of growth factors, such as IGF-1 and PDGF, to enhance cellular differentiation along a mineralized tissue forming phenotype represents a promising approach in the field of periodontal tissue engineering. Additional investigations in combination with other growth factors will be needed to establish the efficacy and utility of growth factor treatments in differentiating progenitor cells along a mineralized forming phenotype.

4.0 Chapter Four:

Cellular Heterogeneity of Periodontal Cells

4.1 Abstract

Back ground: The periodontium is a unique and complex structure comprised of two hard (cementum and bone) and two soft (gingiva and periodontal ligament) tissues. Previous reports have shown that significant heterogeneity exists between (1) cells derived from the same tissue but from different patients (inter-population heterogeneity) and (2) cells derived from the same primary cell culture (intra-population heterogeneity).

Objective: To compare the mineralization potential and expression of bone and cementum genes in cells from the same source in different subjects and to subsequently establish and characterise single cell derived clones from periodontal ligament, and regenerating periodontal tissues.

Method: Two primary periodontal ligament cells (PLC) and two primary regenerating periodontal defect associated cells (RTC) were compared for mineralization formation and bone and cementum markers expression. Three clones from each cell type were established by serial dilution. The primary cells (PLC1 and 2, and RTC1 and 2) and clones (PLCI,PLCII,PLCIII and RTCI,RTCII,RTCIII) were cultured in both mineralization media, consisting of 10^{-7} M ascorbic acid,10mM β glycerophosphate and 50ug/ml dexamethasone, and in control without treatment. Alizarin red S, von Kossa, calcium release and hydroxyapatite formation were tested over 6 weeks in the four primary cell lines PLC 1 & 2 and RTC 1 & 2. For both the primary cell lines and the clones, Real Time-PCR analysis was carried out after 72hr, one, three and six weeks to

determine the expression of the bone and cementum markers, BSP, OCN, Runx2, CAP, CEMP1, and SPON1.

Results: A heterogeneous response in the expression of bone and cementum markers to mineralization treatment was found in the primary cell cultures and clones. Both PLC2 and RTC1 had more mineralization formation than their counterparts PLC1 and RTC2 as demonstrated by histochemical assays. PLC2 had significantly more SPON1 expression and less down-regulation of Runx2, while PLC1 expressed more OCN and CEMP1. RTC1 had greater BSP, OCN and SPON1 expression. The expression of BSP and SPON1 showed the closest correlation to mineralization formation. Among the PLC clones, PLCI and PLCIII showed up-regulation of most markers tested at the early time points of 72hr. Among the RTC clones, RTCIII was found to express more bone and cementum markers than the other two RTC clones.

Conclusion: The results suggest that the periodontal ligament and regenerating periodontal tissue contain heterogeneous cell populations with some cells able to differentiate along a mineralized tissue phenotype as observed by mineralization formation and up-regulation of bone and cementum markers. The expression of mineralized tissue forming markers appears to be greatly influenced by the inherent potential of a given cell line, with only moderate effects exerted by the treatment modality (mineralised or conrol media) or treatment time.

4.2 Introduction

The ideal outcome of periodontal treatment is to regenerate tissues affected by disease to their original state. Periodontal regeneration can be defined as "the reproduction or reconstruction of lost or injured tissue so that the form and function of the lost structures are restored" (Bosshardt and Sculean, 2009b). Recent therapeutic approaches to achieving periodontal regeneration have sought to mimic the natural *in vivo* process of tissue repair by promoting the repopulation of the periodontal defect with progenitor cells capable of differentiating into tissues comprising the periodontal attachment apparatus, namely, bone, periodontal ligament and cementum. Such progenitor cells could be either locally derived or recruited to the periodontal defect whereby they subsequently differentiate into periodontal fibroblasts, cementoblasts and osteoblasts or, alternatively, can be surgically seeded into the periodontal defect (Lin et al., 2008a). Autologous cells represent the material of choice for this approach and oral tissues are an accessible source of cells avoiding the immunologic complications of allogenic or xenogenic materials. Indeed, attempts at implanting periodontal cells into periodontal defects for the purpose of promoting periodontal regeneration have been carried out with promising results (Lang et al., 1998a, Liu et al., 2008, Sonoyama et al., 2006, Seo et al., 2005, Akizuki et al., 2005, Iwata et al., 2009, Park et al., 2011).

Following implantation of cells into a periodontal defect, a key element required for successful periodontal regeneration is the ability of those cells to first proliferate and then subsequently differentiate and acquire the appropriate functional cellular phenotype. Using cloning techniques to study and characterise various cell types, a large number of cells of differing phenotype have been isolated from the periodontal ligament, gingiva, regenerating periodontal tissue and cementum (Ivanovski, 2001a,

Kitagawa et al., 2006a, Hou and Yaeger, 1993, Hakkinen and Larjava, 1992, Fujii et al., 2006, Fujita et al., 2005). More recently, alveolar bone, periodontal ligament, regenerated periodontal defect and gingiva, have all been shown to contain stem cells able to differentiate into different cell types when stimulated appropriately (Seo et al., 2004, Fournier et al., 2010).

In vitro analysis of the differentiation potential of these various periodontal tissue derived cells is important in identifying the appropriate cell type and/or cell treatment combination that would be most suitable for promoting periodontal regeneration, based on the cells ability to differentiate towards an appropriate phenotype. In this regard, cementogenesis is a key process required for successful periodontal regeneration. If the regenerated periodontal tissue is to be fully functional, production of new cementum on the tooth surface is essential to allow subsequent insertion of the periodontal ligament fibres and thereby provide attachment to alveolar bone. Therefore, the capacity of cells repopulating the periodontal defect to achieve a cementoblastic phenotype is of significant importance.

The previous chapters described the bone/cementum phenotypic characteristics of various periodontal cell types. However, the cells used in these studies were shown to be heterogeneous in nature. This is likely due to the fact that the cells were derived from whole tissue explants. Indeed, it has been documented that primary cells derived from periodontal tissues exhibit inter- and intra-population heterogeneity (Giannopoulou and Cimasoni, 1996, Hou and Yaeger, 1993, Irwin et al., 1994, Kuru et al., 1998, Ivanovski, 2001a, Fujita et al., 2005). However, if these cells are to provide reproducible *in vivo* clinical regeneration results, it is essential that their demonstrated bone/cementum

phenotypic potential is consistent from one patient source to the next. Prior to initiating more costly *in vivo* studies, the aim of this study was to describe the inherent heterogeneity in whole tissue derived periodontal ligament and regenerating tissue fibroblasts. Additionally, this study sought to identify a periodontal ligament or regenerating tissue fibroblast single cell derived clone which would consistently differentiate towards a known cementoblast / osteoblast phenotype which could then be subsequently employed for future *in vivo* periodontal regeneration studies in our laboratory.

4.3 Methodology

4.3.1 Cell Isolation and Culture of PLC and RTC

Cell culture procedures were carried out as described in chapter 1. Two primary cell cultures for PLC (1&2) and RTC (1&2), derived from explants obtained from different patients, were compared here to examine the heterogeneity between cells derived from the same tissue type (inter-population heterogeneity).

4.3.2 Transfection and Establishment of Clones

Single cell clones were established from previously transfected primary PLC and RTC primary cell cultures. Briefly, cultures of RTC and PLC were transfected using the putative transforming genes E6 and E7 from the human papilloma virus type 16 prototype, which were contained in the retrovirus vector, pLXSN. The amphotropic virus LXSN16E6E7 was produced by the NIH 3T3-derived mouse fibroblast packaging line PA317, which had been selected in a medium containing G418 (1 mg/ml). Viruses

produced from clonal lines of PA317 were used to infect the two primary fibroblast cell lines (PLC and RTC), which were subsequently selected in standard culture medium containing G418 (1 mg/ml) for 7-10 days (Ivanovski, 2001a).

Transfected cells were retrieved from liquid nitrogen and clones were established by seeding the primary cells into 24-well plates using a limiting dilution of 0.3-0.4 cells/well. Clone growth was assessed microscopically on a daily basis to assure that each clone had originated from a single cell. Following the establishment of the clones, six clonal populations (three from each cell type) were randomly selected for further assessment. All clones were derived from a single RTC and PLC primary cell line.

4.3.3 Mineralization Treatment and Analysis

The primary cells were cultured for 6 weeks in standard culture medium (DMEM, 10%FCS, penicillin/streptomycin, non-essential amino acids) supplemented with 10% FCS, and mineralization media (MM) containing standard media, 50ug/ml ascorbic acid, 10^{-7} M Dexamethasone, and 10mM β-Glycerophosphate. The clones were cultured as described above, with the addition of a group where the standard media included 1% rather than 10% FCS. Q-PCR was performed at baseline (BL), 72hr, one week, three weeks and six weeks. Alizarin red S, von Kossa, Ca^{+2} release and OsteoImage assays were carried out at four time-points (72hr, week 1, week 3 and week 6). These procedures were carried out as described previously in chapters 2.

4.4 Results

4.4.1 von Kossa Staining

Von Kossa staining at 72hr and week1 was very weak. Generally more von Kossa staining was detected in PLC compared to RTC cultures. Furthermore, after 3 weeks of mineralization treatment, no significant difference was noted between the individual primary cell cultures 1 & 2 for both cell types. After six weeks however, PLC2 stained significantly darker than that seen for PLC1. A similar result was also seen for the RTC cells. However, unfortunately the comparison between RTC1 and RTC2 was not ideal as the RTC2 cells had contracted to form a pellet.

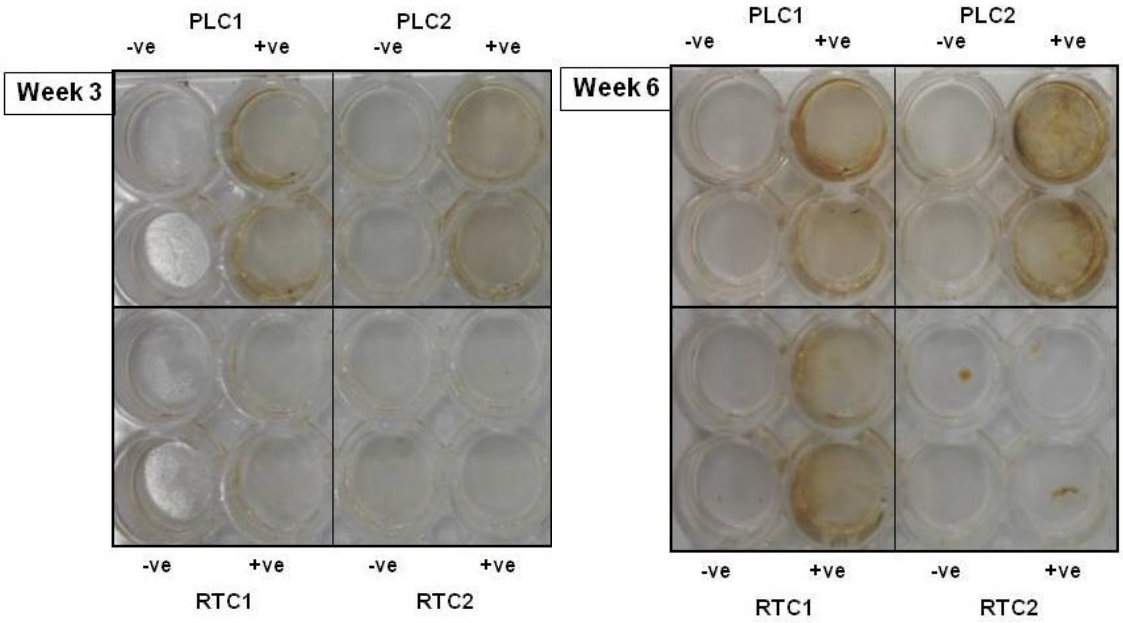


Figure 4.1 Von Kossa staining of PLC and RTC.
Primary cells were cultured in MM (+ve) and control media (-ve) for 3 weeks and 6 weeks.

4.4.2 Alizarin Red S Staining

Alizarin red S staining at 72hr and week 1 was very weak. After three weeks, the staining was generally darker in wells treated with mineralization media (MM) than those without treatment. The same was true after six weeks, but a clear difference was found between different cells lines derived from the same tissue type i.e. PLC2 staining was slightly darker than PLC1, and RTC1 staining was more intense than in RTC2 (Figure 4.2).

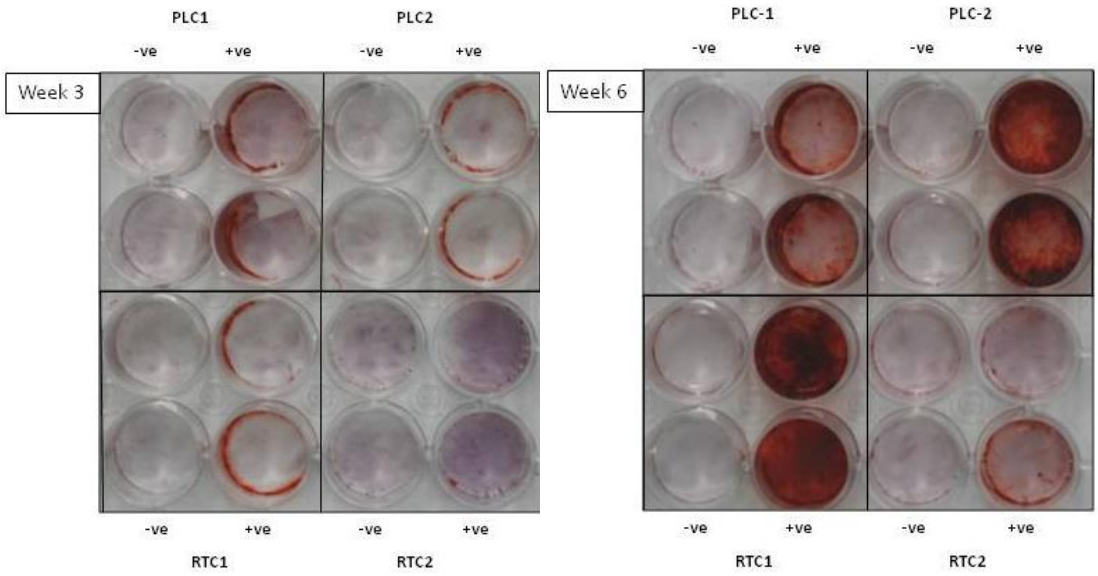


Figure 4.2 Alizarin red S staining in PLC and RTC.
Cells were cultured with (+ve) and without (-ve) mineralization treatment at weeks 3 and 6.

Culturing cells in 10% FCS without the addition of any differentiation factors significantly increased Alizarin red S absorbance readings after six weeks of culture. However, there was no difference between the two cell subgroups i.e. 1 & 2 for either PLC or RTC (Figure 4.3).

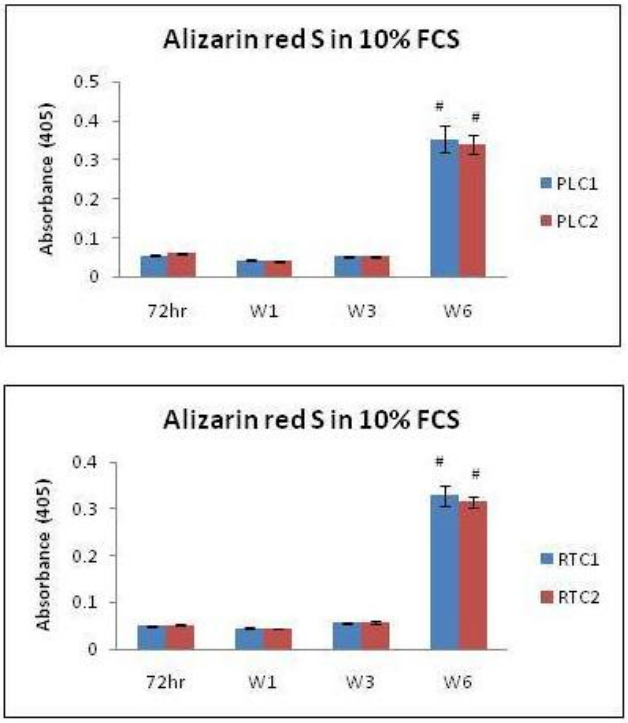


Figure 4.3 Quantitative alizarin red S staining in control media.
(#) Statistically significant compared to earlier time points ($p<0.001$). Values represent mean of quadruplicate measurements ± SEM.

Following culture in mineralization media, Alizarin red S staining increased in both PLC cells over the 6 week study period. Comparison of the two cell populations (1 & 2) for each cell type showed that PLC1 had higher levels of staining at week three comparing to PLC2, although by week 6 this difference was much reduced. The RTC response to mineralization treatment showed even more significant heterogeneity. RTC1 demonstrated significantly increased levels of staining over the 6 week period of

treatment. In contrast to RTC1, RTC2 did not respond significantly at any time-point to mineralization treatment over the 6 week period of culture.

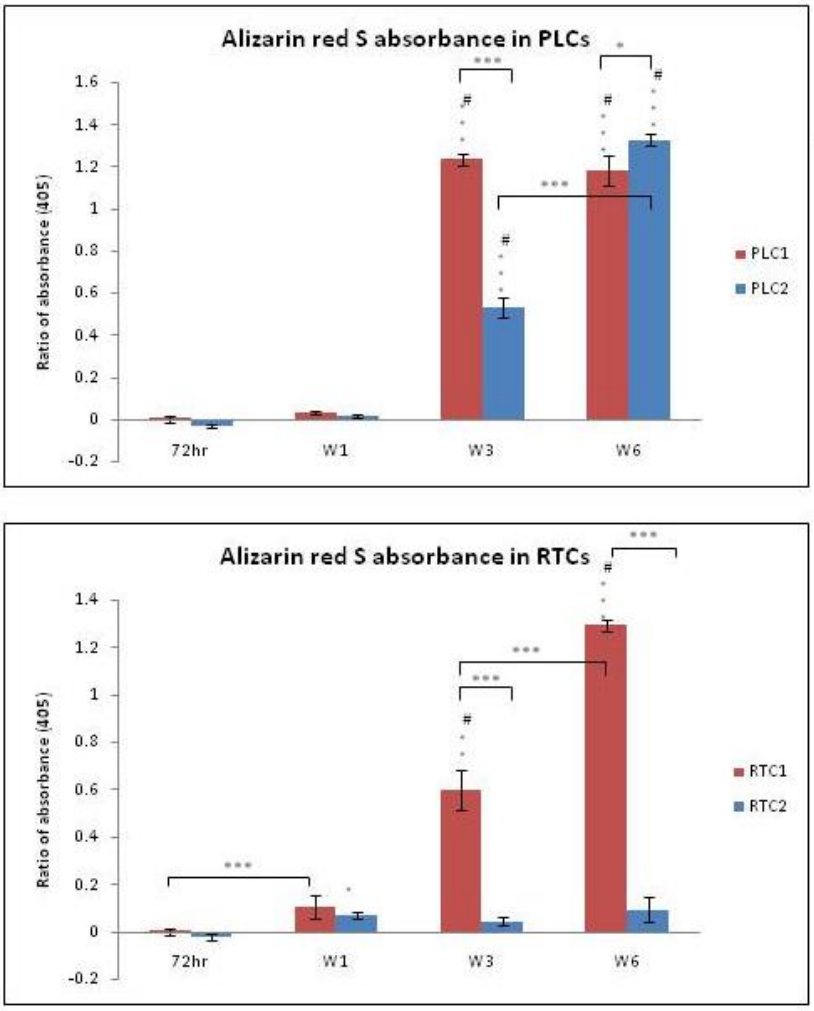


Figure 4.4 Quantitative alizarin red S staining in mineralization media.

