DEVELOPMENT AND CHARACTERISATION OF AN *EX VIVO* MODEL SYSTEM FOR BONE REPAIR

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ABSTRACT

Limitations in current model systems for researching bone repair have hampered the development of alternative clinical therapies. This thesis aimed to develop and validate an *ex vivo* rat mandible model, to investigate specific molecular and cellular processes involved in bone repair. Maintenance of cell and tissue architecture and viability was shown within mandible slices cultured for up to 21 days, both intact and fractured. Autoradiographic studies showed that resident cells were actively synthesising and secreting proteins, and cells of the osteoblast lineage were shown to survive throughout the culture period. The model was responsive to exogenously added growth factors TGF-β1 and BMP-2, with increased cellular migration / proliferation and expression of bone matrix proteins observed. A second model system, an *in vitro* bone slab cell culture system, demonstrated that endogenous growth factors could be released from the matrix of bone by chemicals such as EDTA, calcium hydroxide, and sodium hydroxide. Different growth factor release kinetics were observed with each treatment, and released growth factors were capable of actively influencing the behaviour of osteogenic cells. Pre-treatment of mandible slices with these chemical treatments yielded similar results, with an observed increase in cell number, proliferation, and bone matrix protein expression. The *ex vivo* mandible model developed within this study may represent an ideal system for investigating specific processes of bone repair, as well as a promising alternative to *in vivo* testing of novel clinical therapeutics.

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ABBREVIATIONS

^3H	Tritiated
ABC	Avidin and Biotinylated horseradish peroxidase macromolecular Complex
aFGF	Acidic Fibroblast Growth Factor
ALKs	Activin Receptor-Like Kinases
ANOVA	Analysis of Variance
bFGF	Basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Proteins
BSA	Bovine Serum Albumin
BSP	Bone Sialoprotein
$Ca(OH)_2$	Calcium Hydroxide
CO_2	Carbon Dioxide
Co-Smad	Common mediator Smad
DAB	3,3'-Diaminobenzidine
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
GAG	Glycosaminoglycan

H&E	Haematoxylin & Eosin
IGF	Insulin-like Growth Factor
IL-1	Interleukin-1
IL-6	Interleukin-6
IMS	Industrial Methylated Spirit
LAP	Latency-Associated Peptide
LTBP	Latent TGF-β Binding Protein
MAPK	Mitogen-Activated Protein Kinase
M-CSF	Macrophage Colony-Stimulating Factor
NaOH	Sodium Hydroxide
NF-κB	Nuclear Factor Kappa B
NSAID	Non-Steroidal Anti-Inflammatory Drug
OPG	Osteoprotegerin
PBS	Phosphate-Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PDGF	Platelet-Derived Growth Factor
PDL	Periodontal Ligament
RANK	Receptor Activator of Nuclear Factor κ B
RANKL	Receptor Activator of Nuclear Factor κ B ligand
ROS	Reactive Oxygen Species
R-Smad	Receptor-regulated Smad
SEM	Scanning Electron Microscopy
SIBLING	Small Integrin-Binding Ligand, N-linked Glycoprotein
SLC	Small Latent Complex
SPARC	Secreted Protein, Acidic and Rich in Cysteine

TBS	Tris-Buffered Saline
TGF-β	Transforming Growth Factor-β
TNAP	Tissue Non-specific Alkaline Phosphatase
TNF-α	Tumour Necrosis Factor-α
TRAFs	Tumour Necrosis Factor Receptor-Associated Factors
TRAP	Tartrate-Resistant Acid Phosphatase
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
α-MEM	Alpha-modified Minimum Essential Medium

CHAPTER 1

INTRODUCTION

Bone repair involves a complex cascade of biological events initiated in response to injury or trauma, and the process ultimately results in optimal repair and restoration of function to the bone. It is a recovery process unlike any other tissue regeneration, and the process recapitulates the development and growth of bones observed in the embryo (Zimmermann et al. 2005). In the majority of cases, intramembranous and endochondral ossification lead to normal and natural bone healing, but there are many clinical situations where repair processes are impaired. Examples can be seen in fracture non-unions (Kloen et al. 2002; Trippel et al. 1996), non-healing sockets after tooth extraction, and patients suffering from diseases such as osteonecrosis (Reid 2008), osteoporosis (Namkung-Matthai et al. 2001; Tang et al. 2008), and diabetes. Diabetic patients often show significantly reduced bone healing capabilities, and patients are reported to have a higher failure rate of dental implants due to a reduced capability for osseointegration (Valero et al. 2007). Other contributory factors such as smoking, certain medications, and infection can also result in poor healing (Hayda et al. 1998).

There are a limited number of therapies in clinical practice, with treatments mostly used to aid normal bone healing, or being best suited in healing small defects in maxillofacial surgery and (to a lesser extent) orthopaedic surgery. These therapies are used infrequently, if at all, in the conditions described

above where healing is impaired. There is much interest in refining and developing treatments, and in particular expanding therapies so that they may be used in conditions with impaired bone healing. The current gold standard is the use of bone autograft and allograft as materials for skeletal repair and regeneration (Stevenson 1998). These materials have several drawbacks, including limits in bone availability and infection-associated problems (Cancedda et al. 2007). Research into alternative therapies has focused on the many bone-inducing proteins present in bone (Urist 1965), particularly the bone morphogenetic proteins (BMPs), a group of growth factors known to be potent inducers of bone formation (Bilic et al. 2006; Boden et al. 2000; Friedlaender et al. 2001; Jones et al. 2006). Problems currently associated with this field of clinical treatment are related to the large supraphysiological doses of growth factor required to elicit a therapeutic response in humans.

The development of alternative therapies is hampered by the research model systems currently available for studying bone repair processes. *In vitro* models use one or two cell types at the most (Jiang et al. 2005; Jung et al. 2005), and are unable to recapitulate the spatial arrangement of cells *in vivo*. As such, *in vitro* models can be severely limiting. *In vivo* models have been developed in a variety of animals (Lu and Rabie 2003; Mark et al. 2004; Pearce 2007; Petite et al. 2000) and have yielded considerable information on repair processes. However, such models are extremely expensive, requiring large numbers of animals, it can be difficult to obtain clear data due to systemic influences, and there are ethical implications involved in

undertaking *in vivo* work. One solution to the problems currently associated with modelling of bone repair may be to utilise *ex vivo* organ culture models, where cells and tissues are cultured *in situ*. *Ex vivo* models have significant advantages, as cells and tissue can be cultured in the same spatial arrangement as would be found in the *in vivo* situation, while systemic influences are removed. Furthermore, animal numbers can be significantly reduced, allowing for cheaper and more ethically sound experimentation.

An *ex vivo* tooth slice model, developed by Sloan et al. (1998) has been used successfully instead of traditional *in vitro* and *in vivo* models to investigate a range of dental repair processes (Gonçalves et al. 2007; Murray et al. 2000; Sloan and Smith 1999). The tooth slice model also provided a basis for development of an *ex vivo* rat mandible model to investigate the effects of externally applied forces on the dentine-pulp complex (Dhopatkar et al. 2005). This thesis aims to further develop this *ex vivo* model for investigating specific processes of bone repair. The model will enable the investigation of many bone repair pathways by examining cellular proliferation, migration, and differentiation, as well as the role that specific growth factors play in repair (Lieberman et al. 2002). In conjunction with the mandible model, a bone slab cell culture model will also be developed, to demonstrate proof of principle that growth factors can be chemically released from bone matrix, and that release of such molecules can have profound effects on cell behaviour. The development of such models may be invaluable in the search for ways to improve and enhance the initial stages of bone repair in patients where normal healing does not occur.

1.1 BONE BIOLOGY

1.1.1 General Bone Composition and Organisation

Bone is a dynamic, highly organised tissue, providing a rigid structure for supporting the body, attachment points for muscles and tendons, acting as a reservoir of mineral ions, and producing a vast array of humoral cell types (Ng et al. 1997; Totora and Grabowski 2003). The components of bone can be divided into cellular and acellular categories. The cellular components form the living part of bone tissue and are involved in maintaining both structure and function of the tissue. The acellular components are synthesised by osteoblast cells secreting an organic matrix, which is subsequently mineralised. This mineralised bone matrix provides the structure of the tissue, and is also involved in providing a reservoir of mineral ions which can be accessed as required (Roberts and Hartsfield 2004).

The extracellular matrix (ECM) of bone is comprised predominantly of type I collagen (approximately 90%), as well as a number of non-collagenous proteins including osteonectin, osteopontin, osteocalcin, bone sialoprotein, and the small proteoglycans decorin and biglycan (Nanci 1999; Waddington et al. 2003; Young et al. 1992). Non-collagenous proteins interact within a framework of type I collagen, allowing accumulation of calcium and phosphate ions and subsequent formation of hydroxyapatite crystal which provides an organic framework and gives bone many of its structural

properties. The proteins contained within the matrix can specifically interact with bone cells embedded within the ECM, controlling processes such as survival, proliferation, and differentiation (Schonherr and Hausser 2000). The ECM also acts as a reservoir for a variety of cytokines and growth factors, via proteoglycans and other proteins that bind secreted growth factors and immobilise them within the matrix, enabling the generation of rapid and highly localised signals when required (Hauschka et al. 1986; Ramirez and Rifkin 2003; Schonherr and Hausser 2000; Taipale and Keski-Oja 1997). Interaction of cells with the ECM is therefore fundamental to cellular differentiation and function and may be critical in bringing about rapid healing of damaged bone (Green et al. 1995).

1.1.2 Bone Structure and Types

There are several types of bone, depending on the conditions under which it is produced and also the state of maturity. Mature lamellar bone has an orderly, organised structure, comprising of many collagen fibres running parallel to other fibres in the same layer (Nanci 1999), and provides structural strength and high mineral content (Weiner et al. 1999). Lamellar bone can further be divided into two structural types depending on the location and environment under which it was synthesised: cortical (or compact) bone, and trabecular (or spongy) bone. Cortical bone is rigid and dense, and comprises 80% of skeletal tissue, being found in the walls of bone shafts and on external bone surfaces (Ng et al. 1997). It consists of closely packed osteons

(also known as haversian systems), each one consisting of a central haversian canal surrounded by concentric rings of matrix known as lamellae (Weiner and Traub 1992). The blood vessels of the bone are contained within the haversian canals, and interconnect with blood vessels on the surface of the bone via vascular Volkmann's canals (Figure 1.1). Trabecular bone, located internal to cortical bone and at the end of long bones, has a more porous, lightweight structure, and is composed of trabeculae (thin plates) separated by marrow spaces (Figure 1.2). The bone marrow space contains mesenchymal stem cells, capable of differentiating into cells of the osteoblastic lineage, and as such plays a crucial role in bone repair (Beresford 1989; Krebsbach et al. 1999). Trabecular bone has a very high surface to volume ratio, making it ideal for easy storage and release of calcium and phosphate ions (Buckwalter et al. 1995). The periosteum is the outer layer of cortical and trabecular bone (Figure 1.2), within which reside the stem cells and progenitors which are ultimately responsible for the formation of cells involved in bone repair processes (Malizos and Papatheodorou 2005).

During initial stages of bone repair, rapid bone synthesis is required to bridge the gap left by an injury. Thus an immature bone, known as woven bone, is formed first, in order to restore structural integrity to the injured tissue (Kalfas 2001). This immature bone consists of intertwined mineralised collagen fibres that are orientated in many different directions, and also contains a relatively higher proportion of osteocytes than mature bone (White and Folkens 2000b). As such, woven bone is relatively weak and not optimal for providing

structural strength. In order to provide structural strength, woven bone is slowly remodelled into mature lamellar bone over time (Nanci 1999).

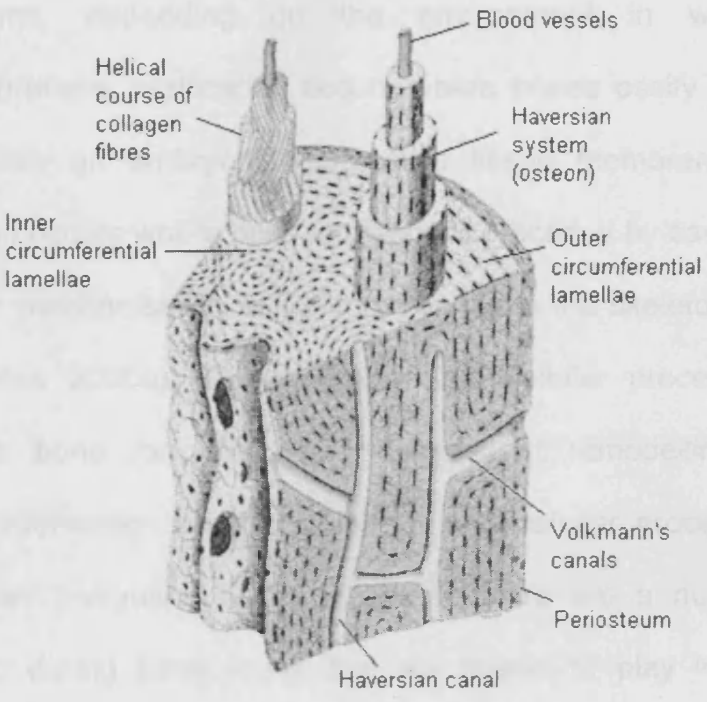

Figure 1.1. Cortical bone structure adapted from (Junqueira and Carneiro 2005).

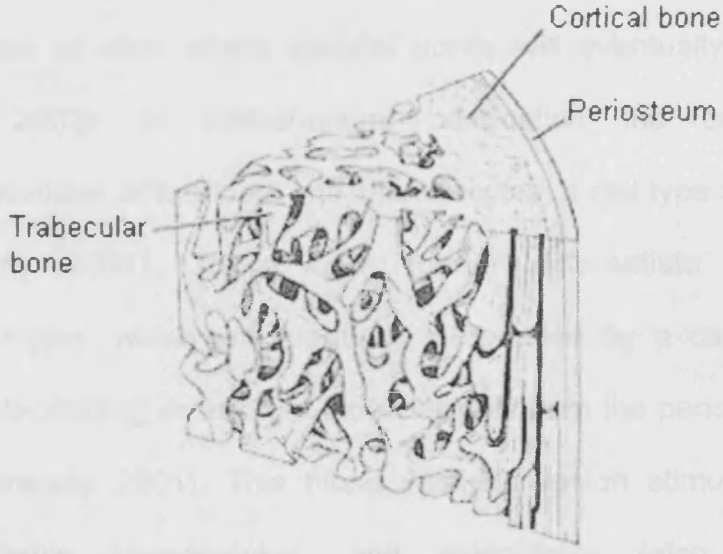

Figure 1.2. Trabecular bone structure adapted from (Spence 1990).

1.1.3 Embryonic Bone Formation

Embryonically, bone development or osteogenesis occurs via two distinct mechanisms, depending on the environment in which it occurs. Intramembranous ossification occurs where bones ossify by apposition on tissue within an embryonic connective tissue membrane. Endochondral ossification occurs where bone formation is preceded by cartilage precursors, and is the mechanism by which most bones in the skeleton develop (White and Folkens 2000a). The molecular and cellular processes involved in embryonic bone formation, and subsequent remodelling, may provide valuable information on the molecular and cellular processes involved in bone repair (Ferguson et al. 1999), as there are a number of proteins expressed during bone repair that are known to play important roles in embryonic bone development (Vortkamp et al. 1998).

At the onset of skeletal development, undifferentiated mesenchymal cells condense at sites where skeletal bones will eventually form (Zelzer and Olsen 2003). In endochondral ossification the cells within these condensations differentiate into chondrocytes, a cell type specific of cartilage (Karsenty 2001). These cells further differentiate into hypertrophic chondrocytes, which are gradually surrounded by a calcified extracellular matrix, facilitating invasion of blood vessels from the perichondrium (Wagner and Karsenty 2001). This blood vessel invasion stimulates apoptosis of hypertrophic chondrocytes, and osteoblasts (also originating from mesenchymal cells) begin to differentiate and deposit osteoid, the type I

collagen-rich extracellular matrix of bone. Any remaining chondrocytes reside in the growth plate, a narrow zone that together with the osteoblasts coordinates longitudinal bone growth (Wagner and Karsenty 2001).

Intramembranous ossification is a process responsible for development of the flat bones, in particular the facial bones, the cranial bones which form the sides and roof of the neurocranium, the mandible and the clavicle (Dixon et al. 1997). During this process, the cells within mesenchyme condensations differentiate directly into the bone-forming osteoblasts, without forming a cartilage precursor, and synthesise osteoid (Kronenberg 2003). Development of the mandible, the bone which forms the basis of the *ex vivo* model to be developed in this thesis, forms by the processes of intramembranous ossification described above, although it is preceded by the development of Meckel's cartilage, thought to initiate and regulate the growth of the primary ossification centre of the mandible (Frommer and Margolies 1971).

1.1.4 Post-natal Bone Remodelling

Following birth, and into adult life, processes of growth and remodelling within bones result in the formation of a skeleton which is well adapted for functions of movement, support, blood cell production, and calcium homeostasis (Zelzer and Olsen 2003). Bone constantly needs to adapt, to facilitate growth within young bones, to respond to environmental stresses, or to stimulate repair processes when damaged. All of these adaptations are

brought about by the control and coordination of two opposing processes: bone formation, coordinated by osteoblasts, and bone resorption, coordinated by osteoclasts. Together these cells orchestrate remodelling processes throughout the skeleton, a process important in the repair of damaged bone (Macdonald and Gowen 1993). Bone remodelling involves the localised removal of aged bone by osteoclasts and replacement with newly formed bone by osteoblasts. It is a complex process, requiring interactive cellular activity between osteoblasts and osteoclasts, and is regulated by a wide range of biochemical and mechanical factors. Remodelling processes occur continuously throughout life, to prevent accumulation of older bone, which is densely mineralised and therefore more brittle than younger bone. Remodelling also occurs to repair microdamage in order to maintain its strength, to adapt to mechanical stresses, and to metabolise minerals stored within its matrix (Ott 2002).

1.2 BONE REPAIR

Unlike other tissue regeneration, which results in fibrous scarring, the process of bone repair involves a complete regeneration of bone with no formation of scar tissue, and ultimately results in optimal skeletal repair accompanied by restoration of skeletal function (Einhorn 1998). The process of bone repair can be divided histologically into direct (primary) healing, and indirect (secondary) healing.

1.2.1 Primary / Direct Healing

Primary (or direct) bone healing occurs where there is rigid internal fixation resulting in anatomic reduction of the bone fragments, and decreased intrafragmentary strain that ensures the stability of this reduction (Dimitriou et al. 2005). The cortex directly attempts to re-establish itself, in order to restore mechanical continuity, by establishing new haversian systems throughout the cortical bone. This is achieved by the formation of 'cutting cones', discrete remodelling units composed of bone-resorbing osteoclasts which tunnel through the cortical bone, and which can infiltrate directly into the opposite bone fragment (McKibbin 1978). These cutting cones provide access for the penetration of blood vessels, accompanied by vascular endothelial cells and perivascular mesenchymal cells (osteoprogenitor cells that will become osteoblasts) and together are able to re-establish the haversian systems (Einhorn 1998). There is very little or no periosteal response during primary fracture healing, so no callus formation is observed (Dimitriou et al. 2005). Since the process of primary bone healing requires extremely high stability of fracture reduction, it is in practice the rarest type of repair. Secondary, or indirect, healing is far more common (Phillips 2005).

1.2.2 Secondary / Indirect Healing

Secondary, or indirect, healing involves both intramembranous and endochondral ossification. The initial stage in secondary healing involves the

formation of an injury-induced haematoma, and inflammation occurs. The haematoma has inherent osteogenic potential (Mizuno et al. 1990), with degranulated platelets in the haematoma releasing growth factors such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) which act initially to recruit mesenchymal stem cells, inflammatory cells and macrophages to the site of injury. In addition, TGF-β promotes recruitment and differentiation of osteoblast progenitors (Barnes et al. 1999). Pro-inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α), are released which recruit inflammatory cells, fibroblasts, and osteoblasts (Dimitriou et al. 2005). The potent angiogenic stimulator vascular endothelial growth factor (VEGF) is released, and this is followed by release of the bone morphogenetic proteins (BMPs), key regulators of bone formation (Gerstenfeld et al. 2003).

Callus formation begins at the periphery of the site of bone injury. Committed osteoprogenitors present in the periosteum stimulate intramembranous ossification at the periphery. Towards the centre, a soft cartilaginous callus is formed by chondrocytes synthesising a matrix with a high concentration of type II collagen fibrils, and deposition of calcium hydroxyapatite crystals into this matrix allows mineralisation of the cartilage (Phillips 2005). Invasion of blood vessels into this matrix recruits osteoblast progenitors, which undergo proliferation and differentiation into osteoblasts and, together with cartilage-resorbing chondroclasts, convert the soft cartilaginous callus into immature woven bone via endochondral ossification (Einhorn 1998). Bone remodelling is the final stage in the secondary bone healing cascade, and functions to

replace the immature woven bone with lamellar bone, through the coupled action of the bone-resorbing osteoclasts and the bone-forming osteoblasts (Schell et al. 2006).

1.2.3 Osteoblasts and their Role in Bone Repair and Remodelling

Osteoblasts originate from multipotent stem cells derived from the embryonic connective tissue mesenchyme. Mesenchymal stem cells are undifferentiated, self-renewing cells found predominantly in the bone marrow but also in the periosteum and connective tissues (Bielby et al. 2007). Being multipotent, they are capable of differentiating into several cell types, namely osteoblasts, chondrocytes, adipocytes, and myoblasts (Pittenger et al. 1999; Prockop 1997). Osteoblasts are found on bone surfaces, usually in a single layer, and are responsible for bone formation, participating in both embryonic skeletal development and post-natal bone remodelling, as well as playing an essential role in bone repair (Mackie 2003).

Osteoblast differentiation is regulated by a complex system of growth factors (Lieberman et al. 2002). The intracellular signals conveyed by these growth factors in turn influence the expression of an array of transcription factors and proteins (Hughes et al. 2006) (Figure 1.3). These transcription factors and proteins can be considered to be biomarkers of osteoblast differentiation, and *in vitro* techniques can exploit expression of these biomarkers to assess the behaviour of cells *in vivo* or *ex vivo* (Kartsogiannis and Ng 2004; Yang and

Karsenty 2002). Numerous growth factors exert their influences on different stages of the differentiation pathway, inducing intracellular signalling processes that in turn affect the expression of transcription factors within the cells. Runx2/Cbfa1 and Osterix, for example, are transcription factors that are essential for osteoblast differentiation, inducing expression of many osteoblast proteins, including osteocalcin and bone sialoprotein (Kim et al. 2004). Bone morphogenetic protein-2 (BMP-2) is capable of inducing expression of these transcription factors, via the upstream regulators Msx-2 and Dlx-5, which are involved in upregulating the expression of Osterix and Runx2 respectively (Kim et al. 2004; Matsubara et al. 2008). It is the transcription factor TAZ, however, expressed early in the pathway, that specifies the osteoblastic cell fate of mesenchymal stem cells (Hong et al. 2005).

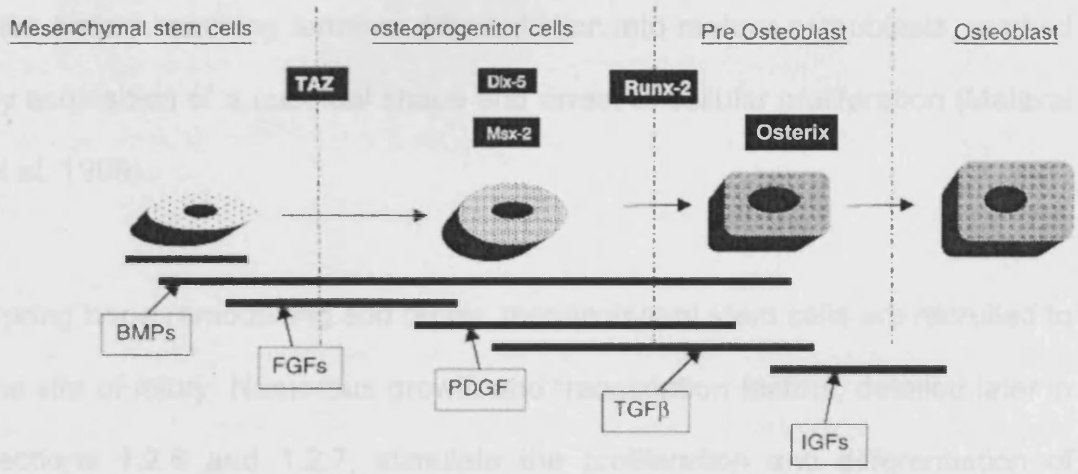

Figure 1.3. Osteoblast differentiation. A host of growth factors act temporally and spatially to drive the stages of differentiation. Intracellular growth factor signalling influences expression of transcription factors which in turn induce expression of osteoblastic proteins. (Adapted from Hughes et al., 2006).

Upon recruitment to the bone surfaces, mesenchymal stem cells give rise to osteoprogenitors, highly proliferative cells with a finite lifespan that are committed to the osteoblast lineage (Aubin and Heersche 2000). Osteoprogenitors further differentiate into pre-osteoblasts, which have a more limited proliferative capacity and begin to resemble the mature osteoblast. This differentiation is associated with a change in morphology, so that the cells take on the cuboidal shape associated with mature osteoblasts, and expression of a number of cell surface markers associated with a mature phenotype, such as osteopontin, alkaline phosphatase and bone sialoprotein, although the cells do maintain a limited ability to proliferate (Mackie 2003). Pre-osteoblasts themselves differentiate into fully mature, functional osteoblasts, synthesising and secreting bone matrix. *In vitro* experiments performed using rat calvaria cells have suggested that osteoprogenitor / pre-osteoblasts must transit though approximately 9-10 population doublings *in vitro* before reaching terminal differentiation into mature osteoblasts, marked by acquisition of a cuboidal shape and arrest of cellular proliferation (Malaval et al. 1999).

During bone remodelling and repair, mesenchymal stem cells are recruited to the site of injury. Numerous growth and transcription factors, detailed later in sections 1.2.6 and 1.2.7, stimulate the proliferation and differentiation of these cells, until a population of fully mature, active osteoblasts are present at the site of trauma (Bielby et al. 2007). As differentiation occurs, the osteoblasts begin to secrete bone matrix proteins. The most abundant of these proteins is type I collagen, but non-collagenous proteins are also

secreted (fully described in section 1.2.8) (Macdonald and Gowen 1993). The synthesis and secretion of this osteoid is initially unmineralised, but osteoblast secretion of proteins such as alkaline phosphatase, bone sialoprotein, osteonectin, and osteocalcin stimulate calcification of this osteoid (Mackie 2003; Whyte 1994). Initially, this mineralised bone matrix takes the form of immature woven bone, but over time this is remodelled into stronger lamellar bone, by osteoblasts working in conjunction with osteoclasts (Tsiridis et al. 2007).

1.2.4 Osteoclasts and their Role in Bone Repair and Remodelling

Osteoclasts are an essential component of bone repair, removing damaged and necrosed tissue, and resorbing the irregular woven bone callus in the remodelling stage, allowing subsequent osteoblast formation of mature lamellar bone (Schindeler et al. 2008). Osteoclasts are large, multinucleated cells created by the differentiation of monocyte / macrophage haemopoietic progenitor cells, found in the bone marrow (Schell et al. 2006). These osteoclast precursors migrate from the bone marrow to the bone surfaces, where they proliferate, differentiate into mononuclear preosteoclasts, and fuse with each other to form multinucleated mature osteoclasts, with around 10-20 individual cells fusing to form each osteoclast (Boyle et al. 2003; Roodman 1999; Suda et al. 1992) (Figure 1.4).

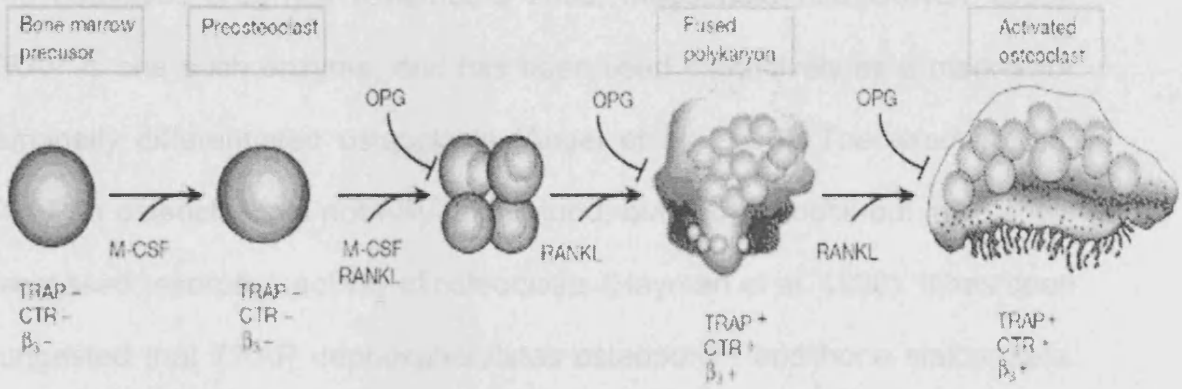

Figure 1.4. Differentiation of haematopoietic precursor cells into mature osteoclasts, via the actions of the osteoblast-derived factors macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B ligand (RANKL). Differentiation is associated with expression of phenotypic markers such as tartrate-resistant acid phosphatase (TRAP), calcitonin receptor (CTR) and the β_3 integrin, which mediate osteoclast migration, attachment, and differentiation. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL and inhibits osteoclast differentiation (Adapted from Boyle et al., 2003).

The differentiation of osteoclasts is dependent on the presence of M-CSF and RANKL. These factors drive proliferation and differentiation of osteoclast precursors, and induce expression of genes that characterise the osteoclast lineage; TRAP, cathepsin K, CTR, and the β_3 integrin (Lacey et al. 1998). Mature osteoclasts are responsible for the resorption of mineralised bone matrix, which they achieve by tightly adhering to bone surfaces via integrins (Crippes et al. 1996; Helfrich et al. 1996). Adherence of mature osteoclasts to a bone surface forms a resorption pit, into which the cells secrete protons

and osteolytic enzymes (Chambers 2000; Macdonald and Gowen 1993). TRAP is one such enzyme, and has been used extensively as a marker for terminally differentiated osteoclasts (Angel et al. 2000). The exact role of TRAP in osteoclasts is not fully understood, but TRAP knock-out mice show decreased resorptive activity of osteoclasts (Hayman et al. 1996). It has been suggested that TRAP dephosphorylates osteopontin and bone sialoprotein, proteins involved in promoting osteoclast attachment, thus TRAP may regulate osteoclast attachment to these proteins and osteoclast migration on the bone surface (Ek-Rylander et al. 1994). TRAP has also been implicated in generation of hydroxyl radicals, iron transport, and as a growth factor for haematopoietic and osteoblast cells (Ljusberg et al. 2005; Sheu et al. 2003). Activation of TRAP is thought to be mediated through cathepsin K, a protease secreted into the acidic microenvironment of the resorption lacunae, facilitating matrix degradation through its proteolytic activity (Drake et al. 1996; Ljusberg et al. 2005; Saftig et al. 1998). Negative regulation of bone resorption is brought about through the actions of the peptide hormone calcitonin, which signals through its cognate receptor abundantly present on osteoclasts (Quinn et al. 1999). Calcitonin inhibits the motility, cytoplasmic spreading, and bone resorption abilities of osteoclasts, preventing bone resorption when required (Lee et al. 1995; Nicholson et al. 1986).

In bone remodelling and repair, osteoclasts function to clear away damaged and necrosed tissue at the site of injury. Resorption of this tissue facilitates release of growth factors bound within the bone matrix, including TGF-β1 and BMPs (Dallas et al. 2002). Release and activation of these molecules

initiates intracellular signalling cascades, which ultimately promote the recruitment and proliferation of mesenchymal stem cells / osteoprogenitors to the site of injury, and stimulates osteoblast differentiation of these cells. This provides a population of cells at the site of injury capable of laying down newly formed bone matrix. To stimulate remodelling of the newly formed bone, osteoblasts produce local signalling molecules that play a role in osteoclast differentiation. The maturation of macrophages into osteoclasts *in vitro* requires the presence of bone marrow stromal cells, or their osteoblastic progeny (Udagawa et al. 1990). These cells produce M-CSF and RANKL, essential for osteoclast differentiation (Quinn et al. 1998; Teitelbaum 2000). M-CSF is a secreted product, and binds to its sole receptor, c-Fms, present on early osteoclast precursors (Kodama et al. 1991; Stanley et al. 1997). Binding of M-CSF initiates dimerization and activation of the receptor tyrosine kinase, leading firstly to autophosphorylation of the receptor (Stanley et al. 1997). This leads to propagation of downstream signals via binding of proteins to the phosphorylated receptor sites that stimulate proliferation and survival of osteoclast progenitors (Ross 2006; Stanley et al. 1997). M-CSF alone, however, is not sufficient to cause differentiation of osteoclasts from their precursors. It is a secreted product, and osteoclast differentiation requires contact between osteoclast precursors and bone marrow stromal cells or osteoblasts. It is therefore the role of RANKL, expressed on the surface of committed osteoblast progenitors, to stimulate the differentiation of osteoclast progenitors into fully mature osteoclasts (Lemaire et al. 2004; Yasuda et al. 1998). RANKL binds to the RANK receptor present on the surface of haemopoietic monocyte progenitors, bringing the two cell types

into contact with each other. This binding initiates recruitment of tumour necrosis factor receptor-associated factors (TRAFs) to the intracellular domain of RANK, activating intracellular signalling cascades. These include stimulation of NF-KB, mitogen-activated protein kinase (MAPK) pathways via Jnk, Erk and p38, and the Akt/protein kinase B pathway (Darnay et al. 1998; Feng 2005). Stimulation of these pathways regulates the fusion of osteoclast precursors, and their survival and differentiation into mature osteoclasts, activating bone resorption (Blair et al. 2007; Burgess et al. 1999; Nakagawa et al. 1998). M-CSF and RANKL therefore work synergistically to induce osteoclast differentiation, with M-CSF driving proliferation of osteoclast progenitors, and RANKL initiating differentiation of this expanded population (Quinn et al. 1998).

Unregulated resorption of bone can lead to severe disease states, and so osteoclast differentiation is tightly regulated via OPG, expressed by stromal cells and osteoblasts (Burgess et al. 1999; Lacey et al. 1998; Vega et al. 2007). OPG blocks the formation of mature osteoclasts by acting as a soluble decoy receptor for RANKL, preventing its binding to RANK and inhibiting osteoclast differentiation (Roodman 1999). Transgenic mice overexpressing OPG show a marked reduction in trabecular osteoclasts, although osteoclast precursor numbers remain at normal levels (Simonet et al. 1997). OPG therefore serves to regulate bone density by modulating the differentiation of osteoclasts from haemopoietic precursors, rather than affecting the proliferative phase. OPG is secreted by osteoblasts in response to osteogenic signals, including TGF-β1 (Centrella et al. 1994; Takai et al.

1998; Thirunavukkarasu et al. 2001; Udagawa et al. 2000). TGF-β1 may inhibit survival and differentiation of osteoclasts not only by inducing OPG, but also by suppressing osteoblast expression of RANKL (Quinn et al. 2001). TGF-β1 is sequestered within the bone matrix, and its release during osteoclastic bone resorption stimulates OPG expression and suppresses RANKL, inhibiting further formation and activity of osteoclasts and ultimately inhibiting bone resorption (Dallas et al. 2002; Taipale and Keski-Oja 1997; Thirunavukkarasu et al. 2001). Through their ability to secrete M-CSF / RANKL and OPG, osteoblasts play a crucial role in both activation and inhibition of bone resorption, creating a complex feedback system where bone formation follows bone resorption and completes a bone remodelling cycle. Therefore, the coordination between osteoblasts and osteoclasts allows the initial and rapid production of woven bone at the site of bone injury or trauma, creating a temporary structure which can be broken down gradually and replaced with a stronger, functional structure. However, the coordinated activities of the osteoblasts and osteoclasts are reliant on a third cell type within the bone system, the osteocyte (Knothe Tate et al. 2004).

1.2.5 Osteocytes and their Role in Bone Repair and Remodelling

Osteocytes coordinate bone remodelling processes by osteoblasts and osteoclasts, by acting as mechanosensors that can respond to mechanical strain and microdamage to signal bone resorption / formation. Osteocytes are terminally differentiated osteoblasts that have become embedded in the

mineralised matrix of bone. Active osteoblasts have a limited lifespan of approximately 3 months, after which time their fate varies, either undergoing programmed cell death (apoptosis), transforming into inactive bone-lining cells, or differentiating into osteocytes (Jilka et al. 1998; Manolagas 2000). The selection of osteoblasts for terminal differentiation to osteocytes is influenced by a variety of factors, including the position of the cell and the presence of local signalling factors (Manolagas 2000). Osteocytes are by far the most abundant cellular component within mammalian bone, and their lifespan of approximately 25 years greatly exceeds that of the osteoblast (Knothe Tate et al. 2004). Once embedded within the bone matrix, the cells cease secretory activity and undergo several phenotypic changes, including a decrease in cell body size, and a reduction in secretory organelles (Knothe Tate et al. 2004). The osteocytes also develop a meshwork of filamentous cellular processes that extend into the surrounding bone tissue, running along the bone canaliculi, and connect the osteocytes to other cell types (Kamioka et al. 2001; Noble et al. 2003). The connections established between osteocytes and surrounding bone cells allow them to regulate bone mass (Franz-Odendaal et al. 2006). Osteocytes are thought to respond to mechanical strain and microdamage to signal processes of bone resorption or formation, through communication between each other and other bone cells within the tissue, via gap junctions at the end of the cellular processes, and through their contact with the bone marrow (Kamioka et al. 2001). Osteocytes can respond to an increase in mechanical loading by producing a number of molecules involved in osteogenesis, such as osteocalcin, and insulin-like growth factor I (IGF-I), known to increase proliferation and

differentiation of osteoblasts, ultimately leading to an upregulation of bone formation (Kawata and Mikuni-Takagaki 1998). Osteocytes can also undergo apoptotic cell death in response to microdamage to the bone, releasing prostaglandins or cytokines which can initiate resorption of the damaged bone by osteoclasts (Sims and Gooi 2008; Verborgt et al. 2002). In this way, osteocytes maintain the balance between bone formation and resorption, and increase local repair mechanisms in response to perceived bone damage (Tomkinson et al. 1997).

1.2.6 Signals Involved in Bone Repair and Remodelling

The differentiation of bone cells, and the processes of bone formation and resorption, are controlled by systemic signals acting though local signalling molecules and growth factors. These molecules act in synergy to recruit progenitor cells to sites of bone injury, to stimulate their proliferation, and to bring about their differentiation into mature bone cells capable of initiating repair processes. The interactions between this 'cocktail' of factors is complex. The way in which these factors interact and influence each other is pivotal to understanding repair processes, and to determine the molecular and cellular events associated with clinical situations of impaired bone healing.

1.2.6.1 Transforming Growth Factor Beta

TGF-β plays a major role in repair processes in bone, stimulating migration and proliferation of osteoprogenitors (Barnes et al. 1999). There are five known isoforms of TGF-β (TGF-β1 through to TGF-β5), and the isoform TGF-β1 is especially prevalent in bone and platelets (Assoian et al. 1983; Janssens et al. 2005; Lieberman et al. 2002). TGF-β is secreted by bone cells in a latent dimeric complex, consisting of TGF-β plus its propeptide, termed latency-associated peptide (LAP) (Saharinen et al. 1999). The association between the TGF-β1 LAP and the growth factor itself, in the small latent complex (SLC), prevents signalling through the TGF-β receptors, keeping the growth factor in an inactive form (Annes et al. 2004). An additional protein, the latent TGF-β binding protein (LTBP-1), promotes binding of the TGF-β complex to the ECM, through covalent cross-linking to ECM proteins via tissue transglutaminase (Annes et al. 2004; Dallas et al. 2002; Todorovic et al. 2005). This matrix storage of growth factor allows for the generation of rapid and highly localised signals when required, which may be critical to bring about rapid healing of damaged bone tissue (Taipale and Keski-Oja 1997; Todorovic et al. 2005). TGF-β release from the matrix in response to bone damage is generally brought about through osteoclast activity, capable of releasing latent TGF-β through cleavage of LTBP-1 (Dallas et al. 2002). The release of the mature TGF-β 25kDa homodimer from this latent complex is required to activate TGF-β, to exert effects on target cells. Osteoclasts themselves are able to activate TGF-β, possibly via

the acidic environment created by secretion of protons into the resorption lacunae (Bonewald et al. 1997). Active TGF-β acts through transmembrane serine/threonine kinase receptors present at the cell surface, classified as type I (activin receptor-like kinases, ALKs) or type II receptors (Derynck and Feng 1997; Janssens et al. 2005). Ligand binding induces the formation of a heteromeric receptor complex, where receptor II phosphorylates receptor I via its constitutively active serine/threonine kinase domain, activating the receptor and initiating intracellular signalling cascades (Massague 1998). The Smad proteins (a family of transcription factors) play a central role in the propagation of signals from activated TGF-β receptors (Janssens et al. 2005; Massague 2000; Miyazawa et al. 2002). Receptor-regulated Smads (Smad2 and Smad3 in the case of TGF-β signalling) are phosphorylated and activated by the type I receptor, to form heteromeric complexes with a common mediator Smad, or Co-Smad, Smad4 (Shi and Massagué 2003). These Smad complexes translocate to the nucleus to regulate target gene expression in conjunction with other nuclear co-factors. TGF-β can also induce transcription of inhibitory Smads (Smad6 and Smad7), forming a negative feedback loop by antagonising TGF-β signalling, either by competing with the R-Smads, or by inducing targeted degradation of the receptors (Massague 2000; Wrana and Attisano 2000). Although Smad proteins are critical in mediating the TGF-β signalling pathway, TGF-β can also activate Smad-independent signalling cascades, including the Erk, JNK, and p38 MAPK pathways, although some of these pathways also regulate Smad activation (Derynck and Zhang 2003).

Release of TGF-β at a site of bone injury stimulates chemotactic recruitment of mesenchymal stem cells and osteoprogenitors from the bone marrow and periosteum, and increases their proliferation (Barnes et al. 1999). There is also evidence that TGF-β promotes the early stages of differentiation within these cells, increasing levels of type I collagen synthesis and inducing expression of Runx2 (Bostrom and Asnis 1998; Janssens et al. 2005). Studies performed on TGF-β null mice have demonstrated a lack of proliferation and differentiation amongst the osteoprogenitor population within these mice with consequential dramatic effects on matrix deposition and mineralisation, with subsequent impaired longitudinal growth and slow mineralisation (Atti et al. 2002). It has also been demonstrated that these TGF-β null mice display reduced elasticity of the bones, and lack material integrity of the long bones when compared to wild-type littermates (Geiser et al. 1998). However, during later stages of osteoblast differentiation, TGF-β has been observed to suppress the expression of Runx2, as well as osteocalcin, thereby acting to inhibit the later stages of osteoblast maturation (Janssens et al. 2005). It appears, therefore, that the role of TGF-β in response to bone injury is to recruit local mesenchymal stem cells and osteoprogenitors to the site of injury, stimulate their proliferation, and induce some early differentiation stages, thereby providing an expanded pool of committed osteoblasts at the site of injury. Other growth factors, such as the BMPs, then take responsibility for the later differentiation stages into fully mature, active osteoblasts capable of bone repair. TGF-β1 also plays a role in regulating osteoclast differentiation, although the osteoclast response to TGF-β1 may depend on the differentiation state (Filvaroff et al. 1999). TGF-

β1 inhibits early differentiation in osteoclast precursors, through either OPG expression or suppression of RANKL (Chenu et al. 1988; Quinn et al. 2001), but in contrast TGF-β1 stimulates bone resorption by mature osteoclasts, possibly through upregulation of prostaglandins, potent stimulators of osteoclast differentiation (Tashjian et al. 1985). Through its actions on both bone formation and resorption, TGF-β1 plays a critical role in repair processes.

1.2.6.2 Bone Morphogenetic Proteins

The BMPs belong to the TGF-β superfamily, and were identified by Urist (1965). The BMPs are key regulators of bone induction, maintenance and repair, and regulate growth and differentiation of cells of the chondroblast and osteoblast lineage (Sakou 1998; Tsiridis et al. 2007; Wozney 1992). Several of the BMPs have also been shown to independently induce ectopic bone formation, a unique ability among growth factor families (Katagiri and Takahashi 2002; Wozney and Rosen 1998). Members of the BMP family play a critical role in repair processes in bone, due to their ability to differentiate mesenchymal stem cells and osteoprogenitors into mature, fully functional osteoblasts. A study by Cheng and colleagues (2003) demonstrated that BMP-2, 6, and 9 exhibit the greatest ability among the BMP family members to induce early and late osteogenic markers, as well as matrix mineralisation, with other members such as BMP-4 and 7 stimulating differentiation of later, committed osteoblasts (Cheng et al. 2003a).

There are over 20 identified and characterised BMP family members which can be further divided into groups based on their structure and function (Biase and Capanna 2005; Cheng et al. 2003a). BMPs are synthesised within bone cells as precursor forms and are sequestered within the ECM, providing an easily accessible reservoir of these growth factors which can be released at times of bone resorption (Mackie 2003). The released BMPs are proteolytically cleaved at the carboxy-terminal region to release the mature protein, which undergoes dimerization to form the active protein (Sakou 1998).

BMPs influence osteoblast differentiation by signalling through a serine-threonine receptor kinase pathway to stimulate the expression of many osteoblast proteins. This pathway, although similar to the TGF-β signalling pathway, involves different type I and type II receptors, with BMPs binding to three distinct type I receptors (ALK2, ALK3, and ALK6) and three distinct type II receptors (type II BMP receptor, type II activin receptor, and type IIB activin receptor) (Derynck and Feng 1997; Miyazono et al. 2005). Intracellular signalling occurs in a similar way to TGF-β signalling (Kawabata et al. 1998), with downstream signalling occurring via phosphorylation of the R-Smads 1, 5, and 8 (Dijke et al. 2003), which translocate to the nucleus (together with the common mediator Smad 4) and regulate transcription of target genes such as Runx2 (Phimphilai et al. 2006). Runx2 is involved in the transcriptional activation of many osteoblast marker genes by interacting with BMP-activated Smads in the nucleus (Barnes et al. 1999; Ducy et al. 1997;

Lieberman et al. 2002). Regulation of Runx2 expression is probably mediated via BMP-induced expression of Dlx5, a homeobox gene that positively regulates later stages of osteoblast differentiation, and stimulates matrix mineralisation, via osteocalcin and alkaline phosphatase upregulation (Miyama et al. 1999). BMPs have also been shown to induce expression of Msx2, another homeobox gene that promotes osteoblast differentiation by inducing expression of the transcription factor Osterix, which in turn upregulates osteocalcin and alkaline phosphatase within osteoblasts (Matsubara et al. 2008; Tu et al. 2006). BMP signalling can also be antagonised, and therefore controlled, by inhibitory Smads (Smad6 and Smad7), or by numerous BMP antagonists including noggin and chordin, which bind to the BMPs and prevent further signalling (Etsuko 2006).

The presence of BMPs around a site of bone injury may indicate the differentiation state of the cells present within the area, depending on which BMPs are expressed. Expression of the early BMPs 2, 6, and 9, may indicate the presence of mesenchymal stem cells or early osteoprogenitors around the site, whereas the expression of the later BMPs, 4 and 7, may indicate the presence of more committed osteoblasts (Cheng et al. 2003a).

1.2.6.3 Platelet Derived Growth Factor

In bone repair processes, PDGF is one of the first factors to be released around the site of injury, from degranulating platelets within the haematoma and from surrounding inflammatory macrophages (Barnes et al. 1999; Bolander 1992; Simmons 1985). PDGF has been shown to play a role in stimulating both migration and proliferation of mesenchymal stem cells and osteoblasts, thus providing a population of osteoblast cells at the site of injury (Graves and Cochran 1990; Rasubala et al. 2003; Ross et al. 1986). In regards to osteoblast differentiation, it has been demonstrated that PDGF inhibits the differentiation of osteoblasts, by decreasing the expression of alkaline phosphatase, osteocalcin, and type I collagen *in vitro* (Yu et al. 1997). Therefore the functions of PDGF as it relates to osteoblast function in bone repair appears to be the recruitment of osteoprogenitors to the site of injury, and stimulation of proliferation of these cells. It has also been demonstrated that PDGF can stimulate osteoclast bone resorption, by inducing expression of prostaglandins (Habenicht et al. 1986; Ross et al. 1986; Tashjian et al. 1982). Thus PDGF may play a role in mediating bone remodelling processes during injury, due to its capability of influencing both bone formation and resorption.

The effects of PDGF on mesenchymal stem cells and osteoblasts are similar to those brought about by TGF-β, and several reports have shown that TGF-β and PDGF actually work in synergy to promote migration and growth of

osteoblasts (Kells et al. 1995). In osteoblast cell cultures it has been reported that TGF-β is capable of inducing the expression of PDGF (Rydziel et al. 1992). This emphasises the need for a 'cocktail' of growth factors to work synergistically to bring about bone repair processes.

1.2.6.4 Vascular Endothelial Growth Factor

VEGF promotes vascular development and adult angiogenesis (Breier and Risau 1996; Ferrara et al. 2003; Senger et al. 1997). Angiogenesis is critical for successful osteogenesis, since the vasculature is responsible for delivering oxygen, nutrients, soluble factors and cells to the bone tissue (Carano and Filvaroff 2003; Hsiong and Mooney 2006; Kanczler and Oreffo 2008). Endothelial cells are the primary target of VEGF, but it has also been shown that VEGF is involved in modulating recruitment, survival, and activity of osteoblasts (Midy and Plouet 1994; Street et al. 2002). VEGF has been reported to directly stimulate the chemotactic migration of primary human osteoblasts, as well as inducing their differentiation (Mayr-wohlfart et al. 2002; Orlandini et al. 2006). Many osteoinductive growth factors induce expression of VEGF, including TGF-β, BMPs and PDGF (Brogi et al. 1994; Carano and Filvaroff 2003; Deckers et al. 2002; Saadeh et al. 1999). VEGF has also been shown to function synergistically with BMP-4 to induce bone formation (Peng et al. 2002). The synergistic activity that VEGF appears to have with many other growth factors makes it an interesting candidate for possible clinical treatments (Ferrara 2004), with studies showing that delivery

of recombinant VEGF (Street et al. 2002), or adenovirus-delivered VEGF (Tarkka et al. 2003) promotes blood vessel formation and ossification in rodent models of bone damage.

1.2.6.5 Fibroblast Growth Factors and Insulin-like Growth Factors

Fibroblast growth factors (FGFs) play critical roles in mediating angiogenesis and mitogenesis of mesenchymal cells (Baird and Walicke 1989; Friesel and Maciag 1995; Ornitz and Itoh 2001). FGF-1 (acidic FGF) and FGF-2 (basic FGF) are the most abundant, expressed in many tissues including bone matrix (Baird and Walicke 1989; Hauschka et al. 1986). FGFs are expressed by osteoblasts (Canalis et al. 1988), and can be detected in early stages of bone repair (Bolander 1992). The exact function of FGFs in osteoblast differentiation is unclear, with effects depending on the maturation of the cell (Debiais et al. 1998). It has been reported that FGFs increase proliferation of early immature osteoprogenitors and prevent apoptosis, increasing the pool of committed osteoblastic cells at the site of injury (Mansukhani et al. 2000). As these cells further differentiate in response to other factors, FGFs induce apoptosis of these cells, perhaps functioning to control the number of osteoblasts that undergo terminal differentiation (Mansukhani et al. 2000). These differentiation-dependent effects of FGF signalling on osteoblast apoptosis are likely to be important in controlling the rate of differentiation and ultimately osteogenesis.

Insulin-like growth factors (IGFs) play critical roles in skeletal development (Lieberman et al. 2002). IGFs are expressed by bone cells (Trippel 1998), although they are also sequestered in the bone matrix (Bautista et al. 1991). IGFs can stimulate osteoblast proliferation and differentiation, particularly the later stages, by upregulating Osterix (Celil and Campbell 2005; Hughes et al. 2006; McCarthy et al. 1989). IGF expression can be increased by both BMP-2 (Canalis and Gabbitas 1994) and TGF-β1 (Okazaki et al. 1995), although it has also been reported that TGF-β1, along with FGF-2 and PDGF, can suppress expression in several *in vitro* models (Gangji et al. 1998). The specific actions of the IGFs in bone repair are not fully elucidated, but may indicate the presence of late-stage differentiation osteoblasts around the site of a bone injury.

1.2.6.6 Wnts

In terms of bone repair, Wnt signalling appears to be involved in mediating only the early osteoblast differentiation stages, inducing expression of early markers Runx2 and alkaline phosphatase, but not inducing osteocalcin expression, associated with mature osteoblasts (Gaur et al. 2005; Gong et al. 2001; Rawadi et al. 2003). Wnts are a group of over 19 secreted, lipid modified glycoproteins that regulate a variety of cellular activities such as proliferation, migration, and gene expression (Moon et al. 2002). They are known to be important in the development and patterning of the skeleton (Greco et al. 1996), but also actively partake in adult bone repair, where they

stimulate osteoblast precursor growth as well as early differentiation events (Rawadi et al. 2003; Ross et al. 2000; Westendorf et al. 2004).

Induction of the Wnt proteins themselves appears to be under the regulation of the TGF-β family, most notably BMP-2, which has been shown to induce Wnt signalling *in vitro* (Bain et al. 2003), and may be required for BMP2-induced expression of alkaline phosphatase within osteoblasts (Rawadi et al. 2003). The exact nature of the interaction between BMPs and Wnts is not yet clear, and neither is the exact role that Wnts play in osteoblast differentiation and bone repair. However Wnt activity present around a site of bone injury would indicate BMP activity, strongly indicating the presence of differentiating osteoblasts and therefore bone formation.

1.2.6.7 Sonic Hedgehog

Members of the hedgehog family of signalling molecules play central roles in the control of pattern formation and cellular proliferation during development (Hammerschmidt et al. 1997; Perrimon 1995). Sonic hedgehog is involved in regulation of skeletal formation (Spinella-Jaegle et al. 2001) and *in vitro* can induce osteoblast differentiation within osteogenic precursors and osteoblasts (Nakamura et al. 1997). Sonic hedgehog has also been shown to increase the commitment of mesenchymal stem cells to the osteoblastic lineage, and inhibits adipocyte differentiation of these cells (Spinella-Jaegle et al. 2001). It has also been shown to act synergistically with BMP-2 during osteoblast differentiation (Nakamura et al. 1997; Spinella-Jaegle et al. 2001;

Yuasa et al. 2002), although the precise nature of the interaction is not yet elucidated.

1.2.7 Major Transcription Factors Involved in Bone Repair

In bone repair, growth factors exert transcriptional regulation via transcription factors which initiate signalling cascades within target cells, shifting their differentiation towards the osteoblast lineage, and away from pathways towards other cell lineages.

TAZ, upregulated by BMP-2, is involved in stimulating differentiation of mesenchymal stem cells into osteoprogenitors, and also suppresses the formation of adipocytes (Hong et al. 2005). TAZ stimulates osteoblast differentiation by acting as a specific transcriptional coactivator of Runx2 (Cui et al. 2003; Kanai et al. 2000), essential for osteoblast differentiation and ultimately repair processes in bone (Ducy and Karsenty 1995; Ducy et al. 1997; Perinpanayagam et al. 2006). TAZ is thought to bind to Runx2 in the cytoplasm, and therefore may play a role in its nuclear translocation, where transcriptional activation can then occur (Cui et al. 2003). Runx2 is able to stimulate the differentiation of cells down an osteoblastic lineage by binding to promoter regions within a number of osteoblast-related genes, such as osteocalcin, osteopontin, type I collagen, and bone sialoprotein, and inducing expression of these proteins (Ducy et al. 1997). Komori and colleagues (1997) demonstrated the absolute requirement for Runx2 expression in bone

formation processes, as Runx2 knockout mice exhibit complete lack of bone formation and die at birth without breathing, probably due to the lack of ossification of the ribs (Komori et al. 1997). Examination of osteoblasts from within these Runx2 knockout mice embryos demonstrate that maturational arrest occurs within these cells at an early stage of osteoblast differentiation, as the cells express osteonectin, an early marker of differentiation, but express only low levels of alkaline phosphatase, and barely detectable levels of the later markers osteopontin and osteocalcin. This indicates that Runx2 plays a critical role in the differentiation of early, immature osteoblasts.

Expression of Runx2 can also be induced via the BMP2-activated Dlx5 transcription factor, a homeobox gene involved in stimulating later phases of the osteoblast differentiation pathway, activating expression of proteins such as alkaline phosphatase and osteocalcin (Kim et al. 2004). Msx2 is another homeobox transcription factor induced by BMP-2 which is associated with stimulation of osteoblast differentiation, and suppression of adipocyte differentiation (Cheng et al. 2003b; Ichida et al. 2004). BMP2-stimulated expression of Msx2 in turn induces expression of Osterix, another transcription factor absolutely required for the differentiation of osteoblasts that upregulates osteocalcin and alkaline phosphatase (Cheng et al. 2003b; Matsubara et al. 2008; Tu et al. 2006). Osterix knockout mice develop a similar phenotype to Runx2 knockout mice (Komori et al. 1997), whereby a complete lack of osteoblast differentiation results in the arrest of both intramembranous and endochondral ossification, leading to an entire lack of skeletal development (Nakashima et al. 2002). Interestingly, however, Runx2

expression was observed in the Osterix knockout mice, at a level comparable to normal wild type mice, suggesting that Osterix is not required for expression of Runx2. Within the Runx2 knockout mice, no expression of Osterix could be detected, suggesting that Osterix acts downstream of Runx2, and that Osterix expression requires the presence of Runx2, which has been demonstrated to bind to the Osterix promoter and stimulate transcription (Nakashima et al. 2002; Nishio et al. 2006).

The signalling pathways involved in regulating bone repair are complex. As such the growth factors and the transcription factors described here are by no means the only factors involved in the osteoblast differentiation process, and many of these signalling pathways are still to be fully elucidated. However, the factors described here are well documented to be significantly involved in the differentiation of cells down an osteoblast lineage, and many of them can be detected by various qualitative and quantitative techniques. As such they are established indicators of osteoblast activity, and therefore bone formation, and they enable a picture of cellular activity to be established when examining repair processes in bone.

1.2.8 Major Proteins Expressed in Bone Repair

The many growth and transcription factors responsible for driving repair processes in bone ultimately regulate production of proteins within target cells. This coordinated secretion dictates functionality of bone repair, and

secreted proteins can be biomarkers for monitoring repair processes. These proteins drive both osteoblast and osteoclast differentiation, thus regulating processes of bone formation and resorption, essential for repair and remodelling. Although each protein has specific roles in bone repair, they work synergistically in order to coordinate aspects of the process. The temporal specificity of proteins in the cellular differentiation pathways make them ideal indicators of cellular behaviour around a site of bone injury.

1.2.8.1 Type I Collagen

Type I collagen is the major protein found within the bone matrix, comprising around 90% of the organic phase, and is secreted by osteoblasts as they differentiate from their precursors (Buckwalter et al. 1995; Kalfas 2001; Seyedin and Rosen 1990). Structurally, each type I collagen molecule is comprised of three helical polypeptide subunits, together forming a triple helix, and these collagen helices associate to form fibrils, which may then further associate to form bundled collagen fibres (Allori et al. 2008). The network of these type I collagen fibres provide the structure onto which bone mineral is deposited (Baht et al. 2008). This is a highly organised process, in which hydroxyapatite mineral is first deposited into gaps between each collagen subunit, which provide nucleation sites for the mineral and allow the process to progress so that eventually mineral deposits occupy all of the available space within the fibrils (Buckwalter et al. 1995). The exact mechanism of bone mineral formation is not yet elucidated, although one

proposed mechanism involves the binding of a protein nucleator to the collagen, perhaps one of the acidic ECM proteins known to be modulators of mineral formation, such as the phosphoprotein bone sialoprotein (Baht et al. 2008).

As well as representing a structural element of the matrix essential for mineralisation, collagen type I also actively contributes to the regulation of cellular activities involved in bone repair and remodelling processes (Ramirez and Rifkin 2003). Collagen type I is able to interact with both osteoblasts and osteoclasts via cell surface integrins, promoting cell attachment to the bone matrix (Clover et al. 1992; Helfrich et al. 1996; Xiao et al. 1998), and is also capable of chemotactically attracting osteoclast precursors (Malone et al. 1982). Collagen is then able to modulate the activity of the cells, by influencing morphology, proliferation, and signal transduction, thus influencing processes of both bone formation and resorption (Green et al. 1995).

1.2.8.2 Osteopontin

Osteopontin is a phosphorylated glycoprotein found within the bone matrix and concentrated at cement (renewal) lines; it is both aspartic-acid rich and highly phosphorylated, resulting in a highly acidic protein (Giachelli and Steitz 2000). It is this highly acidic character, and the presence of a number of putative calcium binding motifs, that enable osteopontin to bind large

amounts of calcium and interact with hydroxyapatite crystals with high affinity, suggesting a role for regulating mineral deposition in bone (Giachelli et al. 1995). It is also able to be crosslinked by transglutaminase, enabling binding to other ECM components such as type I collagen and osteocalcin, thus adding physical strength to the matrix and enabling accumulation of osteopontin within the ECM (Denhardt and Noda 1998). The primary role of osteopontin is thought be as a cell attachment protein due to a number of cell adhesive domains found within its structure, which include RGD domains, anchoring osteoblasts and osteoclasts to bone during repair processes (Denhardt and Noda 1998; Reinholt et al. 1990; Ross et al. 1993). As well as being contained within the bone matrix, expression of osteopontin has been demonstrated by several cell types, including osteoblasts and osteoclasts, as well as their respective progenitors (Denhardt and Noda 1998; Merry et al. 1993; Yamate et al. 1997). Expression of osteopontin subsequently influences activity of the cells themselves, stimulating intracellular signalling pathways that modify cell behaviour and gene expression and influencing bone remodelling (Denhardt and Guo 1993; Miyauchi et al. 1991). Osteopontin expression within cells around a site of bone injury is indicative that osteoblast progenitors have migrated into the site and differentiated into osteoblasts.

1.2.8.3 Osteocalcin

Osteocalcin (bone Gla protein / γ-carboxyglutamate protein) is the most abundant non-collagenous protein within the bone matrix (Lian and Gundberg 1988). Osteoblasts are the only cell type to synthesise osteocalcin, and it is considered to be the most specific protein to bone (Ducy et al. 1997). The protein is released as a 10kD precursor (Pan and Price 1985) and Gla residues are post-translationally synthesised within the protein and can bind calcium. This enables tight binding of osteocalcin to hydroxyapatite, facilitating protein accumulation within the matrix (Kudo et al. 1998; Lian and Friedman 1978). The majority of osteocalcin is incorporated into the bone matrix, although it can also be released into the blood circulation where it can function as an indicator of bone turnover (Polak-Jonkisz and Zwolinska 1998; Price and Nishimoto 1980). The exact functions of osteocalcin are as yet not fully elucidated, but the ability of the protein to bind calcium suggests a role in mediating matrix mineralisation (Lian and Gundberg 1988). Osteocalcin has been shown to be a potent chemoattractant for mesenchymal stem cells, osteoblasts, osteoclasts, and their precursors, indicative of a role in bone repair and remodelling processes (Lucas et al. 1988; Malone et al. 1982). There is a high correlation between circulating serum levels of osteocalcin and bone formation, with elevated levels observed in situations of new bone formation or increased bone turnover (Lian and Gundberg 1988; Taniguchi et al. 2003). Thus osteocalcin both accumulated within the bone matrix, and released into circulation by osteoblasts near a site of bone injury, could

chemotactically attract both osteoblasts and osteoclasts to the site, coupling formation and resorption processes, critical for repair and remodelling (Lucas et al. 1988).

1.2.8.4 Osteonectin

Osteonectin, also known as SPARC (Secreted Protein, Acidic and Rich in Cysteine), is a calcium-binding glycoprotein that is expressed within areas of bone remodelling and repair (Alford and Hankenson 2006). Osteonectin is able to bind to ECM components, including collagen and hydroxyapatite, thus contributing to the organisation of the matrix, and may be involved in regulating cell proliferation and cell-matrix interactions (Brekken and Sage 2000; Young et al. 1992). Mice deficient in osteonectin have been shown to have significantly lower numbers of both osteoclasts and osteoblasts, leading to a dramatic decrease in bone formation and development of a low-turnover osteoporosis-like phenotype (Delany et al. 2000; Delany et al. 2003). Osteonectin present within the bone matrix may stimulate stromal cell differentiation towards the osteoblastic lineage, at the expense of adipogenesis (Bradshaw and Sage 2001; Delany et al. 2003). Osteonectin also appears to provide a survival function for osteogenic cells, particularly in times of stress (Bradshaw and Sage 2001), and may be critical for survival of cells during bone damage, to enable repair.

1.2.8.5 Bone Sialoprotein

Bone sialoprotein (BSP) is an acidic phosphoprotein expressed in abundance by osteoblasts and present at high levels within mineralised bone tissues (Ganss et al. 1999). It is a member of the mineralised tissue-associated SIBLING (Small Integrin Binding Ligand, N-linked Glycoprotein) family (Fisher et al. 2001). BSP contains motifs that enable interaction of the protein with multiple binding partners, including collagen, matrix metalloproteinases, hydroxyapatite, and integrins (Baht et al. 2008; Fisher et al. 2001; Oldberg et al. 1988; Wuttke et al. 2001). This diverse binding ability leaves the exact role of BSP still unknown, although in regards to bone repair it is thought to be involved with the onset of mineralisation (Baht et al. 2008; Chen et al. 1994), and play a role in promoting osteoblast differentiation, particularly the later stages (Gordon et al. 2007).

1.2.8.6 Alkaline Phosphatase

The human alkaline phosphatases are a group of membrane-bound glycoproteins that are expressed throughout the body in four isoforms; three of these isoenzymes are expressed in a tissue-specific distribution and are thus called intestinal, placental, and germ cell (McKenna et al. 1979; Schar et al. 1997). The fourth isoenzyme, tissue non-specific alkaline phosphatase (TNAP), is ubiquitous, but expressed abundantly in liver, kidney, and bone (Moss 1992; Schar et al. 1997). This bone-expressed alkaline phosphatase

enzyme is involved in biomineralisation (Robison 1923), cleaving ester bonds of pyrophosphates, potent inhibitors of hydroxyapatite crystal growth (Whyte 1994), and releasing inorganic phosphate into the matrix (Houston et al. 2004). In terms of bone repair alkaline phosphatase is known to be expressed on the surface of osteoblast progenitors and osteoblasts themselves, and is a widely accepted marker of differentiating osteoblasts (Kim et al. 2004). BMP-2 has been shown to induce expression of alkaline phosphatase (Rawadi et al. 2003; Yamaguchi et al. 1991), and serum levels of the enzyme have been shown to be elevated during fracture healing (Taniguchi et al. 2003).

1.2.9 Impaired Bone Healing

Through the actions of the cells, proteins, growth factors, and transcription factors detailed above, the complex processes involved in bone repair will often lead to normal and natural healing of the injured bone. However, there are many clinical situations where repair processes are impaired. Examples of impaired bone healing can be seen in fracture non-unions (Kloen et al. 2002; Marsh 1998), non-healing sockets after tooth extraction, and patients suffering from diseases such as osteonecrosis, caused by reduced blood flow to the bones (Reid 2008), osteoporosis, where bone mineral density is reduced (Namkung-Matthai et al. 2001; Tang et al. 2008), and diabetes, where patients often show significantly reduced bone healing capabilities (Gandhi et al. 2005; Loder 1988; Macey et al. 1989). Other factors can also contribute to poor healing, such as smoking (Hollinger et al. 1999), infection

at the site of injury, and the use of certain medications, including non-steroidal anti-inflammatory drugs (NSAIDs) (Simon et al. 2002; Zhang et al. 2002).

Current clinical therapies for bone injury are mostly used to aid normal bone healing processes, or to heal small defects. The therapies are used infrequently, if at all, in conditions where normal bone healing does not occur, or occurs at a significantly reduced rate. There is therefore much interest in refining and developing such treatments, and in particular expanding therapies so that they may be used in conditions where healing is impaired.

1.3 BIOMIMETIC MATRICES

Biomaterials are now commonplace in modern clinical approaches towards bone tissue engineering and regeneration (Axelrad et al. 2007; Finkemeier 2002; Mahendra and Maclean 2007). Such materials are osteoconductive scaffolds that passively facilitate bone repair by providing a physical three-dimensional matrix to facilitate attachment of cells and subsequent repair of damaged bone, although they may also be used in cell-based therapies to deliver transplanted cells to a site of injury. Interest has also focused on coupling these scaffolds with exogenous growth factors, or utilising those contained endogenously within the bone matrix, actively inducing bone formation via osteoinduction.

Both synthetic and natural polymers have been extensively investigated for possible uses as biomaterials in tissue regeneration. Biological materials from natural sources have the advantages of being biologically recognisable, can present receptor-binding ligands, and are susceptible to cell-triggered proteolytic degradation and remodelling. Such natural matrices include collagen, hyaluronan, and Bio-Oss®, a natural osteoconductive bovine bone substitute used primarily to fill bone defects in periodontal and maxillofacial surgery (Benke et al. 2001). Natural matrices provide structural scaffolds that can resist both tensile and compressive stresses, via the protein fibrils and hydrated network respectively (Lutolf and Hubbell 2005). The biophysical properties of natural ECMs can also influence cellular functions, as cells can sense mechanical properties of their environment, and convert these mechanical signals into chemical signals. However, significant disadvantages are associated with them, including problems of purification, immunogenicity and transmission of pathogens (Lutolf and Hubbell 2005). Synthetic materials may overcome the problems involved with biological materials, as they attempt to mimic properties of biological ECM material without the side effects outlined above. For example, micro- and nano-fibrillar synthetic materials attempt to recreate the fibrillar architecture of natural ECM components, whereas hydrogels attempt to mimic the hydrated network. Hydrogels are of particular interest, as they can be synthesised in gentle enough conditions for the presence of cells, allowing cell entrapment within the structure for possible delivery to an injury site (Hennink and van Nostrum 2002).

Biomaterials that can actively induce bone formation in an osteoinductive manner are ultimately more successful than those which function as a passive scaffold. The addition of exogenous bioactive molecules to biomaterials, or an ability to release bioactive factors already present within the bone matrix, may increase the potential of biomaterials in bone tissue regeneration therapies.

1.3.1 Clinical Treatments: Bone Autograft and Allograft

Current clinical treatments of bone injury commonly see the use of bone autograft (bone taken directly from the patient) or bone allograft (bone derived from biopsies from other patients or cadavers) as materials to aid in the processes of skeletal repair and regeneration (Finkemeier 2002). Bone grafts can enhance healing where repair does not occur normally, but the use of autografts and allografts has many associated problems. Both types of bone graft are dependent on the surrounding environment to provide cells and a blood supply, as well as mechanical stability, and therefore the success of a bone graft in enhancing bone repair is greatly influenced by the environment in which it is placed (Stevenson 1998). Infection at the site of a graft is also common. The usefulness of autogenous bone is often limited by the amount of material that is available, the necessity of a second operation, and the risk of complications at the donor site (Salkeld et al. 2001). Allograft bone is often the preferred choice for grafting material, as it has a less limited supply, and large structural restorations are possible. However, allograft has a much smaller osteoinductive capacity when compared to autograft, and a

limited capacity to incorporate with host bone (Heiple et al. 1963). Another problem is that allograft is often highly sterilised to reduce pathogen contamination, which can lead to a severe loss of the osteoinductive activity of the material (Lutolf et al. 2003). The problems associated with bone grafts continue to drive the development of new and improved clinical methods for bone regeneration, particularly in situations where repair processes are impaired.

1.3.2 Clinical Treatments: Growth Factor Therapy

Research into alternative therapies has focused on the many bone-inducing proteins present in bone (Urist 1965). Natural ECM modulates tissue repair by binding, storing, and releasing these bioactive growth factors to elicit local signalling responses as required (Hauschka et al. 1986; Taipale and Keski-Oja 1997). Several strategies to engineer the release of growth factors from biomimetic matrices have been presented over the years (Luginbuehl et al. 2004; Malafaya et al. 2002). Animal models have been utilised to investigate the effects of exogenous growth factors on repair, with studies demonstrating that addition of TGF-β can increase callus formation and strength, and bone formation, either on its own or in conjunction with IGF-1 (Lee et al. 2006; Schmidmaier et al. 2004; Srouji et al. 2004). Studies have also demonstrated that recombinant bFGF can promote fracture healing, and prevent non-union (Kawaguchi et al. 2001; Nakamura et al. 1998). Particular attention has focused on the BMPs, potent inducers of bone formation. Recombinant

BMPs have the ability to heal critical sized bone defects in a number of animal species when combined with a carrier of collagen, demineralised bone matrix, biodegradable polymers, or hydroxyapatite (Kang et al. 2004). Recombinant BMP-2 and BMP-7 are now commercially available, and research has focused on their delivery into the site of injury using a biomimetic scaffold or carrier (Gautschi et al. 2007). Successful clinical trials using growth factor therapy have led to the FDA-approval of both BMP-2 and BMP-7 for clinical use in the treatment of non-union in long bones (Friedlaender et al. 2001; Govender et al. 2002) and in the induction of spinal fusion (Boden et al. 2000; Vaccaro et al. 2003).

Problems currently associated with this field of clinical treatment are related to the massive milligram doses of growth factor required to elicit a therapeutic response in humans, which is in stark contrast to the natural situation (Reddi 1998). These problems may be due in part to the treatments containing a single growth factor. This is very different to an *in vivo* situation, where a whole plethora of growth factors work synergistically, activating a complex network of signalling pathways and stimulating optimal repair (Hughes et al. 2006; Lieberman et al. 2002; Lutolf and Hubbell 2005). It may be that clinical treatments that allow for the sequential delivery of multiple growth factors will show more success at clinically treating bone injury (Richardson et al. 2001).

1.4 MODELLING OF BONE REPAIR

1.4.1 Current Models

The development of alternative clinical therapies for bone injury to overcome some of the current associated limitations is hampered by research models currently available for studying bone repair. *In vitro* models use only a single cell system, or at most two in the case of organotypic models. Single cell systems typically involve the isolation of primary osteoblasts or osteoclasts, or their respective progenitors, or the use of established bone cell lines, to investigate the behaviour of these cells in response to varying treatments. This might involve the addition of exogenous growth factors (Abdelmagid et al. 2007; Canalis and Lian 1988; Itonaga et al. 2004; Yamaguchi et al. 1996) or treatment with vitamins (Matsumoto et al. 1991; Shi et al. 2007). Organotypic *in vitro* models co-culture two different cell types, examples being the co-culture of osteoblasts with chondrocytes to investigate the biological integration of cartilage grafts with subchondral bone (Jiang et al. 2005), or the co-culture of osteoblasts and haematopoietic stem cells to investigate physical interactions between the cell types (Jung et al. 2005). While such *in vitro* models can provide information on the behaviour of single cell types in response to varying factors, or the influence of one cell type on another in the case of organotypical models, the information they can yield is limited as they are unable to recapitulate the spatial arrangement of cells *in vivo*. *In vivo* models of bone repair have been developed in a variety of

animals (Lu and Rabie 2003; Mark et al. 2004; Pearce 2007; Petite et al. 2000) and are often critical sized defect models developed in the calvaria, mandible or femur (Fini et al. 2005; Schmitz and Hollinger 1986). While they have yielded considerable information on bone repair, *in vivo* models also have limitations and problems. They are expensive, requiring large numbers of animals, with a typical ethos of one animal equalling one experiment. It can also be difficult to obtain clear data from *in vivo* model systems due to the intrinsic systemic influences, which although important for modulating repair processes can often confuse and cloud the underlying molecular mechanisms. There are also the ethical ramifications associated with *in vivo* work. *Ex vivo* models may have significant advantages over the models above, as cells and tissue are in the same spatial arrangement as *in vivo*, while systemic influences are removed. Furthermore, multiple experiments can be carried out on single animals, reducing animal numbers and experimental costs.

1.4.2 *Ex Vivo* Modelling

In 1998, Sloan and colleagues developed an *ex vivo* tooth slice model (Sloan et al. 1998), with the aim of developing a novel system to investigate dentine repair processes, overcoming some of the problems associated with *in vitro* and *in vivo* models. The model, using cultured rat incisor slices, has been used successfully to investigate a wide range of dental repair processes (Sloan et al. 2000a; Sloan et al. 2000b; Sloan and Smith 1999), including the study of angiogenesis within the pulp (Gonçalves et al. 2007) and

assessment of the cytotoxicity of commonly used dental materials (Murray et al. 2000). The model also provided a basis for the development of another *ex vivo* model; a mandible slice organ culture model (Dhopatkar et al. 2005). This model was developed to investigate the effects of externally applied forces on the dentine-pulp complex, as a means of modelling orthodontic forces. This thesis aims to develop this *ex vivo* mandible slice model for investigating bone repair processes. Cells are more likely to survive and proliferate within mandible slices, since the mandible contains bone cells present within both the endosteum and the trabecular bone, as opposed to cortical bone slices where cells only reside within the endosteum. Also the use of a Trowel type culture system enables natural perfusion of the bone tissue, promoting cellular survival and viability within the system. The periodontal ligament (PDL) within the mandibular tissue aids the fracturing process, ensuring attachment of bone. Use of mandible slices, with the presence of both bone and dental tissue, also allows greater flexibility of the model for future development, such as a model for inflammatory-mediated bone loss in periodontal disease.

1.5 AIMS

This thesis aims to:

- Develop and validate a reproducible and quantitative *ex vivo* rat mandible fracture model that can be used to investigate specific molecular and cellular processes of bone repair;
- Investigate cellular responses by looking at proliferation, migration, and differentiation of cell types present within the model, in particular investigating cellular responses of the osteoblasts and their progenitors;
- Investigate the role that growth factors play in repair and regeneration, in particular cellular responses to these growth factors within the model;
- Develop a reproducible bone slab cell culture model to work in conjunction with the mandible model;
- Use the bone slab model to demonstrate release of bioactive molecules from bone matrix, and their effects on primary bone marrow stromal cells.