The results are presented as the fold change compared to negative control (10% FCS). (#) Statistically significant compared to earlier time points ($p < 0.001$). (*) Represents significance between cells. $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Values represent mean of quadruplicate measurements ±SEM.

4.4.3 Calcium Release

The levels of calcium released into the culture supernatant were very similar for PLC and RTC cells when cultured in 10% FCS without the addition of mineralization media. Although the levels of calcium released from PLC1 and RTC1 were lower at the early time-points of 72hrs and 1 week compared to their counterparts PLC2 and RTC2, by three weeks all four cell types were shown to release very similar levels of calcium (Figure 4.5).

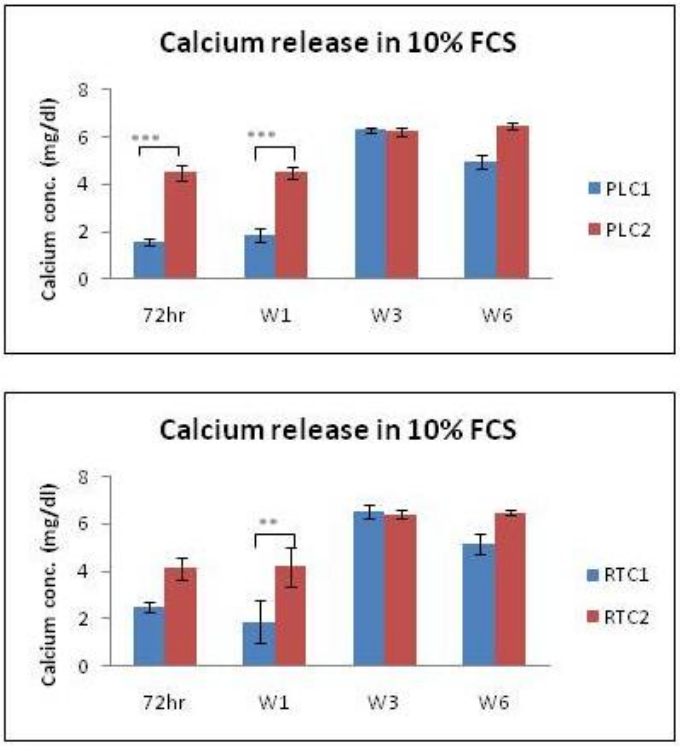


Figure 4.5 Calcium released from PLC and RTC cultured in control media.
(*) Represents a significant difference between cells for each time point @ $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Bars represent mean of quadruplicate measurements ± SEM.

Following treatment with mineralization media, both PLC (1 & 2) and RTC (1 & 2) showed a significant decrease in calcium release between weeks 1 and 3. This corresponds with a significant increase in extracellular matrix mineralization as demonstrated previously with Alizarin red S (Figure 4.2). With respect to specific cell type, PLC 1 and 2 both demonstrated a similar response over time although the magnitude of the response was greater for PLC2 at all time-points. Similarly, and again reflecting the pattern of response to mineralization media seen previously, calcium release from RTC 1 continued to decrease significantly by week 6 while RTC 2 was generally unresponsive at all time-points (Figure 4.6).

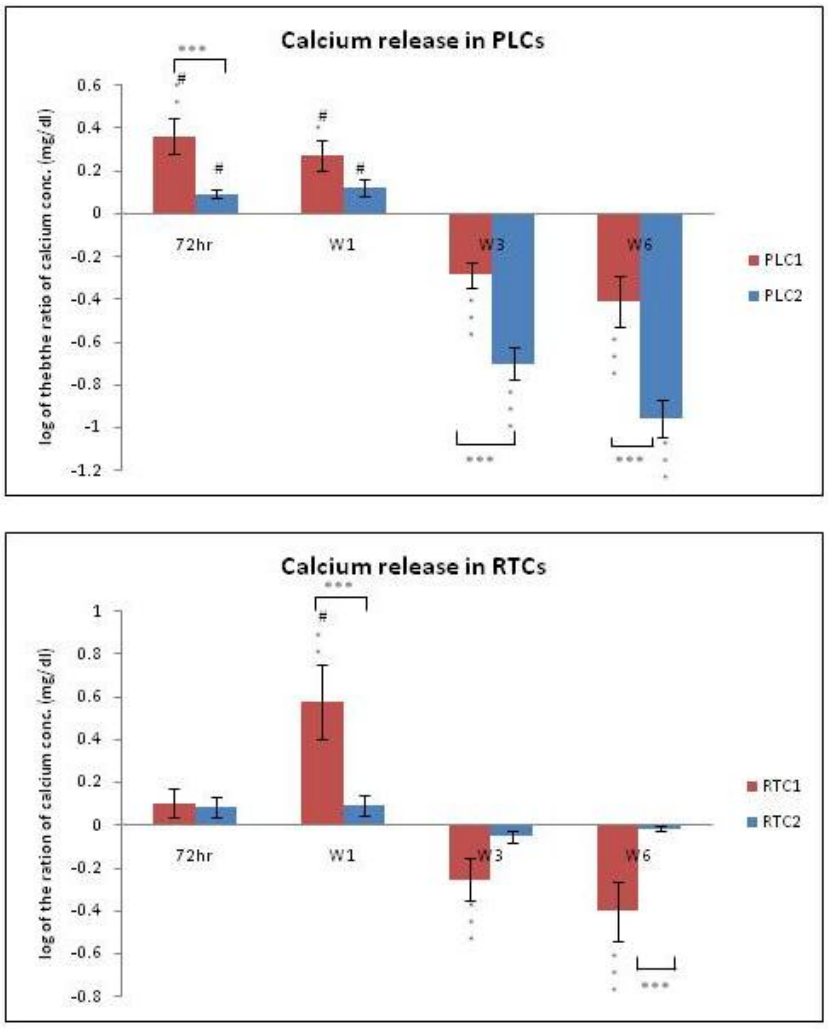


Figure 4.6 Calcium released from PLC and RTC cells cultured in mineralization media. The results are presented as fold changes compared to a negative control (10% FCS). (#) Statistically significant compared to earlier time points ($p < 0.001$). (*) Represents significance between cells for each time point. @ $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Bars represent mean of quadruplicate measurements ± SEM.

4.4.4 Hydroxyapatite Formation

Under control media (10% FCS) conditions, hydroxyapatite production was relatively uniform over the entire 6 weeks in both PLC 1 & 2. Hydroxyapatite production in RTC however increased consistently over the first three weeks. This pattern was the same in both RTC1 and 2, although the magnitude of hydroxyapatite production was consistently higher in RTC1 (Figure 4.7).

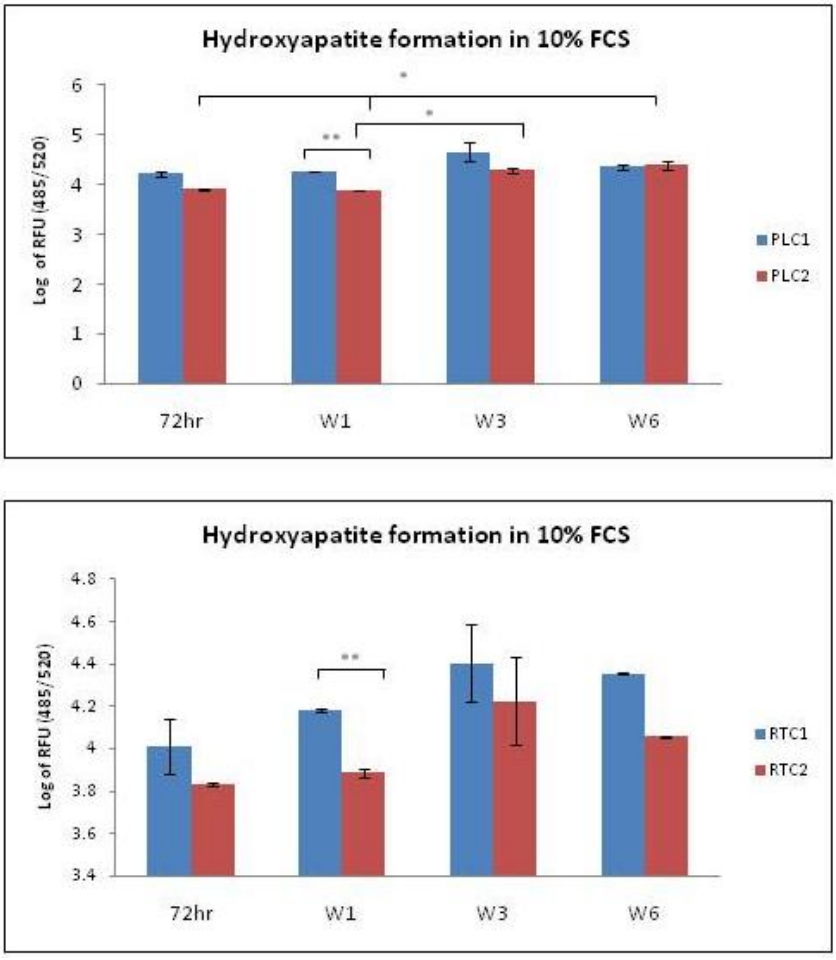


Figure 4.7 Hydroxyapatite formation in PLC and RTC cultured in control media.
(*) Represents significance between time points. $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Bars represent mean of quadruplicate measurements ± SEM.

Cell culture in the presence of mineralization media decreased the production of hydroxyapatite over the first week in both PLC and RTC cells. By week three of treatment, both cell types had significantly increased the production of hydroxyapatite. Whilst this pattern of response was very similar in all cell groups, PLC1 and RTC1 had a consistently larger magnitude change compared to PLC2 and RTC2 respectively (Figure 4.8).

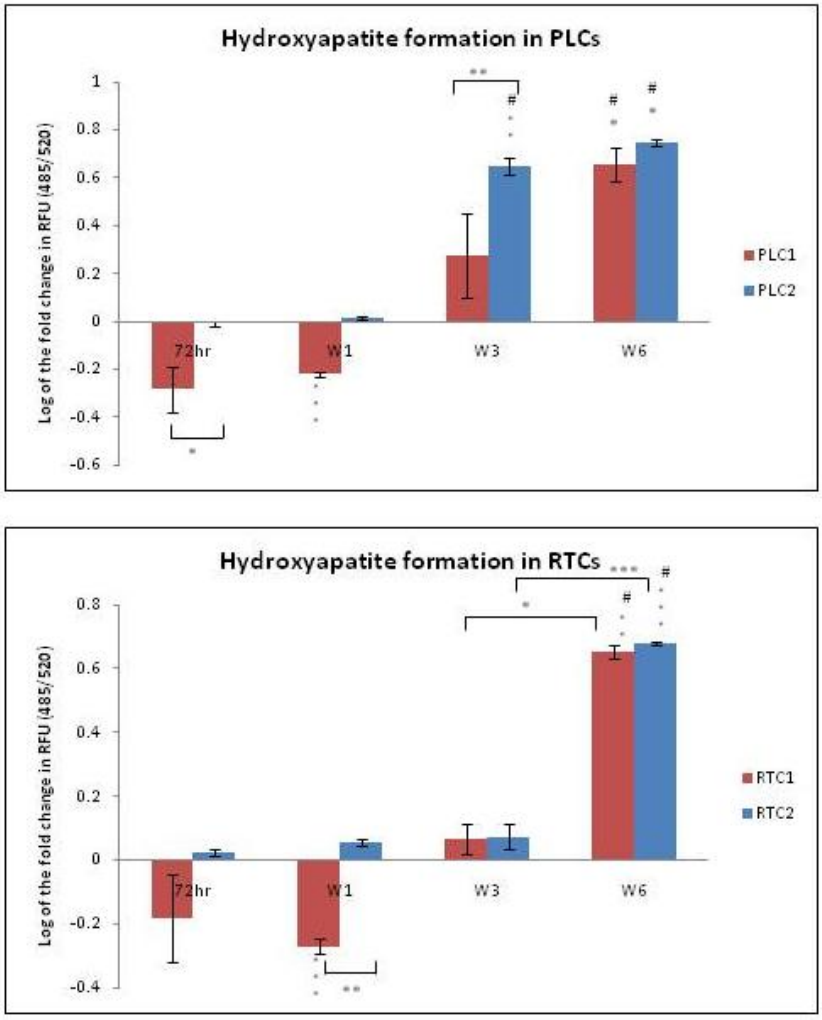


Figure 4.8 Hydroxyapatite formation in PLC and RTC cultured in mineralization media. The results are presented as the fold change compared to the negative control (10% FCS) data. (#) Statistically significant compared to earlier time points ($p < 0.001$). (*) Represents significance between time points. $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Bars represent mean of quadruplicate measurements ± SEM.

4.4.5 Real Time-PCR (Primary cells)

BSP: Cell culture in MM increased the expression of BSP significantly by 6 weeks in both PLC1 and RTC1 when compared to growth in control (10% FCS) media. BSP expression in PLC2 was also increased by 6 weeks, but did not quite reach significance while BSP expression in RTC2 was down-regulated compared to expression in the control media (Figure 4.9).

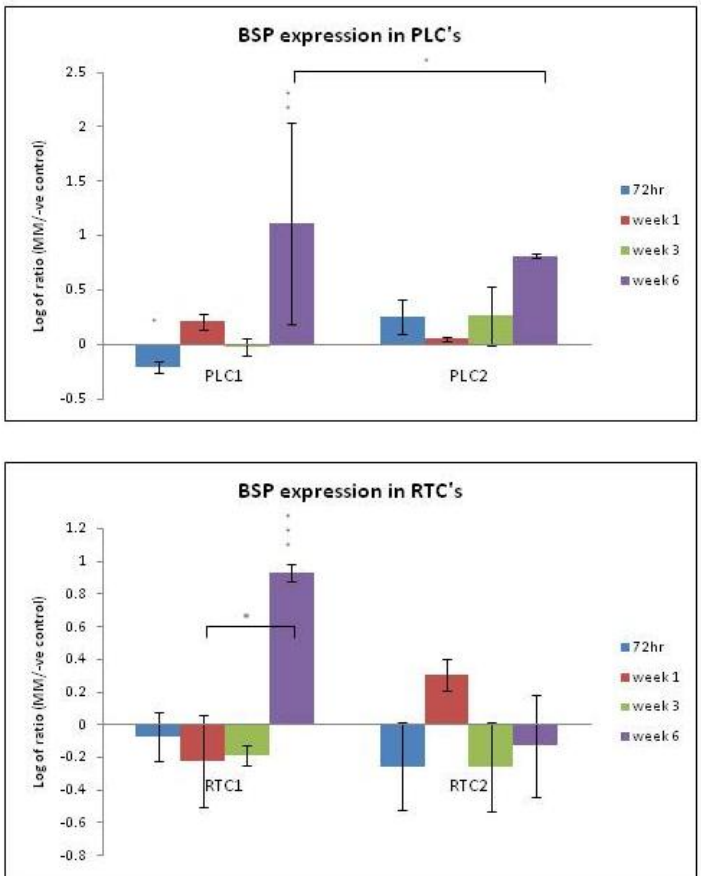


Figure 4.9 BSP gene expression in PLC and RTC.

Fold change differences over 6 weeks (mineralization media/control media).(*) Represents a significant difference compared to the control or between time points, $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Values and bars represent mean of quadruplicate measurements ± SEM.

OCN: Compared to growth in the control (10% FCS) media, OCN gene expression in PLC 1 & 2 after 6 weeks MM treatment were markedly different. Expression in PLC 1 was up-regulated as expected based on the earlier histochemical assays of mineralization, while OCN expression in PLC 2 was down-regulated. Similarly OCN expression in RTC2 was also down-regulated at late time points (Figure 4.10).

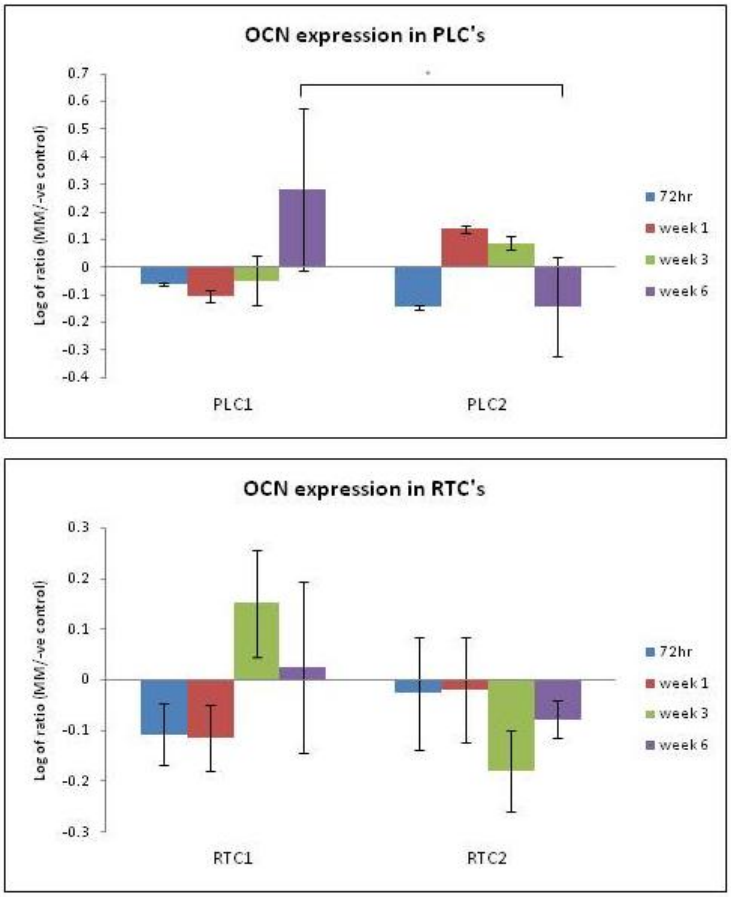


Figure 4.10 OCN gene expression in PLC and RTC.
Fold change differences in OCN gene expression over 6 weeks culture (mineralization media/control media). (*) Represents a significant difference compared to the control or between time points, $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Values and bars represent mean of quadruplicate measurements ± SEM.