CHAPTER 2

ORGAN CULTURE OF INTACT AND FRACTURED RAT MANDIBLE SLICES

2.1 INTRODUCTION

As discussed in the introduction to this thesis, a major limiting factor towards understanding optimal repair processes, and refining and developing therapeutic approaches, is the lack of a suitable model system for studying aspects of bone repair and regeneration. *In vitro* models use a single cell system, for example the culture of bone marrow stromal cells (Yamaguchi et al. 1996), or at the most two cell types in the case of organotypical models, such as the co-culture of osteoblasts and haematopoietic stem cells to investigate physical interactions between the two cell types (Jung et al. 2005). The information such *in vitro* cell culture systems yield is limiting, as they cannot recapitulate the complex interactions that occur *in vivo* between the many cell types present in bone. Neither can such model systems recapitulate the spatial arrangement of cells *in vivo*. *In vivo* models of bone repair have been developed in a number of animals (Pearce 2007; Petite et al. 2000), and while they have yielded considerable information on processes taking place in bone repair, these models can also be limiting. Experiments are extremely costly, requiring large numbers of animals, and results can be somewhat ambiguous due to the complex systemic influences which, although important for modulating bone repair processes, can often confuse

and cloud the underlying molecular mechanisms. There are also the ethical ramifications involved in undertaking such *in vivo* work, especially considering the large numbers of animals that are often used in such experiments.

An attractive alternative to the more limiting *in vitro* and *in vivo* model systems may be *ex vivo* organ culture models. Such models involve the culturing of both cells and tissues *in situ*, in the same spatial arrangement that they would be found *in vivo*, but the systemic influences that often hinder *in vivo* experimentation are removed. Furthermore, the cost of experiments can be significantly reduced, as one animal can yield many experiments, as opposed to the one animal, one experiment policy often adopted in *in vivo* work. An *ex vivo* tooth slice model, involving the culture of rat incisor slices, has been developed (Sloan et al. 1998), and used successfully in the investigation of dental repair processes (Gonçalves et al. 2007; Murray et al. 2000). This thesis chapter aims to build on this tooth slice model, by developing, refining, and characterising a quantitative *ex vivo* fractured rat mandible slice model, to investigate the processes involved in bone repair. Cellular behaviour within the model will be assessed by analysing morphology, proliferation, differentiation, and protein synthesis, to determine if mandible slices (both intact and fractured) can be cultured *ex vivo* while maintaining the viability and behaviour of the cells and tissues. The development of such a model may be invaluable in the search for ways to improve and enhance the initial stages of bone repair in patients, particularly where normal healing does not occur.

CHAPTER TWO

SECTION A:

ESTABLISHMENT OF OPTIMAL CONDITIONS FOR THE ORGAN CULTURE OF INTACT RAT MANDIBLE SLICES

2.2 MATERIALS AND METHODS

2.2.1 Preparation of tissue culture reagents

Washing Medium

Washing medium of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Co Ltd, UK) was prepared containing 1% concentration of a penicillin / streptomycin / amphotericin solution (containing 10,000 units of penicillin, 10mg/ml of streptomycin, and 25µg/ml amphotericin B) (Invitrogen, UK) and 200mM L-glutamine (Invitrogen, UK).

Culture Medium

Culture medium of DMEM (Sigma Chemical Co Ltd, UK) was prepared containing 10% heat inactivated foetal calf serum (FCS) (Invitrogen, UK), 1% concentration of a penicillin / streptomycin / amphotericin solution (containing 10,000 units of penicillin, 10mg/ml of streptomycin, and 25µg/ml amphotericin B) (Invitrogen, UK), 0.15mg/ml of ascorbic acid (Invitrogen, UK), and 200mM L-glutamine (Invitrogen, UK).

Embedding medium for culture of mandible tissue

Embedding medium was prepared by adding 10ml of culture medium to 1% low melting point agar (agarose type VII) (Sigma Chemical Co Ltd) which had been previously sterilised by autoclaving.

2.2.2 Organ culture of intact mandible slices

Mandibles were dissected from 28 day old male Wistar rats, freshly sacrificed by CO_2 asphyxiation. Soft tissue was removed with a sterile scalpel blade, and the mandible placed into sterile washing medium. After removal of the condyle, ramus, and molars with a segmented diamond-edged rotary saw, sections of mandible approximately 2mm thick were cut using the rotary saw cooled with washing medium at 4°C (Figure 2.1). Sterility of the disc was achieved by washing with 70% ethanol and distilled water prior to use. Mandible slices were placed immediately into fresh washing medium.

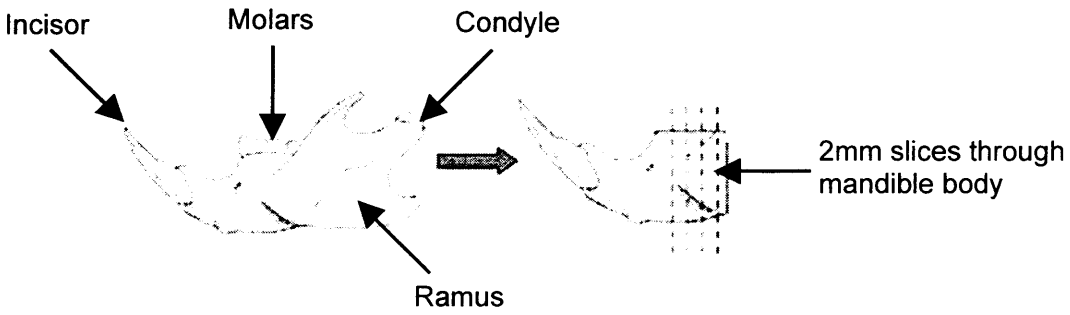

Figure 2.1: Preparation of mandibles.

Two different culture methods were used for the organ culture of the intact mandible slices: base type cultures; and Trowel type cultures (Bègue-Kirn et al. 1992; Sloan et al. 1998). For base type cultures, the intact mandible slices were transferred from the washing media into individual wells of a plastic 24-well plate (Greiner Bio-One, UK), and 2ml of culture media added to each well (Figure 2.2). For Trowel type cultures, the intact mandible slices were transferred from the washing media into individual wells of a plastic 96-well plate (Greiner Bio-One, UK) and embedded in 100µl sterile molten embedding media. Slices were left at room temperature for 10 min until semi-solid, after which time the embedded mandible slices were transferred from the multiwell dish to a sterile 25mm diameter Millipore filter (mixed esters of cellulose acetate and nitrate). With the aid of a plastic ring support, the Millipore filter was floated on the surface of 2ml of culture medium in Trowel type cultures (Trowell 1959) in individual wells of a plastic 24-well plate (Greiner Bio-One, UK) (Figure 2.3). Mandible slices in both base and Trowel type cultures were incubated at 37°C, in a humidified atmosphere of 5% CO_2 in air, for 7, 14, or 21 days.

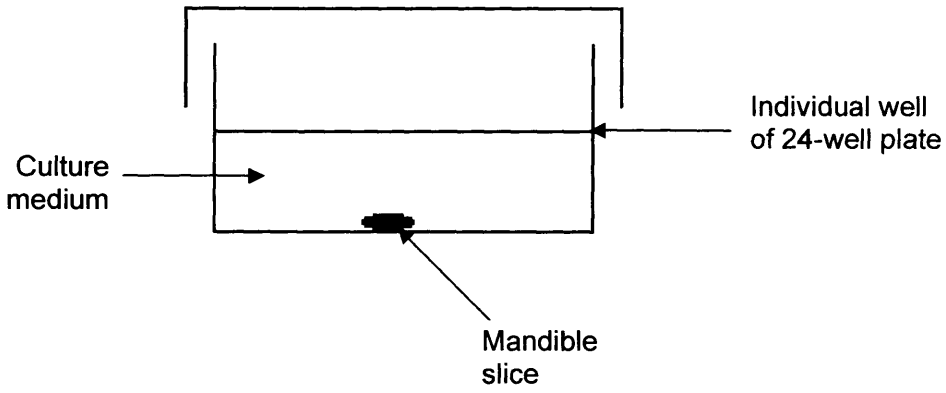

Figure 2.2: Base type culture.

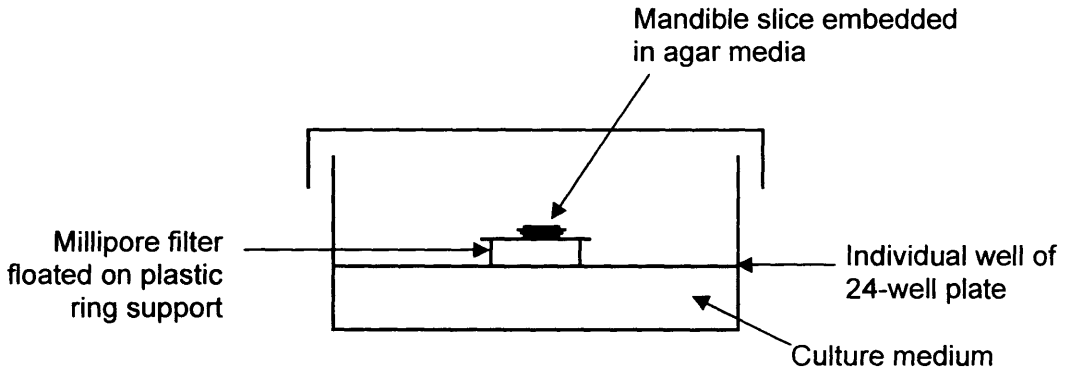

Figure 2.3: Trowel type culture.

2.2.3 Histological examination

Following culture, mandible slices were fixed in 10% (w/v) neutral buffered formalin at room temperature for 24 hrs. Slices were then demineralised in 10% (w/v) formic acid at room temperature for 72 hrs, while being gently agitated. Mandible slices were processed through a series of 70, 90, and 100% graded alcohols, cleared through xylene, and taken to molten paraffin wax (Raymond Lamb, UK) on an automatic tissue processer (Shandon Pathcentre). Slices were embedded in paraffin wax in a metal mould prior to sectioning. Sections of 7μm thickness were cut on a rotary microtome (Leica) at different levels throughout the tissue and mounted onto glass SuperFrost microscope slides (Fisher Scientific Ltd, UK). In order to improve adhesion of sections to the slides, sections were dried in an oven at 65°C. Sections were then rehydrated by taking to xylene, a series of graded alcohols, and water. Sections were stained with haematoxylin, washed and blued in Scott's tap water, and differentiated in 1% acid alcohol. After a further wash in tap water, sections were stained with eosin, washed in tap water again, and finally dehydrated through alcohol and cleared with xylene. Sections were mounted under a coverslip with DPX mounting medium (Raymond Lamb, UK), and examined on an Olympus AX40 light microscope. Photographs were taken using a Nikon DXM digital camera and ACT-1 imaging software. Cell numbers were automatically counted using Image ProPlus software within 100μm^2 areas of the PDL and the pulp of haematoxylin and eosin (H&E) stained mandible sections. 100μm^2 areas within five random fields of view were counted per section. PDL and pulp cell counts were obtained for 7, 14,

and 21 day cultures (n=12 for each time point), cultured in base and Trowel type cultures, and standard errors of the mean were calculated. Mean values were analysed using one-way analysis of variance (ANOVA) and Tukey's post hoc test, to analyse differences between the cell numbers in different culture types and culture periods.

2.2.4 Cell viability assessment

At the end of a culture period, culture media was removed from each well, and mandible slices in Trowel type cultures were removed from the filters and placed onto the base of the well. 2ml of acridine orange / ethidium bromide solution (100µg/ml in DMEM prepared from 2.5mg/ml and 10mg/ml stock solutions respectively, Sigma Chemical Co, UK) was added to each culture, and incubated for 5 s. Mandible slices were then washed briefly in 2ml phosphate buffered saline (PBS) before being placed onto a microscope slide and examined immediately under UV light using an Olympus AX70 fluorescence microscope. Viable cells actively extruded ethidium bromide and exhibited green fluorescence, whereas non-viable cells fluoresced red. Photographs were taken using a Nikon DXM digital camera and ACT-1 imaging software.

2.2.5 Tritiated proline pulse chase

Cellular secretion of collagen can be shown by incorporation of tritiated proline (Kameyama 1975; Weinstock and Leblond 1974). Proline is an amino

acid essential for the synthesis of collagen, and to a much lesser extent other proteins. Therefore the addition of a radiolabelled proline enables the visualisation of protein secretion.

Freshly prepared mandible slices were placed into individual wells of a plastic 96-well dish (Greiner Bio-One, UK). 100µl of embedding media containing 0.37MBq of ^{3}H proline (Amersham, UK; L – [5 ^{3}H] proline, specific activity 925GBq/mmol; 25Ci/mmol) was added to each well, using a dedicated radioactive tray and sterile disposable pipettes to prevent contamination. Once semi-solid, slices were transferred into Trowel type culture containing 2ml of culture media with 0.185MBq of ^{3}H proline / ml of media. Slices were cultured at 37°C in 5% CO_2 in air in a humidified incubator for 48 hrs. After 48 hrs, culture media was removed, and replaced with fresh working media containing no radiolabel. Cultures were continued for up to 7 or 14 days. Control cultures were prepared in the absence of radiolabel within the embedding or working media. Mandible slices were placed into individual wells of a plastic 96-well plate, into which 100µl of embedding media was added and left to solidify. Once semi-solid, the embedded slices were transferred into Trowel type cultures containing 2ml of culture media. The control slices were cultured at 37°C in 5% CO_2 in air in a humidified incubator for 7 or 14 days.

After a culture period of 7 or 14 days, slices were processed, embedded, and cut into 7µm sections (described in 2.2.3). Sections were dewaxed in xylene for 5-10 min, hydrated in industrial methylated spirit (IMS) for 5 min and left to

air-dry before autoradiography was carried out in a light-tight darkroom using a photographic safelight to provide suitable illumination. All glassware was washed in 90% ethanol, followed by distilled water, prior to use. A glass dipping chamber containing molten LM-1 nuclear emulsion (Amersham, UK) was placed into a 45°C water bath. Experimental slides containing the histological sections of cultured tissue were dipped vertically into the dipping chamber for 5 s, withdrawn at a steady rate, and excess liquid removed from the back of the slide. Slides were placed face-up on an ice block, to gel the emulsion, for 5-10 min. Following this, slides were placed into a light-tight box and left at room temperature for 1-2 hrs to allow the slides to dry. Silica gel was placed into the box to provide a dry atmosphere and the slides were left in the dark at 4°C for 12 days to expose. Control slides were prepared to assess for false-positive signals, by dipping and exposing slides from unlabelled control cultures. To assess for false-negative signals, slides of sections incorporating radiolabel were dipped in nuclear emulsion and then exposed to the light for a few seconds. Control slides were exposed as previously described.

After a 12 day exposure, the slides to be developed were placed into Phenisol developing solution (Ilford Photo, UK; diluted 1:4 with distilled water) for 5 min with gentle agitation, under darkroom conditions. Slides were then placed into 0.5% (w/v) acetic acid stop solution for 1 min with gentle agitation, before being transferred into 30% (w/v) sodium thiosulphate fixing solution for 4 min, again with gentle agitation. Under light conditions, slides were soaked in fixing solution for a further 4 min, and then washed in gently

running tap water (at room temperature) for 15 min. Slides were washed twice in distilled water (10ml/slide) for 15 min, and then counterstained with H&E (as described in 2.2.3).

The number of silver grains deposited were automatically counted within 100μm^2 areas of the PDL matrix, or within the predentine, of radiolabelled mandible sections, using Image ProPlus software. Silver grain numbers were also calculated within similar areas in unlabelled control cultures. 100um^2 areas within five random fields of view were counted per section. PDL and predentine grain counts were obtained for 7 and 14 day cultures (n=10 for each time point), cultured in base and Trowel type cultures, and standard errors of the mean were calculated. Mean values were analysed using one-way ANOVA and Tukey's post hoc test, to analyse silver grain deposition over the culture period.

2.2.6 Immunohistochemistry

Immunohistochemical staining was carried out on mandible slices cultured in Trowel type cultures for 7 or 14 days. Following histological fixation, demineralisation, processing and embedding of mandible slices (as described in 2.2.3), 7μm thick sections were cut on a rotary microtome at different levels throughout the tissue and mounted onto glass SuperFrost microscope slides (Fisher Scientific Ltd, UK). Sections were dried overnight in an oven at 65°C prior to immunolocalisation, in order to improve adhesion of sections to the slides.

Sections were deparaffinised and hydrated by washing with xylene for 5-10 min, IMS for 5 min, and rinsing in distilled water for 5 min. Sections were then incubated in 3% hydrogen peroxide solution for 10 min to quench endogenous peroxidase activity and washed in tris-buffered saline (TBS) buffer for 2x 2.5 min. The staining for proliferating cell nuclear antigen (PCNA), an indicator of cellular proliferation, and osteopontin and alkaline phosphatase, markers of osteoblast differentiation, was carried out using a Vectastain ABC peroxidase kit (Vector Laboratories Ltd). The kit included normal horse serum for blocking, a universal biotinylated secondary antibody and the 'ABC' reagent – a preformed avidin and biotinylated horseradish peroxidase macromolecular complex. The horseradish peroxidase is visualised by the development of a peroxidase substrate that produces a colour.

Sections were blocked using the normal horse serum diluted in TBS (50µl serum in 5ml TBS) for 20 min. Excess serum was wiped from the slides and sections incubated for 1 hr with the appropriate primary antibody, diluted appropriately in TBS / 1% bovine serum albumin (BSA) (Table 2.1). For negative controls, a non-immunogenic IgG isotype control antibody (Sigma Chemical Co; diluted to working concentration of primary antibody) was used instead of the primary, as well as exclusion of the primary antibody and replacement with TBS.

Following primary antibody incubation, sections were washed for 2x 2.5 min in TBS buffer and then incubated for 30 min with the universal biotinylated

secondary antibody. Following a further TBS buffer wash, sections were incubated for 30 min with the VECTASTAIN 'ABC' reagent followed by incubation in DAB peroxidase substrate solution (Vector Laboratories) for 2-5 min. Sections were rinsed in tap water, and counterstained with 0.1% light green for 1 min. Finally sections were dehydrated in IMS for 5-10 min, cleared in xylene for 5-10 min, and glass coverslips applied using DPX mounting medium (Raymond Lamb, UK). Sections were examined under an Olympus AX70 light microscope, and photographs taken using a Nikon DXM digital camera and ACT-1 imaging software.

Antibody	**Source of antibody**	**Isotype**	**Source / species reactivity**	**Clone ID**	**Antibody Dilution**
PCNA	Santa Cruz Biotech	IgG	Monoclonal mouse anti-rat	PC10	1:20
Osteopontin	Kind gift from Larry Fisher (Fisher et al. 1995)	IgG	Monoclonal mouse anti-human	LF-124	1:250
Alkaline Phosphatase	Santa Cruz Biotech	IgG	Polyclonal rabbit anti-human	H-300	1:50

Table 2.1: Immunohistochemistry primary antibodies.

2.3 RESULTS

2.3.1 Histology and viability of intact mandible slices

2.3.1.1 Uncultured intact mandible slices

Studies were first performed on mandible slices that had not been cultured. H&E histology performed on these slices showed maintenance of architecture throughout the tissue, with the pink eosin staining highlighting intact bone, dentine and PDL (Figure 2.4). Morphological analysis of cells within all areas of the tissue indicated intact, viable cells with darkly stained, round nuclei. Cell types observed included osteoblasts and osteocytes within the bone, bone marrow stromal cells, PDL cells, odontoblasts and pulp cells. All cell types were observed to be residing within the appropriate areas of the tissue, where they would be found *in vivo*. Acridine orange / ethidium bromide vital dye staining of the surface of slices prior to culture showed significant cell death throughout the tissue (Figure 2.5). Since H&E histology demonstrated healthy viable cells further into the tissue, this may indicate surface damage due to the slicing procedure.

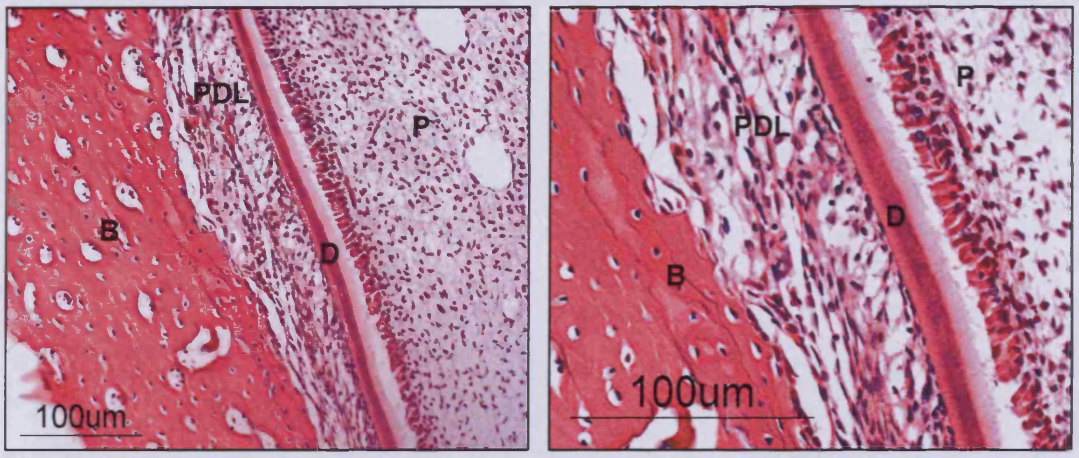

Figure 2.4: Histology of uncultured mandible slices, showing maintenance of tissue and cells of the bone (B), periodontal ligament (PDL), dentine (D), and pulp (P).

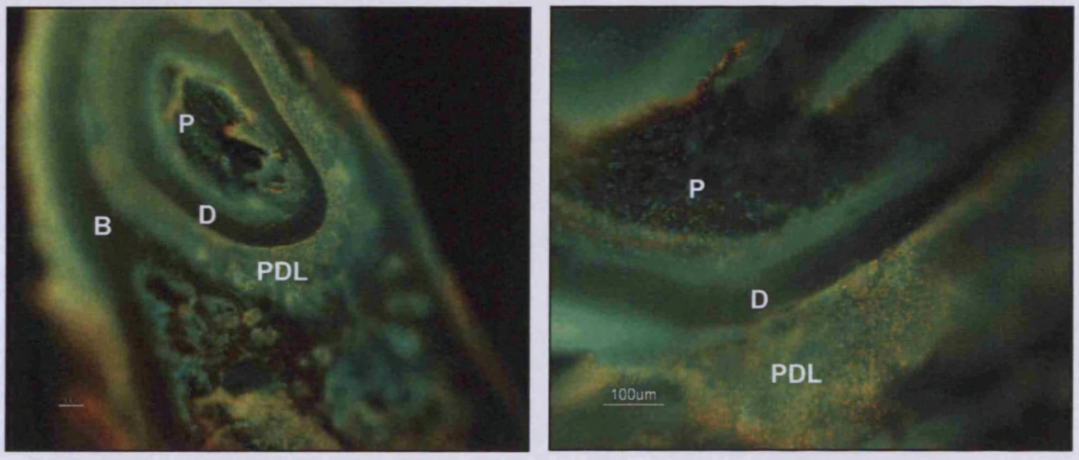

Figure 2.5: Acridine orange / ethidium bromide vital dye staining of an uncultured mandible slice showing bone (B), periodontal ligament (PDL), dentine (D), and pulp (P).

2.3.1.2 24 hour culture of intact mandible slices

Intact mandible slices were prepared from 28 day old male Wistar rats and placed into either base type culture or Trowel type culture for 24 hours. H&E histology performed on base type cultures after this 24 hour period showed maintenance of tissue architecture within all areas, including the bone, dentine, and PDL, as indicated by the pink eosin stain highlighting these intact structures (Figure 2.6). Morphological analysis of cells within the tissue indicated the presence of intact, viable cells with darkly stained, round nuclei. Mandible slices cultured in Trowel type appeared morphologically very similar to those cultured in base type, with maintenance of tissue and cell morphology observed throughout the tissue (Figure 2.7). Generally, the morphological appearance of the tissues and cells closely paralleled that of the uncultured control mandible slices. Acridine orange / ethidium bromide vital dye staining of the surface of slices in both base type cultures (Figure 2.8) and Trowel type cultures (Figure 2.9) demonstrated significant cell death, although again the H&E stains indicated healthy viable tissue further into the mandible slice. Taken together these results demonstrated that the observed cell death was present on the surface only, probably due to damage from the slicing procedure, indicating that the surface damage had not been resolved after 24 hours. Acridine orange / ethidium bromide staining of intact mandible slices after 4 days in culture, however, showed significantly improved cell viability, with the vast majority of cells across the surface of the tissue fluorescing green (Figures 2.10 and 2.11), indicating resolution of surface damage.

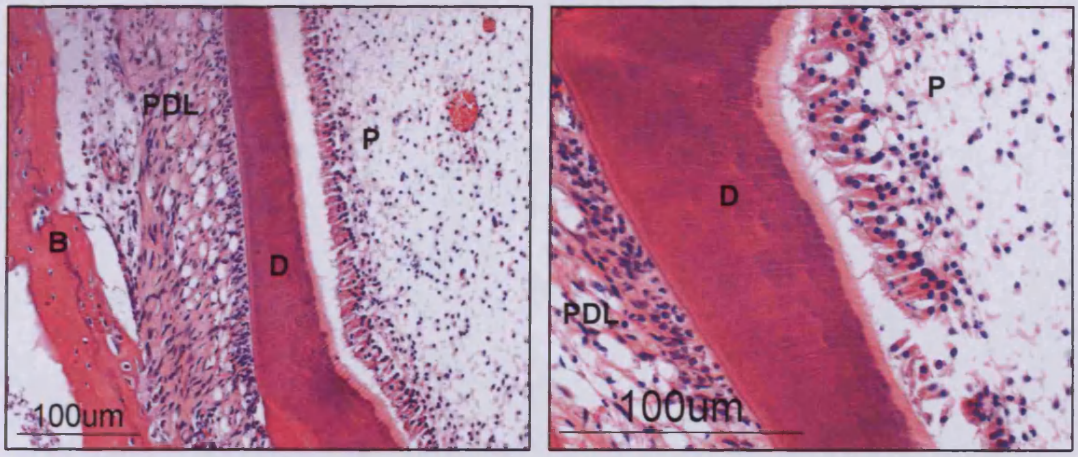

Figure 2.6: Histology of 24 hour base type culture showing maintenance of cell and tissue architecture of the bone (B), periodontal ligament (PDL), dentine (D), and pulp (P).

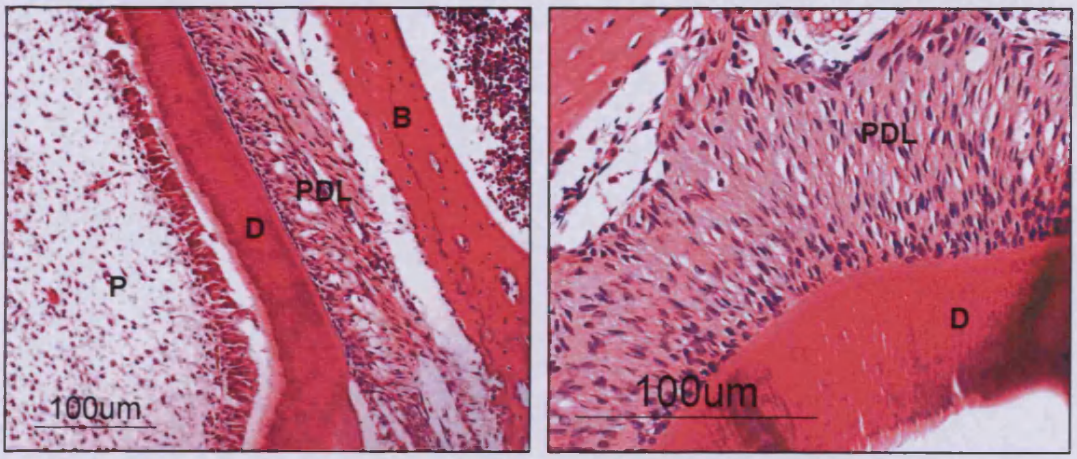

Figure 2.7: Histology of 24 hour Trowel type culture showing maintenance of cell and tissue architecture of the bone (B), periodontal ligament (PDL), dentine (D), and pulp (P).

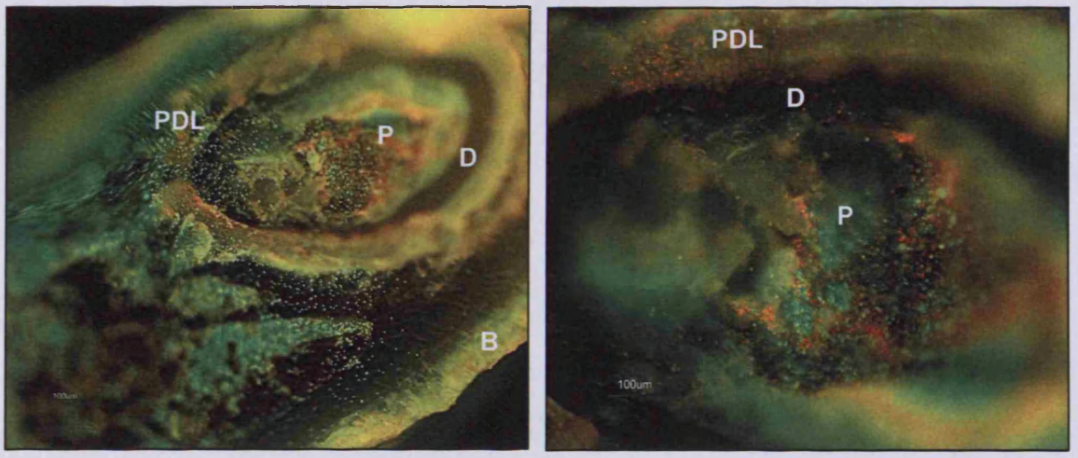

Figure 2.8: Acridine orange / ethidium bromide vital dye staining of a mandible slice cultured for 24 hours in base type culture, showing bone (B), periodontal ligament (PDL), pulp (P) and dentine (D).

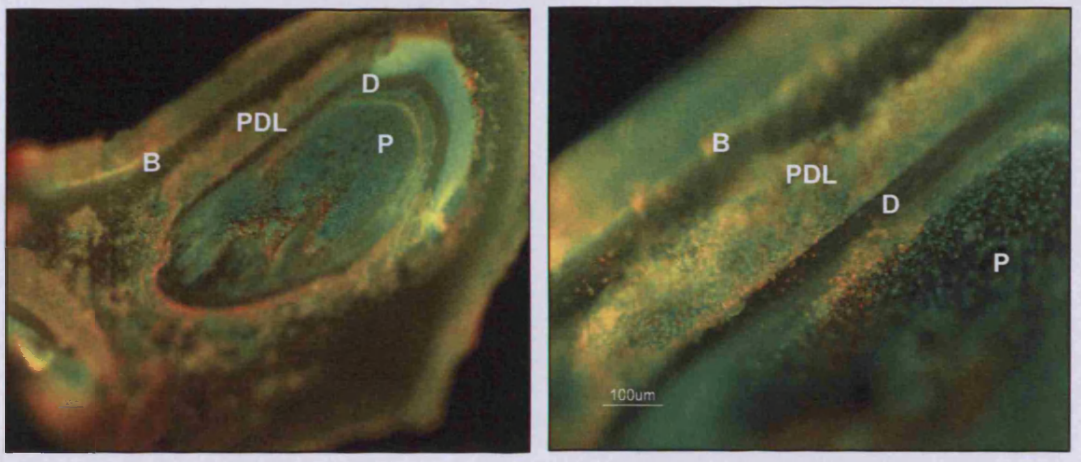

Figure 2.9: Acridine orange / ethidium bromide vital dye staining of a mandible slice cultured for 24 hours in Trowel type culture, showing bone (B), periodontal ligament (PDL), pulp (P) and dentine (D).

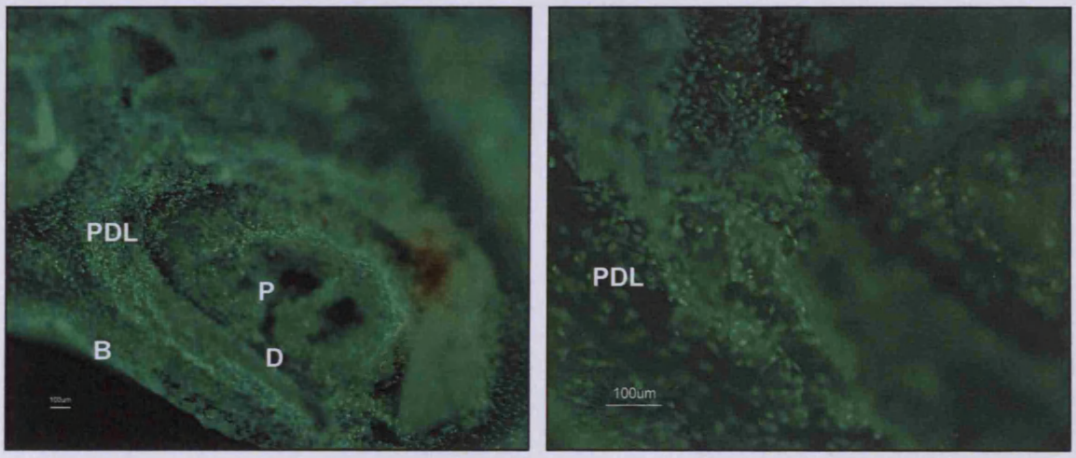

Figure 2.10: Acridine orange / ethidium bromide vital dye staining of a mandible slice cultured for 4 days in base type culture, showing bone (B), periodontal ligament (PDL), pulp (P) and dentine (D).

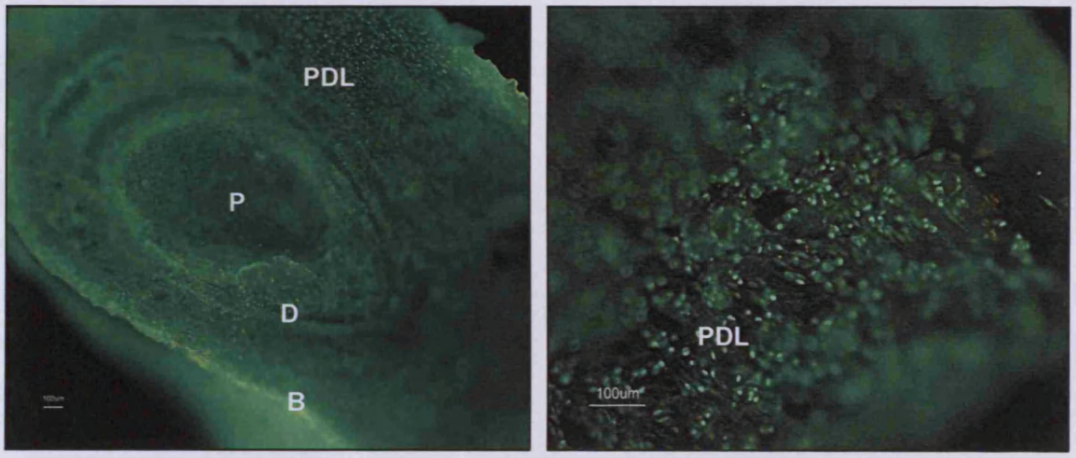

Figure 2.11: Acridine orange / ethidium bromide vital dye staining of a mandible slice cultured for 4 days in Trowel type culture, showing bone (B), periodontal ligament (PDL), pulp (P) and dentine (D).

2.3.1.3 7 day culture of intact mandible slices

After 7 days in base type cultures, H&E histology showed maintenance of architecture across the mandibular tissue, and was comparable to the uncultured and 24 hour cultured mandible slices (Figure 2.12). Cells in all areas appeared with darkly stained round nuclei and were observed to be morphologically similar to uncultured controls. Tissue and cell architecture appeared to be maintained to a slightly higher degree in Trowel type cultures (Figure 2.13). Acridine orange / ethidium bromide vital dye staining indicated a high level of cellular viability maintained throughout the tissue, in both base type cultures (Figure 2.14) and Trowel type cultures (Figure 2.15). The majority of cells exhibited green fluorescence, with only a few cells fluorescing red, and viability was comparable to slices cultured for 4 days.

Automated cell counts performed on H&E stained sections of base type cultures using Image ProPlus software calculated an average number of 91 ± 3.1 cells per 100μm^2 area within the PDL, and an average number of 132 ± 3.3 cells per 100μm^2 area within the pulp. The same cell counts performed on Trowel type cultures calculated an average of 102 ± 5.4 cells per 100μm^2 area within the PDL, and an average number of 133 ± 2.8 cells per 100μm^2 area within the pulp (Figures 2.24 and 2.25). Statistical analysis showed no significant differences in cell numbers between base and Trowel type cultures, either in the PDL or the pulp ($p>0.05$).

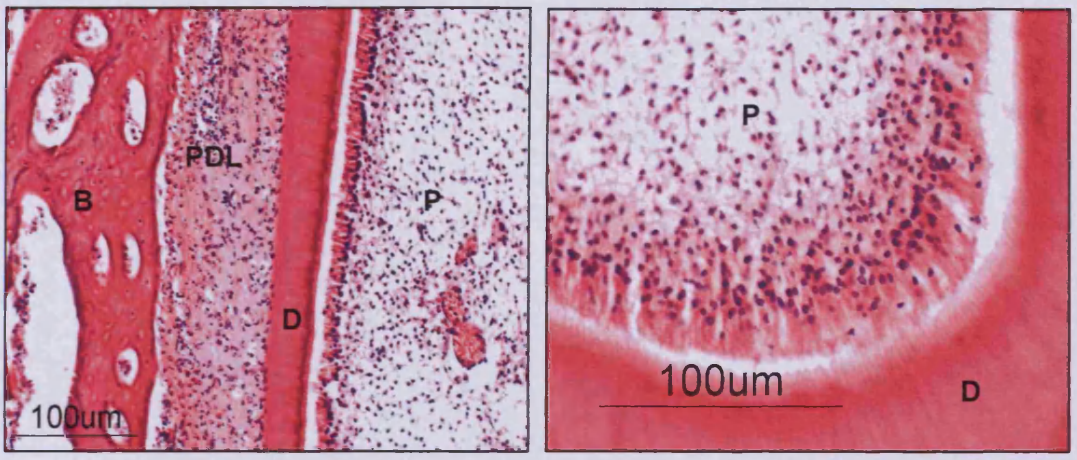

Figure 2.12: Histology of 7 day base type culture. Maintenance of cell and tissue architecture can be observed throughout the bone (B), periodontal ligament (PDL), dentine (D) and pulp (P).

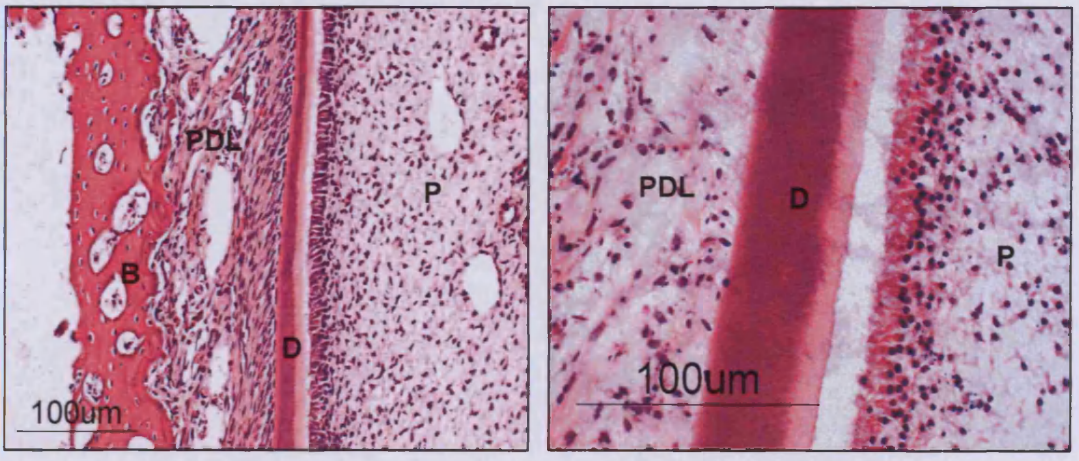

Figure 2.13: Histology of 7 day Trowel type culture. Maintenance of cell and tissue architecture can be observed throughout the bone (B), periodontal ligament (PDL), dentine (D) and pulp (P).

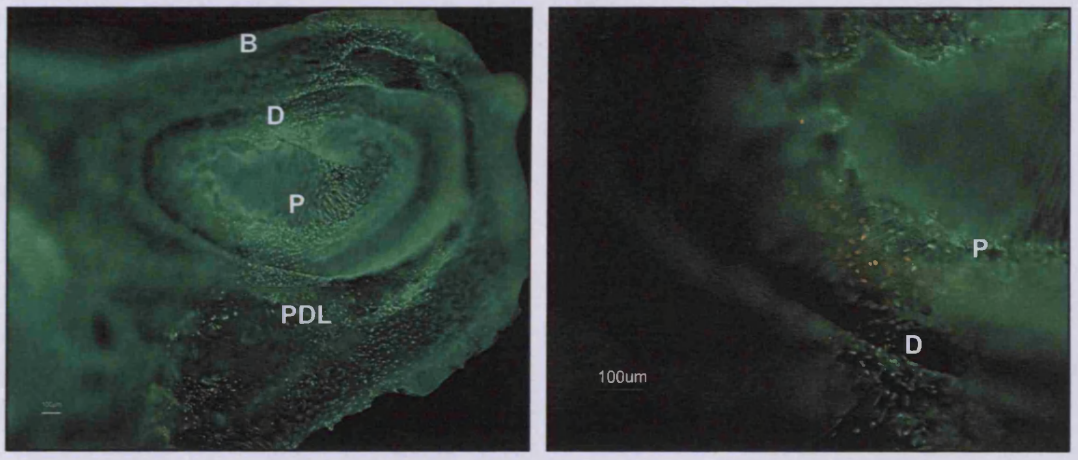

Figure 2.14: Acridine orange / ethidium bromide vital dye staining of a mandible slice cultured for 7 days in base type culture, showing bone (B), periodontal ligament (PDL), pulp (P) and dentine (D).

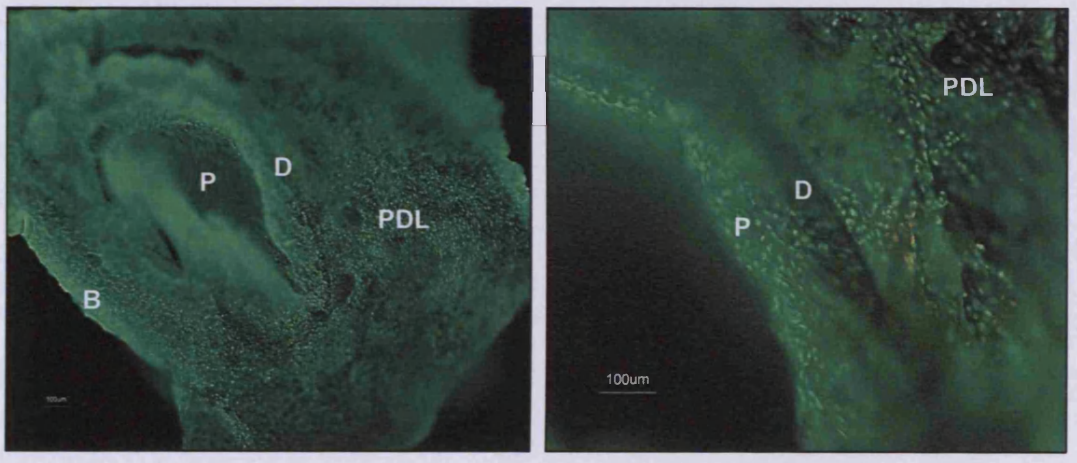

Figure 2.15: Acridine orange / ethidium bromide vital dye staining of a mandible slice cultured for 7 days in Trowel type culture, showing bone (B), periodontal ligament (PDL), pulp (P) and dentine (D).

2.3.1.4 14 day culture of intact mandible slices

Maintenance of tissue architecture was observed throughout mandible slices after 14 days in base type culture, and was comparable to the 7 day cultured mandible slices (Figure 2.16). Cells within the slices demonstrated darkly stained, round nuclei, and were morphologically similar to 7 day cultures. Similar results were observed with mandible slices in Trowel type cultures, with maintenance of cell and tissue architecture throughout the tissue, although as with the 7 day cultures, maintenance of tissue architecture appeared to be better within these Trowel type cultures (Figure 2.17). There did appear to be a slight decrease in cell numbers in the 14 day cultures, when compared with the 7 day cultures, and this was confirmed with automated cell counting (Figure 2.24 and 2.25). Within base type cultures, there were an average of 79 ± 1.4 cells per 100μm^2 area of the PDL, and 126 ± 6.3 cells per 100μm^2 area of the pulp. Trowel type cultures had an average number of 89 ± 2.2 cells per 100μm^2 area of the PDL, and 129 ± 3 cells per 100μm^2 area of the pulp. However, statistical analysis did not show this slight decrease in cell number to be significant ($p>0.05$). Also, acridine orange / ethidium bromide vital dye staining indicated high cellular viability across the tissue in both base type cultures (Figure 2.18) and in Trowel type cultures (Figure 2.19), indicating that although cell numbers had slightly decreased, the vast majority of the population remaining were still highly viable. Viability appeared to be higher within the Trowel type cultures when compared with the base type cultures, and there were slightly higher cell numbers within Trowel type cultures, although this was not shown to be significant ($p>0.05$).

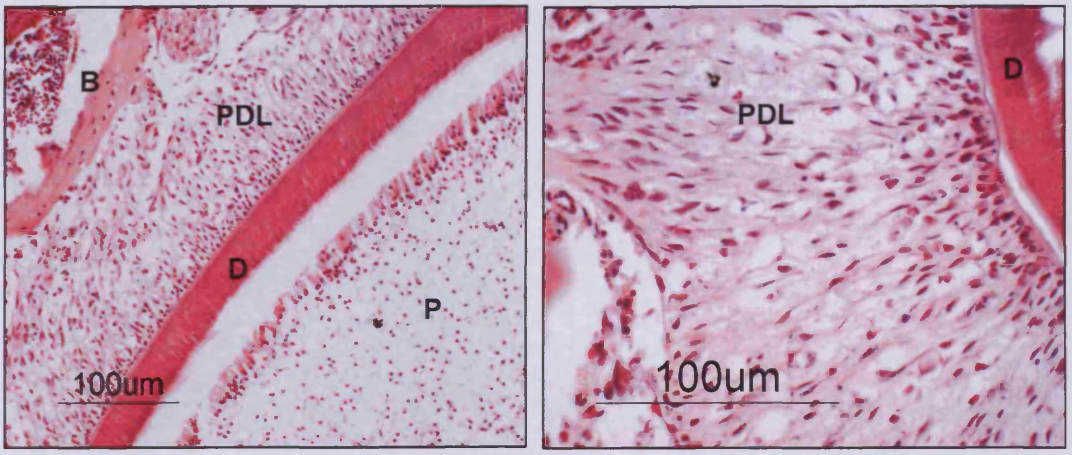

Figure 2.16: Histology of 14 day base type culture. Maintenance of cell and tissue architecture can be observed throughout the bone (B), periodontal ligament (PDL), dentine (D) and pulp (P).

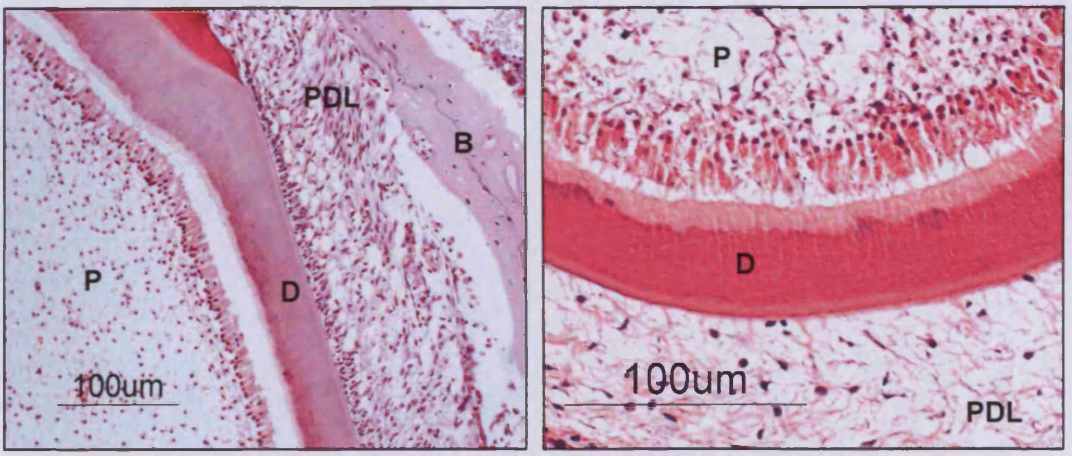

Figure 2.17: Histology of 14 day Trowel type culture. Maintenance of cell and tissue architecture can be observed throughout the bone (B), periodontal ligament (PDL), dentine (D) and pulp (P).

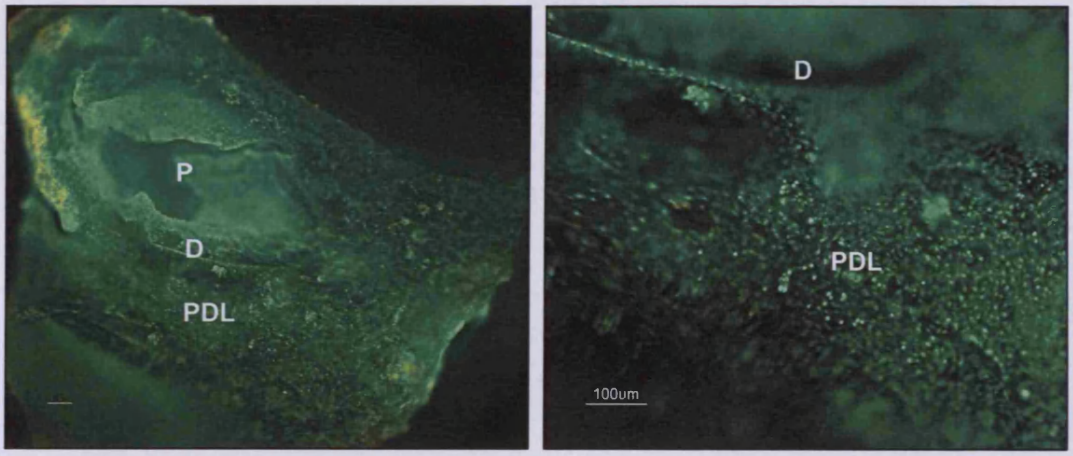

Figure 2.18: Acridine orange / ethidium bromide vital dye staining of a mandible slice cultured for 14 days in base type culture, showing bone (B), periodontal ligament (PDL), pulp (P) and dentine (D).

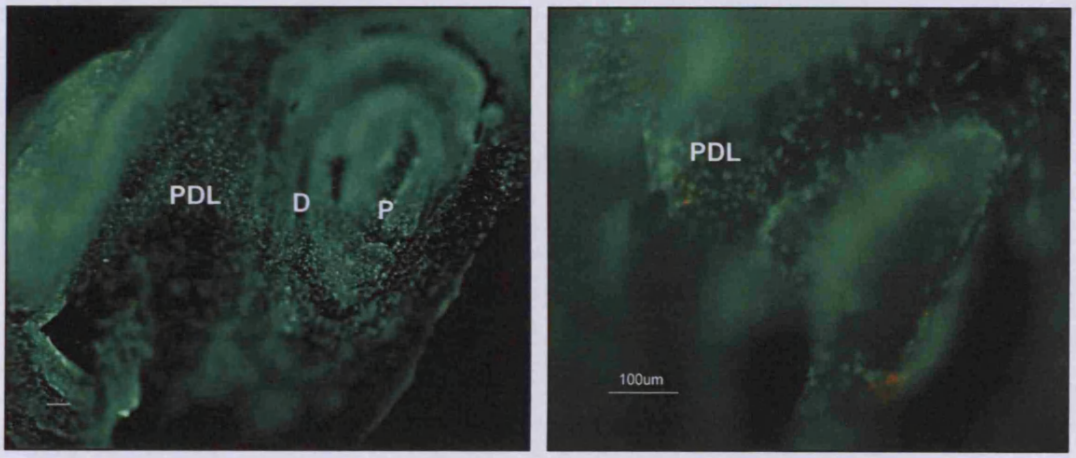

Figure 2.19: Acridine orange / ethidium bromide vital dye staining of a mandible slice cultured for 14 days in Trowel type culture, showing bone (B), periodontal ligament (PDL), pulp (P) and dentine (D).

2.3.1.5 21 day culture of intact mandible slices

After 21 days of culturing, maintenance of cell and tissue architecture could still be observed throughout both base type cultures (Figure 2.20) and Trowel type cultures (Figure 2.21). Tissue structure was well preserved and cells still appeared morphologically intact with round, darkly stained nuclei. Slices in Trowel type cultures appeared to demonstrate slightly better maintenance of tissue architecture and cellular viability. Results were comparable with 7 and 14 day cultures, although cell numbers again appeared to have decreased. This was confirmed with automated cell counting (Figure 2.24 and 2.25), and statistical analysis confirmed that there were significant differences in cell numbers within the 21 day cultures, when compared to the 7 day cultures ($p<0.001$). This was shown in both base and Trowel type cultures, within both the PDL and the pulp. Within base type cultures, automated cell counts calculated an average of 47 ± 2.6 cells per 100μm^2 area of the PDL, and 105 ± 2.5 cells per 100μm^2 area of the pulp. Trowel type cultures had an average of 53 ± 1.2 cells per 100μm^2 area of the PDL, and 107 ± 4 cells per 100μm^2 area of the pulp. However, despite this decrease in cell number, acridine orange / ethidium bromide vital dye staining indicated high cellular viability across the tissue in both base type cultures (Figure 2.22) and in Trowel type cultures (Figure 2.23). This indicated that a highly viable population of cells were still present after 21 days in culture. Viability appeared to be higher within the Trowel type cultures when compared with the base type cultures, as indicated by the presence of fewer dead cells, although there was no significant difference in cell numbers between the types of culture.

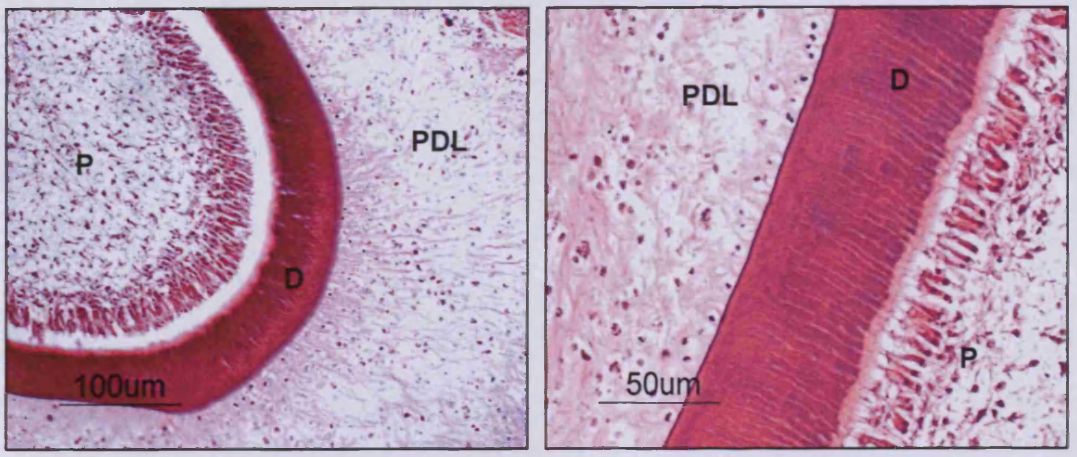

Figure 2.20: Histology of 21 day base type culture. Maintenance of cell and tissue architecture can be observed throughout the bone (B), periodontal ligament (PDL), dentine (D) and pulp (P).

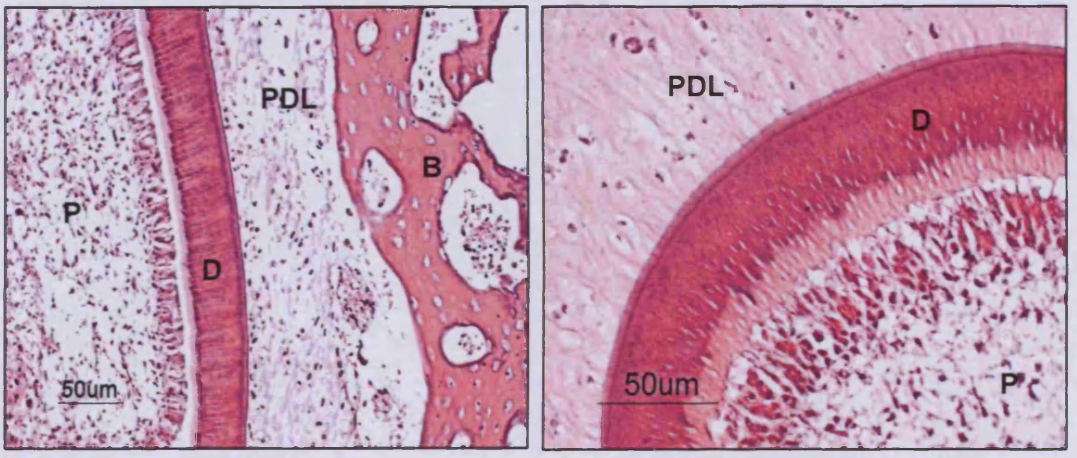

Figure 2.21: Histology of 21 day Trowel type culture. Maintenance of cell and tissue architecture can be observed throughout the bone (B), periodontal ligament (PDL), dentine (D) and pulp (P).

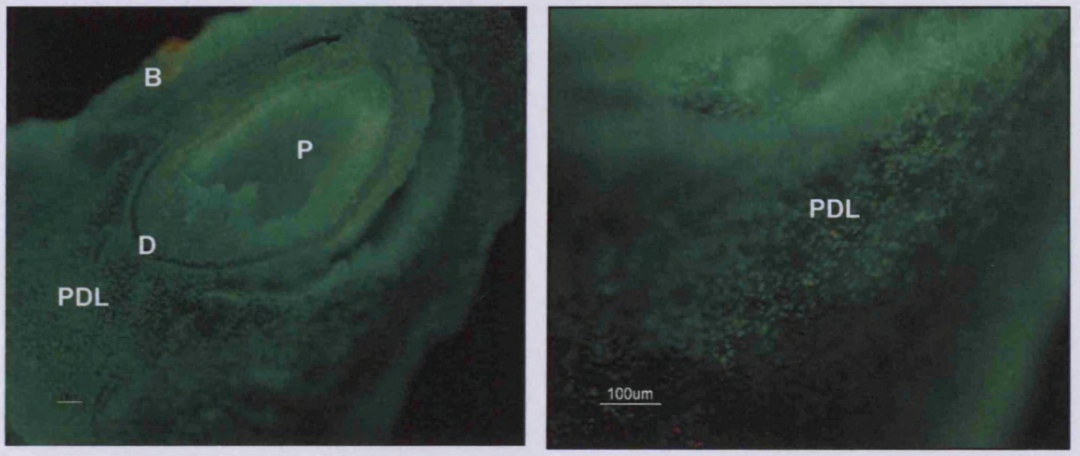

Figure 2.22: Acridine orange / ethidium bromide vital dye staining of a mandible slice cultured for 21 days in base type culture, showing bone (B), periodontal ligament (PDL), pulp (P) and dentine (D).

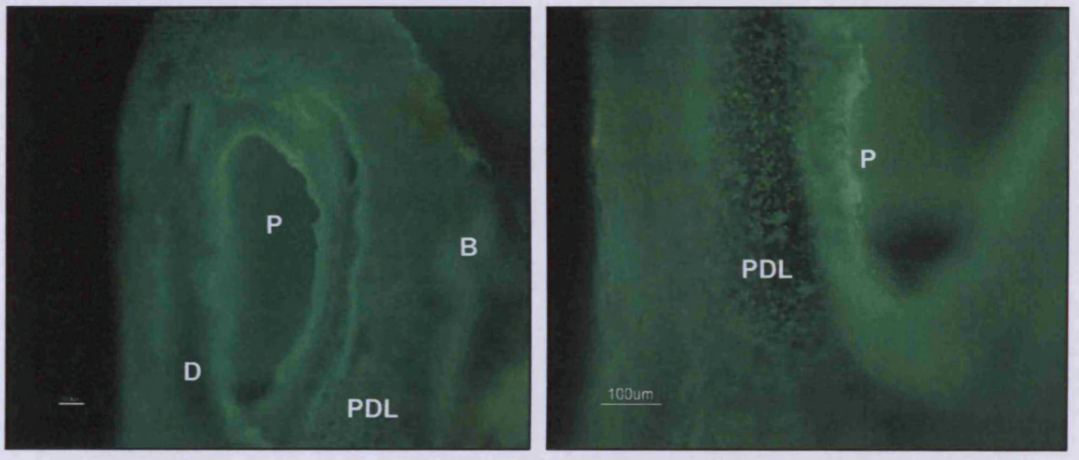

Figure 2.23: Acridine orange / ethidium bromide vital dye staining of a mandible slice cultured for 21 days in Trowel type culture, showing bone (B), periodontal ligament (PDL), pulp (P) and dentine (D).

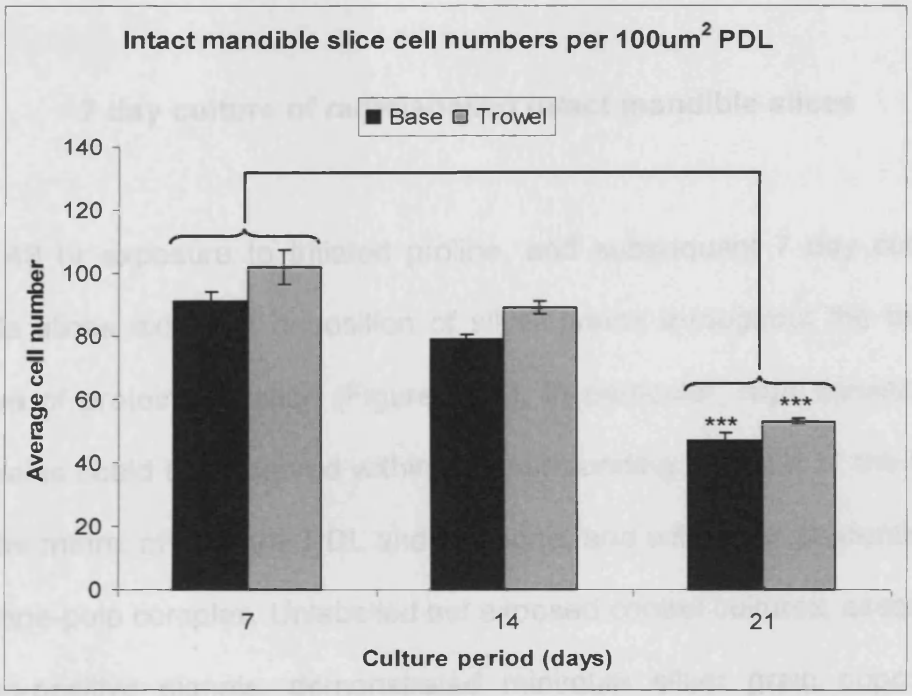

Figure 2.24: Average cell numbers per 100µm² area within the periodontal ligament after 7, 14 or 21 days of culture.
*** p<0.001

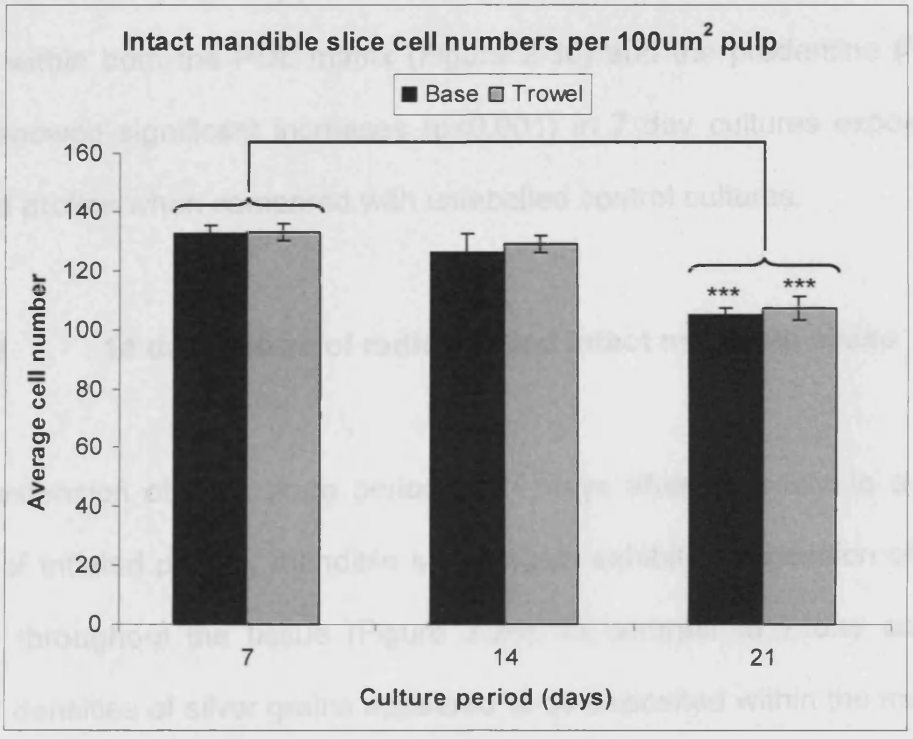

Figure 2.25: Average cell numbers per 100µm² area within the pulp after 7, 14 or 21 days of culture.
*** p<0.001

2.3.2 Synthetic and secretory activity of intact mandible slices

2.3.2.1 7 day culture of radiolabelled intact mandible slices

After a 48 hr exposure to tritiated proline, and subsequent 7 day culture, mandible slices exhibited deposition of silver grains throughout the tissue, indicative of protein secretion (Figure 2.26). In particular, high densities of silver grains could be observed within and surrounding the cells of the PDL, within the matrix of both the PDL and the bone, and within the predentine of the dentine-pulp complex. Unlabelled but exposed control cultures, assessing for false-positive signals, demonstrated minimum silver grain deposition within the tissue, and the small amount that could be observed did not follow any distinctive deposition patterns (Figure 2.27). Automated counts of silver grains within both the PDL matrix (Figure 2.30) and the predentine (Figure 2.31) showed significant increases ($p<0.001$) in 7 day cultures exposed to tritiated proline when compared with unlabelled control cultures.

2.3.2.2 14 day culture of radiolabelled intact mandible slices

After extension of the culture period to 14 days after exposure to a 48 hr pulse of tritiated proline, mandible slices again exhibited deposition of silver grains throughout the tissue (Figure 2.28). In contrast to 7 day cultures, higher densities of silver grains appeared to be deposited within the matrix of the PDL, and less associated with the actual cells of the PDL. There also appeared to be a higher density of silver grains within the predentine of 14

day cultures, when compared with 7 day cultures. Unlabelled but exposed control cultures, assessing for false-positive signals, exhibited minimum deposition of silver grains within the tissue (Figure 2.29). The small amount of deposition that could be observed within these control cultures did not follow any distinctive deposition patterns, and was comparable to 7 day control cultures.

Automated counts of silver grains showed significant increases ($p<0.001$) in 14 day cultures exposed to tritiated proline, when compared with unlabelled control cultures, both within the PDL matrix (Figure 2.30) and the predentine (Figure 2.31). Comparisons between 7 day cultures and 14 day cultures revealed significantly higher silver grain numbers within the PDL matrix of 14 day cultures ($p<0.001$). There was also a slightly higher increase in silver grains within the predentine of 14 day cultures when compared with 7 day cultures, but this increase was not shown to be significant.

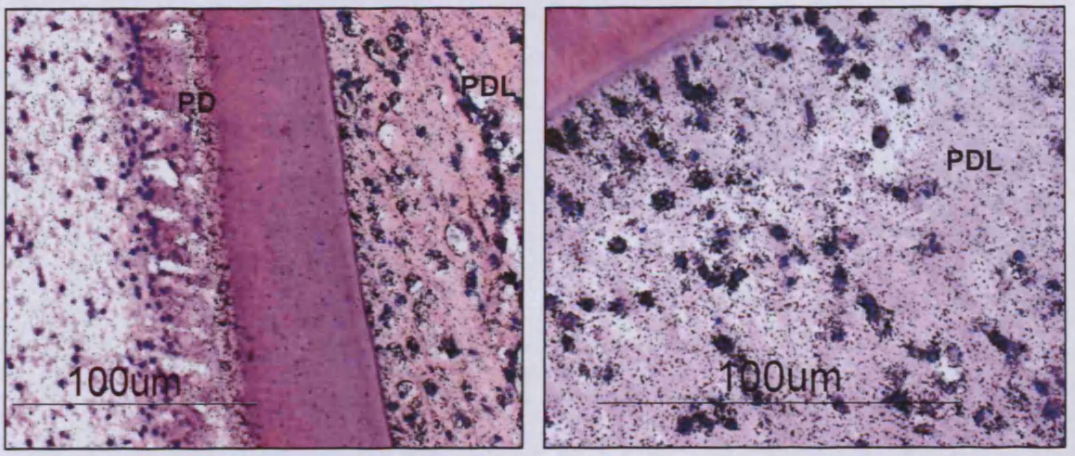

Figure 2.26: Tritiated proline radiolabelled mandible slices cultured for 7 days. Silver grains can be seen within the cells and matrix of the periodontal ligament (PDL), and within the predentine (PD) of the dentine-pulp complex.

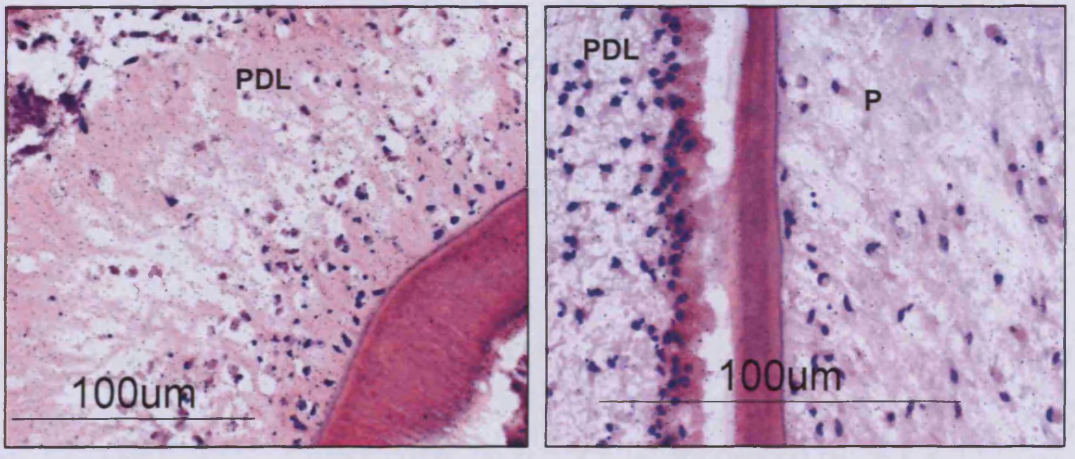

Figure 2.27: Radiolabel false positive controls. Mandible slices cultured for 7 days with no addition of radiolabel to the culture.

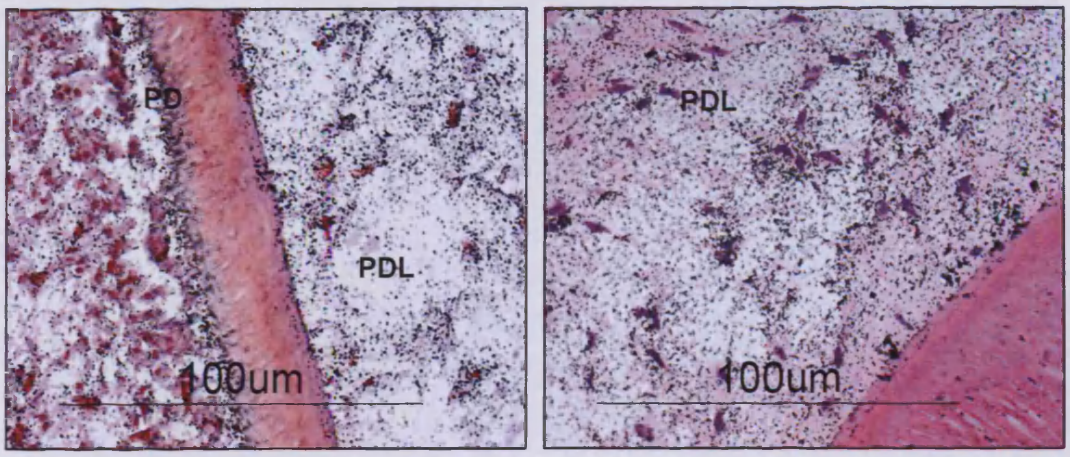

Figure 2.28: Tritiated proline radiolabelled mandible slices cultured for 14 days. Silver grains can be seen within the cells and matrix of the periodontal ligament (PDL), and within the predentine (PD) of the dentine-pulp complex.

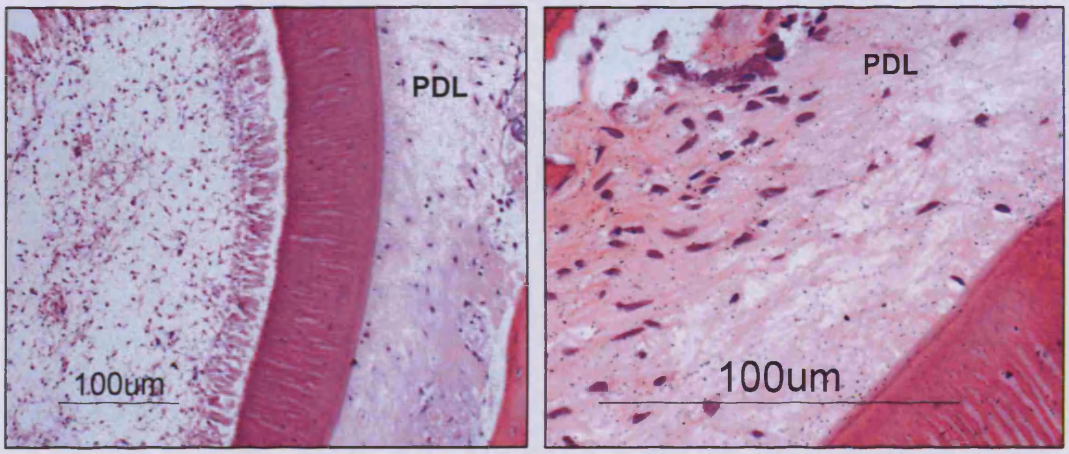

Figure 2.29: Radiolabel false positive controls. Mandible slices cultured for 14 days with no addition of radiolabel to the culture.

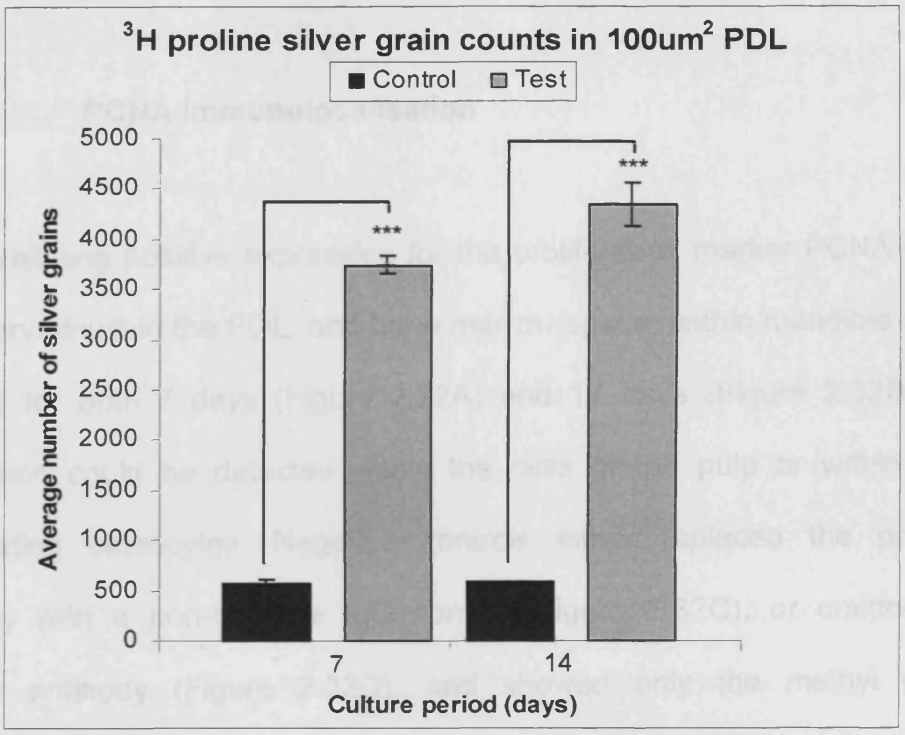

Figure 2.30: Average number of silver grains per 100 µm^2 area within the periodontal ligament after 7 or 14 days of culture.
*** p<0.001

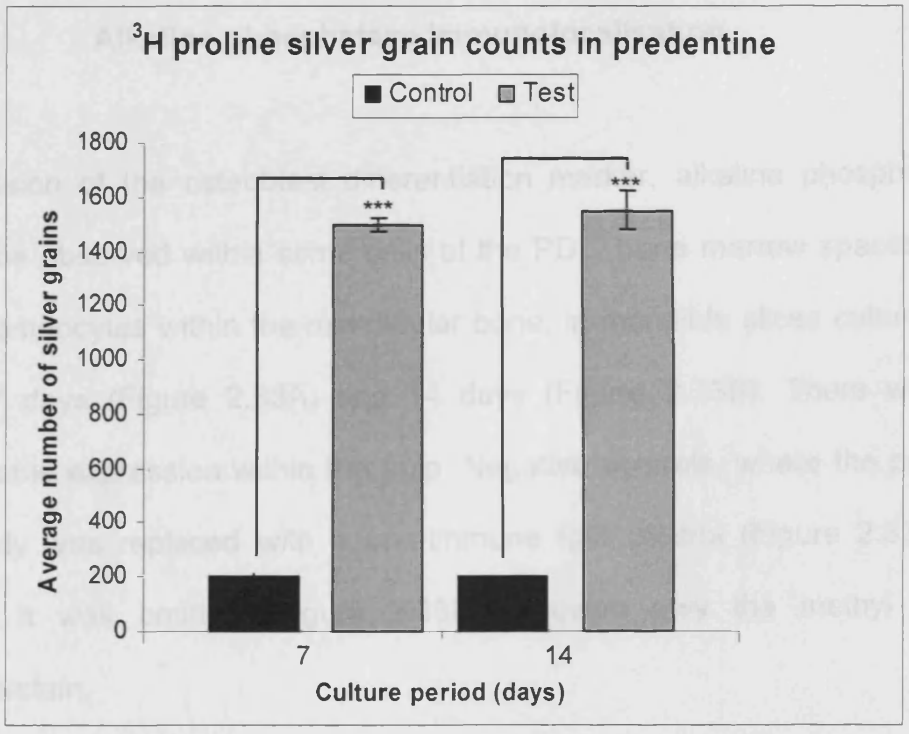

Figure 2.31: Average number of silver grains within the predentine after 7 or 14 days of culture.
*** p<0.001

2.3.3 Immunohistochemical characterisation

2.3.3.1 PCNA immunolocalisation

Cells exhibiting positive expression for the proliferation marker PCNA could be observed within the PDL, and bone marrow space, within mandible slices cultured for both 7 days (Figure 2.32A) and 14 days (Figure 2.32B). No expression could be detected within the cells of the pulp or within non-proliferating osteocytes. Negative controls either replaced the primary antibody with a non-immune IgG control (Figure 2.32C), or omitted the primary antibody (Figure 2.32D), and showed only the methyl green counterstain.