Runx2: While expression of the transcription factor RUNX2 was significantly down-regulated in both PLC 1 & 2 for the first three weeks following treatment with MM compared to growth in control (10% FCS) media, there was a significant difference in the temporal patterns of expression. Down-regulation in PLC1 increased over this time while it decreased in PLC2. However by six weeks treatment in MM, Runx2 expression was up-regulated in both PLC 1 & 2. Runx2 expression was down-regulated in all RTC cells which was not statistically significant (Figure 4.11).

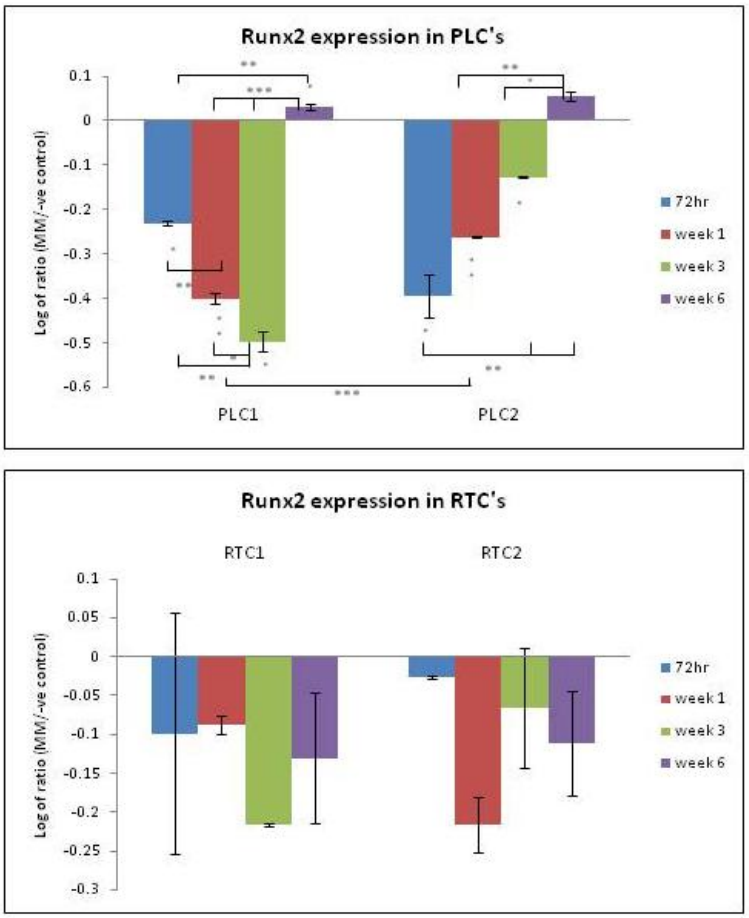


Figure 4.11 Runx2 gene expression in PLC and RTC.

Fold change differences in Runx2 gene expression over 6 weeks culture (mineralization media/control media). (*) Represents a significant difference compared to the control or between time points, $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Values and bars represent mean of quadruplicate measurements ± SEM.

CAP: After six weeks of treatment with MM, CAP expression, which was initially up-regulated at the early time-points (72hr and 1week) in all cell cultures, had decreased significantly compared to CAP expression in the control (10% FCS) media (Figure 4.12).

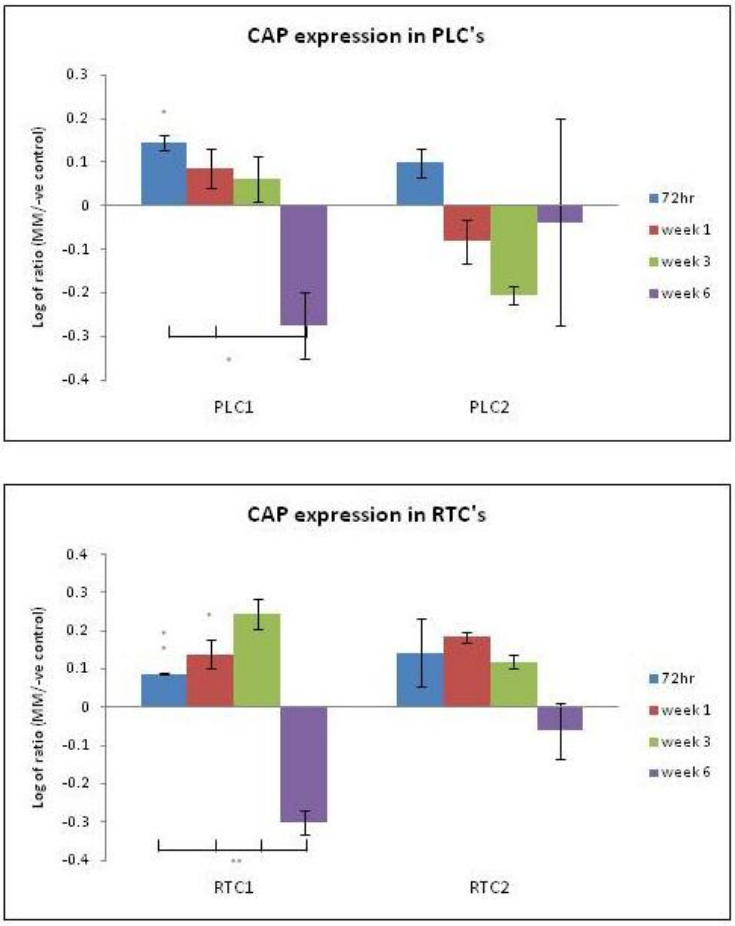


Figure 4.12 CAP gene expression in PLC and RTC.

Fold change differences in CAP gene expression over 6 weeks culture (mineralization media/control media). (*) Represents a significant difference compared to the control or between time points, $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Values and bars represent mean of quadruplicate measurements ± SEM.

CEMP1: Treating PLC1 with MM down-regulated the expression of CEMP1 significantly at week 3, and in PLC2 at week 1. Moreover, at week 6, PLC1 had more CEMP1 expression than PLC2. A general CEMP1 down-regulation was noted in all RTC cells, although this was not statistically significant (Figure 4.13).

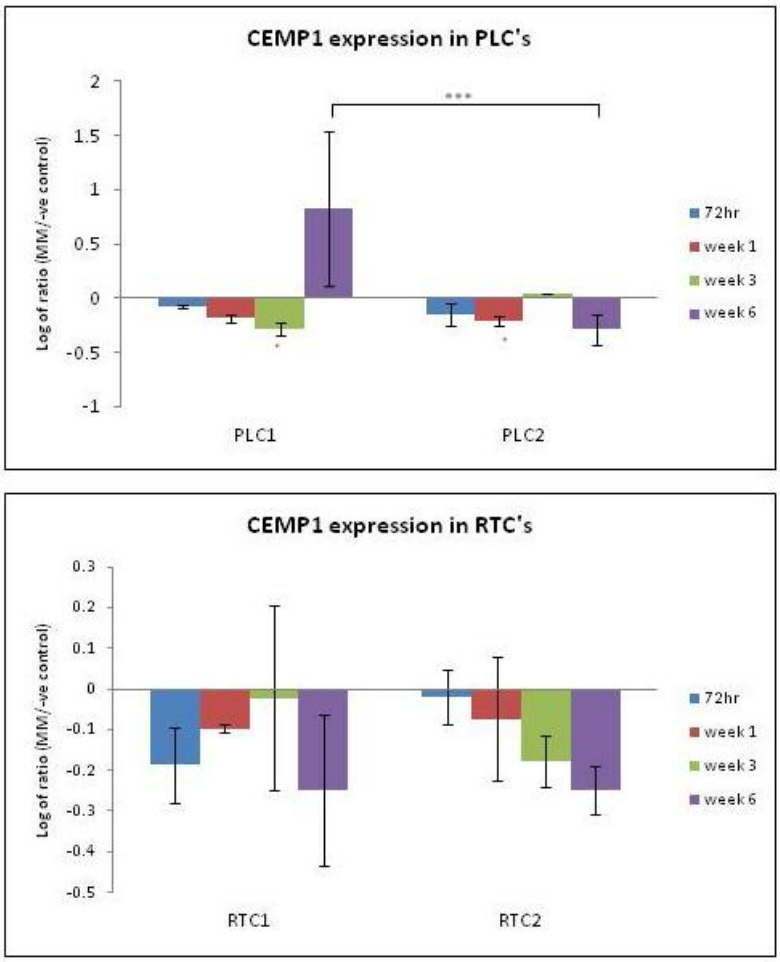


Figure 4.13 CEMP1 gene expression in PLC and RTC.

Fold change differences in BSP gene expression over 6 weeks culture (mineralization media/control media). (*) Represents a significant difference compared to the control or between time points, $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Values and bars represent mean of quadruplicate measurements ± SEM.

SPON1: Overall, the temporal patterns of SPON1 expression in PLC and RTC in response to MM treatment were quite different. SPON1 expression in both PLC 1 & 2 was up-regulated over the 6 weeks of MM treatment (albeit in PCL1 there was some down-regulation between 1 and 3 weeks). SPON1 expression was also up-regulated in both RTC 1 & 2, this was particularly notable at earlier time points, ie. in RTC1 at 72hr and week 1, and in RTC2 at 72hr (Figure 4.14).

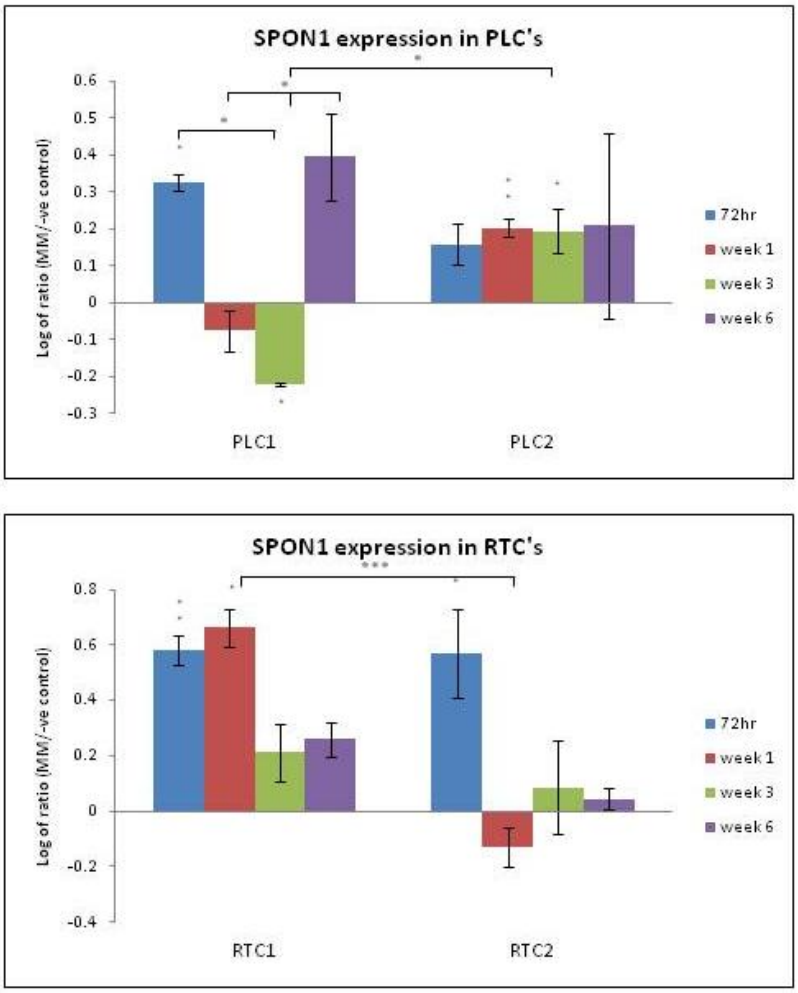


Figure 4.14 SPON1 gene expression in PLC and RTC.

Fold change differences in SPON1 gene expression over 6 weeks (mineralization media/control media). (*) Represents a significant difference compared to the control or between time points, $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Values and bars represent mean of quadruplicate measurements ± SEM.

4.4.6 Real Time-PCR (Single cell Clones)

Control media: Single cell derived clones obtained from both PLC and RTC primary cell lines were cultured in 1% FCS and 10% FCS for 6 weeks. Overall, the relative levels of bone/cementum gene expression observed were variable, ranging from quite low (BSP expression) to high relative expression levels (SPON1). Moreover, a general trend of higher gene expression in 1% FCS as compared to 10% FCS condition is demonstrated (Figure .4.15 and 4.16). In general, similar patterns of expression were seen between the three clones derived from PLC. One obvious exception was the relative expression of BSP in PLCIII which was increased after 6 weeks of 10% FCS compared to the other PLC clones where expression did not increase. There was a general trend for both the bone and cementum genes examined to be more highly expressed when PLC's were cultured in 1% FCS (Figure 4.15). Similarly, RTC clones had a higher relative expression of the marker genes in 1% FCS than 10% FCS. However, RTCI appeared to have more BSP, OCN and CAP expression compared to the other two RTC clones (Figure 4.16).

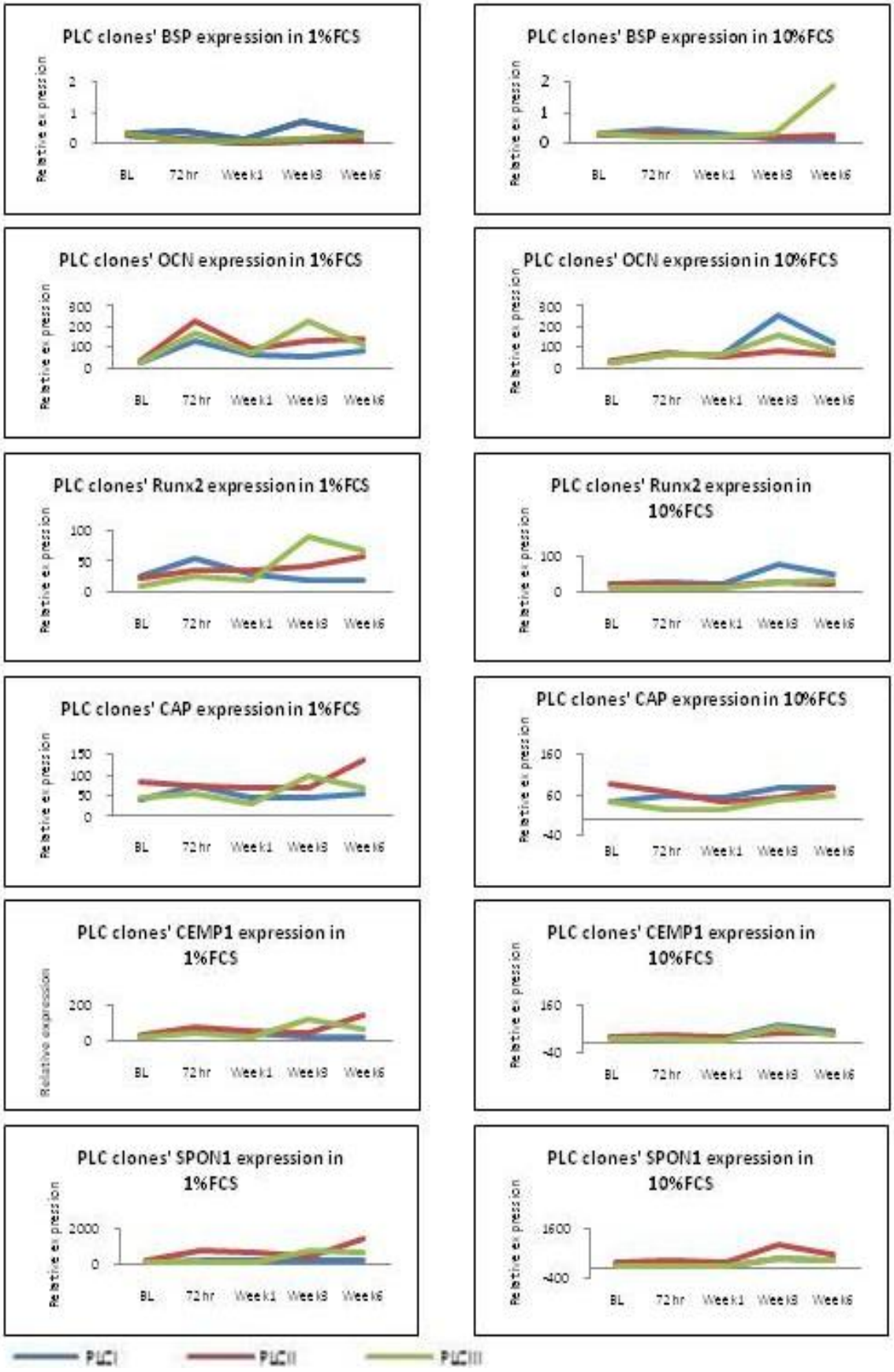


Figure 4.15 PLC clone gene expression in 1% FCS and 10% FCS.