2.3.3.2 Alkaline phosphatase immunolocalisation

Expression of the osteoblast differentiation marker, alkaline phosphatase, could be observed within some cells of the PDL, bone marrow spaces, and some osteocytes within the mandibular bone, in mandible slices cultured for both 7 days (Figure 2.33A) and 14 days (Figure 2.33B). There was no detectable expression within the pulp. Negative controls, where the primary antibody was replaced with a non-immune IgG control (Figure 2.33C) or where it was omitted (Figure 2.33D), showed only the methyl green counterstain.

2.3.3.3 Osteopontin immunolocalisation

Osteopontin is a protein thought to be involved in cell adhesion and matrix mineralisation. It is expressed by a variety of cell types, including osteoprogenitors, osteoblasts and osteoclasts, as well as being localised to the mineralised bone matrix, especially concentrated at cement lines (Giachelli and Steitz 2000). Expression of this protein could be detected in mandible slices cultured for both 7 days (Figure 2.34A) and 14 days (Figure 2.34B). Expression was localised to cells within the PDL and bone marrow spaces. The pattern of expression was similar to that observed for immunolocalisation of alkaline phosphatase, with immunopositivity identified within cells of the PDL and bone marrow spaces. Negative controls either replaced the primary antibody with a non-immune IgG control (Figure 2.34C), or omitted the primary antibody (Figure 2.34D), and these controls showed only the methyl green counterstain.

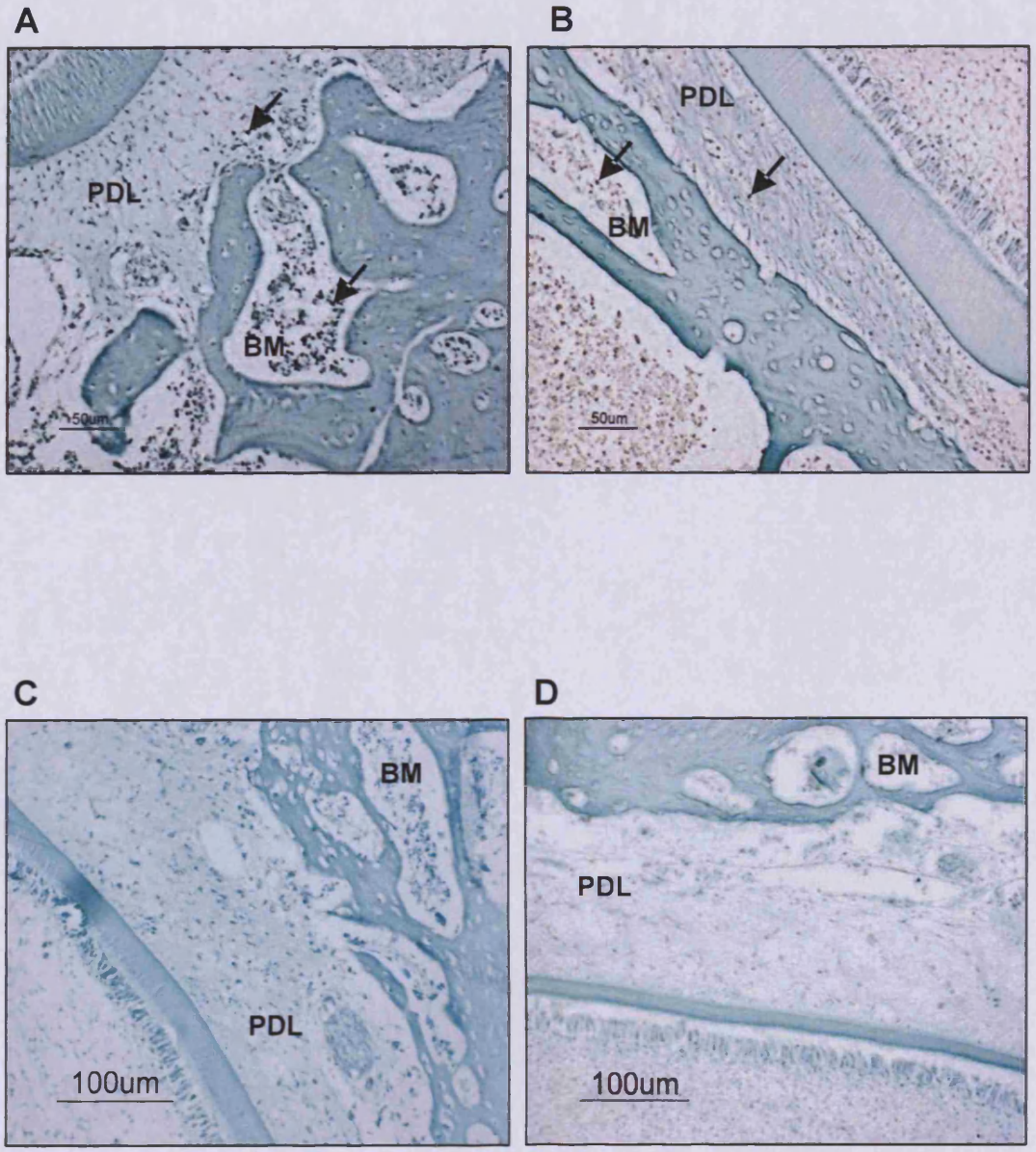

Figure 2.32: PCNA immunohistochemistry. Positive cellular expression (examples indicated by arrows) can be observed within cells of the periodontal ligament (PDL) and bone marrow (BM) within (A) 7 day cultures and (B) 14 day cultures. Negative controls using (C) a non-immune IgG or (D) omitting the primary antibody exhibit only the methyl green counterstain.

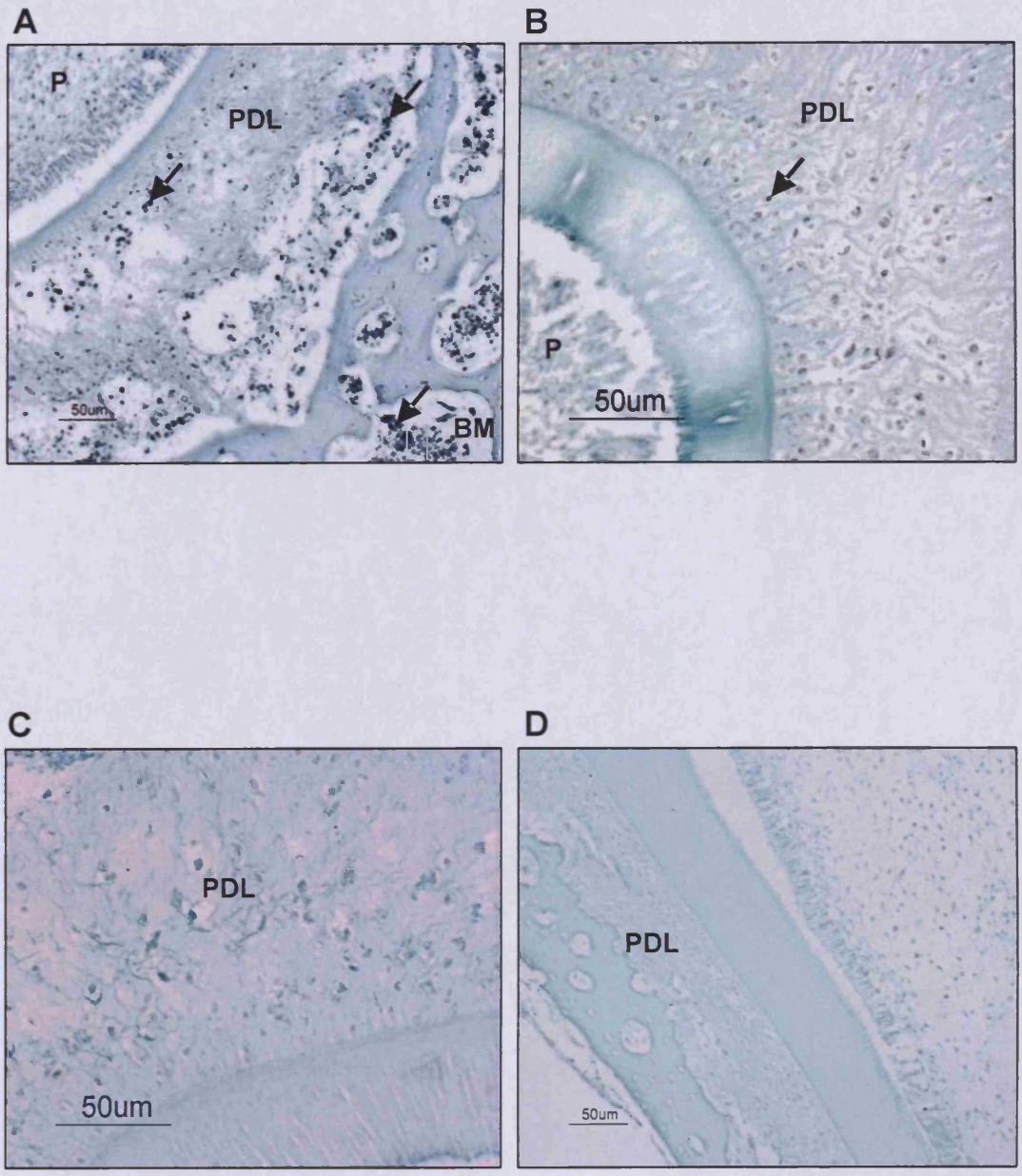

Figure 2.33: Alkaline phosphatase immunohistochemistry. Positive cellular expression (examples indicated by arrows) can be observed within some cells of the periodontal ligament (PDL) and bone marrow (BM), and some osteocytes, within (A) 7 day cultures and (B) 14 day cultures. Negative controls using (C) a non-immune IgG or (D) omitting the primary antibody exhibit only the methyl green counterstain.

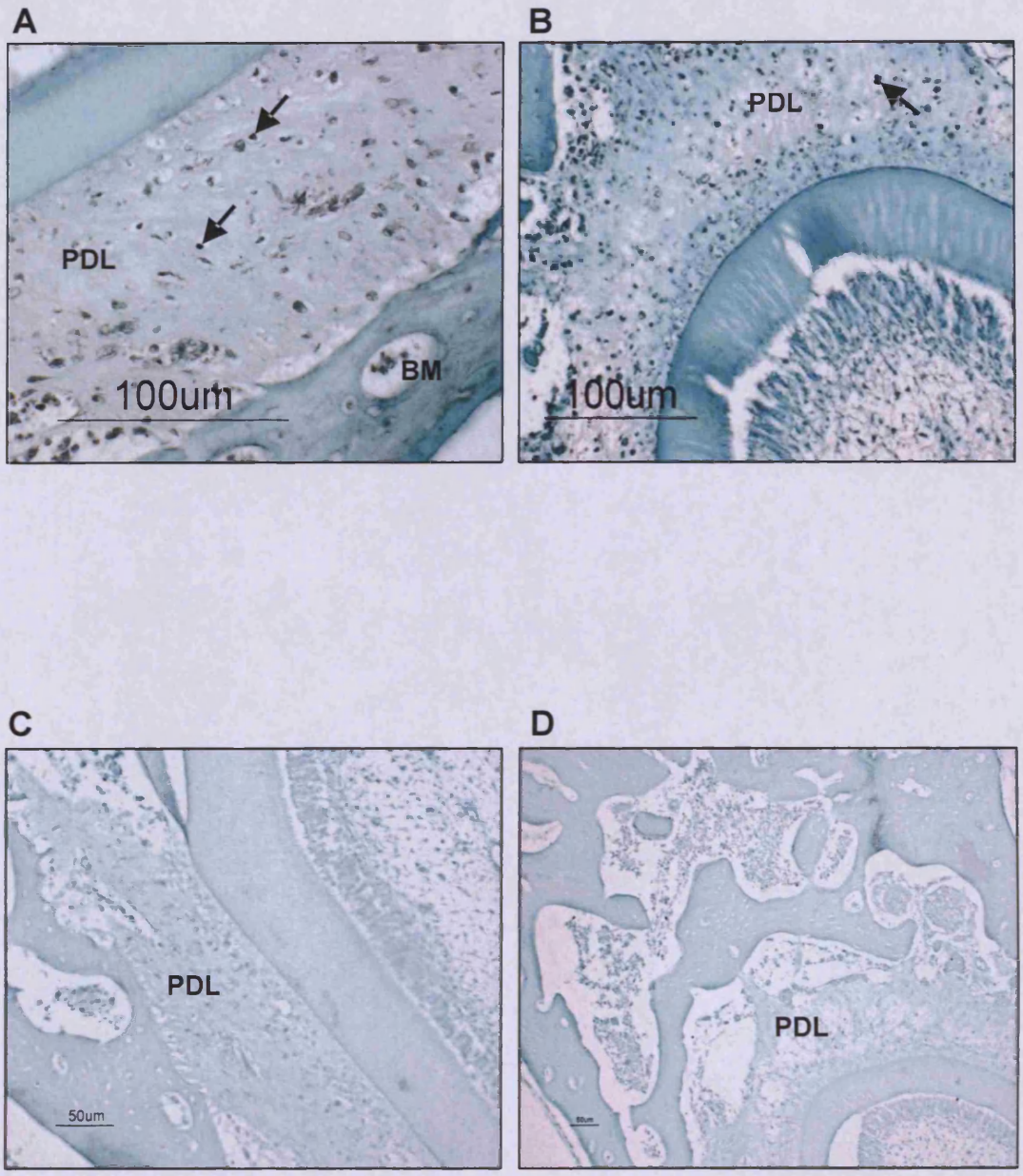

Figure 2.34: Osteopontin immunohistochemistry. Positive cellular expression (examples indicated by arrows) can be observed within some cells of the periodontal ligament (PDL) and bone marrow (BM), within (A) 7 day cultures and (B) 14 day cultures. Negative controls using (C) a non-immune IgG or (D) omitting the primary antibody exhibit only the methyl green counterstain.

2.4 DISCUSSION

This study has demonstrated the successful establishment of an *ex vivo* rat mandible culture model. Culture conditions have demonstrated that intact rat mandible slices can be successfully cultured for up to 21 days, with maintenance of tissue architecture, cellular morphology, and viability during this time. Cells have been shown to be actively synthesising and secreting matrix during the culture period and expression of typical *in vivo* proliferation markers and bone markers have been demonstrated within the various cell types.

Mandibles that had been initially sliced but not cultured, and mandible slices cultured for 24 hours, showed significant cell death across their surfaces. This was most likely due to initial surface damage caused by the slicing procedure, as histological stains indicated healthy viable tissue deeper into the mandible slice. However, 4 day cultures demonstrated significantly improved cellular viability, suggesting that after this time period the surface damage had been resolved, perhaps by apoptosis or necrosis of the cells and tissue being sloughed off to reveal healthy viable tissue underneath. In base type cultures these cells are likely to be sloughed off into the surrounding media, but in Trowel type cultures it is likely that these cells remain within the agar plug. After 4 days mandible slices remained highly viable for up to 21 days in culture, with very little observed decrease in cellular viability across the time period. Automated cell counts of these mandible slices did, however, reveal a decrease in cell numbers across the

culture period, although the decrease was only statistically significant after 21 days of culture. Such a decrease in cell number is probably due to cell death within the PDL and pulp across the extended time in culture, a problem often encountered during the culture of cells and tissues (Arden and Betenbaugh 2004). There are a number of reasons for this decrease in cell number. Programmed cell death will occur due to the finite lifespan of the cells residing in the tissue, ultimately decreasing the number of resident cells within the mandible cultures. This aging of cells over time in culture will also leave them more susceptible to stress, particularly to the effects of reactive oxygen species (ROS) (Fatokun et al. 2008; Tan et al. 1998). These highly reactive molecules are a natural by-product of oxygen metabolism, playing important roles in cell signalling, but can cause significant structural damage in aged cells which have a declining ability to defend themselves. Cell death due to tissue hypoxia may also be a contributing factor to the decrease in cell numbers, particularly within the base type cultures, where the mandible slices were fully submerged in media and thus not cultured at the liquid-gas interface. It is likely that this type of culture technique would lead to a reduction in oxygen tension within the mandible tissues, important for maintaining essential cellular functions. A number of studies have demonstrated that hypoxia can affect cellular behaviour and viability, reducing cellular proliferation and viability (Park et al. 2002; Utting et al. 2006), and decreasing expression of osteoblastic genes (Park et al. 2002). The cell death occurring during the extended culture period, observed as a decrease in cell numbers, is not apparent within the acridine orange / ethidium bromide viability stains, with the 21 day viability stains showing no

significant increase in cell death. Since the stain only shows clearly the surface of the tissue, this is most likely due to the dead cells on the surface of the tissue being sloughed off, as previously seen at the beginning of the culture period, where damage from slicing was removed over time. H&E stains, which represent sections beneath the surface of the tissue, do however show some cell death occurring within the tissue. Importantly, although there is a decrease in cell numbers, both the viability stain and the histology stain demonstrate the presence of a remaining population of highly viable cells, even after 21 days of culture.

Two culture methods were utilised in this study: submerged base type cultures, where mandible slices are cultured on the base of a culture dish and submerged in media, and Trowel type cultures (Trowell 1959), where the slices are embedded in a semi-solid agar and cultured at the liquid-gas interface. Both methods are widely used in organ culture techniques, but the Trowel type culture has been previously reported to promote better viability and tissue morphology, both in culture of tooth tissue (Bègue-Kirn et al. 1992; Sloan et al. 1998) and bone (Nifuji and Noda 1999). In this study, similar results have been observed, with Trowel type cultures yielding slightly higher cellular viability and maintenance of tissue architecture within the mandible slices. These observations are most likely due to the mandible slices being cultured at the liquid-gas interface, which allows for gaseous diffusion, thereby increasing oxygen tension within the tissues. As mentioned earlier, maintaining an appropriate oxygen tension is important for the viability of cells and tissues, and within bone cells it has been reported that hypoxia can

inhibit both osteoblast viability (Utting et al. 2006), and osteogenic differentiation by decreasing the expression of the essential transcription factor Runx2 (Park et al. 2002). Therefore, a lower oxygen tension, such as that which may occur within submerged cultures, may affect both survival and differentiation of bone cells. Cell survival may also be affected within these submerged cultures due to the mandible slices being positioned on the base of the culture dish, resulting in one surface of the slice being in contact with plastic during the entire culture period. This may facilitate the out-growth of cells onto the tissue culture dish, as seen in classical explant cultures, and also may affect viability since the surface in contact with the dish may not be receiving suitable nutrient diffusion from the surrounding media. In contrast, mandible slices in Trowel type cultures reside in a three-dimensional agar medium, allowing for optimum nutrient diffusion into all areas of tissue. Another significant advantage of Trowel type cultures is that the system can be easily manipulated by addition of components to this semi-solid agar media that surrounds the mandible slice. The advantages of the Trowel type cultures are such that they were used in all further experiments involving the mandible model.

In order to confirm the activity of the cells within the mandible tissue during culture, the synthetic and secretory abilities of these cells were assessed using a tritiated proline pulse chase experiment. Proline is an amino acid essential for the production of collagen, as well as other proteins, albeit to a much lesser extent, and by incorporating a radiolabelled form of proline into the mandible cultures, it is possible to visualise cellular synthesis and

secretion of protein (Sloan et al. 1998; Weinstock and Leblond 1974). After 7 days of culture, the vast majority of protein secretion (as indicated by silver grain deposition throughout the tissue) was apparent within, and closely surrounding, the cells of the PDL, the matrix of both the PDL and the bone, and also within the predentine of the dentine-pulp complex. This suggests that the cells residing within the PDL and bone actively synthesise and secrete proteins into their surrounding matrix throughout the first 7 days of culture, and the odontoblasts present within the pulp secrete proteins into the predentine matrix. Since a large proportion of silver grains are closely associated with the cells after this time, and a much lesser proportion found within the matrix itself, it is likely that over the 7 days of culture the cells have taken up the radiolabelled proline, and begun to utilise it to synthesise proteins, but secreted only a small proportion. However, after extending the culture period to 14 days, a significantly greater proportion of silver grain deposition could be observed within the matrix of the PDL and bone (Figure 2.28), with less being associated with the actual cells themselves. This suggests that the proteins synthesised by the cells residing in the PDL and bone during the first 7 days, were secreted into the matrix after 14 days of culture. These observations provide evidence that the cells present within the mandible model are capable of actively synthesising and secreting proteins throughout their period in culture, suggesting that the cells are actively functioning in culture, rather than simply laying dormant. The distribution of radiolabel detected by this autoradiographic method was similar to that observed in a similar *ex vivo* tooth slice model (Sloan et al. 1998), where after 7 days silver grain deposition were closely associated with the

odontoblasts, and after 14 days distribution was predominately observed within the predentine.

Immunohistochemical characterisation of the model was carried out to investigate cell behaviour in the tissue under *ex vivo* culture conditions. Firstly, cellular proliferation was characterised by investigating the expression of PCNA, an established marker of cellular proliferation. PCNA, a co-factor for DNA polymerase δ, accumulates during the G1 phase of mitosis and is most abundant during the S phase, where DNA replication takes place, while little or no PCNA is expressed in resting cells (Celis et al. 1987). This temporal specificity of PCNA makes it a good marker for proliferation in cells. Within an *in vivo* situation, PCNA expression would be observed within the cells of the PDL, which is comprised predominantly of fibroblasts, which have a high proliferative capacity, as well as highly proliferative osteoprogenitor cells and mesenchymal stem cells (Lekic and McCulloch 1996; Seo et al. 2004). These mesenchymal stem cells, which have the capability of differentiating down the osteoblastic linage in response to appropriate stimuli, are also present within the bone marrow spaces of the mandibular bone (Prockop 1997). It could be hypothesised, therefore, that PCNA expression within the mandible model would be localised to the cells within these areas of the PDL and bone marrow spaces. Immunohistochemistry data from the mandible slices confirmed expression of PCNA within these areas, both after 7 and 14 days of culture. No PCNA expression was observed within the post-mitotic odontoblast cells within the pulpal area (Arana-Chavez and Massa 2004), and limited expression was observed within the cells of the pulp itself,

where the resident cells have a slow rate of turnover (Casasco et al. 1997; Pinzon et al. 1966).

Immunohistochemical localisation of alkaline phosphatase and osteopontin was carried out to investigate the type of cells residing within the cultured mandible tissue. Alkaline phosphatase is known to be expressed *in vivo* within osteoblasts, as well as their precursors, and as such is a widely accepted marker of the presence of differentiating osteoblasts (Kim et al. 2004). Osteopontin is another protein expressed by cells of an osteoblastic lineage, thought to be involved in cell adhesion and matrix mineralisation (Merry et al. 1993; Yamate et al. 1997), and as such is also a marker of differentiating osteoblasts (Denhardt and Noda 1998; Giachelli and Steitz 2000). Expression of both of these bone markers has been demonstrated within the *ex vivo* mandible model, within cells residing within the PDL and bone marrow spaces, after both 7 and 14 days of culture. The expression patterns observed in the model indicate the presence of cells of the osteoblastic lineage within the mandible tissues when cultured *ex vivo*, which may be osteoprogenitors, or fully differentiated osteoblast cells. This correlates with *in vivo* studies showing the presence of osteoblasts and their progenitors within both the PDL and bone marrow spaces (Isaka et al. 2001; Krebsbach et al. 1999; Lekic et al. 1996). However, the presence of these cells within the cultured mandible tissue is likely to represent cells remaining in the tissue from the start of culture, rather than demonstrating active differentiation occurring during culture, as no significant alterations in bone marker expression are observed. This data does however indicate that

osteoblast cells can be maintained within the mandible tissue during culturing, within areas that would naturally contain these cells *in vivo*.

This chapter has demonstrated that intact mandible slices can be cultured for up to 21 days in culture, with maintenance of cell and tissue architecture, and cellular viability. Resident cells within the tissues were active throughout the culture period, secreting proteins into the matrix and predentine. The observed expression patterns of PCNA, alkaline phosphatase, and osteopontin suggest that the cells residing within the *ex vivo* model are behaving in a similar manner to that seen *in vivo*, both in terms of their cellular proliferation, and the localisation of osteoblast cells within the tissues. This suggests that the culture conditions are not significantly altering the cellular behaviour within the tissues. The development of this *ex vivo* mandible model has significant advantages over more traditional *in vitro* and *in vivo* modelling systems. Whereas *in vitro* models use only one or two cell types (Yamaguchi et al. 1996), the *ex vivo* mandible model enables the culture of many cell and tissue types together, allowing many of the complex interactions that occur *in vivo* to be recapitulated, but without the presence of the complex systemic influences that can often hinder *in vivo* experimentation. Furthermore, the *ex vivo* model system can significantly reduce costs and ethical objections, with one animal yielding up to eight experiments, as opposed to the one animal, one experiment ethos often used for *in vivo* work. The next part of this chapter aims to further develop and characterise this mandible culture system, to produce a quantitative model for investigating specific processes of bone repair.

CHAPTER TWO

SECTION B:

ORGAN CULTURE OF FRACTURED RAT MANDIBLE SLICES

2.5 MATERIALS AND METHODS

2.5.1 Organ culture of fractured mandible slices

Mandibles were dissected from 28 day old male Wistar rats, and slices prepared as in section 2.2.2. After preparation, a fracture was introduced into the mandible slices by placing pressure onto the central portion of the bone of the lingual plate using a sterile scalpel blade. Fractures were prepared in a similar location in each mandible slice. Fractured mandible slices were placed into Trowel type culture, as described previously (section 2.2.2) using prepared culture medium and embedding medium as described in section 2.2.1. Slices were cultured at 37°C, in an atmosphere of 5% CO_2 in air, in a humidified incubator, for 7, 14, or 21 days.

2.5.2 Histological examination

After each culture period, mandible slices were fixed, demineralised, processed, sectioned and stained for H&E as described in section 2.2.3. Cell numbers were automatically counted within 100μm^2 areas of the PDL and the pulp of H&E stained mandible sections, using Image ProPlus software.

$100\mu m^2$ areas within five random fields of view were counted per section. PDL and pulp cell counts were obtained for 7, 14, and 21 day cultures (n=12 for each time point), and standard errors of the mean were calculated. Mean values were analysed using one-way ANOVA and Tukey's post hoc test, to analyse differences between the cell numbers in different culture periods, as well as between the fractured and the intact mandible slices.

2.5.3 Cell viability assessment

Cellular viability within fractured mandible slice cultures was assessed with an acridine orange / ethidium bromide vital dye stain, as described in section 2.2.4.

2.5.4 Tritiated proline pulse chase

The synthetic and secretory activity of cells within the fractured mandible slice cultures was investigated with tritiated proline pulse chase experiments, as described previously in section 2.2.5. Quantification and statistical analysis of silver grain deposition was also performed as in section 2.2.5.

2.5.5 Immunohistochemistry

Fractured mandible slices cultured for 7 or 14 days were stained immunohistochemically for the expression of the proliferation marker PCNA, and the bone markers alkaline phosphatase and osteopontin, using the

method described in section 2.2.6. Positive cells for each of the makers were automatically counted within 100μm^2 areas of the PDL using Image ProPlus software (n=5 for each marker). Positive cells were counted within a 100μm^2 area of PDL adjacent to the site of fracture, 100μm^2 areas of PDL either side of the fracture, and a 100μm^2 area of PDL from the opposite side to fracture, to assess if the fracturing process was influencing the behaviour of the cells of the PDL in any way. Standard errors of the mean were calculated, and mean values were analysed using one-way ANOVA and Tukey's post hoc test, to analyse differences between areas of PDL adjacent to the fracture site, and opposite the fracture site.

2.6 RESULTS

2.6.1 Histology and viability of fractured mandible slices

Optimum culture conditions were established for the mandible model in section A of this chapter. It was observed that Trowel type cultures appeared to be more favourable in maintaining tissue architecture and cell viability, therefore this Trowel type system was used for all fractured mandible slices.

Mandible slices were firstly prepared and fractured, but not cultured. H&E histology of these slices showed maintenance of tissue architecture throughout all areas, including the bone, PDL, and dentine (Figure 2.35). Cells appeared viable, with darkly stained, round nuclei, and many cell types could be observed within the tissue. These included osteoblasts and osteocytes within the bone, bone marrow stromal cells, PDL cells, odontoblasts and pulp cells. Fractures were of a similar size and position in all slices. Cell and tissue architecture adjacent to the fracture site were well maintained, with no excess damage caused by the fracturing procedure. Acridine orange / ethidium bromide vital dye staining of uncultured tissues showed major cell death across the surface of the slice, including around the surface of the fracture site indicated by the presence of red fluorescence (Figure 2.36). Since H&E histology demonstrated healthy tissue further into the mandible slice, this may be due to surface damage due to the slicing procedure. Results for both H&E histology, and acridine orange / ethidium bromide staining, were comparable with those for uncultured intact slices.

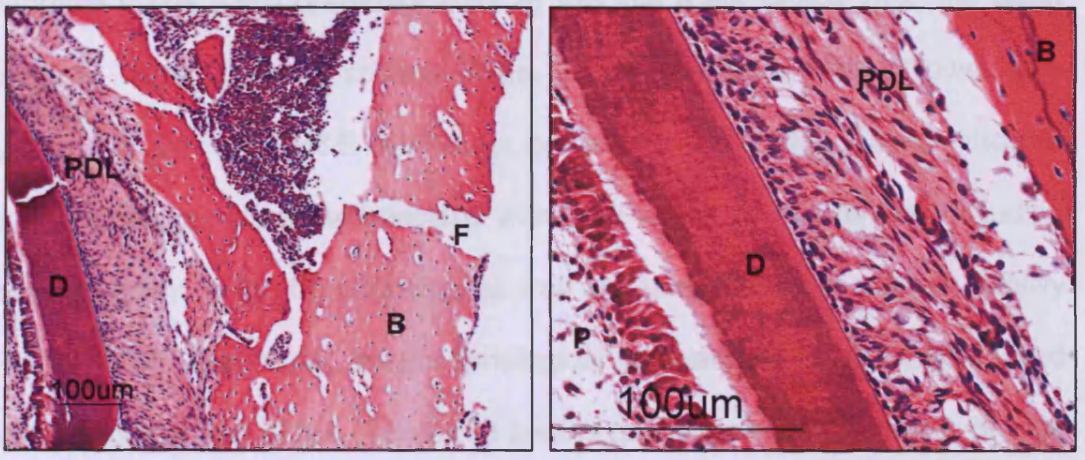

Figure 2.35: Histology of uncultured fractured mandible slices showing maintenance of tissue and cells of the bone (B), periodontal ligament (PDL), dentine (D), pulp (P), and around the site of fracture (F).

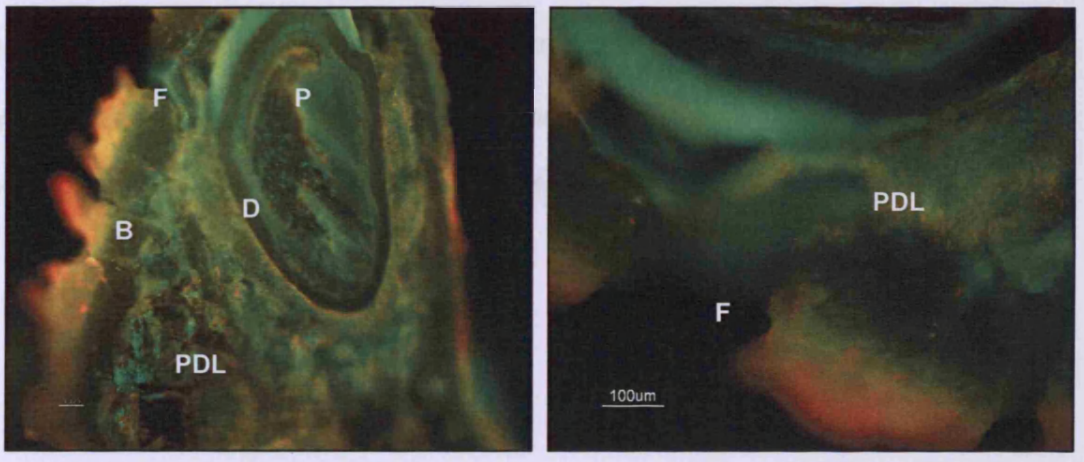

Figure 2.36: Acridine orange / ethidium bromide vital dye staining of an uncultured fractured mandible slice, showing bone (B), periodontal ligament (PDL), pulp (P), dentine (D) and the fracture site (F).

2.6.1.1 24 hour culture of fractured mandible slices

Mandible slices were prepared from 28 day old male Wistar rats, the bone fractured using a sterile scalpel blade, and slices placed into Trowel type culture for 24 hrs. H&E histology performed on these slices showed maintenance of tissue architecture within all areas of the tissue, including areas adjacent to the fracture site, as well as maintenance of cellular viability (Figure 2.37). Generally, the morphological appearance of the tissues and cells paralleled that of the uncultured control mandible slices. Acridine orange / ethidium bromide staining again demonstrated significant levels of cell death throughout the tissue, indicating that surface damage had not been resolved after 24 hrs (Figure 2.38). However, there did appear to be an increase in the number of viable cells fluorescing green when compared with the uncultured control slices, and acridine orange / ethidium bromide staining of fractured mandible slices cultured for 4 days showed vastly improved cell viability throughout the entire of the surface (Figure 2.39).

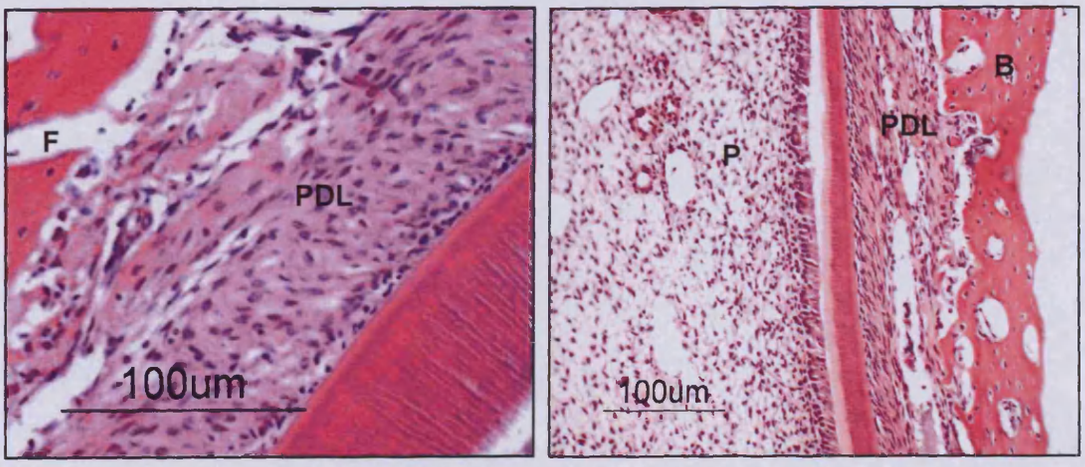

Figure 2.37: Fractured mandible slices after 24 hours culture.

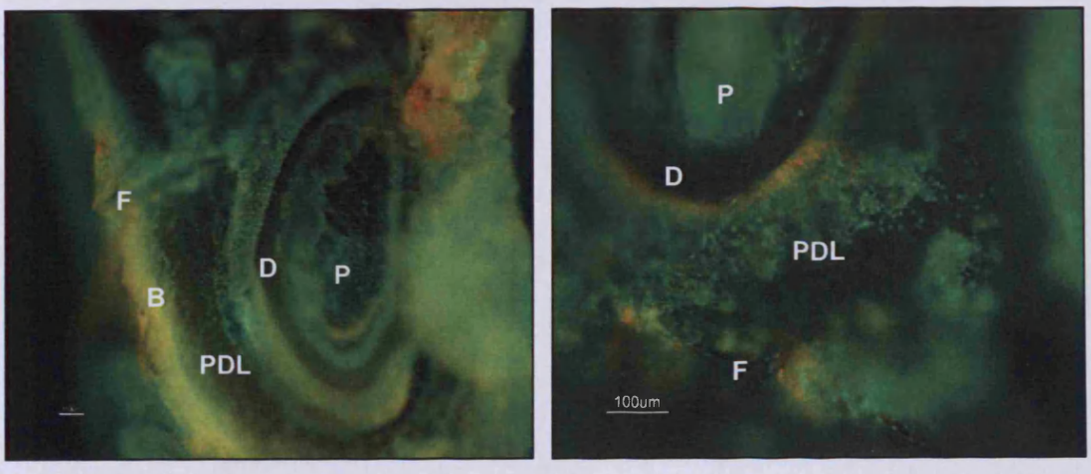

Figure 2.38: Acridine orange / ethidium bromide vital dye staining of a fractured mandible slice cultured for 24 hours.

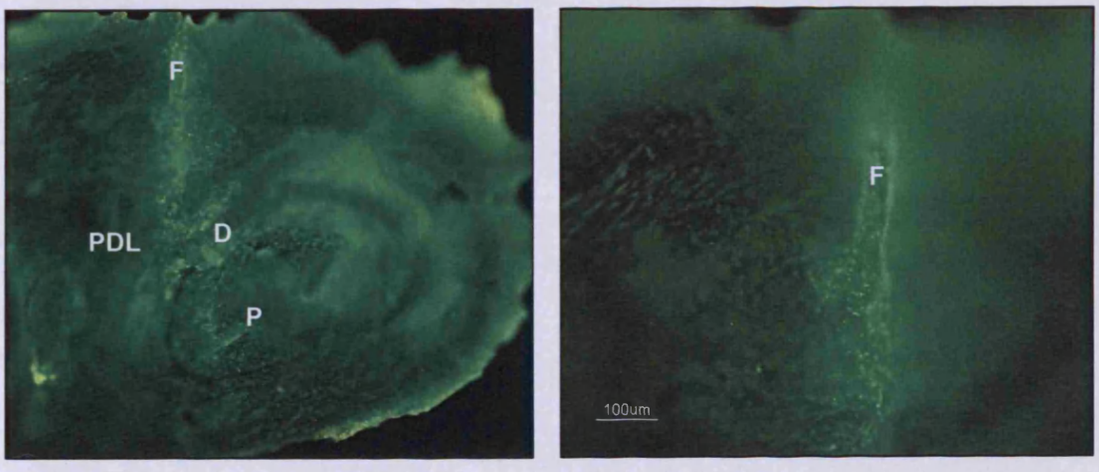

Figure 2.39: Acridine orange / ethidium bromide vital dye staining of a fractured mandible slice cultured for 4 days.