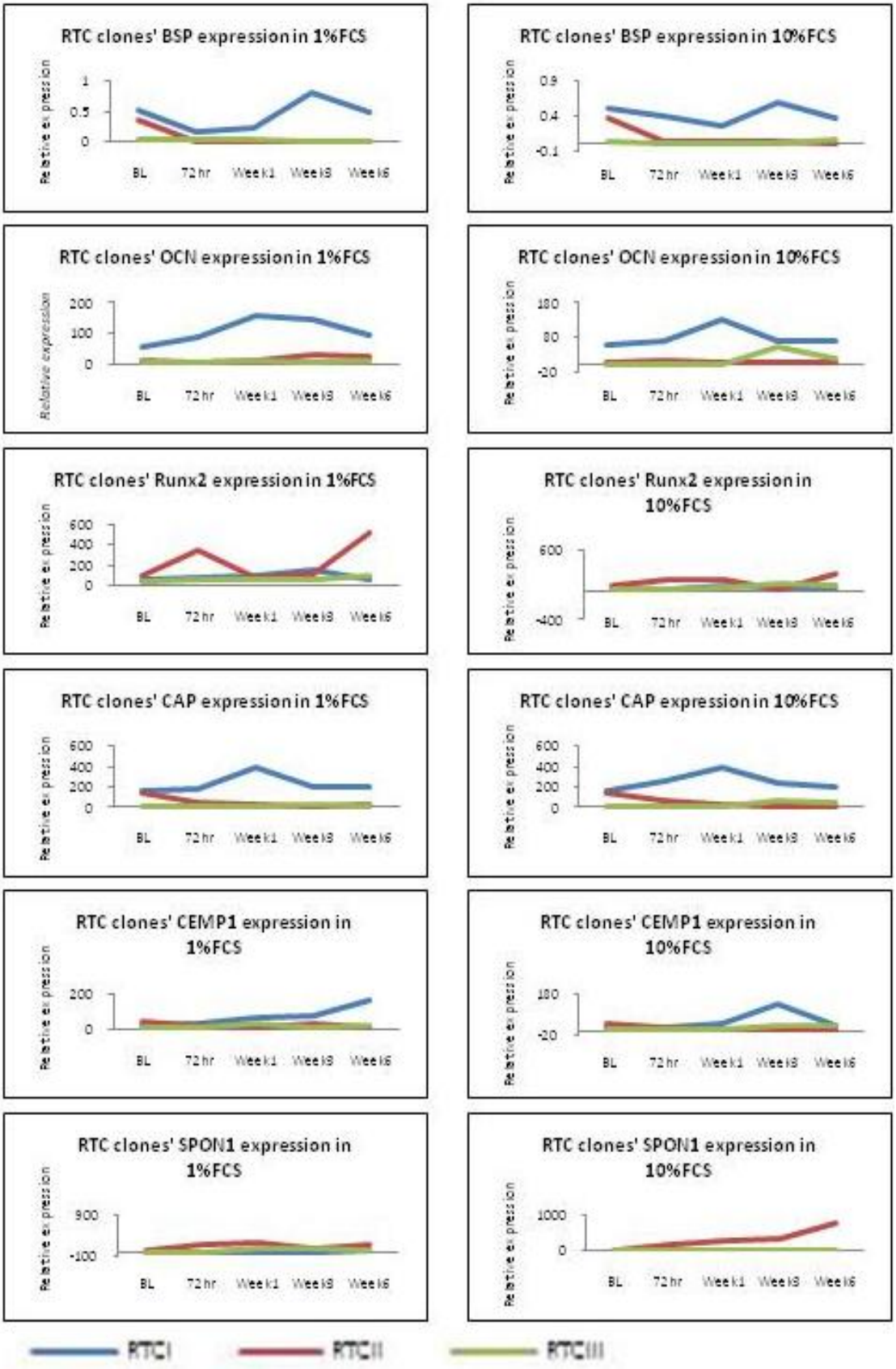


Figure 4.16 RTC clone gene expression in 1% FCS and 10% FCS.

The gene expression data of all six clones over the six weeks of 1% FCS and 10% FCS culture are compared together in Figure 4.17. This then allows a relative assessment of the effect of cell type, treatment (growth conditions) and time of treatment on the expression of bone and cementum genes. Clearly, some clones appear to have higher inherent expression levels of particular genes. For example, Runx2 is particularly highly expressed by RTC 2, while OCN is expressed in all clones except RTC 2 and 3. Similarly, CAP is also strongly expressed by all PLC clones, but only in one RTC clone (RTC 1). While there is a trend for the expression of the various genes to increase over time with continuing treatment, this effect does not appear to be consistent. Taking these results together, it appears that the inherent ability for a clonal line to express a given bone/cementum marker may play more of a deterministic role in the subsequent gene expression than either the treatment or length of the treatment time.

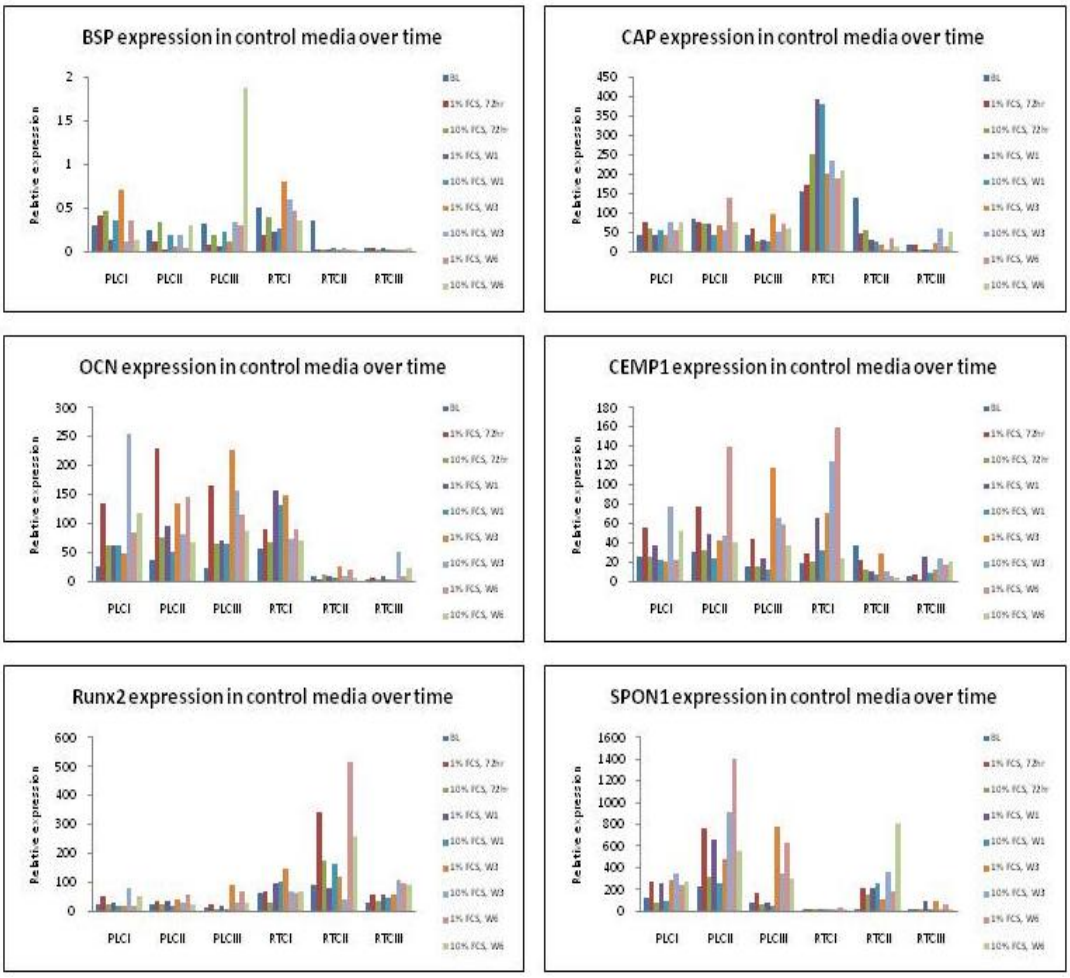


Figure 4.17 Gene expression from all clones cultured in 1% FCS and 10% FCS.

Mineralization media: No consistent pattern of bone or cementum gene expression was observed in the PLC clones cultured in MM for six weeks. This highlights further, the heterogeneity observed in periodontal cellular responses. Nevertheless, Runx2, CAP, CEMP1 and SPON1 expression were all initially up-regulated at the early time-point of 72hrs in both PLCI & III. Expression of these genes was subsequently down-regulated by week 6. Interestingly, OCN expression in clones PLCII and III was also down-regulated at each time-point (Figure 4.18).

GOI	Fold change in gene expression											
	PLCI				PLCII				PLCIII			
	72hr	W1	W3	W6	72hr	W1	W3	W6	72hr	W1	W3	W6
BSP	-2.0	-	-	7.7	-2.5	-	-	-	-2.0	-	-	-
OCN	-	-	-	-5.0	-3.3	-5.0	-3.3	-3.3	-3.3	-5.0	-10.0	-2.5
Runx2	2.6	-	-	-	-	-	-	-	2.0	-	-	-
CAP	2.7	-	-	-	-	-	-	-	3.0	2.4	-	-
CEMP1	2.6	-	-	-3.3	-	-	2.0	-	2.2	-	-2.0	2.1
SPON1	5.7	-	-	-2.0	-	-	-	-	6.6	3.3	-	2.6

Figure 4.18 PLC clone gene expression after mineralization treatment.
The table shows the ratio of the up-regulated (red) or down-regulated (blue) genes (threshold of two fold change).

Similarly, a heterogeneous pattern of gene expression was observed in the RTC clones and in particular RTCI. Gene expression in RTC clone II was broadly down-regulated in the first three weeks after which there was a switch to up-regulation in 4 of the six genes examined (BSP, CAP, CEMP1, SPON1). RTC clone III was the most responsive cell line demonstrating strong up-regulation in expression of all gene markers at the early time-point of 72 hrs which was subsequently maintained over the 6 week period (Figure 4.19).

GOI	Fold change in gene expression											
	RTCI				RTCII				RTCIII			
	72hr	W1	W3	W6	72hr	W1	W3	W6	72hr	W1	W3	W6
BSP	-2.0	3.3	-	-	-	-	-10.0	92.7	8.1	-	-3.3	2.6
OCN	-	-	2.1	-2.0	-	-	-10.0	-	2.0	2.3	-2.5	-
Runx2	2.9	-	-	2.3	-	-10.0	-	-2.5	2.1	-	-2.0	-
CAP	-	-	2.1	-	-	-3.3	-10.0	2.4	9.3	5.1	-	2.3
CEMP1	-	2.1	-3.3	5.3	-	-	-3.3	3.5	3.0	2.4	-	
SPON1	-3.3	-	-	7.0	-	-	-3.3	2.4	10.3	6.9	3.6	12.3

Figure 4.19 RTC clone gene expression after mineralization treatment.
The table shows the ratio of the up-regulated (red) or down-regulated (blue) genes (threshold of two fold change).

4.5 Discussion

It is generally accepted that the periodontal ligament consists of different cell populations in various stages of differentiation. Recent studies have demonstrated that periodontal ligament (Seo et al., 2004), gingival (Fournier et al., 2010) and regenerated periodontal tissue fibroblasts (Lin et al., 2008b) contain stem cells that have the potential to generate tissues similar to both cementum and periodontal ligament. In previous chapters, we have shown that cells derived from PLC, RTC and OB have the potential to differentiate in response to mineralization treatment and form mineralized tissue. Moreover, PLC, RTC and OB responded to MM, IGF, PDGF and the combination of IGF/PDGF by up-regulating bone and cementogenic gene expression.

In the present study, this cellular response was further analysed by comparing cells derived from the same cell type, but from different individuals. The results confirmed that a significant heterogeneity in cellular and gene expression response to mineralization treatment can occur within cells derived from the same tissue type. Moreover, it was interesting to find that culturing PLCs and RTCs in control media alone enhanced the osteogenic differentiation of some of those cells. For example, Alizarin red S staining was significantly increased at week 6 compared to earlier time points, while, hydroxyapatite formation was found to increase in PLC2 over time when cells were cultured in 10% alone. When the two cells from the same tissue (PLC1 vs PLC2 and RTC1 vs RTC2) were compared, both PLC2 and RTC1 were found to have more potential to differentiate along a mineralized phenotype as suggested by the histochemical and genetic expression results. Moreover, both BSP and SPON1 expression appeared to be the genes that were most consistently associated with the mineralization process.

Periodontal ligament contains a heterogeneous cell population at various stages of differentiation and lineage commitment. Previous studies indicate that periodontal ligament cells can form mineralized nodules *in vitro* (Lekic et al., 2001b) and express the bone-related proteins, bone sialoprotein and osteocalcin (Ivanovski, 2001a). Cells derived from the periodontal ligament were found also to respond to mineralization treatment with increased ALP activity and bone nodule formation (Arceo et al., 1991, Cho et al., 1992, Seo et al., 2004). To further examine this heterogeneity in cellular response among periodontal cells, we have established single cell derived clones from periodontal ligament- and regenerating tissue- derived cells. As the clones were established from 'immortalized' cell lines, they should provide a stable phenotype with a consistent response to stimuli, and are therefore ideal to study and characterize the mineralization response in different cell types which can then be used for further periodontal tissue engineering studies both *in vitro* and *in vivo.* The successful transfection of PLC and RTC primary cell populations prolonged the life span of the cultures, facilitated the cloning process and helped in retaining the characteristics of the parent cells (Parkar et al., 1999, D'Errico et al., 1999, Fujii et al., 2006, Pi et al., 2007).

The bone and cementum marker expression data (Figure 4.17) shows a pattern of 'clustering' in individual clones, and this data suggests that cell differentiation ability may be more predominantly dependent on the inherent characteristics of a given cell phenotype, with treatment type and duration being secondary considerations. In the current study, when the clones were cultured in 10% FCS and 1% FCS, the cells expressed bone and cementum markers to varying degrees, however, a trend of higher expression was found when cells were cultured in 1%FCS, suggesting that nutrient-deprivation may induce cell differentiation, possibly at the expense of proliferation. In the previous chapters, the PLC response (i.e mineralization and gene expression) to the

various treatments (i.e. MM, IGF and PDGF) was more than that observed in RTC cells. When PLC clones were treated with mineralization media, PLCII showed the least response in terms of osteogenic differentiation and expression of bone and cementum markers. Both PLCI and PLCIII showed an up-regulation of some of the genes tested especially at 72hr. In the previous chapters, the RTC response to treatment was very minimal, with only minor levels of BSP and SPON1 expression. In this chapter, the heterogeneous results of RTC after mineralization treatment was evident, and may thus explain the previous observed response in this population. RTCIII responded to the mineralization treatment with up-regulation of most of the gene markers at various time points, while both RTCI and RTCII had a lesser response. These results may help in isolating good candidates for osteogenic/cementogenic differentiation for use in future periodontal tissue engineering trials.

Moreover, the results obtained from the single cell clones clearly demonstrates intra-population heterogeneity showing that cells derived from the same primary cell population have significant heterogeneity. The establishment of these clonal cell lines with diverse gene expression profiles confirms the presence of heterogeneous cell populations in primary periodontal ligament and regenerated tissue derived cell lines harvested from GTR treated defects (Bartold et al., 2006a, Fujii et al., 2008, Lekic et al., 2001a, Nojima et al., 1990, Pitaru et al., 1994, Piche et al., 1989, Haase et al., 2003, Ivanovski, 2001b, Ivanovski, 2001a, Hakkinen and Larjava, 1992, Fujita et al., 2005, Hou and Yaeger, 1993, Singhatanadgit et al., 2009). As outlined above, all of the studied periodontal derived cell clones showed a response to mineralization treatment in a different manner. The expression of differentiation-related genes, including bone and cementum markers was variable between the different clones, including those from the same source. The ability of the differentiation treatment to guide

osteoblastic/cementoblastic differentiation may be based on the inherent differentiation potential of individual cell lines. Moreover, there is good evidence to suggest that not all cells in a mixed explant culture will respond to osteogenic/cementogenic treatment (Bartold and Raben, 1996a).

5.6 Conclusion

Heterogeneity is evident between cell lines obtained from the same tissue. Furthermore, the analysis of single cell derived clones demonstrated significant intra-population heterogeneity of cells derived from the same primary cells. The expression of bone/cementum markers appears to be an inherent property of a given cell line, and may have a greater influence on gene expression than the cell culture treatment or the time in culture.

5.0 Chapter Five

In vivo Cementogenesis: A Pilot Study

5.1 Abstract

Background: The management of the periodontal defect has always been a challenge. Recent research has focused on the potential of periodontal tissue engineering techniques using autologous progenitor cells grown *in vitro* and subsequently implanted into the defect, with the ultimate goal of achieving periodontal regeneration. Cementogenesis is a key requirement for periodontal regeneration, and hence the ability of cells to form cementum on the tooth surface is an important consideration in choosing an appropriate phenotype for periodontal regeneration.

Objective: To establish an animal model in order to test the ability of cell sheets comprising cells differentiated along the cementoblast/osteoblast phenotype to promote cementum formation *in vivo*.

Method: Human periodontal ligament fibroblasts were treated with mineralization media (50ug/ml ascorbic acid, 10^{-7} M Dexamethasone, and 10 mM β-Glycerophosphate) for one week. Cell sheets were subsequently fabricated using temperature responsive tissue culture plates and transplanted together with a dentine slice into a subcutaneous pouch in athymic rats. After 6 weeks, the samples were retrieved and examined histologically and immunohistochemically for new cementum and/or periodontal ligament formation.

Results: Histological analysis showed the presence of an intact cell sheet and mineralised tissue deposition on the dentine slice facing the cell sheet in the samples treated with mineralization media. Further, immunohistochemical staining showed that

CEMP1 protein expression was strongly localised in cells contiguous with the dentine suggesting that these cells may be undergoing differentiation into cementoblasts and forming cementum..

Conclusion: Using an athymic rat model, this pilot study has shown that cell sheets containing progenitor cells derived from periodontal tissue can support cementogenesis *in vivo*.

5.2 Introduction

Tissue engineering is an emerging field of science aimed at developing techniques for the fabrication of new tissue(s) to replace those lost or damaged as a result of trauma and/or disease. These techniques are based on the principles of cell biology, developmental biology and biomaterial science (Vacanti et al., 1991, Narem and Sambanis, 1995, Reddi, 1997, Reddi, 1998, Bartold et al., 2000a) Tissue engineering techniques usually combine the transplantation of biomaterials containing appropriately selected and primed cells, together with an appropriate mix of regulatory factors, with the ultimate goal of promoting cell growth and specialization. As such, this process has also been utilized to facilitate periodontal regeneration (Yang et al., 2009, Chen et al., 2008, Liao, 2010, Park et al., 2011, Ishikawa et al., 2009).

The three main ingredients for optimal tissue engineering are molecular signals, responding stem cells, and the assembly of an extracellular matrix (Bartold et al., 2006b). For example, bone formation requires an osteoinductive signal, a suitable substratum which acts as a scaffold for new bone tissue to form, and responding host cells capable of differentiation into bone cells. All three of these components are amenable to manipulation for use in the tissue engineering of periodontal tissues (Reddi, 1997). However, regeneration of periodontal tissue is more complicated than bone as it is a complex structure composed of related, but distinct hard and soft tissues. Cultured PDL cells applied in various scaffolds or in suspension have been shown to be able to induce a new periodontal tissue apparatus on root dentin surfaces (Lang et al., 1998a, Lekic et al., 2001c, Dogan et al., 2002, Nakahara et al., 2004). Moreover, cementum engineering *in vitro* with 3D pellets, consisting of cultivated periodontal ligament cells incorporated into three-dimensional biodegradable polymers, has also been attempted

(Jin et al., 2003, Yang et al., 2009). Furthermore, the slow release of growth factors such as BMP2 and PDGF from transfected cells has been shown to further promote the regeneration of all the components of the periodontal attachment apparatus (Chen et al., 2008, Jin et al., 2004).

Cell sheet engineering is a new, alternative approach to traditional tissue engineering methods that omits the need for a biomaterial carrier (scaffold). In this method, cell sheets are constructed *in vitro* using temperature-responsive cell culture surfaces which respond reversibly to changes in temperature. Developed in 1990 by Yamada and co-workers (Yamada et al., 1990), the cell culture surface is coated with poly N-isopropylacrylamide (Hirose et al., 2000, Nishida et al., 2004a). Poly N-isopropylacrylamide is hydrophilic below 32°C and hyodrophobic above this temperature. By simply lowering the temperature, an intact confluent cell monolayer may be harvested *in toto* i.e. without disrupting the extracellular matrix. The use of this non-enzymatic cell harvesting system has been shown to be non-invasive, gentle, and harmless to cells (Yamato et al., 1998, Kushida et al., 1999, Yamato et al., 1999). Recent *in vivo* animal studies have shown that this cell sheet technique has been successfully utilized in the treatment of corneal dysfunction (Nishida et al., 2004b), myocardial infarction (Miyahara et al., 2006), and oesophageal ulceration (Ohki et al., 2006).

Periodontal ligament fibroblasts cultured on the temperature-responsive surface were able to be harvested from culture dishes as a contiguous cell sheet with abundant extracellular matrix and were shown to retain intact integrins (Hasegawa et al., 2005). Moreover, no contamination with microorganisms or malignant transformation was

detected when human periodontal ligament fibroblasts were cultured *in vitro* and transplanted *in vivo* to mice (Washio et al., 2010). The cell sheet method has also been used successfully to promote periodontal regeneration in animal studies (Akizuki et al., 2005, Hasegawa et al., 2005, Gomez Flores et al., 2008, Washio et al., 2010, Iwata et al., 2010). Using human periodontal ligament fibroblast (PLF) cell sheets transplanted together with dentine slices into athymic rats, Gomez Flores et al (2008) demonstrated the formation of both new immature cementum-like tissue, and periodontal ligament with perpendicularly oriented fibres inserted into the newly deposited cementum-like tissue (Gomez Flores et al., 2008). Washio et al (2010) also used a similar model to test the safety and efficacy of using human periodontal ligament cell (PLC) sheets for periodontal regeneration, and showed that PLF sheets implanted with dentin blocks induced the formation of cementum and periodontal ligament-like tissue in immunodeficient mice (Washio et al., 2010).

Similar findings were also observed when PLF cell sheets were transplanted into periodontal bony defects and buccal bone dehiscences in animal models (Hasegawa et al., 2005, Akizuki et al., 2005, Flores et al., 2008, Iwata et al., 2009), suggesting that this technique can be useful for periodontal regeneration. More recently, cell sheets derived from three different populations (Periodontal ligament cells, iliac bone marrow mesenchymal stromal cells and alveolar periosteal cells) were compared in a severe one wall periodontal defect in dogs, and the periodontal ligament cells were again found to be able to regenerate both new cementum and periodontal ligament fibres (Tsumanuma et al., 2011).

Previous chapters in this thesis have identified and characterised, periodontal cells with the potential to form a cementogenic phenotype following osteogenic induction *in vitro*. Therefore, the aim of this study was to evaluate and confirm the *in vivo* efficacy of this novel cell sheet technology to promote cementogenesis in an athymic rat model using primary periodontal ligament cells cultured in mineralization media.

5.3 Methodology

5.3.1 Periodontal Cell Isolation and Characterization

Periodontal ligament cells were isolated and cultured as previously described in chapter 2. PLC1 from the previous chapter was chosen for the current experiment. Extracellular mineralization in this cell line following mineralization treatment was demonstrated using Von Kossa, Alizarin red S staining, calcium release and hydroxyapatite nodule formation as reported in previous chapters (Figure 5.1, 5.2 and 5.3). Furthermore, mRNA expression of bone and cementum marker expression was also carried out as previously described (Figure 5.4).

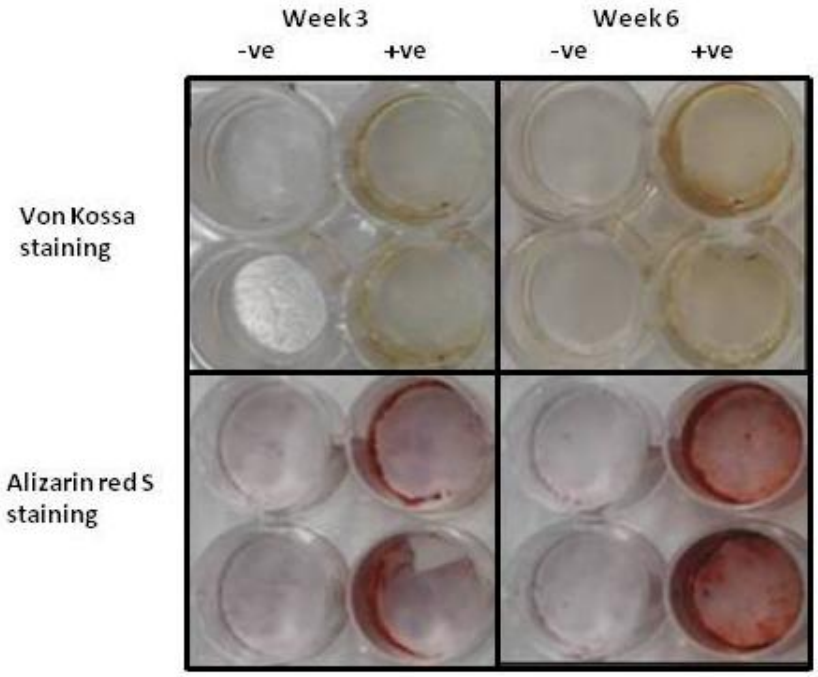


Figure 5.1 Von Kossa staining and Alizarin red S staining for PLC1.

PLC1 was cultured for three and six weeks with (+ve) and without (-ve) mineralization treatment.

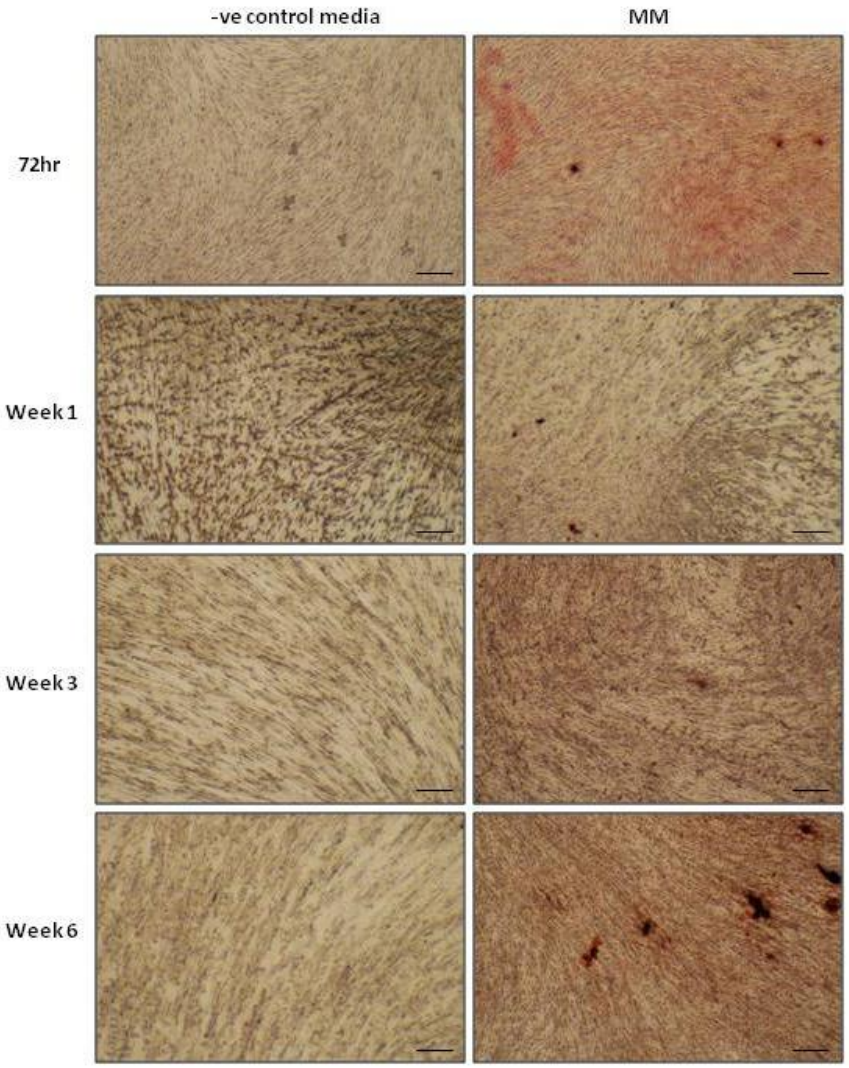


Figure 5.2 Qualitative alizarin red S staining of PLC1.

PLC1 was cultured with mineralization media (MM) and without (–ve control media). Scale bar is 100µm.

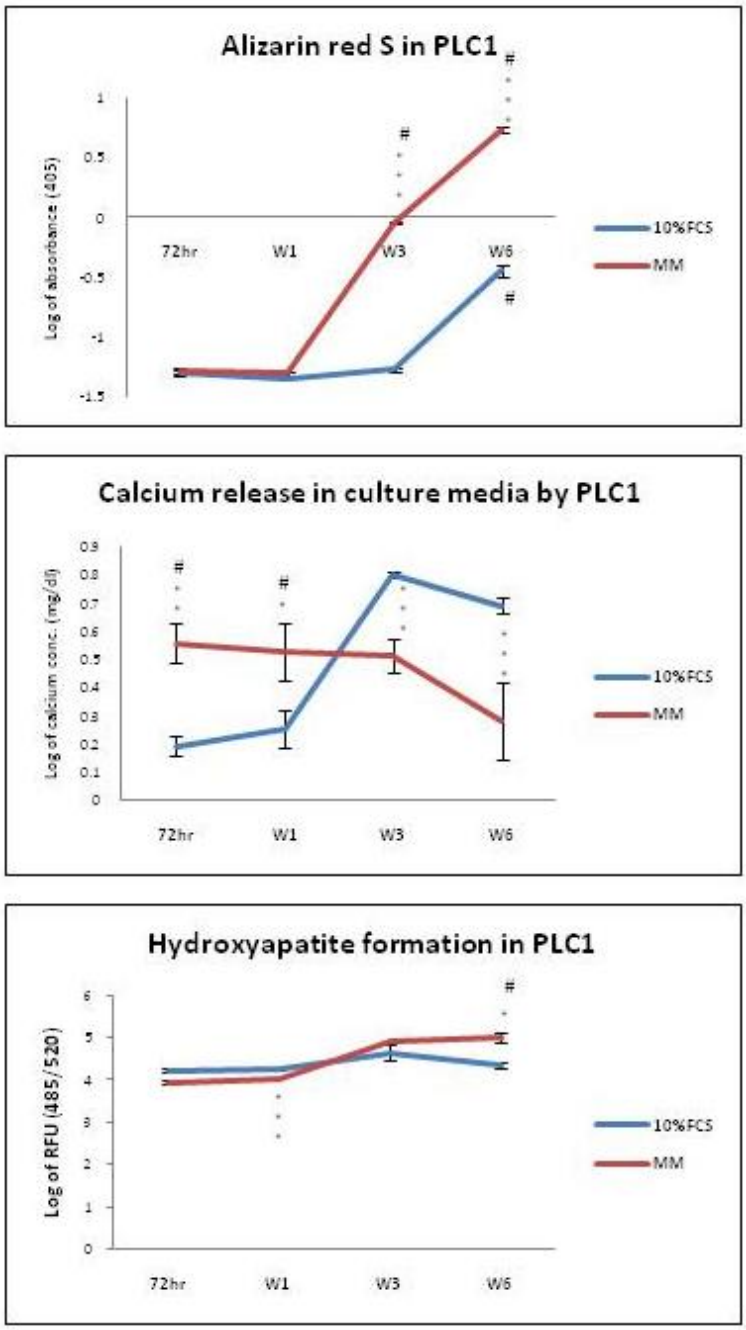


Figure 5.3 Histochemical analysis of PLC1.

Quantitative alizarin red S staining, calcium release and hydroxyapatite formation from PLC1 cultured in mineralization media (MM) and control media (10% FCS). (*) Represents significance comparing to –ve control. $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. (#) Represents significance compared to the other time points. Values represent mean of duplicate measurements ± SEM.

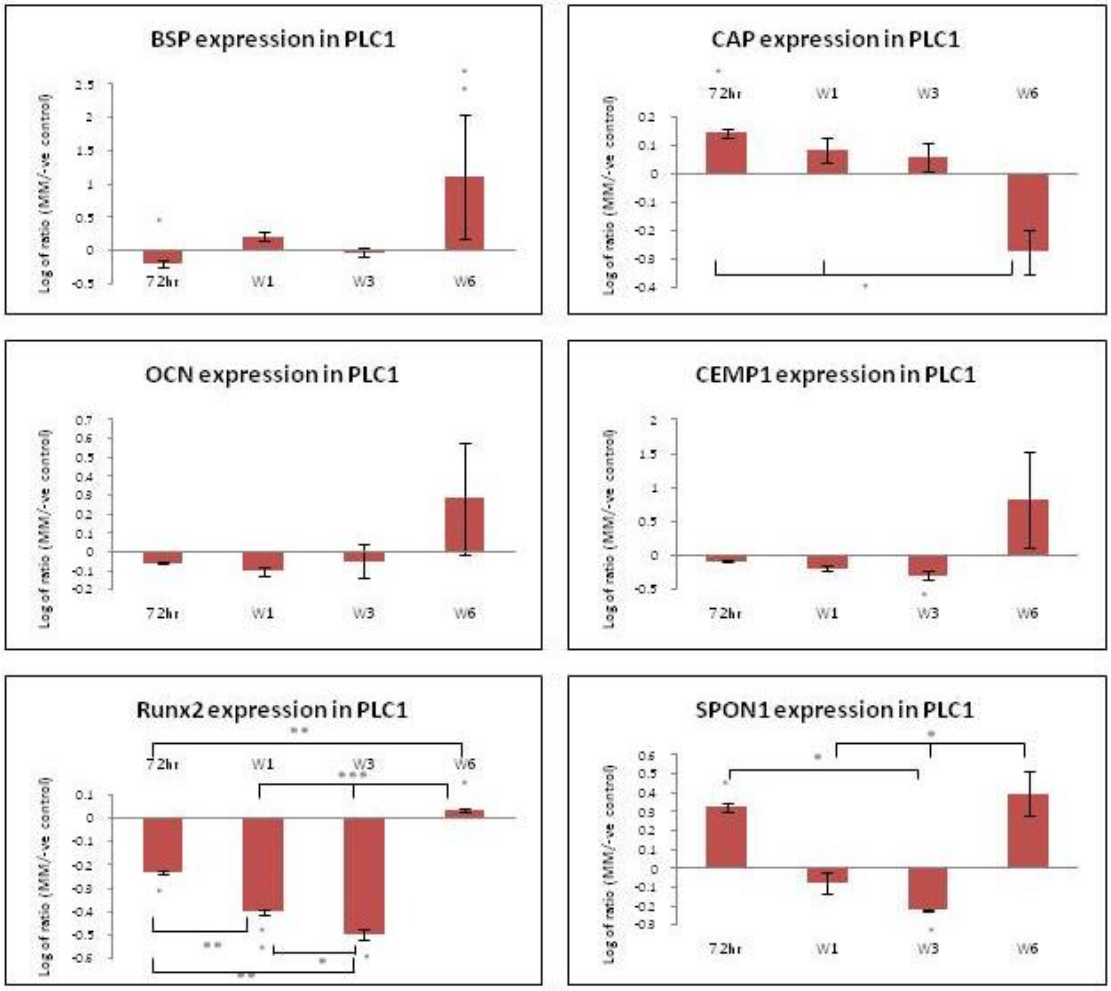


Figure 5.4 PLC1 gene expression.

Real-time-PCR results in PLC1 over 6 weeks of mineralization treatment. (*) represents a significant difference compared to the control and between time points, $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$. Bars represent mean of duplicate measurements ±SEM.

5.3.2 Periodontal Ligament Cell Sheets

Periodontal ligament cells were grown to confluence in standard tissue culture plates as described previously. The cells were then detached from the surface using 0.2% trypsin and plated on 35 cm diameter temperature-responsive culture dishes (Thermo Scientific Nunc UpCell^{TM} surface, U.S.A.) at a concentration of 10^5 cells/well and cultured at 37°C for one week. The cell sheets were subsequently harvested according to the method described by (Gomez Flores et al., 2008). Briefly, a polycarbonate membrane (provided with the Thermo Scientific UpCell^{TM} surface, USA) was used to support the cell sheet during the harvesting phase. The membrane was placed on the upper surface of the cultured cells and incubated at 20°C for 1 h. The cell sheet and supporting membrane were then placed onto the dentin slices. Both test and control cell sheets were prepared as described. The test cell sheets were cultured in mineralization media (50ug/ml ascorbic acid, 10^{-7} M Dexamethasone, and 10 mM β-Glycerophosphate) for one week prior to harvesting while the control PLC sheets received no MM treatment (control media only). Both control and test cell sheets were finally transplanted subcutaneously into the back of immunodeficient athymic rats.