2.6.1.2 7 day culture of fractured mandible slices

After 7 days in culture, fractured mandible slices stained for H&E histology showed maintenance of cell and tissue architecture across the mandibular tissue (Figure 2.40). Cells appeared viable within all areas of the mandible slices, exhibiting darkly stained round nuclei, and both cell and tissue morphology and viability were maintained around the site of fracture. The fractured slices appeared morphologically similar to uncultured and 24 hour cultured slices. Acridine orange / ethidium bromide vital dye staining indicated maintenance of a high level of cellular viability across the surface of the tissue (Figure 2.41). The vast majority of cells exhibited green fluorescence, indicating their viability, and only a relatively few number of cells displayed red fluorescence. A large number of green fluorescing cells adjacent to the site of fracture indicated high viability at this area, with no increase in cell death associated with the fracturing process. Viability was comparable to slices cultured for 4 days.

Automated cell counts using Image ProPlus software were performed on H&E stained sections of fractured mandible slices cultured in Trowel type cultures for 7 days. The software calculated an average number of 99 ± 2.8 cells per 100μm^2 area within the PDL, and an average number of 126 ± 1.8 cells per 100μm^2 area within the pulp (Figures 2.46 and 2.47).

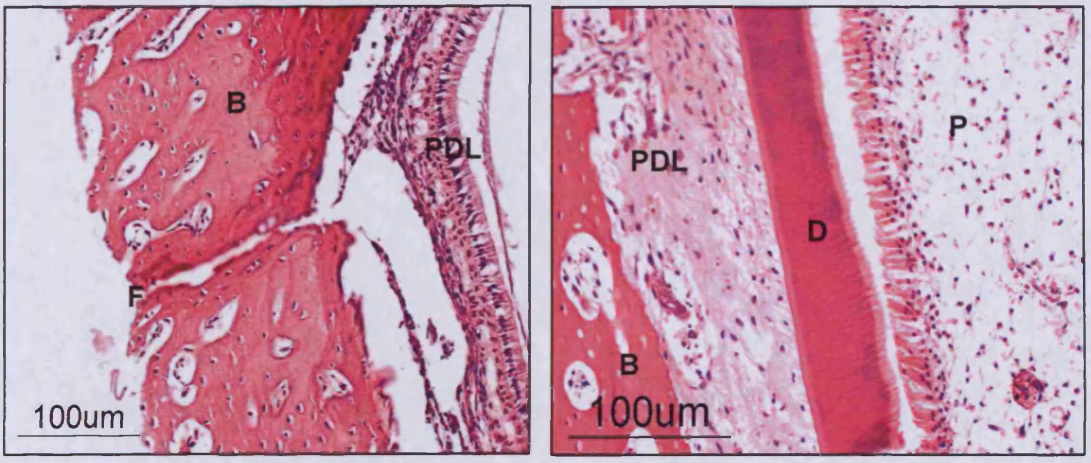

Figure 2.40: Fractured mandible slices cultured for 7 days. Maintenance of cell and tissue architecture can be observed throughout the bone (B), periodontal ligament (PDL), dentine (D) and pulp (P). Cell and tissue architecture is maintained around the fracture site (F).

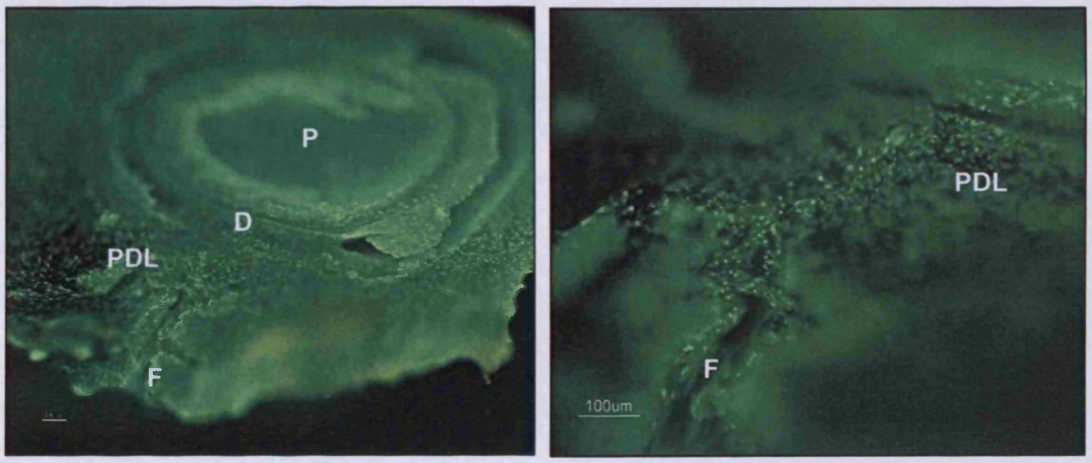

Figure 2.41: Acridine orange / ethidium bromide vital dye staining of a fractured mandible slice cultured for 7 days, showing bone (B), periodontal ligament (PDL), pulp (P), dentine (D) and the fracture site (F).

2.6.1.3 14 day culture of fractured mandible slices

After 14 days of culture, H&E histology demonstrated maintenance of tissue architecture throughout the mandible slice, including around the site of fracture (Figure 2.42). Cells appeared viable with darkly stained, round nuclei, and results were comparable with those for 7 day cultures. There appeared to be a slight decrease in cell numbers in the 14 day fractured cultures, when compared with the 7 day fractured cultures, and this was confirmed with automated cell counting, with an average of 86 ± 1.5 cells per 100μm^2 area counted within the PDL, and an average of 115 ± 1.7 cells per 100μm^2 within the pulp (Figure 2.46 and 2.47). However, statistical analysis did not show this decrease in cell number to be significant ($p>0.05$). Also, acridine orange / ethidium bromide vital dye staining demonstrated high cellular viability across the tissue surface (Figure 2.43), indicating that although there was a slight decrease in cell numbers, the vast majority of the cell population present were still highly viable. There was again a large population of viable cells adjacent to the site of fracture, with no increased cell death associated with this area, as shown by limited red fluorescence.

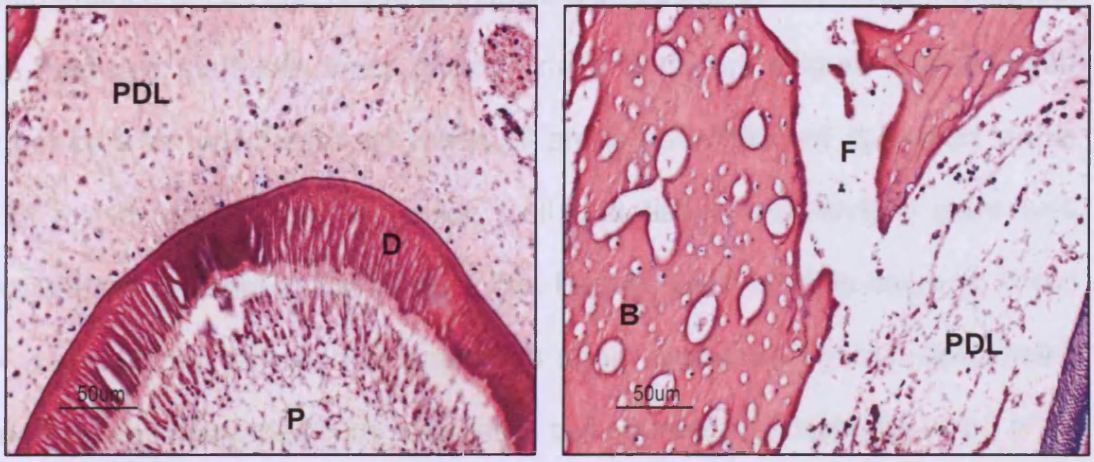

Figure 2.42: Fractured mandible slices cultured for 14 days. Maintenance of cell and tissue architecture can be observed throughout the bone (B), periodontal ligament (PDL), dentine (D) and pulp (P). Cell and tissue architecture is maintained around the site of fracture (F).

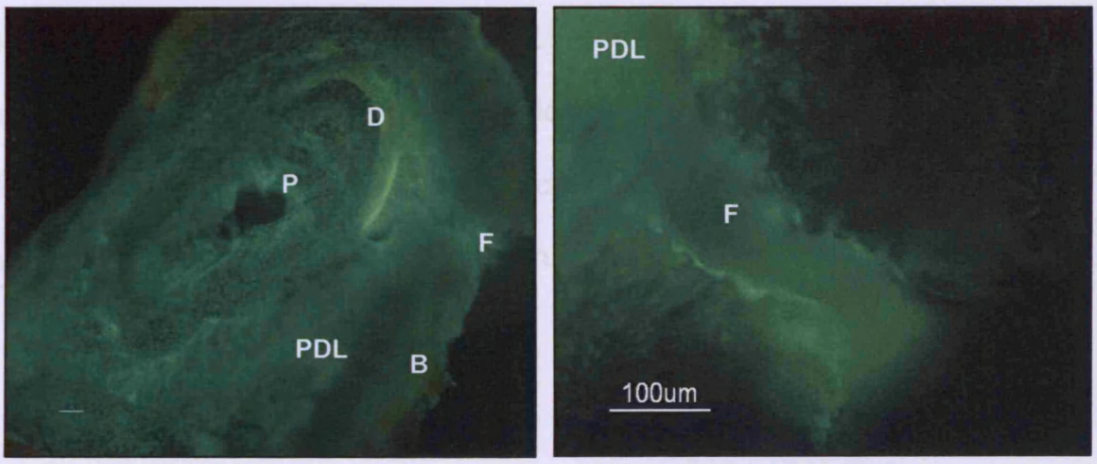

Figure 2.43: Acridine orange / ethidium bromide vital dye staining of a fractured mandible slice cultured for 14 days, showing bone (B), periodontal ligament (PDL), pulp (P), dentine (D) and the fracture site (F).

2.6.1.4 21 day culture of fractured mandible slices

Maintenance of cell and tissue architecture could still be observed throughout the fractured mandible slices after 21 days of culture (Figure 2.44). Tissue structure was well preserved within all areas and cells still appeared viable with round, darkly stained nuclei. Cell and tissue architecture were well maintained adjacent to the fracture site. Results were comparable with 7 and 14 day cultures, although cell numbers again appeared to have decreased, and this was confirmed with automated cell counting. An average of 52 ± 1.3 cells per 100μm^2 area within the PDL was calculated, and an average of 113 ± 4.3 cells per 100μm^2 was calculated within the pulp (Figure 2.46 and 2.47). Statistical analysis performed on these cell counts demonstrated that the cell numbers within the PDL of 21 day cultures were significantly decreased when compared to the 7 day cultures ($p<0.001$). However, there was no significant difference in cell numbers within the pulp. Also, despite the decrease in PDL cell number after 21 days of culture, acridine orange / ethidium bromide vital dye staining indicated high cellular viability across the tissue surface (Figure 2.45). This indicated the presence of a highly viable population of cells even after 21 days in culture.

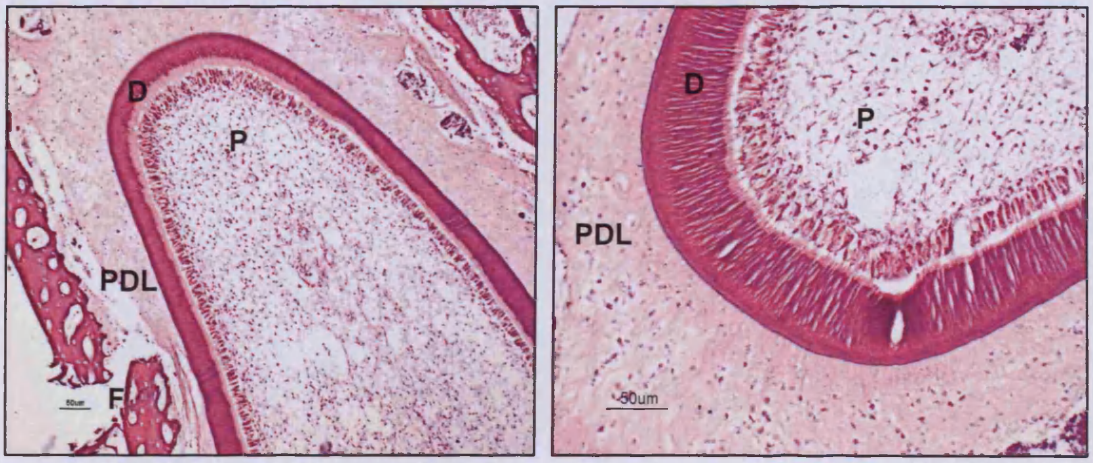

Figure 2.44: Mandible slices cultured for 21 days. Maintenance of cell and tissue architecture can be observed throughout the bone (B), periodontal ligament (PDL), dentine (D), pulp (P), and around the site of fracture (F).

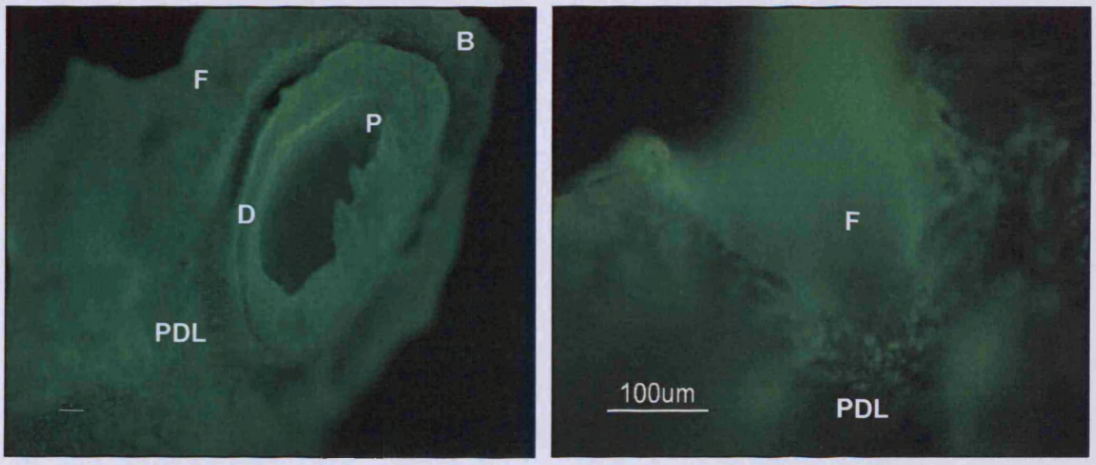

Figure 2.45: Acridine orange / ethidium bromide vital dye staining of a fractured mandible slice cultured for 21 days, showing bone (B), periodontal ligament (PDL), dentine (D), pulp (P), and the site of fracture (F).

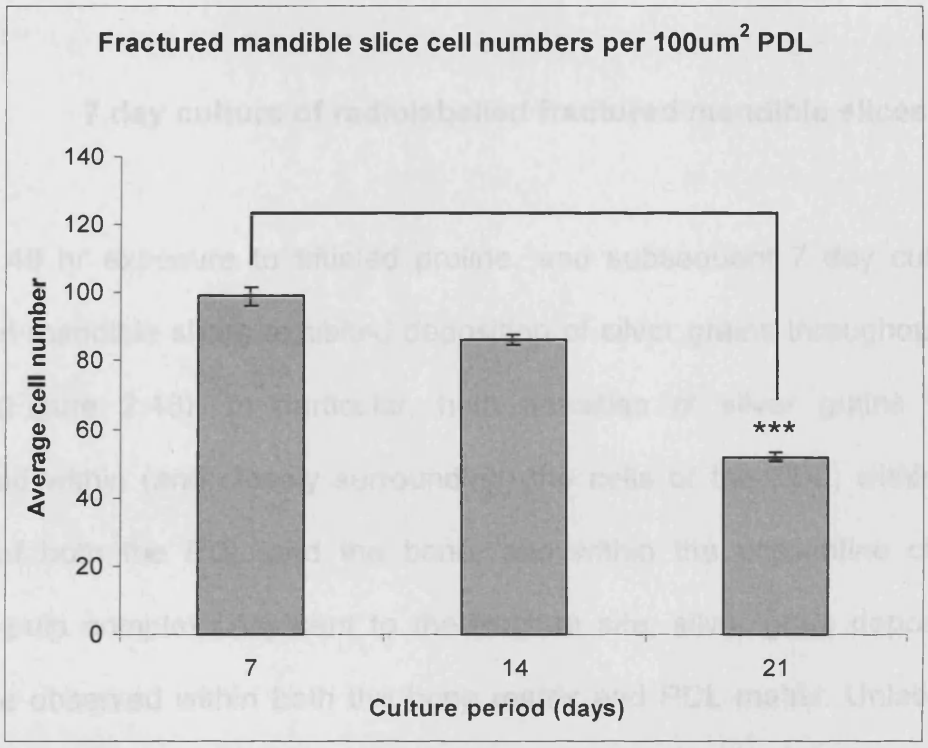

Figure 2.46: Average cell numbers per 100µm² area within the periodontal ligament after 7, 14 or 21 days of culture.

*** $p<0.001$

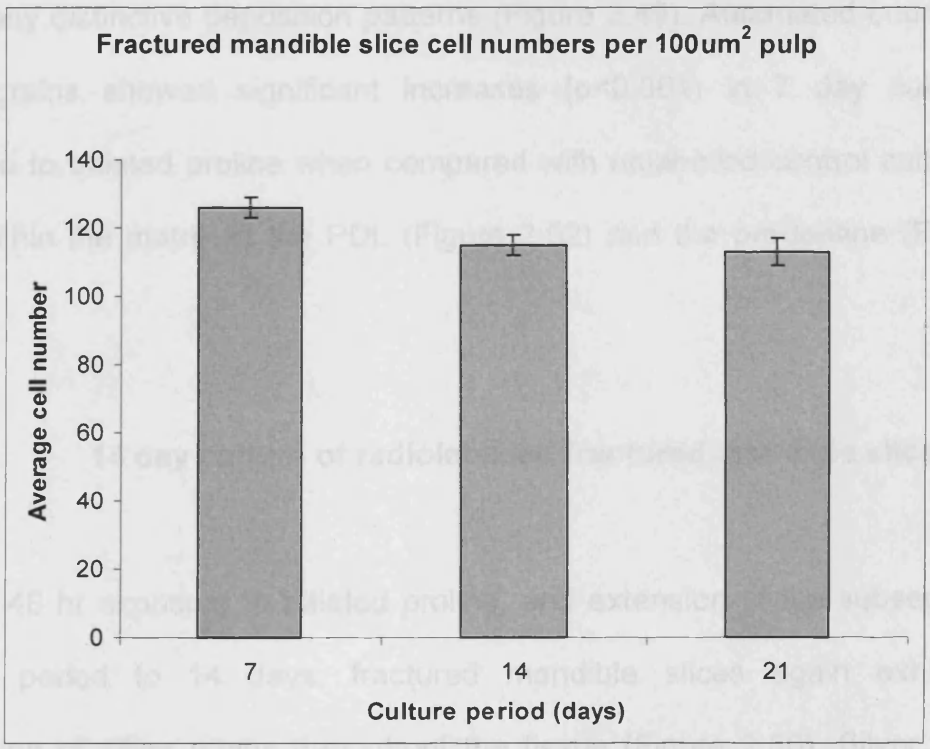

Figure 2.47: Average cell numbers per 100µm² area within the pulp after 7, 14 or 21 days of culture.

2.6.2 Secretory and synthetic activity of fractured mandible slices

2.6.2.1 7 day culture of radiolabelled fractured mandible slices

After a 48 hr exposure to tritiated proline, and subsequent 7 day culture, fractured mandible slices exhibited deposition of silver grains throughout the tissue (Figure 2.48). In particular, high densities of silver grains were deposited within (and closely surrounding) the cells of the PDL, within the matrix of both the PDL and the bone, and within the predentine of the dentine-pulp complex. Adjacent to the fracture site, silver grain deposition could be observed within both the bone matrix and PDL matrix. Unlabelled but exposed control cultures, assessing for false-positive signals, demonstrated minimum silver grain deposition within the tissue, and did not follow any distinctive deposition patterns (Figure 2.49). Automated counts of silver grains showed significant increases ($p<0.001$) in 7 day cultures exposed to tritiated proline when compared with unlabelled control cultures, both within the matrix of the PDL (Figure 2.52) and the predentine (Figure 2.53).

2.6.2.2 14 day culture of radiolabelled fractured mandible slices

After a 48 hr exposure to tritiated proline, and extension of the subsequent culture period to 14 days, fractured mandible slices again exhibited deposition of silver grains throughout the tissue (Figure 2.50). Silver grain deposition could again be seen within the bone matrix and PDL matrix

adjacent to the site of fracture. Higher silver grain density appeared to be deposited within the matrix of the PDL, and less associated with the actual cells of the PDL; a contrast to the results obtained after 7 days of culture. There also appeared to be a higher density of silver grains within the predentine of 14 day cultures, when compared with 7 day cultures. Unlabelled, but exposed control cultures, assessing for false-positive signals, exhibited minimum deposition of silver grains within the tissue (Figure 2.51). The small amount that could be observed did not follow any distinctive deposition patterns, and was comparable to 7 day control cultures.

Automated counts of silver grains with Image ProPlus software indicated significant increases ($p<0.001$) in 14 day cultures exposed to tritiated proline, when compared with unlabelled control cultures. This was observed both within the matrix of the PDL (Figure 2.52) and the predentine (Figure 2.53). Comparisons between 7 day cultures and 14 day cultures revealed significantly higher silver grain numbers within the PDL matrix of 14 day cultures ($p<0.05$). A slightly higher increase in silver grains was also observed within the predentine of 14 day cultures when compared with 7 day cultures, but this increase was not shown to be significant.

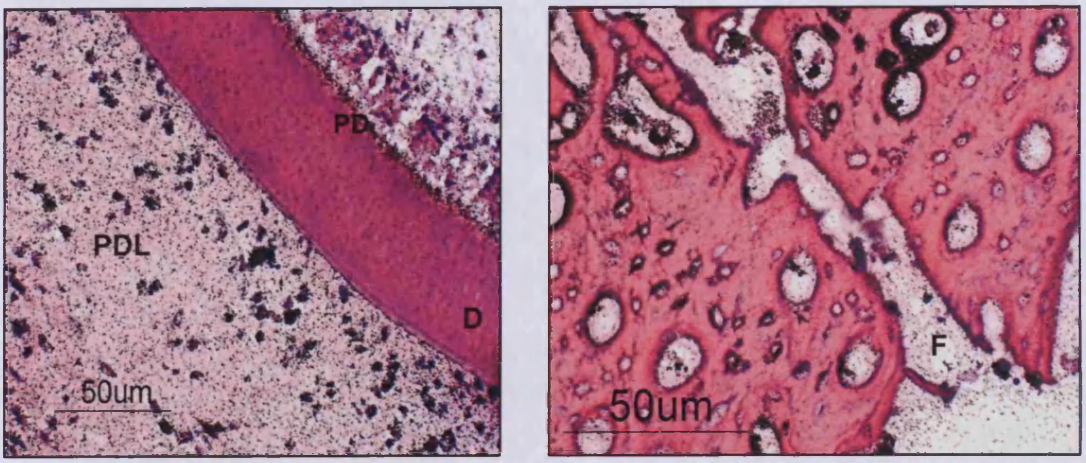

Figure 2.48: Tritiated proline radiolabelled fractured mandible slices cultured for 7 days. Silver grains can be seen within the cells and matrix of the periodontal ligament (PDL), within the predentine (PD) of the dentine-pulp complex, and within the matrix around the fracture site (F).

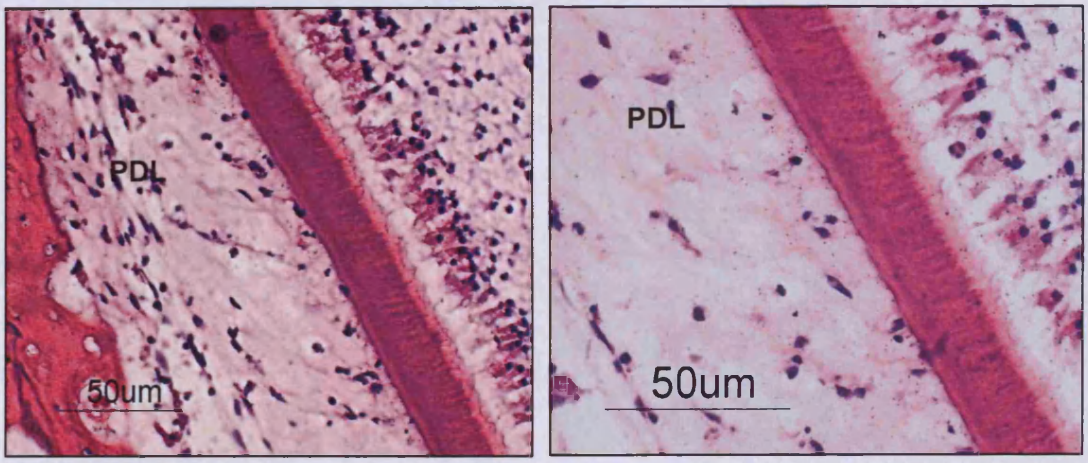

Figure 2.49: Radiolabel false positive controls. Slices were exposed, but no radiolabel was added to the culture.

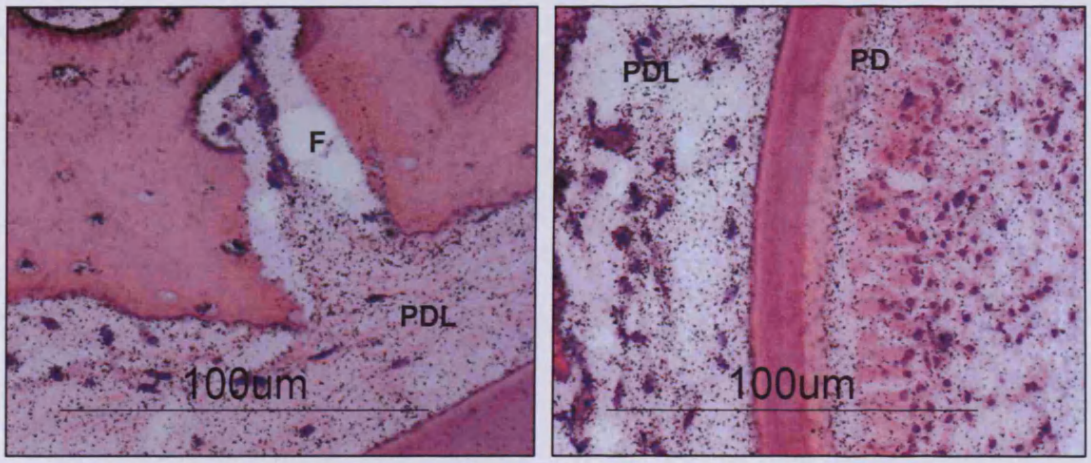

Figure 2.50: Tritiated proline radiolabelled fractured mandible slices cultured for 14 days. Silver grains can be seen within the cells and matrix of the periodontal ligament (PDL), within the predentine (PD) of the dentine-pulp complex, and within the matrix surrounding the fracture site (F).

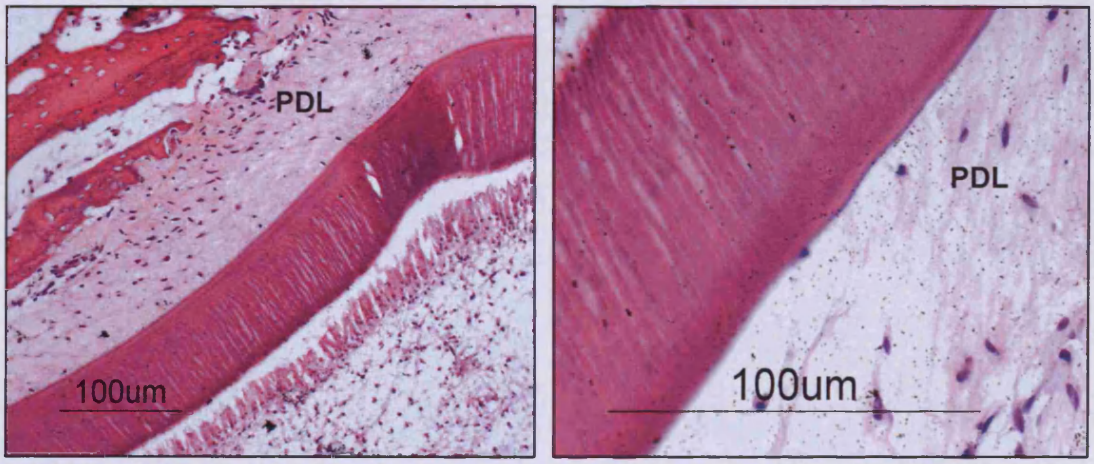

Figure 2.51: Radiolabel false positive controls. Slices were exposed, but no radiolabel was added to the culture.

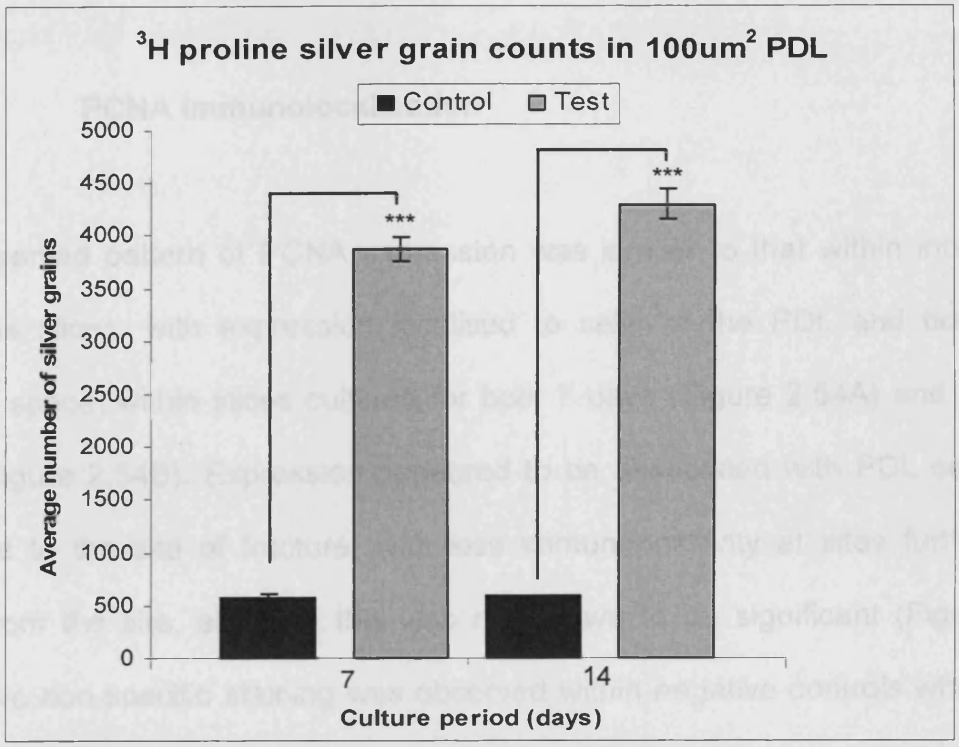

Figure 2.52: Average number of silver grains per 100µm² area within the periodontal ligament of fractured mandible slices after 7 or 14 days of culture.
*** p<0.001

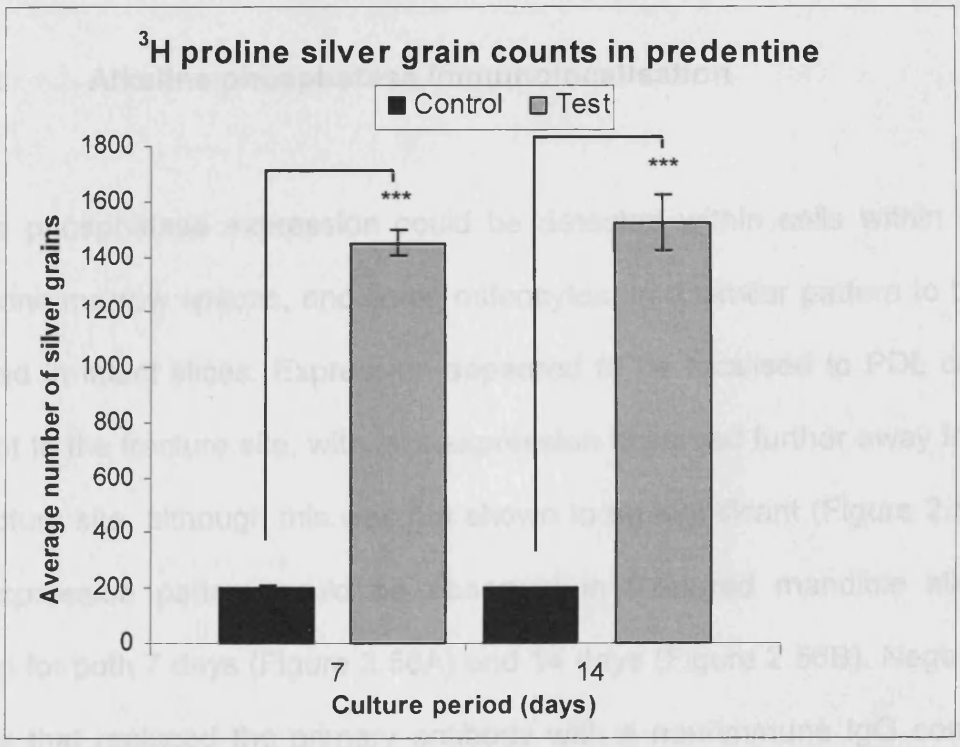

Figure 2.53: Average number of silver grains within the pulp of fractured mandible slices after 7 or 14 days of culture.
*** p<0.001

2.6.3 Immunohistochemical characterisation

2.6.3.1 PCNA immunolocalisation

The observed pattern of PCNA expression was similar to that within intact mandible slices, with expression localised to cells of the PDL and bone marrow space, within slices cultured for both 7 days (Figure 2.54A) and 14 days (Figure 2.54B). Expression appeared to be associated with PDL cells adjacent to the site of fracture, with less immunopositivity at sites further away from the site, although this was not shown to be significant (Figure 2.55). No non-specific staining was observed within negative controls which either replaced the primary antibody with a non-immune IgG control (Figure 2.54C) or omitted the primary antibody altogether (Figure 2.54D).

2.6.3.2 Alkaline phosphatase immunolocalisation

Alkaline phosphatase expression could be detected within cells within the PDL, bone marrow spaces, and some osteocytes, in a similar pattern to that observed in intact slices. Expression appeared to be localised to PDL cells adjacent to the fracture site, with less expression observed further away from the fracture site, although this was not shown to be significant (Figure 2.57). This expression pattern could be observed in fractured mandible slices cultured for both 7 days (Figure 2.56A) and 14 days (Figure 2.56B). Negative controls that replaced the primary antibody with a non-immune IgG control

(Figure 2.56C), or omitted the primary antibody (Figure 2.56D), showed only the methyl green counterstain with no non-specific staining.

2.6.3.3 Osteopontin immunolocalisation

Expression of the bone marker osteopontin could be detected around the fracture site, within cells of the PDL and bone marrow spaces, within fractured mandible slices cultured for both 7 days (Figure 2.58A) and 14 days (Figure 2.58B). Positive expression could also be detected in cells within the fracture site itself. In a pattern similar to that of PCNA and alkaline phosphatase, osteopontin expression appeared to be localised to PDL cells adjacent to the fracture site, and was increased compared to areas opposite the site of fracture, although this was not shown to be significant (Figure 2.59). Negative controls, where the primary antibody was replaced with a non-immune IgG control (Figure 2.58C) or where it was omitted (Figure 2.58D), showed no non-specific staining.

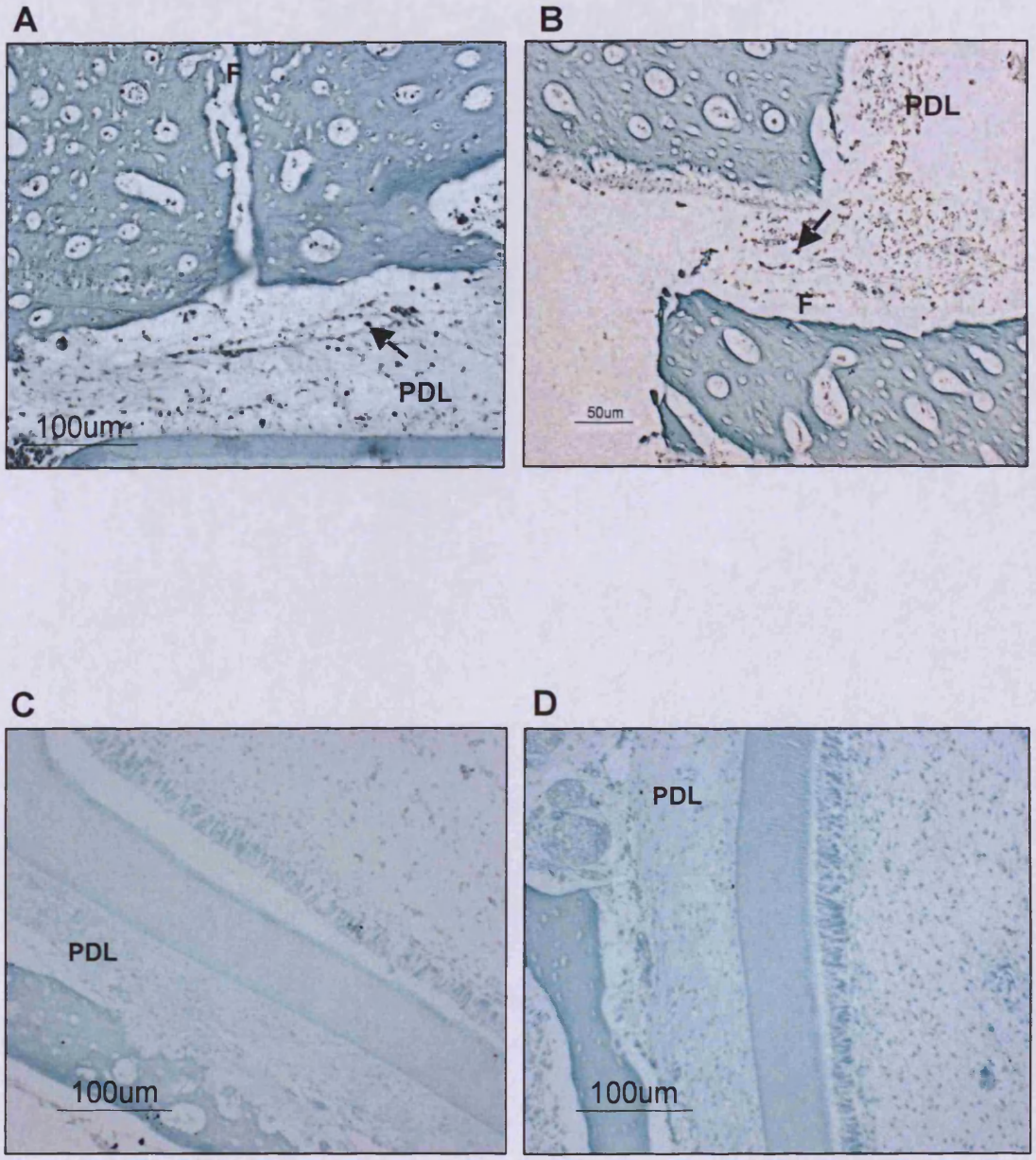

Figure 2.54: Mandible slices immunohistochemically stained for expression of proliferation marker PCNA. Positive cellular expression (examples indicated by arrows) can be observed within cells of the periodontal ligament (PDL) and bone marrow (BM) within (A) 7 day cultures and (B) 14 day cultures. Negative controls using (C) a non-immune IgG or (D) omitting the primary antibody exhibits only the methyl green counterstain.