5.3.3 Preparation of Dentine Slices

Teeth were collected from patients undergoing extraction for orthodontic reasons at the Griffith University dental clinic. Ethical approval was attained through the Griffith University Human Research Ethics Committee (DOH/17/7/HREC). Human dentine slices were prepared using a Leica SP1600 Saw Microtome (Leica Microsystems, Germany), (Figure 5.5). First the teeth were mounted in the machine using a custom

made acrylic base (Figure 5.6) whereby 0.5mm dentine slices were cut under constant water irrigation. The cementum layer was then completely removed using a polishing disc mounted on a portable micro-motor again with adequate irrigation. The dentine slices were then conditioned with saturated citric acid (pH1) for 1 min to remove the smear layer and expose the dentine fibrils (Babay, 1997) and finally washed in an ultrasonic water bath for 10min.

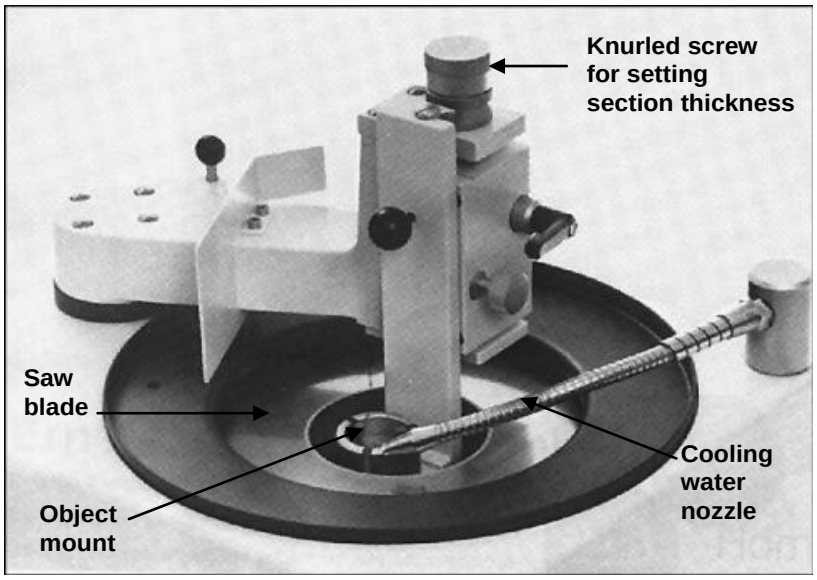


Figure 5.5 Leica SP1600 Saw Microtome.
Teeth were mounted on the "object mount" and the saw set at 0.5mm thick slices.

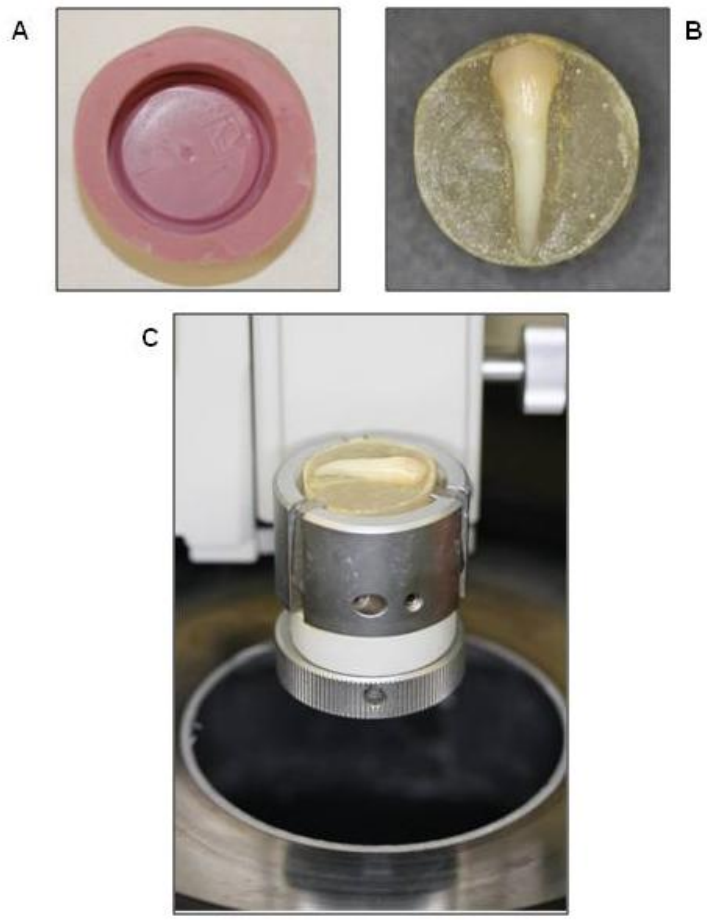


Figure 5.6 Mounting and sectioning the dentine chips.

A. Dental impression putty mould used to support the acrylic. B. Self cure orthopolymer was used to support the tooth, C. the tooth supported on the mount and ready for cutting.

5.3.4 Animal Surgery and Histology

The research protocol for this study was approved by the Griffith University Animal Ethics Committee, (protocol number DOH/01/08/AEC). This pilot investigation was carried out using three male, 8 week old athymic rats (Rattus norvegicus Strain - CBH /rnu/rnu) purchased from the Animal Resources Centre, Murdoch, Western Australia. Isofurane (Attane™, Bomac Animal Health Pty Ltd, Australia) inhalation was used to achieve anaesthesia and three surgical approaches were performed. In the first rat (Figure 5.7) the surgical technique used was as described previously by Gomez Flores *et al* (Gomez Flores et al., 2008). A mid-sagittal incision was made on the dorsum of the rat's back and two cell sheet/dentin constructs were placed with the cell sheets facing the muscle surface, and the incision was sutured using 5-0 Prolene sutures (polypropylene, monofilaments suture, Ethicon, U.S.A.). The two constructs used were (a) dentine chip with PLC1 cells cultured in control media (no treatment) and (b) dentine chip with PLC1 cells treated with MM. The rat was given prophylactic antibiotic cover, 200µl/day of Enrofloxacin (Baytril® 25 antibacterial oral solution, active ingredient 25mg/ml Enrofloxacin, Bayer Australia Ltd) for the first week post-surgery. No post-operative complications were observed and the animal tolerated the implanted constructs very well. Wound healing was allowed to occur for six weeks before the animal was sacrificed and the constructs were collected. The only problem encountered using this technique however was movement of the constructs across the midline during the 6 weeks post-surgery, making it somewhat difficult to identify each construct upon collection. The constructs were found to be encapsulated in fibrous tissue.

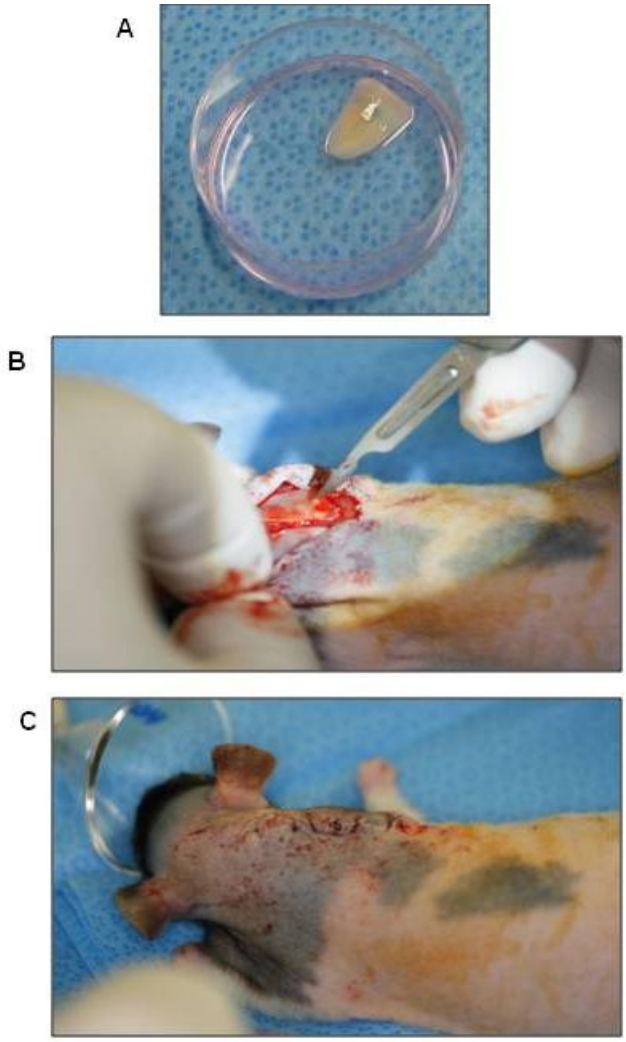


Figure 5.7 First surgical procedure using midsagittal incision.

A. Dentin chip covered with a periodontal ligament fibroblast cell sheet. B. The dentine chip/cell sheet construct is transplanted in into the back of an athymic rat. C. The midsagittal incision sutured back together.

In the second surgical technique (Figure 5.8), two smaller separate incisions were performed, one on each side of the midsagittal line creating a small pouch where the constructs were placed. This time there was no significant movement of the construct across the midsagittal plane. Again the constructs were found to be encapsulated in fibrous tissue.

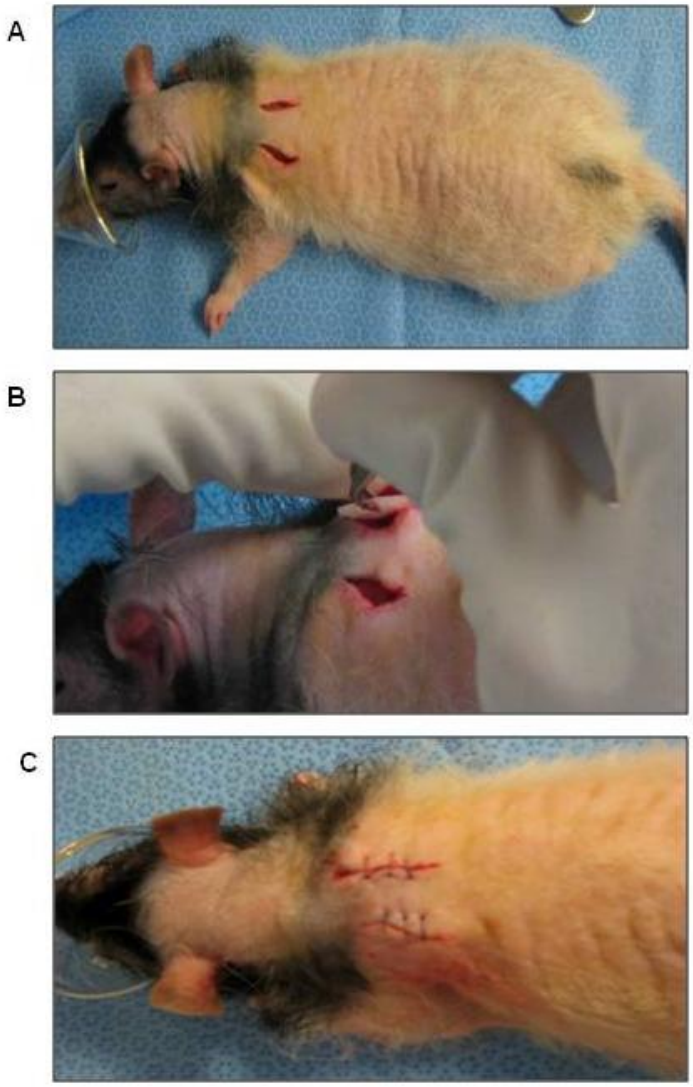


Figure 5.8 Second surgical procedure using two separate pouches.
A. Separate incisions were performed on each side of the midsagittal plane, B. The dentine/cell sheet constructs were placed in the pouch created, C. The incisions sutured back together.

In the third surgical procedure (Figure 5.9), two holes were made in each dentine chip prior to implantation using a tapered, pointed end diamond bur. Four cell sheet/dentine constructs were prepared; two each of PLC with and without mineralization treatment. After making a midsagittal incision as described in the first surgical procedure, the constructs were secured in place by suturing them to the back muscles using 5-0 Prolene sutures. This removed any problem of interference with the wound healing due to movement of the construct.

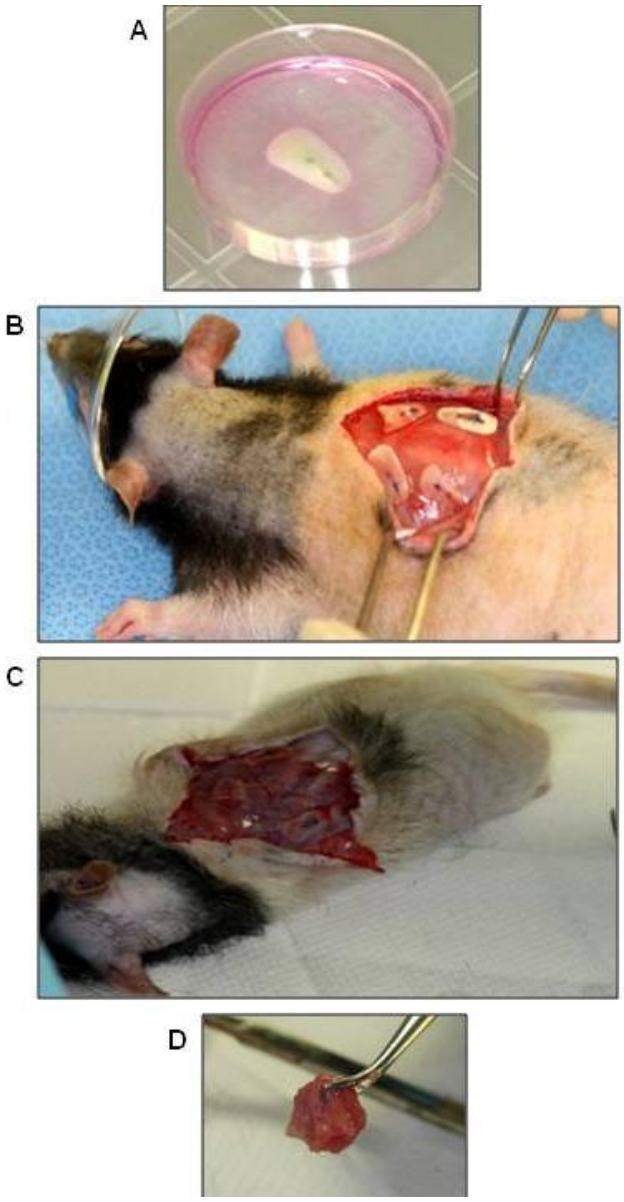


Figure 5.9 Third surgical procedure using two holes in the dentine.

A. Two holes were drilled in each dentine chip, B. Dentine/cell sheet constructs were secured on the dorsal muscles using monofilament suture, C. Re-entry procedure, D. retrieval of the construct after 6 weeks of healing.

5.3.5. Histology and Immunohistochemistry

The excised tissues were fixed in 10% neutral-buffered formalin for 24 hrs. After rinsing the fixed tissue, the dentine slices were decalcified using 10% EDTA (Ethylenediaminetetraacetic acid) for 6 weeks. The EDTA solution was changed regularly (twice a week) and a control dentine chip was examined regularly for decalcification using a small diameter needle to find any resistance to perforation. The constructs were subsequently embedded in paraffin wax and 5um thick sections were prepared for standard histology and immunohistochemistry.

For standard histological examination, after de-waxing and hydration, routine haematoxylin and eaosin staining was carried out. The cell nuclei were stained with haematoxylin and matrix stained with eosin.

For immunohistochemical staining, following de-waxing and hydration, endogenous peroxidase activity within the sections was quenched by incubating with 3% H_2O_2 for 20 minutes, then blocked with 10% swine serum for 1 hour. The samples were incubated with the CEMP1 primary antibody (diluted 1:200, sc-164032, Santa Cruz, California, USA) overnight at 4^{o}C, followed by incubation, at room temperature with a biotinylated universal swine-anti-mouse, rabbit, goat secondary antibody (DAKO, CA, USA) for 15 min, and then with horseradish perioxidase-conjugated avidin-biotin complex (DAKO, CA, USA) for another 15 minutes. The antibody complexes were visualized by the addition of a buffered diaminobenzidine (DAB) substrate for 4 minutes. Mayer's haematoxylin (HD Scientific Pty Ltd.) was used for counter staining.

5.4 Results

5.4.1 Periodontal cell characterization

The results of von Kossa and Alizarin red S staining, calcium release and hydroxyapatite formation all suggested that the cell line chosen for the study (PLC1) was able to form mineralized matrix following stimulation with mineralization media *in vitro* (Figures 5.1, 5.2 and 5.3). Moreover, RT-PCR showed a trend of up-regulation in all osteogenic and cementogenic genes tested (except CAP) after 6 weeks of treatment (Figure 5.4).

5.4.2 Histology

After 6 weeks transplantation, H&E staining of the paraffin embedded sections identified the presence of the cell sheets in all samples. As frequently described in studies using decalcified sections to study cementogenesis, the newly formed tissue was sometimes found to be detached from the adjacent dentine slice. This was particularly the case when the sheet was grown in the control media. The cell sheet was found to be attached to more intensely stained areas close to the dentine surface in the MM treated cell sheets (Figure 5.10). The encapsulating fibrous tissue was also evident in the sections.

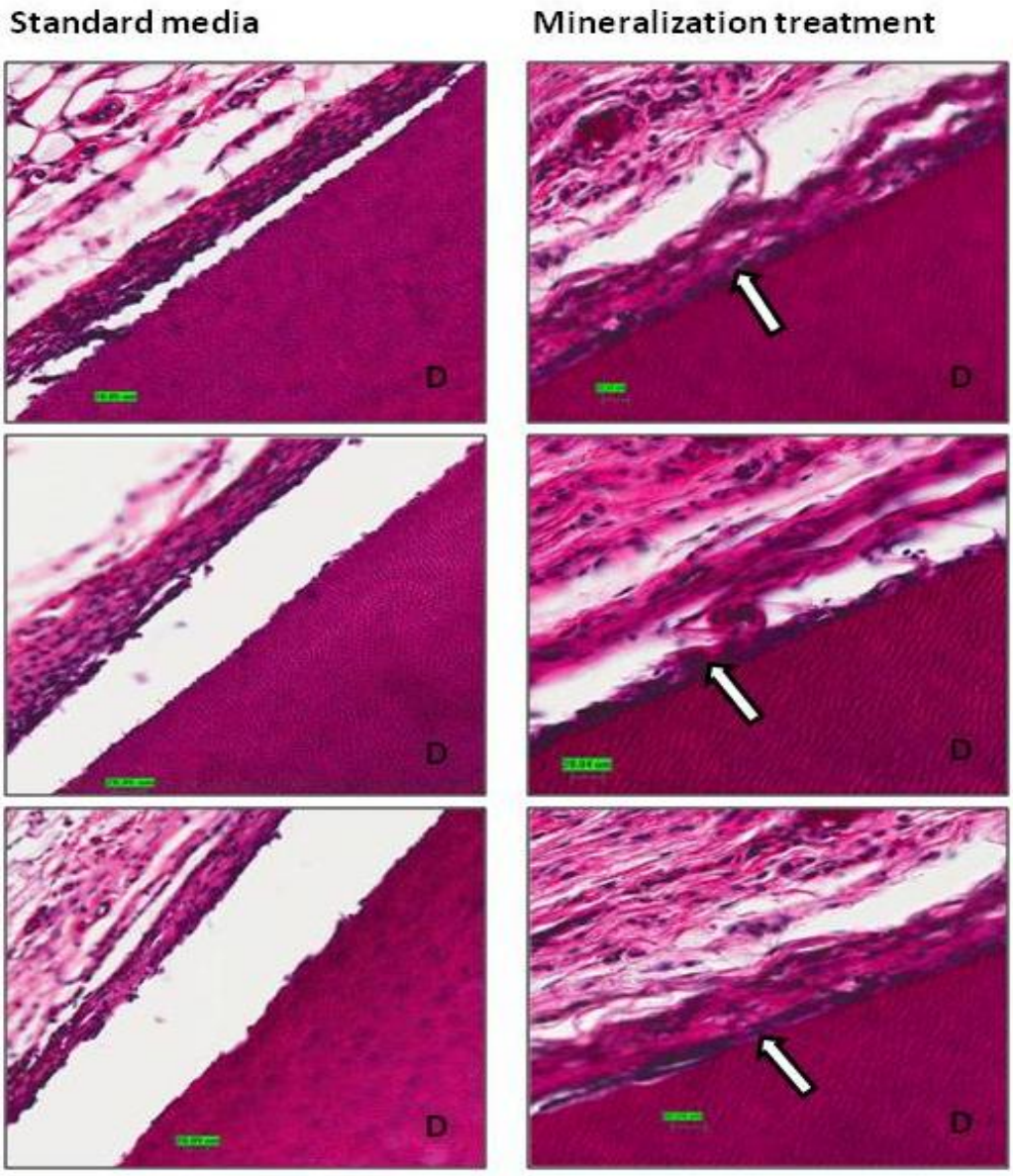


Figure 5.10 Haematoxylin and Eosin staining.

Paraffin embedded sections obtained 6 weeks post-transplantation of a dentine/periodontal fibroblast cell sheet construct in an athymic rat. In some areas the mineralized treated periodontal cell sheet is observed attached to the dentine chip with darker staining at the interface (arrow), whereas no such observations were found in the control sections. D, dentin. Green scale bar is 20µm

5.4.3 Immunohistochemistry

To further characterize the cells attached to the dentine after MM treatment, immunohistochemical staining with CEMP1 antibody was performed. CEMP1 was found to be localized in the cell sheet attached to the dentine chip, with particularly strong staining of the cells and matrix adjacent to the dentine, Figure 5.11.

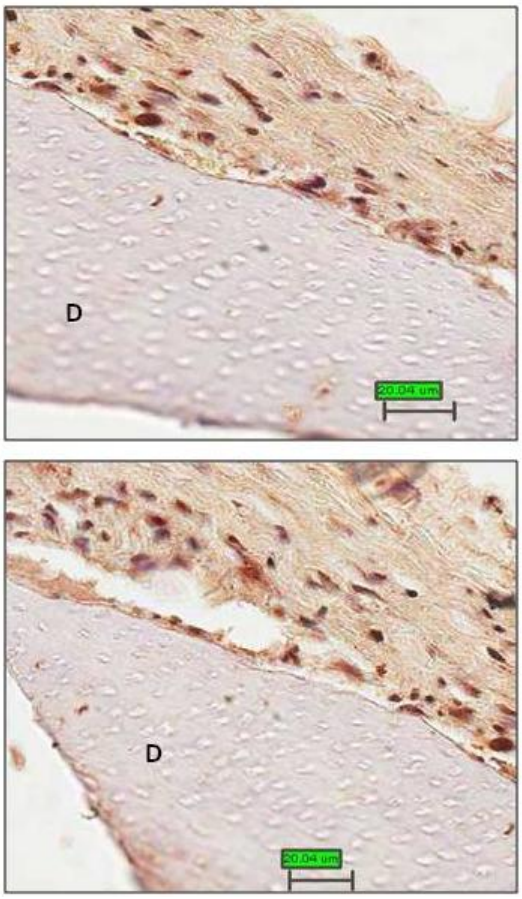


Figure 5.11 CEMP1 immunohistochemistry.

Paraffin embedded sections obtained 6 weeks post-transplantation of a dentine/periodontal fibroblast cell sheet construct in an athymic rat. CEMP1 antigen was detected in the cells attached to the dentine chip. Green scale bar is 20µm.

5.5 Discussion

One of the major goals of periodontal therapy is to regenerate or reconstitute the tissue damaged or destroyed as a result of the disease process, thus restoring its original architecture and function. Many surgical procedures have been advocated for periodontal regeneration. Most recently, cell based therapies which aim to increase the appropriate progenitor cell population at the wound site have been receiving increased attention. This approach has successfully demonstrated regeneration of the periodontal ligament, root surface cementum and alveolar bone (Seo et al., 2004, Lang et al., 1998a, Dogan et al., 2002, Nakahara et al., 2004, Hasegawa et al., 2005).

One objective of this pilot study was to establish a surgical procedure that can be used to screen cell lines with demonstrated *in vitro* cementogenic potential for future use in tissue engineering experiments in our laboratory. Stability of the transplanted construct using the procedure described by Gomez Flores et al (2008) was a point of concern as any movement in the early healing stages could possibly disrupt the attachment of the cell sheet to the dentine surface. Gomes Flores et al used a polymer membrane to secure the dentine construct in the surgical site which we thought may ultimately compromise blood supply to the construct, which is an important consideration for tissue healing and regeneration (Slavkin and Bartold, 2006). Construct stability is also important for cell sheet stabilization and subsequent cell differentiation and tissue regeneration. These difficulties were not reported in similar *in vivo* studies using the same principle of subcutaneous transplantation of a cell sheet construct (Washio et al., 2010, Iwata et al., 2010, Gomez Flores et al., 2008). We experienced some difficulties in stabilising the construct, although these were ultimately overcome by suturing the dentine chip to the dorsal muscle.

In the previous chapter, we were able to demonstrate that mineralization media (MM) treatment of periodontally derived cells was able to stimulate differentiation of the cells along a mineralized forming phenotype, with up-regulation of bone and cementum gene expression after 3-6 weeks. Therefore, in this chapter we sought to examine the ability of a MM treated periodontal ligament cell line (PLC1) with a demonstrated *in vitro* cementogenic potential to achieve *in vivo* cementogenesis.

Histological analysis showed that the periodontal cell sheet treated with MM remained closely positioned and/or adhered to areas of the dentine slice after 6 weeks *in vivo*. Some darker staining which resembled mineralised tissue was found on the cell sheet side of the dentine chip. However, the cell sheets from the control samples cultured in control media were not firmly adhered to the dentine chip. These results support the earlier findings of the previous chapters, which suggested that the cell type used in this *in vivo* pilot study (PLC1) had the potential to differentiate into a hard tissue forming phenotype. Further, the addition of a negative control dentine chip without cells was unnecessary as the cell sheets were placed on one side of the dentine chip only. Hence the opposite side was considered as the negative control with no cell sheet. The opposite side without cells was demonstrated to be covered with fibrous tissue with no obvious cell layer or mineralization.

Moreover, CEMP1 protein analysis showed strong staining in the cells attached to the dentine chip, which suggests cementoblastic differentiation of those cells. This finding correlates with other reports that CEMP1 protein has been shown to localise in cementum and the adjacent cementoblast cell layer. Furthermore, CEMP1 mRNA is

highly expressed in cementoblast subpopulations, progenitor cells of the human periodontal ligament and human root derived cells (Alvarez-Perez et al., 2006, Arzate et al., 2002, Nunez et al., 2010, Park et al., 2011).

Currently, our laboratory is testing various constructs of different biomaterial/dentine/cell sheet combinations using this technique. Moreover, transplantation of cell sheets into larger animal models is also currently being investigated in our laboratory to test periodontal cell sheet capacity for achieving periodontal tissue regeneration.

5.6 Conclusion

The present study demonstrates that cells with osteogenic/cementogenic potential *in vitro,* as demonstrated by mineralized tissue formation and bone and cementum marker (CEMP1) expression, can participate in cementum formation *in vivo*.

6.0 Chapter six

General Discussion and Future Directions

Periodontitis is a chronic inflammatory disease that leads to irreversible damage to both soft and hard tissue components of the periodontium. Therefore, the most desirable outcome of periodontal therapy is the full restoration or reconstitution of the form and function of the lost tissues, which is defined as periodontal regeneration. In general, the regeneration of any tissue type requires the interaction of three factors: the availability of the appropriate cell type(s), soluble mediators of cell function that activate these cells and an evolving extracellular matrix (Bartold et al., 2000b). The periodontium is a complex structure comprising both soft and hard tissue components and hence, successful periodontal regeneration requires a cascade of temporally and spatially coordinated interactions. During periodontal regeneration, cells such as fibroblasts, cementoblasts and osteoblasts must not only be attracted in appropriate numbers, but subsequently induced, at the right time and in the right place, to synthesize their appropriate extracellular matrices and restore the lost alveolar bone, cementum and periodontal ligament (Bartold et al., 2000a).

It is generally accepted that cementoblast / osteoblast progenitor cells are located in the endosteal spaces of alveolar bone as resident paravascular cells. During regeneration these cells rapidly migrate into the contiguous periodontal ligament and differentiate into cementoblasts and osteoblasts (McCulloch, 1987). Moreover, there is also growing evidence that undifferentiated multipotent ‘stem’ cells are present in the different components of the periodontium (Seo et al., 2004, Fournier et al., 2010, Lin et al.,

2008a, Wu et al., 2009, Xu et al., 2009). Therefore, in this thesis we hypothesised that primary cells obtained from alveolar bone, regenerated periodontal tissue and periodontal ligament derived explants would contain cells of the cementoblastic phenotype in various stages of differentiation. This is significant as cementogenesis is a critical stage in the process of periodontal regeneration, as it's the newly formed cementum into which the fibres of the periodontal ligament are inserted that provides functional attachment between the tooth and alveolar bone. We propose that an improved understanding of the cells involved in the regenerative process, as well as the effect of various growth factors on these cells, may ultimately result in the development of novel, more predictable regenerative therapies.

The presented series of studies aimed to characterize the cementoblastic differentiation potential of various cells derived from the periodontium during differentiation induced by mineralization media, and the growth factors IGF-1 and PDGF. The rationale was that the most effective treatment / cell combination could then be identified for use in regenerating periodontal tissue *in vivo*. To this end, a number of different assays were performed to measure cellular differentiation and to determine the effect of the growth factors in this process.

More specifically, in Chapter 2, the differentiation of periodontal derived ligament cells (PLC), regenerated periodontal tissue (RTC) and alveolar bone cells (OB) along a mineralized tissue forming phenotype after induction by mineralization media was examined *in vitro* using a combination of functional biochemical assays and real time PCR (RT-PCR) quantification of cementum and bone associated gene expression. In Chapter 3, the effect of the growth factors IGF-1 and PDGF, as well as the combined

effect of these growth factors on cementum and bone associated gene expression was also examined *in vitro* using RT-PCR. In Chapter 4, we sought to characterize the heterogeneity that was observed between and within cell populations derived from the same tissue type. These studies culminated in Chapter 5, whereby cells chosen on the basis of their cementogenic phenotype were induced to differentiate, and subsequently implanted as a cell sheet in an athymic rat animal model in order to examine their cementogenic potential *in vivo*.

The periodontal ligament has been shown to contain undifferentiated mesenchymal stem cells, which under defined culture conditions differentiate into cementoblast-like cells, adipocytes, and collagen-forming cells (Seo et al., 2004, Chen et al., 2006, Somerman et al., 1988, Cheng et al., 1994). These stem cells also show a capacity to form mineralized nodules under osteogenic culture conditions, and to generate a cementum/periodontal ligament-like structure when transplanted *in vivo* (Seo et al., 2004, Gronthos et al., 2006, Gay et al., 2007, Lindroos et al., 2008, Xu et al., 2009). Another possible source of osteoblast and cementoblast precursors are the endosteal spaces of alveolar bone from which cells have been observed to adopt a paravascular location in the periodontal ligament of mice (McCulloch, 1987). More recent studies have also identified progenitor cells in the regenerated tissue harvested from periodontal defects treated using guided tissue regeneration (Lin et al., 2008b). Guided tissue regeneration (GTR) is based on the observation that the periodontal ligament, but not gingival connective tissue or bone, contains cells capable of establishing new attachment fibers between cementum and bone (Nyman et al., 1980, Karring et al., 1980, Nyman et al., 1982a). GTR thus advocates the use of synthetic barrier membranes to encourage appropriate progenitor cells from the periodontal ligament to re-populate the defect site and to exclude other cell types which would promote repair rather than

true regeneration of a new periodontal attachment. Therefore, cells cultured from these regenerating defects (RTC) were investigated based on the rationale that this population would be enriched in progenitor cells capable of facilitating periodontal regeneration.

The process of biomineralization is initiated by osteoblasts which secrete a range of extracellular matrix proteins including type I collagen, several glycoproteins, and proteoglycans. Some of these proteins, notably osteocalcin (OCN) and certain glycoproteins, bind Ca^{2+} ions with high affinity, thus raising the local concentration of these ions. Osteoblasts also release very small membrane-enclosed matrix vesicles with which alkaline phosphatase (ALP) and other enzymes are associated. These enzymes hydrolyze PO_4^{3-} ions from various macromolecules, creating a high concentration of these ions locally. The high ion concentrations cause crystals of $Ca_3(PO_4)_2$ to form on the matrix vesicles. The crystals grow and mineralize further with formation of small growing masses of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] which surround the collagen fibers and all other macromolecules. Eventually the masses of hydroxyapatite merge as a confluent solid bony matrix and calcification of the matrix is completed (Junqueira, 2003).

In chapter 2, biochemical assays were used to characterise the differentiation of primary cells (PLC, RTC and OB) along a mineralized tissue forming phenotype. Both Alizarin red S and von Kossa staining were used to demonstrate deposition of the two major components of mineralized nodules i.e. calcium and phosphate ions. Moreover, as mineral deposits in osteoblast cultures are mostly $Ca_3(PO_4)_2$ substituted hydroxyapatite similar to that seen in bone, cartilage and teeth (Ebberink et al., 1989), a commercially

available assay kit was used to detect hydroxyapatite formation in the differentiating cells.

All three cells formed mineralized tissue to various degrees. Both PLC and OB showed more mineralization than RTC, which was still able to form mineralized tissue, but at a later time point. These findings suggest that the population of PLC, RTC and OB tested in this study were able to differentiate into a mineralized tissue forming phenotype, which is in accordance with other reports in the literature (Arceo et al., 1991, Cho et al., 1992, Khanna-Jain et al., 2010, Lin et al., 2008a).

The biochemical assays were carried out at four time points following mineralization treatment i.e. after 72hr, 1, 3, and 6 weeks. Mineralization was found at weeks three and six. These findings are consistent with and complementary to other *in vitro* mineralization studies, where the maturation phase of the mineralized nodules occurred in the second (Wang et al., 1999, Somerman et al., 1988) or third (Quarles et al., 1992, Bellows et al., 1992, Cheng et al., 1994) week. Moreover, all of the biochemical tests, Alizarin red S staining, von Kossa staining, calcium release and hydroxyapatite formation were consistent in the temporal pattern of mineralization that was observed.

To examine the bone and cementum gene expression profile during differentiation, real time PCR was also performed at each time point. The majority of information regarding gene expression during PLC and OB differentiation has been carried out at only a few early time points (Hayami et al., 2007, Hiraga et al., 2009, Hou et al., 2000, Lamour et al., 2007, Pi et al., 2007, Wu et al., 2009). Furthermore, no published data could be

found regarding gene expression during RTC differentiation. Indeed, the expression of cementum associated markers has not been previously investigated during the differentiation of periodontal cells *in vitro*. Therefore, our study design sought to gain new information regarding the expression of bone and cementum associated gene expression during the mineralization of periodontal cells *in vitro*.

Real time PCR was used to quantify the relative expression of cementum and bone markers, some of which are expressed at relatively low levels. Real-time PCR allows rapid quantification of a target gene sequence during the extension phase of PCR amplification (Ong and Irvine, 2002). Unlike traditional PCR techniques, which rely on the assessment of a product following amplification for a predetermined number of cycles, real-time PCR is able to precisely quantify DNA because it measures the PCR product during the extension phase of amplification, rather than a pre-specified endpoint (Kim, 2001). Therefore, we used RT-PCR to quantify the relative expression of the genes of interest. Comparisons of the up or down-regulation of genes according to cell type, length of treatment time and treatment type could then be made. Using the sensitive RT-PCR method also allowed us to detect the expression of the marker genes at baseline, before any differentiation treatment began.

A set of guidelines outlining the minimum information required for reporting quantitative RT-PCR experiments has recently been published to ensure the relevance, accuracy, correct interpretation, and reproducibility of studies using this methodology (Bustin et al., 2009). One essential criterion is the justification of the number and choice of reference genes used for normalisation of the data obtained. As it is often difficult to select a reference gene that will have a stable, i.e. consistent level of expression across

different cell types and following different treatments, an average value obtained from a number of reference genes is currently the accepted best method (Vandesompele, 2002). We used three reference genes (GAPDH, Rps13 and β-MG) for the current experiment.