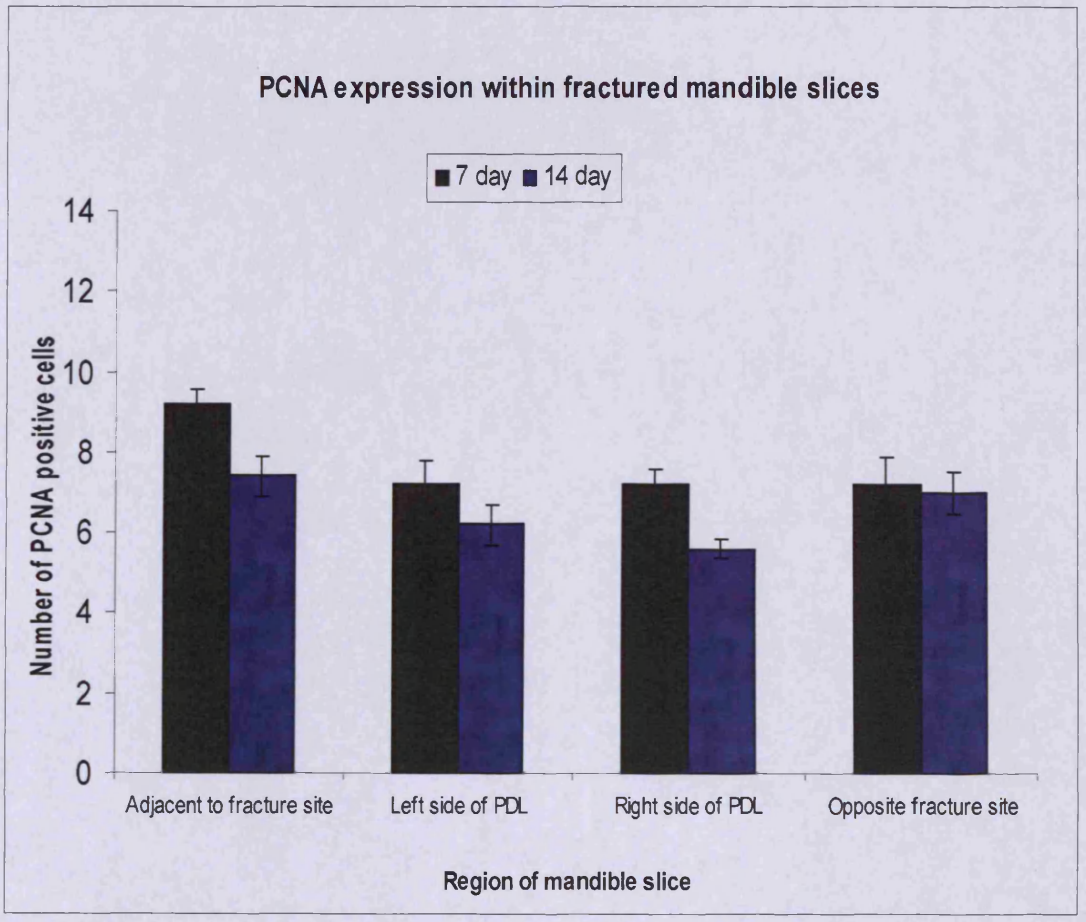

Figure 2.55: Number of PCNA positive cells within various 100μm² areas of periodontal ligament in fractured mandible slices cultured for 7 or 14 days.

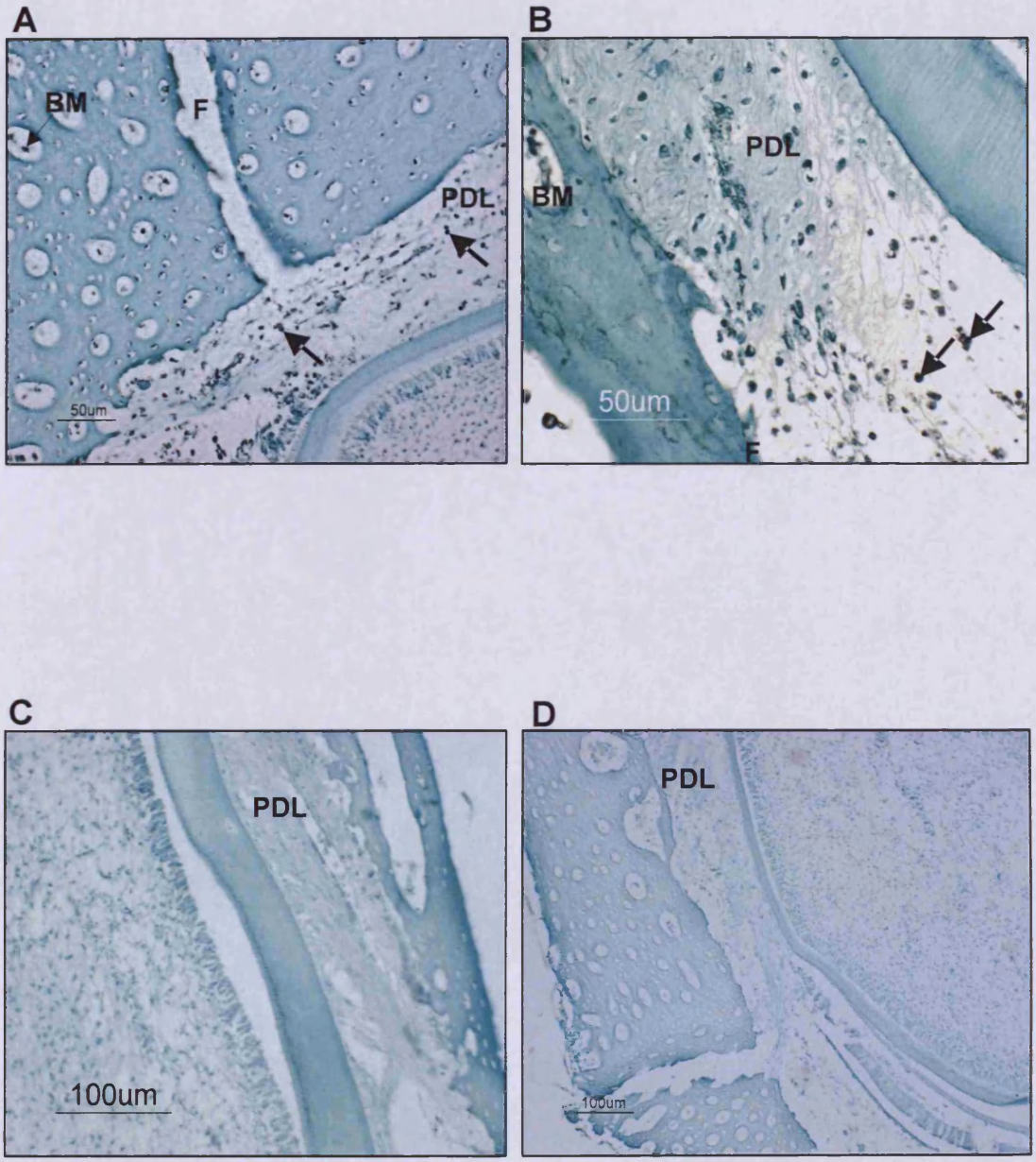

Figure 2.56: Mandible slices immunohistochemically stained for expression of alkaline phosphatase. Positive cellular expression (examples indicated by arrows) can be observed within cells of the periodontal ligament (PDL) and some cells of the bone marrow (BM), within (A) 7 day cultures and (B) 14 day cultures. Negative controls using (C) a non-immune IgG or (D) omitting the primary antibody exhibits only the methyl green counterstain.

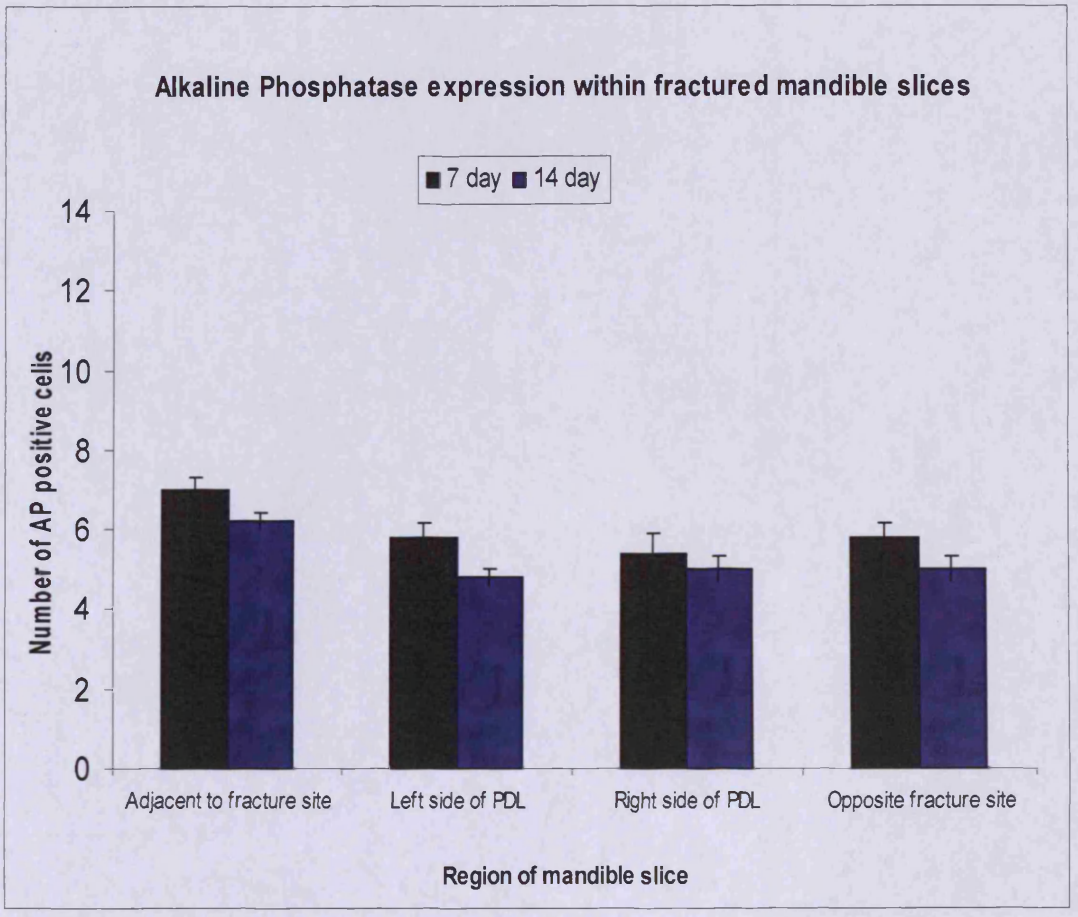

Figure 2.57: Number of alkaline phosphatase positive cells within various 100μm^2 areas of periodontal ligament in fractured mandible slices cultured for 7 or 14 days.

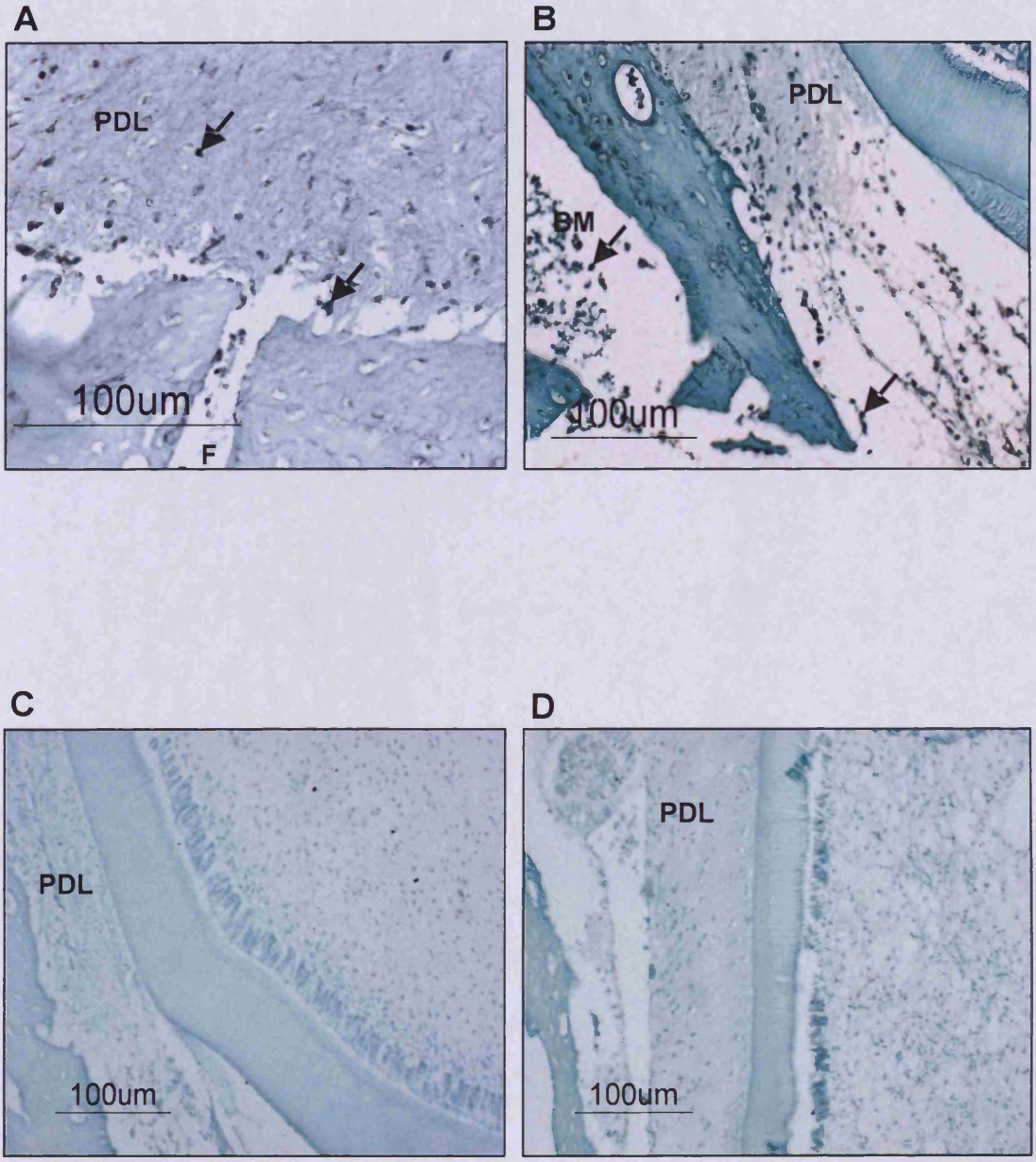

Figure 2.58: Mandible slices immunohistochemically stained for expression of osteopontin. Positive cellular expression (examples indicated by arrows) can be observed within some cells of the periodontal ligament (PDL) and bone marrow (BM), within (A) 7 day cultures and (B) 14 day cultures. Negative controls using (C) a non-immune IgG or (D) omitting the primary antibody exhibits only the methyl green counterstain.

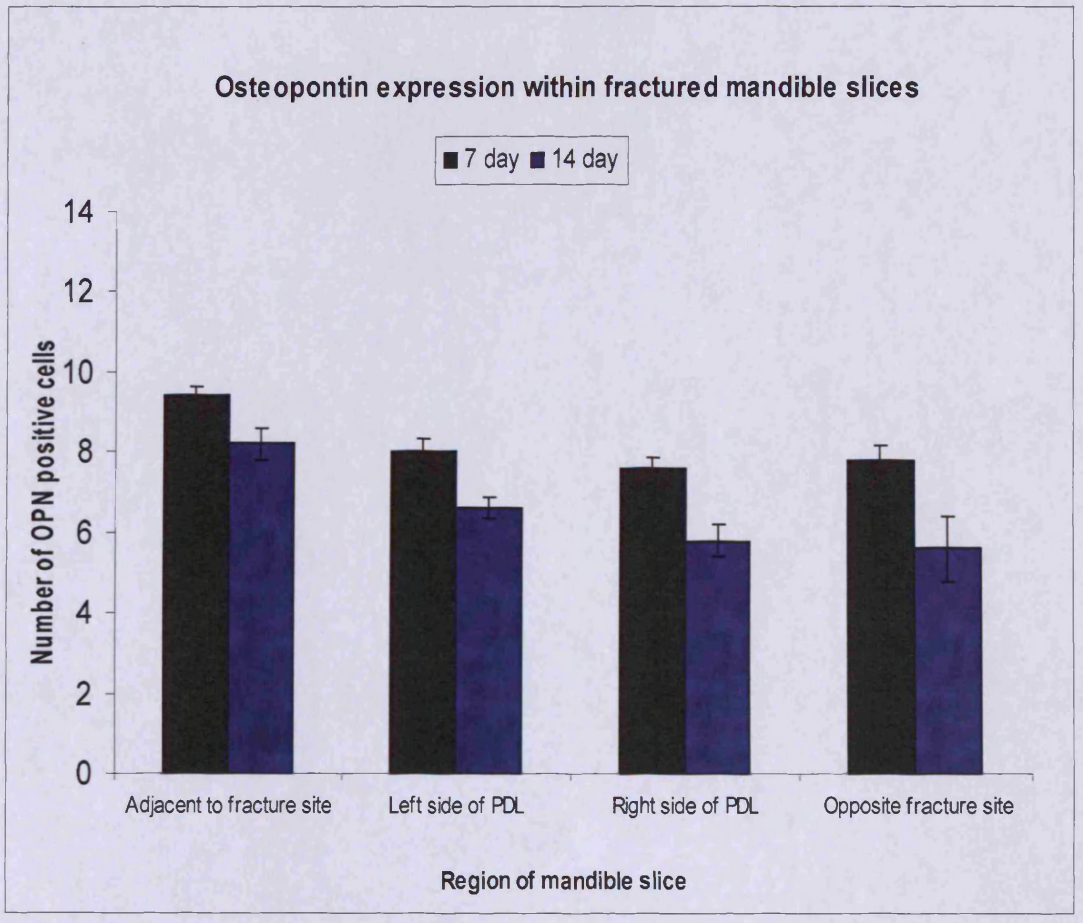

Figure 2.59: Number of osteopontin positive cells within various 100μm² areas of periodontal ligament in fractured mandible slices cultured for 7 or 14 days.

2.7 DISCUSSION

This second part of this chapter has demonstrated that the *ex vivo* mandible model can be successfully fractured and cultured, without sustaining further damage to the tissue or cells. In similar results to those observed within intact mandible slices, fractured mandible slices could be successfully cultured for up to 21 days, with maintenance of cell and tissue architecture, and cellular viability adjacent to the site of fracture. As with the intact slices, cells within the fractured slices were shown to be actively synthesising and secreting proteins throughout the culture period, and expression of proliferation markers and bone markers were observed within various cell types adjacent to the site of fracture.

Mandibles that had been initially sliced and fractured, but not cultured, and fractured mandible slices cultured for 24 hrs, showed significant cell death across the tissue surface. This cell death was present throughout all areas of the tissue, including adjacent to the site of fracture. Since histological stains indicated healthy viable tissue further into the mandible slice, this damage was probably due to initial trauma to the tissue caused by the slicing procedure, as was previously observed within the intact slices. 4 day cultures of fractured mandible slices demonstrated a recovery in viability of surface tissue, again suggesting that by this time surface damage had been resolved, probably by damaged cells and tissue being sloughed off to reveal healthy viable tissue underneath. After the 4 day culture period, fractured mandible slices remained highly viable for up to 21 days in culture. There was very little

decrease in cellular viability observed across this extended culture period, and importantly no decrease in viability was associated with areas of tissue in close proximity to the site of fracture. This demonstrates that the mandible slices can be successfully fractured without sustaining excessive damage to the cells and tissues within adjacent areas, thus providing a healthy and viable population of cells adjacent to the site of fracture with which to study early repair processes within the model. Automated cell counts of the fractured mandible slices did reveal a slight decrease in cell numbers across the culture period, both within the PDL and pulp, although statistical analysis only revealed a significant difference after 21 days of culture, and only within the PDL. This decrease in cell numbers was seen throughout the tissue, and was not significantly associated with the site of fracture, thus suggesting that the decrease was probably due to cell death caused by the extended time in culture, as seen previously in the intact mandible slices, and often encountered during cell and tissue culture (Arden and Betenbaugh 2004). Despite the decrease in cell numbers after 21 days in culture, both the viability stain and the histology stain demonstrate that after 21 days of culture there was still a significant presence of a highly viable cell population within the tissue, including adjacent to the fracture site.

The synthetic and secretory capabilities of the cells within the fractured mandible slices were assessed using a tritiated proline pulse chase experiment, to ensure that the fracturing procedure was not affecting the activity of the cells. After 7 days of culture, silver grain deposition (indicative of protein secretion) could be seen adjacent to the fracture site, both within

and closely surrounding cells residing in this area. Some silver grain deposition could also be observed within the matrix itself, of both the bone and the PDL. After extension of the culture period to 14 days, a significantly greater proportion of silver grain deposition could be observed within the matrix of both the bone and the PDL adjacent to the site of fracture, with less being associated with the cells themselves. This suggests that the cells residing in the bone and PDL adjacent to the site of fracture synthesise proteins during the first 7 days, which remain closely associated with them, but by 14 days these proteins are secreted into the matrix. This provides evidence that these cells are actively synthesising and secreting proteins, showing their functionality. The distribution of radiolabel within the PDL of the fractured mandible slices was similar to the distribution observed in intact slices, and to that observed in the *ex vivo* tooth slice model (Sloan et al. 1998), suggesting that fracturing does not detrimentally affect the activity of the cells, and cells can still actively function within the tissue.

Immunohistochemical staining was carried out to characterise the cells residing in areas adjacent to the site of fracture, to assess if the fracturing procedure was altering cell behaviour. Cellular proliferation was firstly assessed by investigating the expression of the cellular proliferation marker PCNA, followed by investigation of expression of the bone markers alkaline phosphatase and osteopontin. Cells positive for PCNA, alkaline phosphatase, and osteopontin could be observed within the PDL and bone marrow spaces adjacent to the site of fracture, after both 7 and 14 days of culture. Osteopontin positive cells could also be observed within the fracture

site itself, which may have migrated from the PDL during the culture period, or may have been an artefact of the fracturing process which may have disrupted cells in the area. There was a slight increase in PCNA, osteopontin and alkaline phosphatase positive cells within areas adjacent to the fracture site, compared to mandible regions opposite to the site of fracture, although this was not shown to be significant. These observed increases, although not significant, may indicate stimulation of some early reparative processes within the tissue in response to the fracturing process, stimulating migration and proliferation of cells within the PDL. However, despite the presence of these responsive osteoprogenitors present within the mandible tissue, it is unlikely that any further repair mechanisms would be initiated during the culture period. The mandible model has a distinct lack of blood supply, and therefore lacks the inflammatory and haemopoietic cell lineages that *in vivo* would infiltrate the site of injury and bring about further repair responses, including osteoclastic bone resorption (Colnot et al. 2006; Mizuno et al. 1990; Oe et al. 2007). Therefore the mandible model probably lacks sufficient stimulus for bone repair within the tissue, in response to the fracture alone. However, this model provides a valuable tool with which to investigate specific early repair processes in bone in the absence of the systemic influences seen *in vivo*, which can often interfere with obtaining clear data from such experiments. Stimulation of the model, with growth factors or chemical treatments to release bone matrix components, will enable the study of the specific roles of these factors in repair processes in bone.

CHAPTER 3

GROWTH FACTOR STIMULATION OF THE *EX VIVO* FRACTURED MANDIBLE MODEL

3.1 INTRODUCTION

During normal bone repair the processes of intramembranous and endochondral ossification will ultimately result in optimal repair of the damaged tissue and restoration of function (Vortkamp et al. 1998). Bone healing can, however, fail or be severely delayed in clinical situations such as fracture non-union and non-healing sockets following tooth extraction. Significant research over the last 25 years has sought to develop alternative therapies to aid in bone healing processes through the utilisation of osseoinductive or osseoconductive factors at the site of injury. The process of bone formation may be augmented by the clinical application of bone-inducing growth factors, which have long been known to govern successful bone healing processes. These have included the BMPs and other members of the transforming growth factor family, such as TGF-β1, using a scaffold or carrier (Boden et al. 2000; Friedlaender et al. 2001; Ramoshebi et al. 2002; Westerhuis et al. 2005). The clinical potential for these bioactive molecules is, at present, limited due to the supraphysiological milligram doses of the growth factor that are currently needed to elicit an effect in healing small bone defects, making them a highly expensive clinical treatment (Simpson et al. 2006).

Recent studies using an *ex vivo* tooth slice culture system (Sloan et al. 1998) have demonstrated that the bioactive growth factor TGF-β1, applied to the tooth slice within either alginate hydrogels or agarose beads, has mitogenic effects on cells within the subodontoblast layer, and is capable of inducing differentiation of odontoblast-like cells within the dentine-pulp complex with subsequent upregulation of dentine matrix secretion (Dobie et al. 2002; Sloan et al. 1998; Sloan and Smith 1999). TGF-β1, as well as being present in the matrix of dentine (Sloan et al. 2000a), is the most abundant growth factor in bone (Fox and Lovibond 2005; Kanaan and Kanaan 2006). It is known to play key roles in bone repair processes, stimulating recruitment and proliferation of mesenchymal stem cells and osteoprogenitor cells at sites of injury (Barnes et al. 1999), as well as promoting early stages of differentiation within these cells (Bostrom and Asnis 1998; Janssens et al. 2005). It has also been demonstrated that serum levels of TGF-β1 are significantly reduced in patients displaying delayed fracture healing (Zimmermann et al. 2005). BMP-2 is another growth factor that also plays a critical role in the repair of bone, as it is capable of stimulating the differentiation of mesenchymal stem cells and osteoprogenitor cells into mature, fully functional osteoblast cells, capable of laying down new bone (Cheng et al. 2003a; Lieberman et al. 2002). A substantial amount of research has been conducted into the clinical use of BMP-2 in bone repair, and successful clinical trials have led to the FDA-approval of BMP-2 in the treatment of non-union in long bones (Govender et al. 2002) and in the induction of spinal fusion (Boden et al. 2000). Thus, it is apparent that these two growth factors are highly involved in the bone repair cascade, and it is likely that they work synergistically. TGF-

β1 stimulates recruitment and proliferation of mesenchymal stem cells and osteoprogenitors, providing a pool of osteogenic cells at the site of bone injury, while BMP-2 serves to differentiate these cells down an osteoblastic lineage, thus providing a population of fully mature osteoblast cells capable of producing new bone to repair the damaged area.

The development of an *ex vivo* mandible slice culture system in this study provides a model for investigating specific mechanisms involved in growth factor regulation of bone repair processes, particularly within a delayed or non-healing clinical setting, and may provide a functional model for testing novel therapeutic agents. Thus the aim of this chapter of the study was to examine the effects of adding single exogenous TGF-β1 and BMP-2 to the fracture site of the mandible model, on the response and behaviour of the cells within the PDL during culture, to demonstrate proof of principle of the functionality of the model for testing novel therapeutics.

3.2 MATERIALS AND METHODS

3.2.1 Cellular stimulation of fractured mandible slices with TGF-β1

Human recombinant TGF-β1 (PeproTech EC Ltd, UK) was solubilised in 10mM citric acid (pH 3) to a concentration of 50μg/ml, and then further diluted to a working concentration in 0.1% BSA in PBS and stored at -20°C. 150μm sized Affi Gel Blue agarose beads (BioRad Laboratories, UK) were divided into 100 bead aliquots and incubated with 50μl 1.6μg/ml TGF-β1 in 0.1% BSA in PBS for 30 min at 37°C. As a control, 100 agarose beads were also incubated in 0.1% BSA in PBS without TGF-β1.

Fractured mandible slices were prepared from 28 day old male Wistar rats immediately before bead treatment (as described in 2.5.1). Prior to embedding in semi-solid agar medium the fracture site was packed with 6 agarose beads ± TGF-β1 using sterile forceps and needles, with the aid of a dissecting microscope (Bausch and Lomb). The numbers of beads used were the optimal number that could be placed within the fracture site of the mandible slice without overlap. Once the beads were transferred, the mandible slices were cultured in Trowel type cultures (as described in 2.2.2) for 7 days. Control fractured mandible slices which had no beads transferred into the fracture site were also prepared and cultured in Trowel type cultures as above.

3.2.2 Cellular stimulation of fractured mandible slices with BMP-2

Human recombinant BMP-2 (Invitrogen, UK) was solubilised in 20mM acetic acid to a concentration of 1mg/ml, and then further diluted to a working concentration in 0.1% BSA in TBS and stored in -20°C. 150µm sized Affi Gel Blue agarose beads (BioRad Laboratories, UK) were divided into 100 bead aliquots and incubated with 50µl 100ng/ml BMP-2 in 0.1% BSA in PBS for 30 min at 37°C. Control agarose beads were prepared as previously described (3.2.1).

Fractured mandible slices were prepared from 28 day old male Wistar rats immediately before bead treatment (as described in 2.5.1). Prior to embedding in semi-solid agar medium the fracture site was packed with 6 agarose beads ± BMP-2 using sterile forceps and needles. Once the beads were transferred, the mandible slices were cultured in Trowel type cultures (as described in 2.2.2) for 7 days. Control fractured mandible slices which had no beads transferred into the fracture site were also prepared and cultured in Trowel type cultures.

3.2.3 Histological examination

After culture, fractured mandible slices stimulated with TGF-β1 or BMP-2 (with respective control slices) were prepared for histological staining with H&E (as described in section 2.2.3). A total of 10 mandible slices were cultured for each treatment type and control type. Cell numbers were

automatically counted within randomly selected 100μm^2 areas of the PDL using Image ProPlus software. Cells were counted within the fracture site itself, within 100μm^2 of the PDL adjacent to the site of fracture, and within 100μm^2 areas of the PDL to the left and right of the fracture site. A control count was also taken within a randomly selected 100μm^2 area of the PDL at a mandible region opposite the site of stimulation. Standard errors of the mean were calculated, and mean values were analysed using one-way ANOVA and Tukey's post hoc test, to analyse differences between treatment types and controls. Statistical analysis was also used to compare between TGF-β1 and BMP-2 stimulation.

3.2.4 Immunohistochemistry

Fractured mandible slices stimulated with TGF-β1 or BMP-2 (with respective control slices) were stained immunohistochemically for expression of the proliferation marker PCNA, and the bone marker osteopontin, as described in section 2.2.6. Expression of an additional bone marker, bone sialoprotein, was also examined using the same method as described in 2.2.6 (rabbit anti-rat BSP, clone LF-87; diluted 1:50) (Fisher et al. 1995). A total of 5 mandible slices were cultured for each treatment type and control type, for each marker. Cells positive for each of the markers were automatically counted within randomly selected 100μm^2 areas of the PDL using Image ProPlus software. Positive cells were counted within 100μm^2 of the PDL adjacent to the site of fracture, and within 100μm^2 areas of the PDL to the left and right of the fracture site. A control count was also taken within a 100μm^2 area of the

PDL at a mandible region opposite the site of stimulation. Standard errors of the mean were calculated, and mean values were analysed using one-way ANOVA and Tukey's post hoc test, to analyse differences between treatment types and controls. Differences were also analysed between mandible slices stimulated with TGF-β1 and BMP-2, for each marker.

3.3 RESULTS

3.3.1 Histology of TGF-β1 and BMP-2 stimulated mandible slices

Fractured mandible slices stimulated with TGF-β1 and subsequently cultured for 7 days showed aggregation of cells within the PDL in close proximity to the site of fracture and stimulation (Figure 3.1A). This aggregation of cells was not observed within regions of PDL opposite the site of stimulation (Figure 3.1B) or within control mandible slices, either cultured in the presence of bead alone (Figure 3.1C) or in the absence of either bead or growth factor (Figure 3.1D). Similar results were observed in fractured slices stimulated with BMP-2, with aggregation of cells observed within the PDL in close proximity to the BMP-2 soaked bead (Figure 3.2A). As with TGF-β1 stimulation, regions of PDL opposite the site of BMP-2 stimulation (Figure 3.2B) or within control mandible slices (Figures 3.2C and 3.2D) did not exhibit such increased cell numbers.

Statistical analysis of cell counts within 100μm^2 areas of the PDL (Figure 3.3) indicated that the increase in cell numbers within the PDL of TGF-β1 stimulated mandible slices was significant, both adjacent to the site of fracture ($p<0.001$), 100μm to the left of the site of fracture ($p<0.001$) and 100μm to the right of the site of fracture ($p<0.01$). These results were observed when compared with both control mandible slices. There was no significant difference in cell numbers within regions of mandible slices opposite the site of TGF-β1 stimulation, when compared to control slices

($p>0.05$). Cell counts within 100μm^2 areas of the PDL in BMP-2 stimulated slices (Figure 3.4) showed similar significant increases in cell numbers, both adjacent to the site of fracture ($p<0.001$), 100μm to the left of the site of fracture ($p<0.01$) and 100μm to the right of the site of fracture ($p<0.05$). These results were observed when compared with both control mandible slices. There was no significant difference in cell numbers within regions of mandible slices opposite the site of stimulation, when compared to control slices. Statistical analysis of cell counts in TGF-β1 and BMP-2 stimulated mandible slices showed a decrease in PDL cell numbers within BMP-2 treated mandible slices when compared to those treated with TGF-β1, but this was not shown to be significant ($p>0.05$).

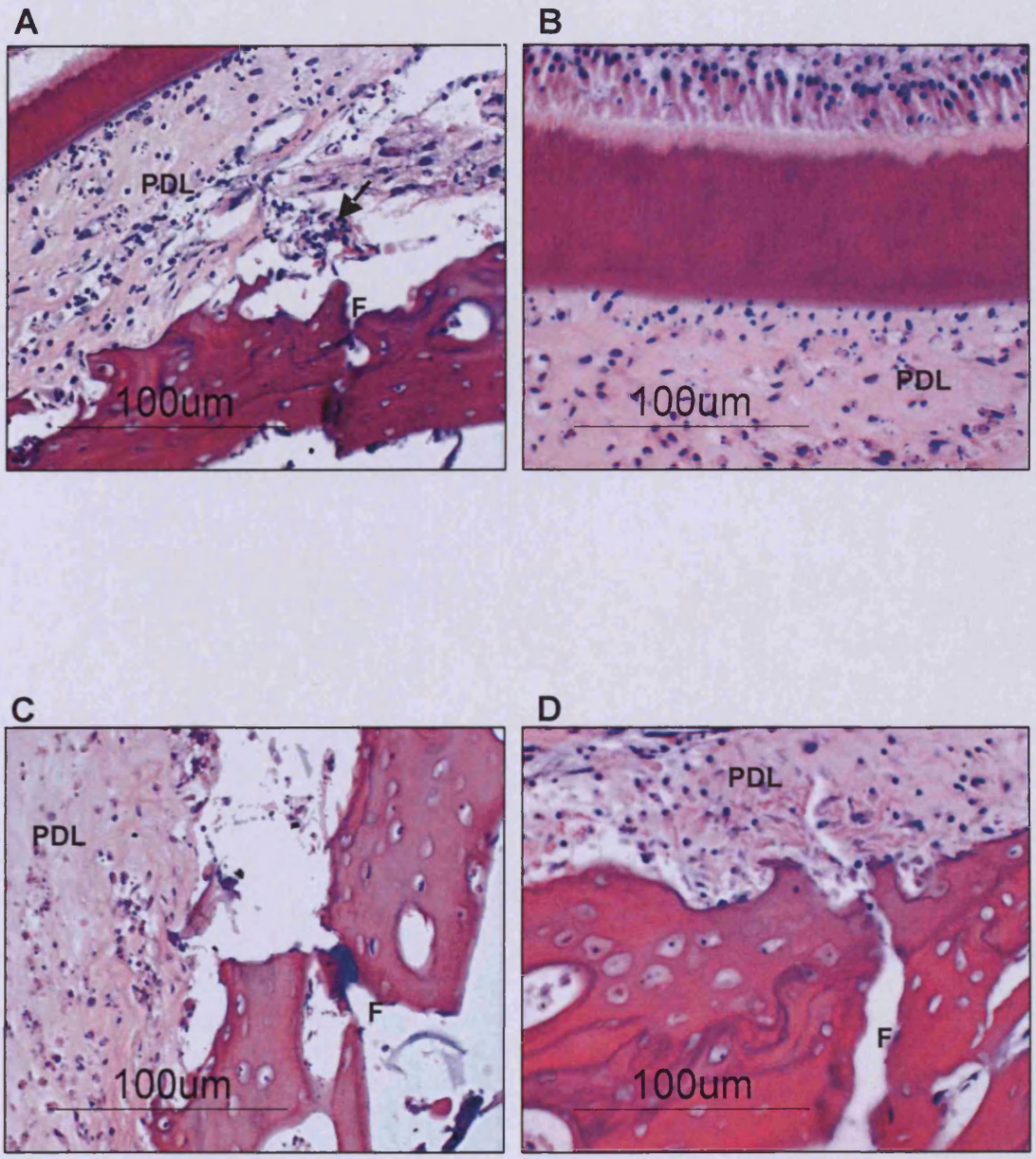

Figure 3.1: Mandible slices cultured for 7 days following stimulation with TGF-β1 with agarose beads. (A) Aggregation of cells within the periodontal ligament (PDL) can be observed within close proximity to the site of fracture (F) in TGF-β1 stimulated slices. Similar aggregation cannot be observed within (B) regions of PDL at the opposite side of stimulation, (C) in control slices cultured in the presence of agarose beads alone, or (D) in control slices cultured in the absence of either bead or growth factor.