The formation of cementum is a crucial step towards periodontal regeneration as it is the cementum into which fibers of the periodontal ligament insert, thereby providing functional attachment between the alveolar bone and the tooth. However, one major impediment to investigating cementum formation *in vitro* is a lack of specific cementum markers (Alvarez-Perez et al., 2006, Bosshardt, 2005). As cementoblasts share many molecular properties with osteoblasts, markers previously used for osteoblasts have also been used to try and identify cementoblasts. These markers include type I collagen, non-collagenous proteins such as OCN, OPN and BSP, and the transcription factor, Runx2 (D'Errico et al., 1997, Bosshardt, 2005, Kitagawa et al., 2006a). In the current project we chose three bone markers (BSP, OCN and Runx2) commonly associated with cementogenesis as well as periodontal regeneration (D'Errico et al., 1997, Kitagawa et al., 2006a, Yamamoto et al., 2007, Berry, 2003, Nanci, 1999). BSP constitutes between 8 and 12% of the total non-collagenous proteins in bone and cementum (Fisher et al., 1983, Fisher et al., 1987, Fisher et al., 1990b). OCN expression has been described in both dental root formation and early cementogenesis (Bronkers et al., 1994, Bosshardt et al., 1998). Runx2 has been found to act as an inducer of osteoblast differentiation and has all the characteristics of a differentiation regulator in the osteoblast lineage (Ducy et al., 1997, Komori et al., 1997). It has been shown to be expressed in periodontal ligament fibroblasts (Jiang et al., 1999) and in human cementoblast cell lines (Kitagawa et al., 2006a).

However, these molecules are not cementum-specific (Bosshardt, 2005). Many efforts have been carried out to identify cementum specific markers from extracts of cementum and cementoblastoma derived medium. This has lead to the identification of the only cementum markers available to date and which we have included in our investigations: cementum attachment protein (CAP) and cementum protein 1 (CEMP1 or CP23), (Arzate et al., 1992, Arzate et al., 2002, Alvarez-Perez et al., 2006).

More recent studies have now identified other putative markers which may be useful in identifying cementoblasts and cementum formation, including the enamel associated proteins ameloblastin, amelogenin, enamelin, and tuftelin (Nunez et al., 2010), a heparin-binding glycoprotein F-Spondin (Kitagawa et al., 2006) and glucose transporter 1 (GLUT1) (Koike et al., 2005). However, further studies are needed to confirm the specific localization and expression of these markers by cementoblasts during cementogenesis. We have included the assessment of F-Spondin expression in the current study as there is strong evidence in the literature that this protein may provide the specificity required to identify cells with cementoblastic potential . For example, F-Spondin has been suggested to play a role in the early stages of PDL formation (Nishida et al., 2007), is highly expressed in human cementoblast-like cell lines (HCEM), was not found in osteoblast cell lines, and when human periodontal ligament cells were transfected with F-Spondin, they changed their morphology and exhibited increased expression of OCN, BSP, CEMP1 mRNA and ALP activity (Kitagawa et al., 2006).

Therefore, in chapter 2, we assessed the gene expression of six putative bone/cementum markers, over a 6 week time period. Our findings are consistent with other studies

demonstrating that PLC can be osteogenic in nature and differentiate into mineralized tissue forming cells, which may be either osteoblasts or cementoblasts (Mukai et al., 1993, Bartold et al., 2000a, Cho et al., 1992, Arceo et al., 1991). As expected, alveolar bone cells were also shown in our study to differentiate and express bone markers.

The RTC results suggest that this population may contain cells able to differentiate along a mineralized tissue forming phenotype. This is not surprising since these cells were derived from periodontal defects treated via GTR, a clinical technique which aims to promote the re-population of the periodontal defect with cells from the periodontal ligament and alveolar bone. The results are consistent with other studies which have described the 'progenitor' nature of these cells and their ability to differentiate along an osteogenic phenotype (Wakabayashi et al., 1996, Ivanovski, 2001a, Lin et al., 2008b).

Moreover, all of the primary cells tested expressed the osteogenic/cementogenic markers at baseline and over time even without any induction media, suggesting that the cell population tested might already contain differentiated cells and might also raise a question as to whether these markers are truly bone/cementum specific. Mineralization treatment up-regulated further both BSP and SPON1 expression in the three cell types, which might suggest that both of these markers are good candidates as markers of cell differentiation along a mineralized tissue forming phenotype. The two putative cementum genes regularly used as cementum specific markers, CAP and CEMP1 were up-regulated only in PLC, which is in agreement with the hypothesis that cells residing in the periodontal ligament are able to form cementum and facilitate the establishment of new periodontal attachment (Nyman et al., 1980, Karring et al., 1980, Nyman et al., 1982a).

When discussing the significance of studies of this nature, the expression of various genes *in vitro* often assumes that the mRNA produced is ultimately translated into active protein expression. Indeed, recent studies have found a positive correlation between mRNA and protein expression levels in many normal human cells (Gry et al., 2009, Guo et al., 2008, Anderson and Seilhamer, 1997). The results from these studies also suggest that mRNA expression might correlate with cellular protein expression. Further studies need to be carried out to verify any correlation between mRNA expression and protein synthesis during periodontal cell differentiation.

The findings reported in Chapter 2 suggest that PLC, RTC and OB contain cells able to respond to mineralization treatment, form mineralized matrix and express bone/cementum genes. Moreover, these cells may play an important role in periodontal hard tissue (bone and cementum) formation and hence periodontal regeneration. To elucidate the effect of the most commonly used growth factors in periodontal cell differentiation and periodontal tissue regeneration, the effect of IGF-1 and PDGF, alone and in combination, on periodontal cells differentiation was examined in chapter 3.

Most of the published literature on the effect of IGF-1 and/or PDGF on osteogenic differentiation has been carried out in cell lines (Strayhorn et al., 1999, Wildemann et al., 2007, Koch et al., 2005). However, it's been suggested that commercially available cell lines, which are generally transfected cells, are model systems that differ in various respects from primary cells, notably in that many of the regulatory pathways are not present and the chromosomal arrangements are beyond the normal patterns found in healthy tissues (Carlson et al., 2007). Although there is increasing data on the effect of

IGF-1 and/or PDGF on the regeneration of bone (Vikjaer et al., 1997, Lynch et al., 1994), periodontal defects (Wang et al., 1994, Cho et al., 1995, Howell et al., 1997, Park et al., 1995, Nevins et al., 2003, Nevins et al., 2005, Nevins et al., 2007), and peri-implant bone defects (Becker et al., 1992, Lynch et al., 1991a), there is a lack of understanding of the molecular effect that these factors exert during periodontal cell differentiation.

The published *in vitro* and *in vivo* data on the effect of IGF-1 and/or PDGF on cementoblasts, osteoblasts and mesenchymal cell line gene expression is inconsistent and confusing (Saygin et al., 2000, Strayhorn et al., 1999, Tanaka et al., 2002, Tanaka and Liang, 1995, Koch et al., 2005, Chung et al., 2009). For example, it appears that the effect of PDGF and IGF-1 on osteoblast-associated genes is dependent on a variety of factors including growth factor concentrations, duration of application, serum concentration, source and phenotype of the cells under investigation, as well as the stage of cell differentiation. The only available data on the effect of IGF-1 and/or PDGF on primary cells derived from the periodontal ligament is from a study carried out by Ivanovski *et al* (2001) which found no effect of these growth factors on osteogenic gene expression (OCN, OPN and BSP) following short term application (24hrs). A number of significant differences between this study and that described in this thesis may explain these discrepancies in reported observations Ivanovski, *et al* (2001) used standard end-point PCR while we have used a more sensitive real-time PCR methodology. Also while the same concentration of the growth factors was used, the cells were cultured in a 0.2% FCS, while we used a slightly higher concentration (1% FCS). To choose the minimum concentration of FCS for our study, we performed a preliminary experiment (data not shown) whereby cells were cultured in a series of different FCS concentrations (0.2%, 1%, 5% and 10%) for six weeks and found that 1%

FCS was minimum concentration needed for cell survival over the six weeks course of our study. Furthermore, we found that cells cultured in 1 and 10% FCS showed similar patterns of gene expression over time although 1% FCS appeared to induce higher levels of relative gene expression compared to the 10% FCS medium. This observation is in agreement with the current literature which suggests that low serum concentrations can promote differentiation in different cell types, for example oligodendrocytes (Raff et al., 1983) and hepatic progenitor cells (He et al., 2011).

Limited information is available in the literature on the effect of IGF-1 and PDGF on the differentiation of periodontal derived cells i.e. periodontal ligament, regenerated periodontal defect and alveolar bone cells (OBs) and their expression of bone (BSP, OCN and Runx2) and cementum genes (CAP, CEMP-1 and SPON1). Overall, the results of chapter 3, suggest a positive effect of IGF-1 and PDGF on periodontal cell differentiation and the expression of bone/cementum related genes. Osteoblasts were the most responsive cell type to the growth factor treatment tested, with up-regulation of most of the genes tested, which was in agreement with the current literature suggesting a differentiation effect of IGF-1 and/or PDGF on osteoblasts (Koch et al., 2005, Jonsson et al., 1993, Tanaka et al., 2002). The effect of these growth factors on PLC may explain the current growing evidence of the positive effect of these factors on periodontal regeneration described in clinical trials (Giannobile et al., 1996, Jin et al., 2004, Cho et al., 1995, Lynch et al., 1989, Nevins et al., 2003, Nevins et al., 2005, Nevins et al., 2007, Mellonig et al., 2009, Camelo et al., 2003, Howell et al., 1997).

Moreover, the effect of the growth factors on osteogenic/cementogenic gene expression was noted at the four different time points studied, however, more up-regulation was

found after 3 and 6 weeks of cells culture. The up-regulation that is noted is relative to the expression in the 'untreated' cells at the same time point. The cells and the surrounding extracellular matrix would evolve over a period of 3 and 6 weeks in culture, and it appears that the cells' increased 'maturity' and the associated extracellular environment make the cells more responsive to the growth factors than at the earlier time-points.

The combination of IGF-1/PDGF was found to have either a synergistic effect or no effect on the expression of the markers tested in PLC, RTC and OB. Indeed, the addition of IGF-1 to PDGF has been found to have a synergistic effect on cell mitogenesis (Mott et al., 2002, Wildemann et al., 2007, Giannobile et al., 1997). However, their effect on cellular differentiation and bone formation was conflicting (Pfeilschifter et al., 1990, Wildemann et al., 2007, Tanaka et al., 2002, Lynch et al., 1994).

Both BSP and SPON1 were found to be up-regulated in the three cell types after growth factors treatment, suggesting that these two markers could be associated with cellular differentiation and there could be a shared mechanism between these two genes. Furthermore OCN was only up-regulated in OB cells at later time points with all treatments. However, the two putative cementum markers, CAP and CEMP1 were not affected by IGF-1 or PDGF alone, but were up-regulated when the two growth factors were combined. These results suggest that both IGF-1 and PDGF might contribute to osteogenesis, and to a lesser degree to cementogenesis *in vitro*.

It is noteworthy that the studies described in Chapters 2 and 3, which utilized a number of primary cell lines, were characterized by significant heterogeneity in the response of cells derived from the same cell type. This extensive heterogeneity in mineralized tissue formation and bone and cementum associated gene expression by the different cell populations studied is perhaps not surprising given the cells inhabiting the periodontium are noted for their considerable heterogeneity (McCulloch and Bordin, 1991, Ko et al., 1984, Phipps et al., 1997, Lekic et al., 1997, Roberts and Chamberlain, 1978, Ivanovski, 2001a, Fujita et al., 2005). To investigate this issue of heterogeneity among cells derived from the same tissue source, we established single cell derived clones. This experimental approach allowed an examination of specific clonal patterns of gene expression during mineralization/differentiation inducing treatment. Although, there is always the possibility of the transfection process changing the nature and characteristics of the original primary cell lines, primary periodontal ligament and gingival fibroblast cell lines immortalized by HPV16 transfection have been shown to consistently retain normal periodontal ligament and gingival fibroblast phenotypes, respectively, and periodontal ligament markers and osteogenic differentiation in the immortalized periodontal ligament cells are distinct from immortalized gingival fibroblast cells (Pi, et al. 2007).

Indeed, the mineralization media effect on the expression of osteoblast/cementoblast-associated genes was more pronounced in certain clones, which further illustrates the intra-population heterogeneity that exists within primary cultures. Interestingly, high levels of expression of the bone and cementum genes by some clones when cultured only in control media suggests that they may belong to a subpopulation of cells with inherent osteogenic/cementogenic potential. Moreover, the clonal response to mineralization treatment covered the gamut of possible responses with some genes not

affected, and others down-regulated or up-regulated. For example, clone PLCIII had high Runx2, CAP, CEMP1 and SPON1, and low BSP and OCN expression, while RTCIII showed up-regulation in almost all markers at almost all time points. The clone results not only demonstrate the heterogeneity of cells derived from the same source, but also the inherent ability of some clones to express differentiation markers, irrespective of the time in culture or the culture conditions.

The observed response of the cells described in chapters 2-4 may also explain the large variability in the response of primary cells derived from the same cell type and hence the difficulty that this poses during statistical analysis. Differences in cellular proliferation have been studied previously. HGF were found to grow more rapidly than PLF (Mariotti and Cochran 1990), and RTC cells derived from regenerated periodontal defects proliferate faster than PLF (Ivanovski *et al*., 2001). However, it was apparent in our study that differences in the response to osteogenic media or growth factors were independent of inherent cellular differences in proliferation rate. Previous work using these cells did not find any correlation between proliferation and the expression of bone markers (Ivanovski *et al*., 2001a, Ivanovski *et al*., 2001b).

These findings emphasize the importance of potential cellular heterogeneity when extrapolating results from *in vitro* cell culture analysis to an *in vivo* tissue response, especially when transfected commercially available cell lines are used. The results also reinforce the necessity for investigating means to facilitate periodontal regeneration through the promotion of a selective repopulation of the defect by cells with a regenerative phenotype. One approach may be via *in vitro* selection of appropriate cells

with the required characteristics to promote differentiation and regeneration of the lost tissues.

Although osteogenic/cementogenic gene expression was found to generally increase over time after the various inducing treatments, a particular sequence or a clear pattern of gene expression was very hard to determine, and hence a general conclusion regarding the temporal pattern of gene expression in the three cell types examined was difficult. That might be attributed to the heterogeneity among the primary cells used in this study which could suggest the presence of cells at different stages of differentiation, cells with no differentiation potential or cells which might be fully differentiated.

To this end, the primary cell line PLC1 was chosen to study the formation of cementum *in vivo* as PLC1 had shown significant mineralization formation potential demonstrated by both Alizarin red S staining and hydroxyapatite formation analysis *in vitro*. This cell line also expressed a variety of cementum and osteogenic genes which increased over time (apart from CAP). In chapter 5, these cells were treated with mineralization media for one week, allowed to adhere to a slice of human dentine and transplanted subcutaneously into immunodeficient rats. Subsequent histological assessment of the transplanted PLC1 cell sheet/dentine construct showed attachment of the cell sheet to the dentine chip in the mineralization media treated sections after six weeks of *in vivo* transplantation. Fibroblast attachment to the root surface has been shown to be essential, along with cell migration and differentiation, for the formation of functional periodontal attachment (Raulin et al., 1987). Interestingly, the control sheet (without mineralization treatment) did not show a direct attachment of the cell sheet to the dentine chip after six weeks of transplantation. This difference may be attributed to the increased extracellular

matrix deposition of the mineralization media treated cells, due to the presence of ascorbic acid which increases collagen production and hence facilitates cellular adhesion and attachment (Quarles et al., 1992).

Moreover, the recognition of CEMP1 antigen within the cells and tissue attached to the dentine chip also suggests the presence of cementoblastic differentiation. It is likely and indeed desirable for the host cells to participate in the 'regenerative' response. The presence of the transplanted cells in this case can be tracked by using a specific human gene (eg. *alu*) which would identify human from rat cells. However, the use of a cell sheet in our study made it possible to localise the transplanted cells as the cell sheet structure could be identified in the histological sections. This pilot study therefore confirmed that primary cells that were identified as having the ability to differentiate along an osteogenic/cementogenic phenotype based on their gene expression profile *in vitro* were able to induce cementum formation when transplanted subcutaneously *in vivo*. Further analysis is required to fully characterize the cell sheet/tissue adhered to the dentine chip after 6 weeks of *in vivo* transplantation.

In conclusion, the results of this thesis demonstrate that cells residing in the periodontal ligament, alveolar bone and regenerated periodontal tissue can be induced to differentiate along a mineralized tissue forming phenotype as demonstrated by the formation of mineralized nodules and the temporal up-regulation of various bone and cementum genes. Furthermore, the growth factors IGF-1 and PDGF were demonstrated to enhance osteogenic and cementogenic gene expression in those cells. *In vivo* cementogenesis could then be achieved using a cell population with known characteristics in terms of cementogenic potential.

More specifically, with regards to the aims of the series of studies presented in this thesis outlined in Chapter 1, the following conclusions can be made:

1- Cells derived from periodontal ligament (PLC), periodontal regenerated tissue (RTC) and alveolar bone (OB) contain progenitor cells able to differentiate into mineralized tissue forming phenotype as demonstrated by the formation of mineralized extracellular matrix after 3 weeks of treatment with mineralization media along with the up-regulation of bone and cementum marker genes. PLC appeared to be the most responsive in terms of the up-regulation of cementum gene expression and hence appears to be the most suitable candidate for use in periodontal regeneration *in vivo*. BSP and SPON1 gene expression were found to be more closely associated with the mineralization process.

2- Both IGF-1 and PDGF were found to up-regulate BSP and SPON1 gene expression in all three cells types tested. OB followed by PLC were the most responsive cell types. Both growth factors had less impact on RTC gene expression. Moreover, OCN was up-regulated only in OB following growth factor treatment. Low concentrations (1%) of FCS also induced more cellular differentiation compared to standard media containing 10% FCS.

3- Both PLC and RTC are a heterogeneous cell population. Clones established from these cells had different responses to mineralization treatment with some clones able to differentiate along a mineralized tissue phenotype. Although the expression of the gene markers could be influenced by culture conditions, the over-riding determinant on gene expression levels appeared to be the individual cell lines' inherent ability to express a given bone/cementum marker.

4- Cementogenesis was demonstrated *in vivo* using a PLC previously demonstrated to have significant *in vitro* cementogenic potential.

The results of this series of studies presented in this thesis highlight the importance of understanding the heterogeneity inherent among primary cells and therefore we would recommend researchers to characterize their cells under investigation before moving to more advanced periodontal regeneration studies. Moreover, the effect of mineralization media, IGF-1, PDGF and the sequence of bone/cementum gene expression during periodontal cell differentiation over time should be clarified further to facilitate future periodontal regeneration approaches.

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