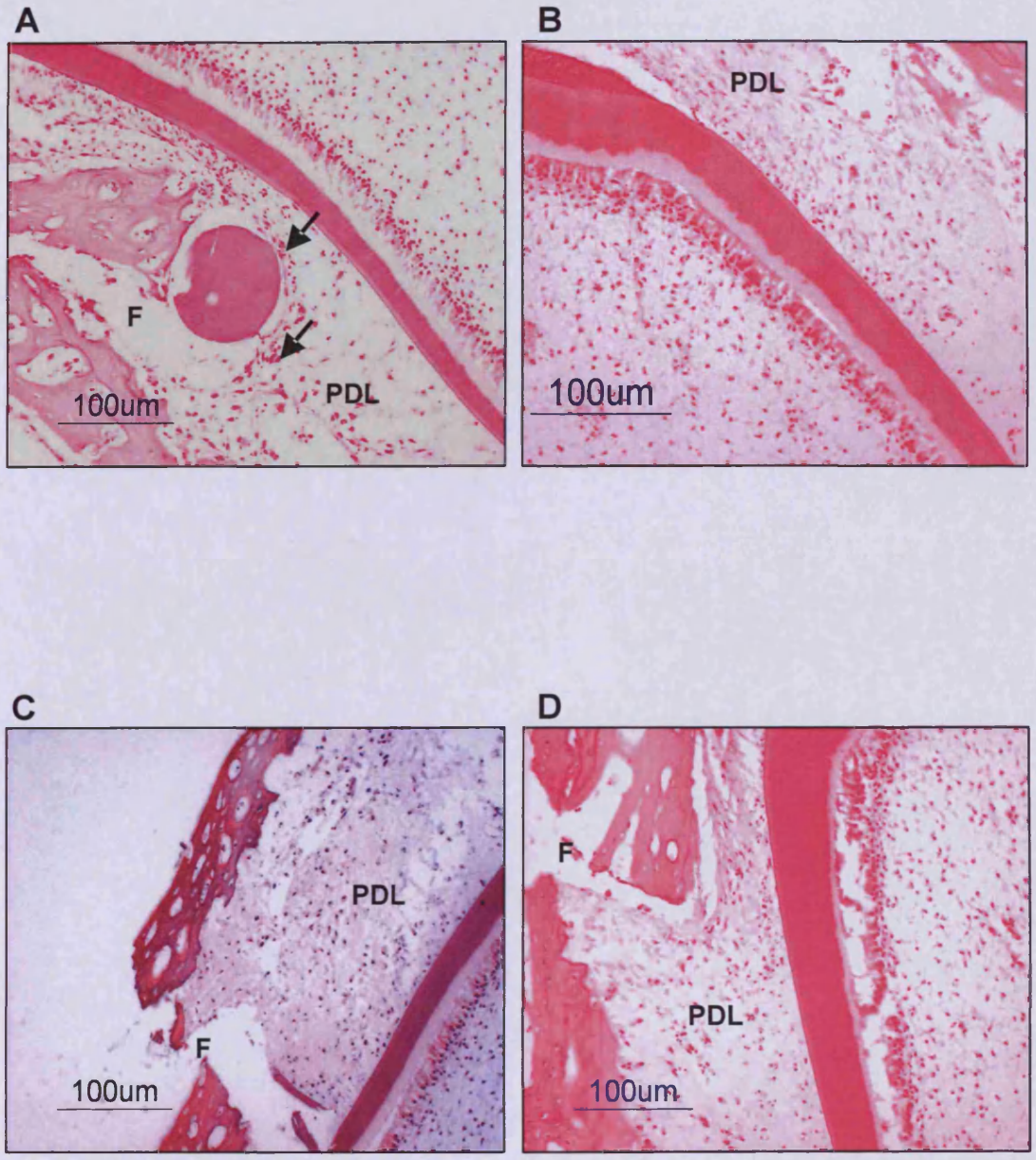

Figure 3.2: Mandible slices cultured for 7 days following stimulation with BMP-2 with agarose beads. (A) Aggregation of cells within the periodontal ligament (PDL) can be observed within close proximity to the bead in BMP-2 stimulated slices. Similar aggregation cannot be observed within (B) regions of PDL at the opposite side of stimulation, (C) in control slices cultured in the presence of agarose beads alone, or (D) in control slices cultured in the absence of either bead or growth factor.

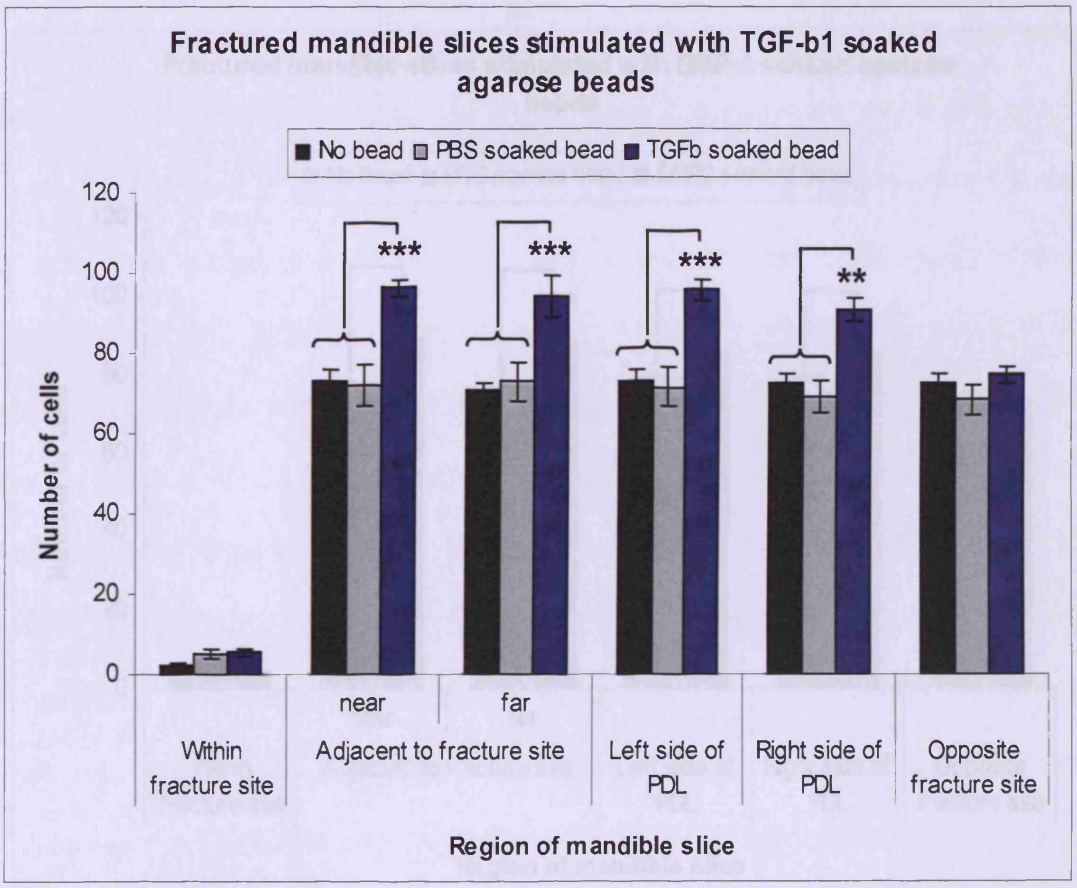

Figure 3.3: Average cell numbers within 100μm² areas of periodontal ligament in fractured mandible slices stimulated with TGF-β1, compared with control slices cultured in the presence of bead alone, or in the absence of bead.

** $p<0.01$, *** $p<0.001$

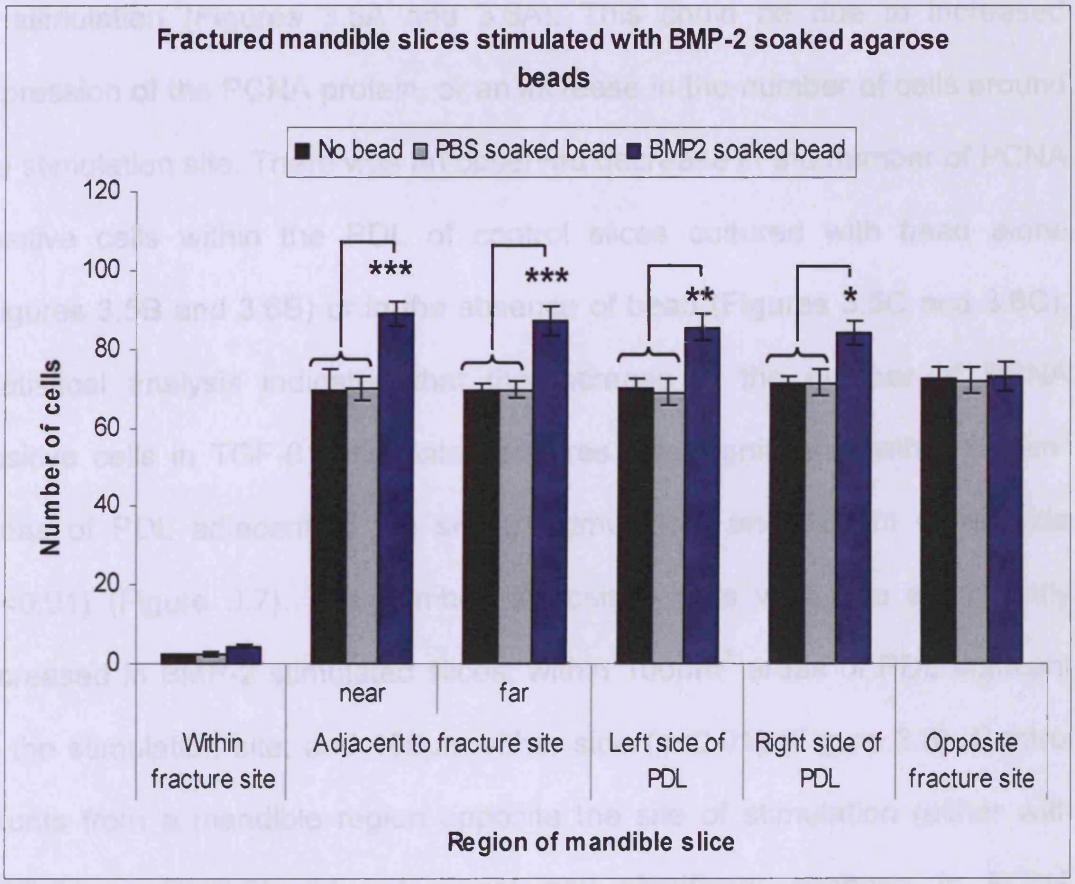

Figure 3.4: Average cell numbers within various 100μm² areas of periodontal ligament in fractured mandible slices stimulated with BMP-2, compared with control slices cultured in the presence of bead alone, or in the absence of bead.

* p< 0.05, ** p<0.01, *** p<0.001

3.3.2 Immunohistochemical characterisation of stimulated cells

3.3.2.1 PCNA immunolocalisation

Fractured mandible slices stimulated with TGF-β1 or BMP-2 exhibited higher expression of PCNA within cells of the PDL, closely associated with the site of stimulation (Figures 3.5A and 3.6A). This could be due to increased expression of the PCNA protein, or an increase in the number of cells around the stimulation site. There was an observed decrease in the number of PCNA positive cells within the PDL of control slices cultured with bead alone (Figures 3.5B and 3.6B) or in the absence of bead (Figures 3.5C and 3.6C). Statistical analysis indicated that the increase in the number of PCNA positive cells in TGF-β1 stimulated cultures was significant, within 100μm^2 areas of PDL adjacent to the site of stimulation, and 100μm either side ($p<0.01$) (Figure 3.7). The number of positive cells was also significantly increased in BMP-2 stimulated slices, within 100μm^2 areas of PDL adjacent to the stimulation site, and 100μm either side ($p<0.01$) (Figure 3.8). Control counts from a mandible region opposite the site of stimulation (either with TGF-β1 or BMP-2) did not reveal any significant changes in PCNA expression, when compared with control slices. Statistical comparisons between TGF-β1 and BMP-2 stimulated slices indicated a higher number of PCNA immunopositive cells within TGF-β1 stimulated slices, but this was not shown to be significant ($p>0.05$). Negative controls replaced the primary antibody with a non-immune IgG control and showed the methyl green counterstain with no non-specific staining (Figures 3.5D and 3.6D).

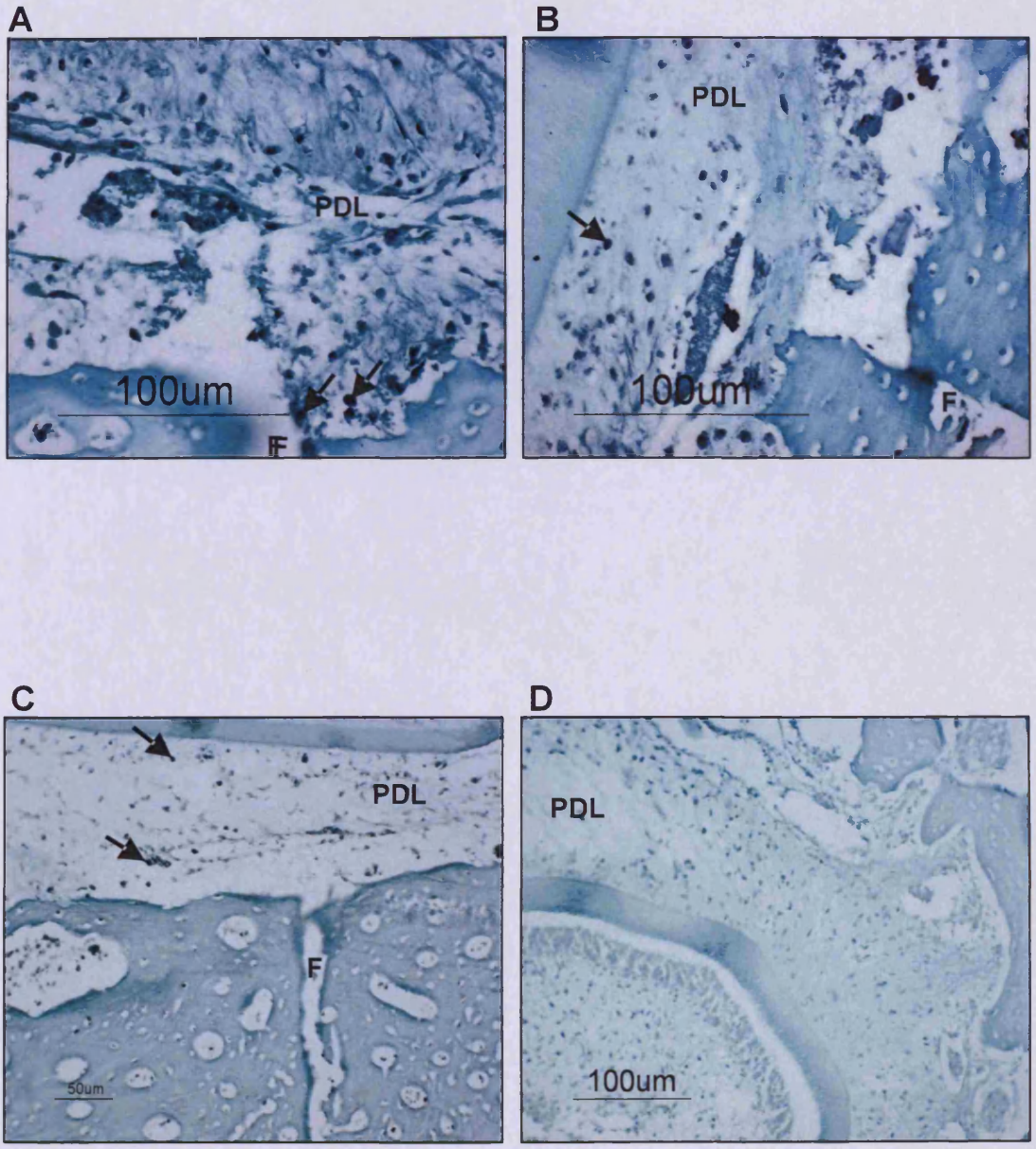

Figure 3.5: Mandible slices immunohistochemically stained for expression of PCNA (examples of PCNA immunopositivity indicated by arrows). Increased cellular expression can be observed within (A) cells of the periodontal ligament (PDL) in mandible slices stimulated with TGF-β1, when compared with (B) control mandible slices cultured with bead alone or (C) control mandible slices cultured in the absence of bead. (D) Negative controls using a non-immune IgG exhibits only the methyl green counterstain.

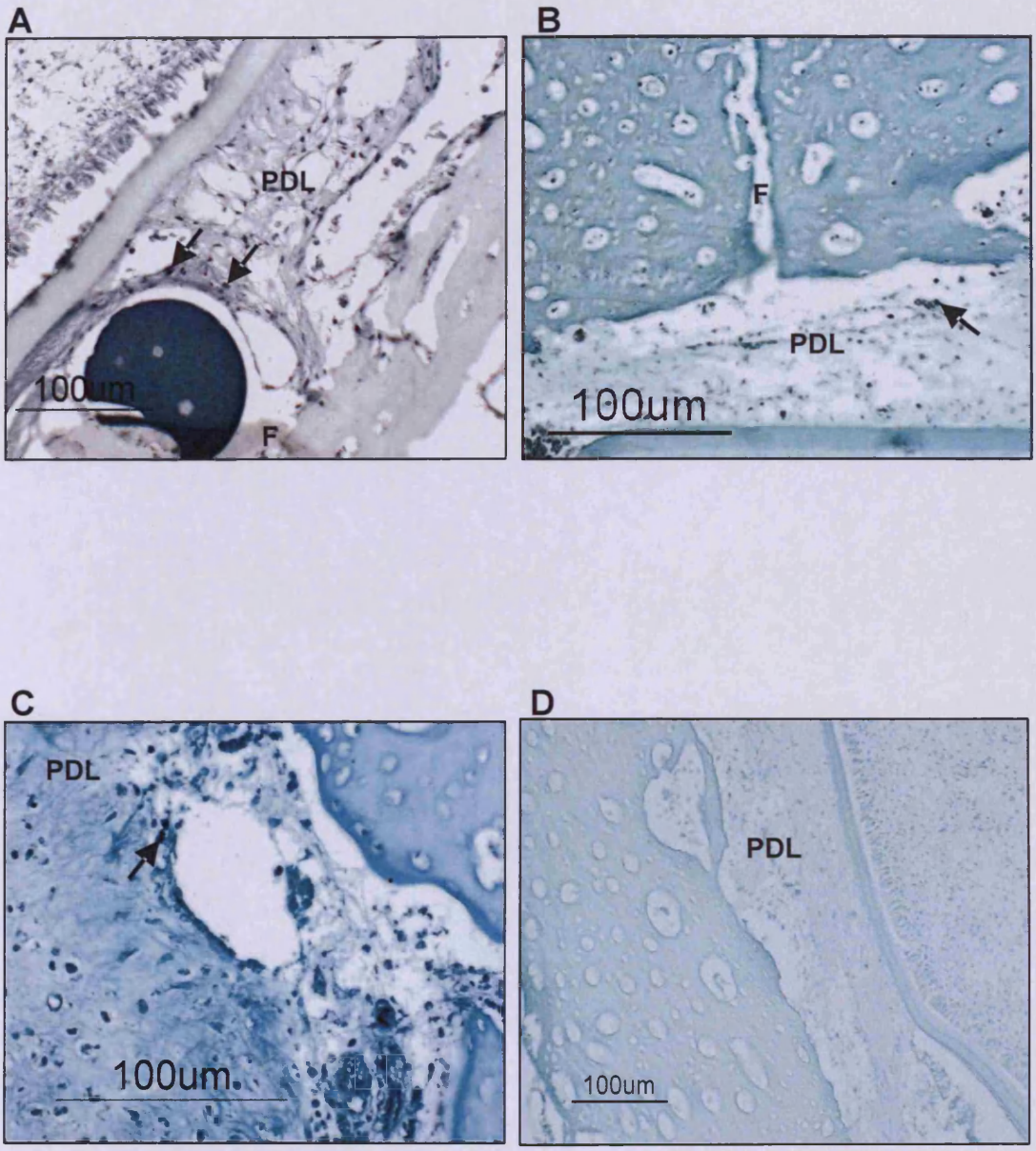

Figure 3.6: Mandible slices immunohistochemically stained for expression of PCNA (examples of PCNA immunopositivity indicated by arrows). Increased cellular expression can be observed within (A) cells of the periodontal ligament (PDL) in mandible slices stimulated with BMP-2, when compared with (B) control mandible slices cultured with bead alone or (C) control slices cultured in the absence of bead. (D) Negative controls using a non-immune IgG exhibits only the methyl green counterstain.

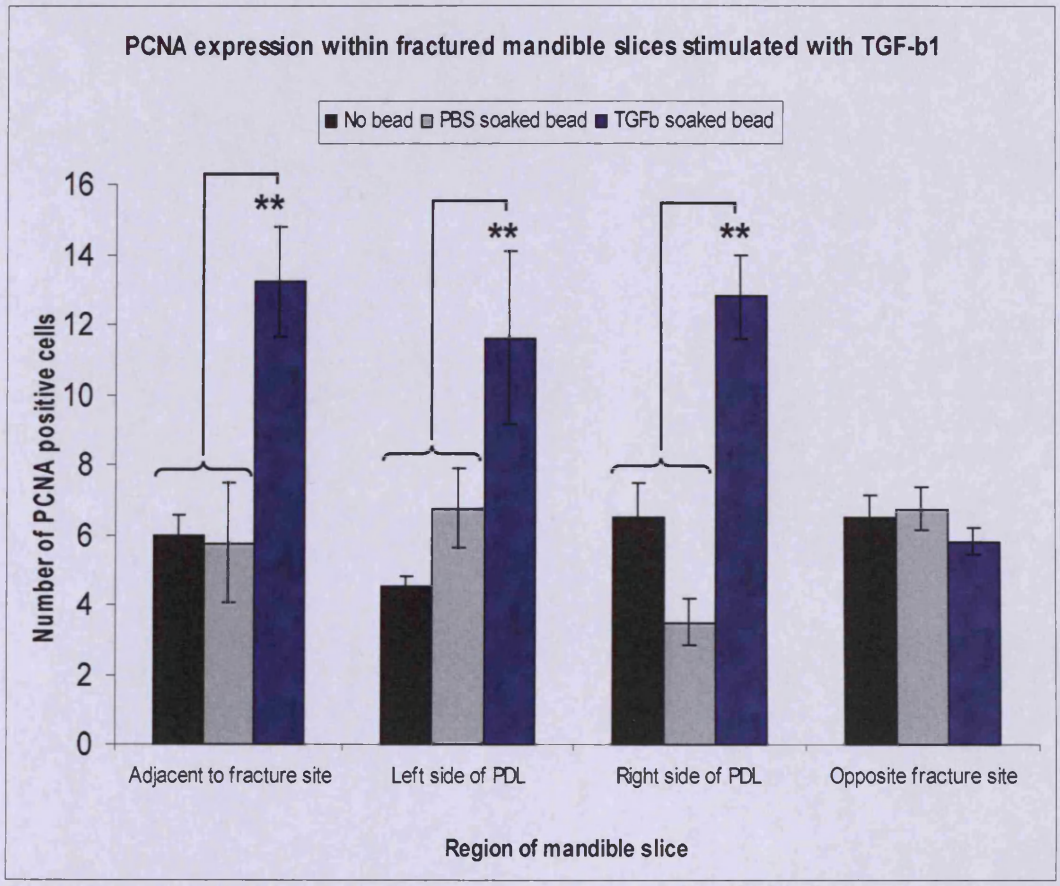

Figure 3.7: Number of PCNA positive cells within various 100μm² areas of periodontal ligament in fractured mandible slices stimulated with TGF-β1, compared with control slices cultured in the presence of bead alone, or in the absence of bead.

** $p<0.01$

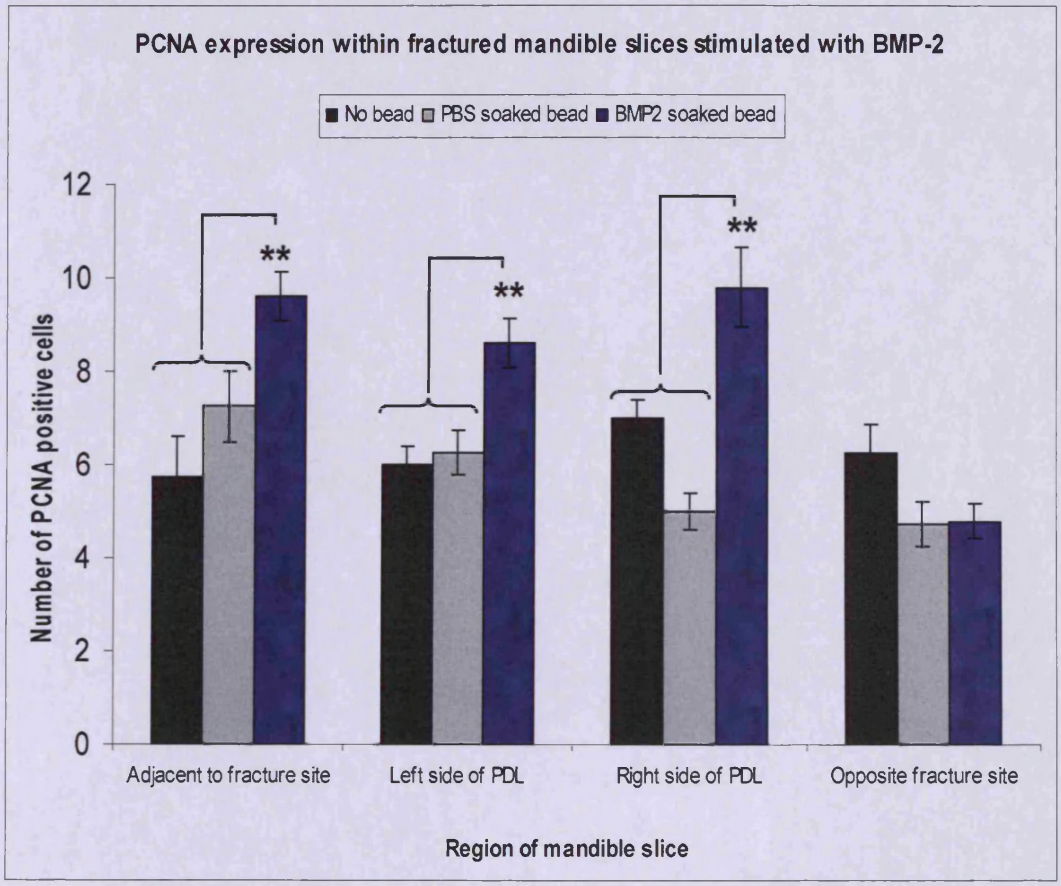

Figure 3.8: Number of PCNA positive cells within various 100μm² areas of periodontal ligament in fractured mandible slices stimulated with BMP-2, compared with control slices cultured in the presence of bead alone, or in the absence of bead.

** $p<0.01$

3.3.2.2 Osteopontin immunolocalisation

Expression of the bone marker osteopontin appeared to be increased in mandible slices stimulated with either TGF-β1 or BMP-2, within cells of the PDL closely associated with the site of fracture and therefore stimulation (Figures 3.9A and 3.10A). Expression was lower within the PDL of control slices cultured with bead alone (Figures 3.9B and 3.10B) or in the absence of bead (Figures 3.9C and 3.10C), although this may be due to a reduction in cell number rather than a decrease in expression. Statistical analysis demonstrated that the number of osteopontin positive cells was significantly increased in mandible cultures stimulated with TGF-β1, within 100μm^2 areas of PDL adjacent to the site of stimulation, and also 100μm either side ($p<0.05$) (Figure 3.11). Similar significant increases were observed within BMP-2 stimulated mandible slices, also within 100μm^2 areas of PDL adjacent to the site of stimulation, and also 100μm either side ($p<0.001$) (Figure 3.12). Control counts from a mandible region opposite the site of stimulation (either with TGF-β1 or BMP-2) did not reveal any significant changes in expression of osteopontin. Treatment with BMP-2 appeared to elicit a greater response than TGF-β1 treatment, with a greater observed increase in osteopontin immunopositivity, but this was not shown to be significant ($p>0.05$). Negative controls (as prepared in 3.3.2.1) showed only the methyl green counterstain with no non-specific staining (Figure 3.9D and 3.10D).

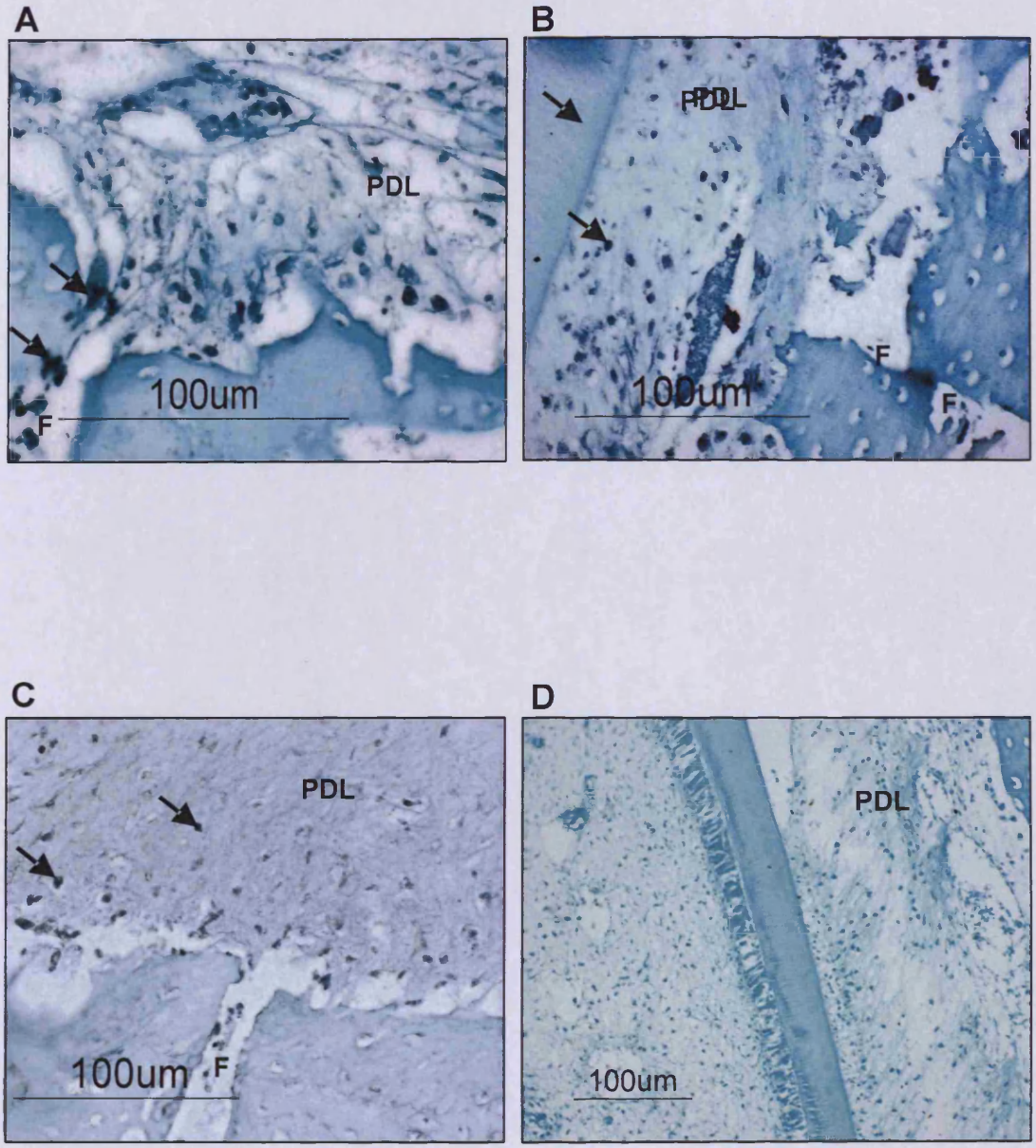

Figure 3.9: Mandible slices immunohistochemically stained for expression of osteopontin (examples of osteopontin immunopositivity indicated by arrows). Increased cellular expression can be observed within (A) cells of the periodontal ligament (PDL) in mandible slices stimulated with TGF-β1, when compared with (B) control mandible slices cultured with bead alone or (C) control slices cultured in the absence of bead. (D) Negative controls using a non-immune IgG exhibits only the methyl green counterstain.

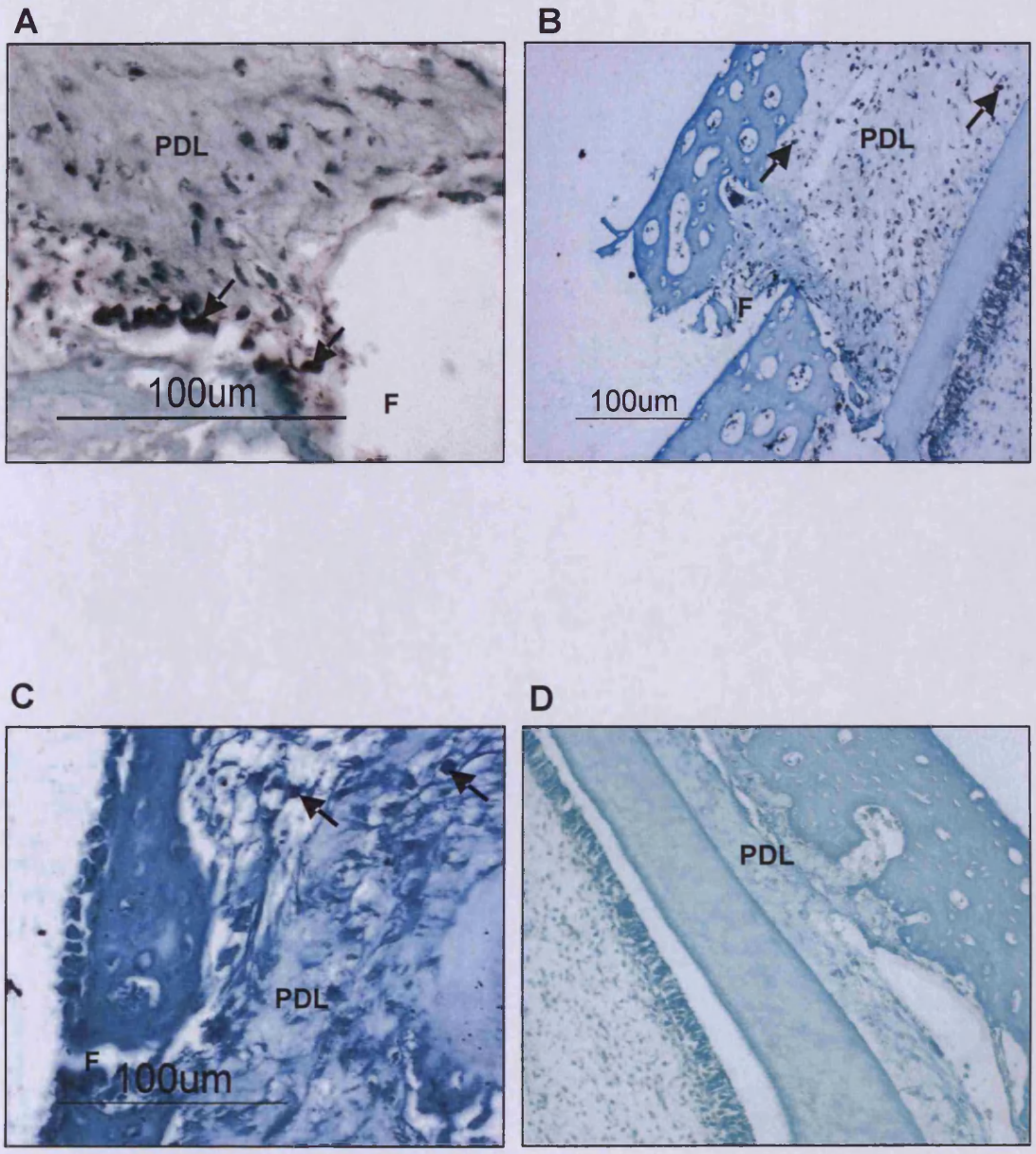

Figure 3.10: Mandible slices immunohistochemically stained for expression of osteopontin. Increased cellular expression (examples indicated by arrows) can be observed within (A) cells of the periodontal ligament (PDL) in mandible slices stimulated with BMP-2, when compared with (B) control mandible slices cultured with bead alone or (C) control slices cultured in the absence of bead. (D) Negative controls using a non-immune IgG exhibits only the methyl green counterstain.

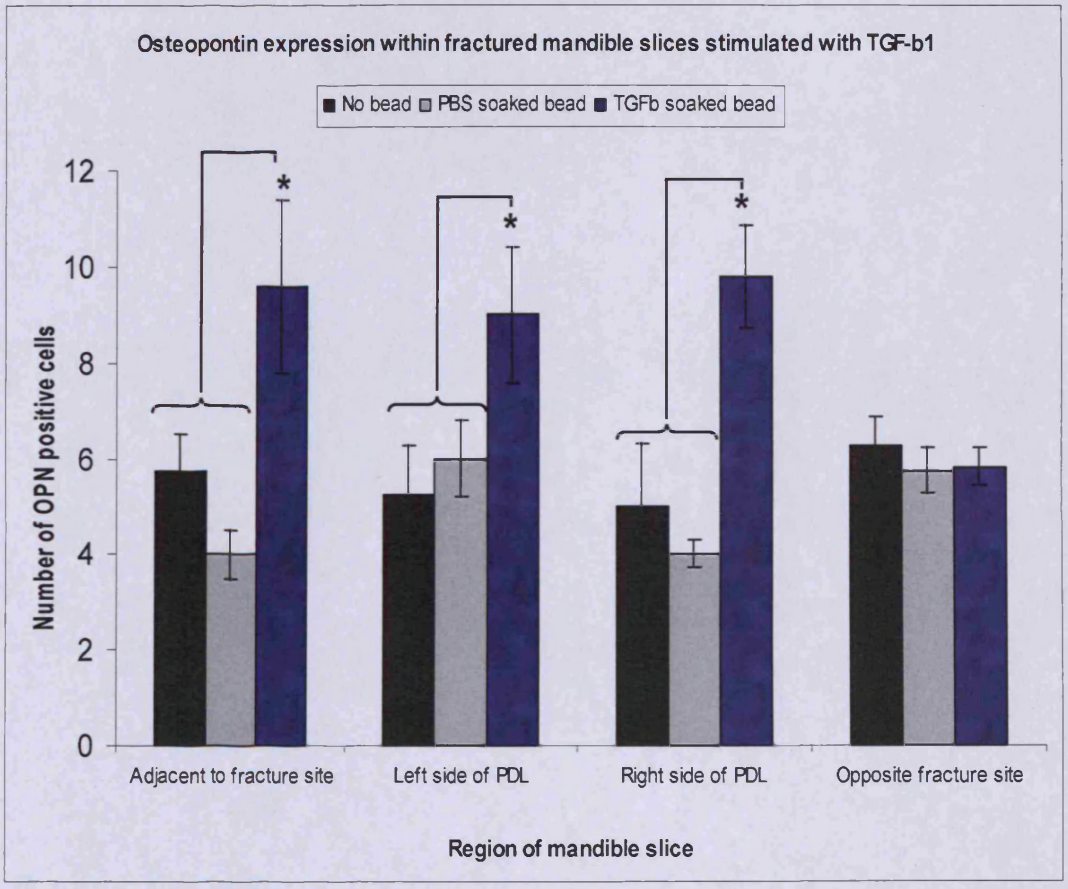

Figure 3.11: Number of osteopontin positive cells within various 100μm² areas of periodontal ligament in fractured mandible slices stimulated with TGF-β1, compared with control slices cultured in the presence of bead alone, or in the absence of bead.

* $p<0.05$

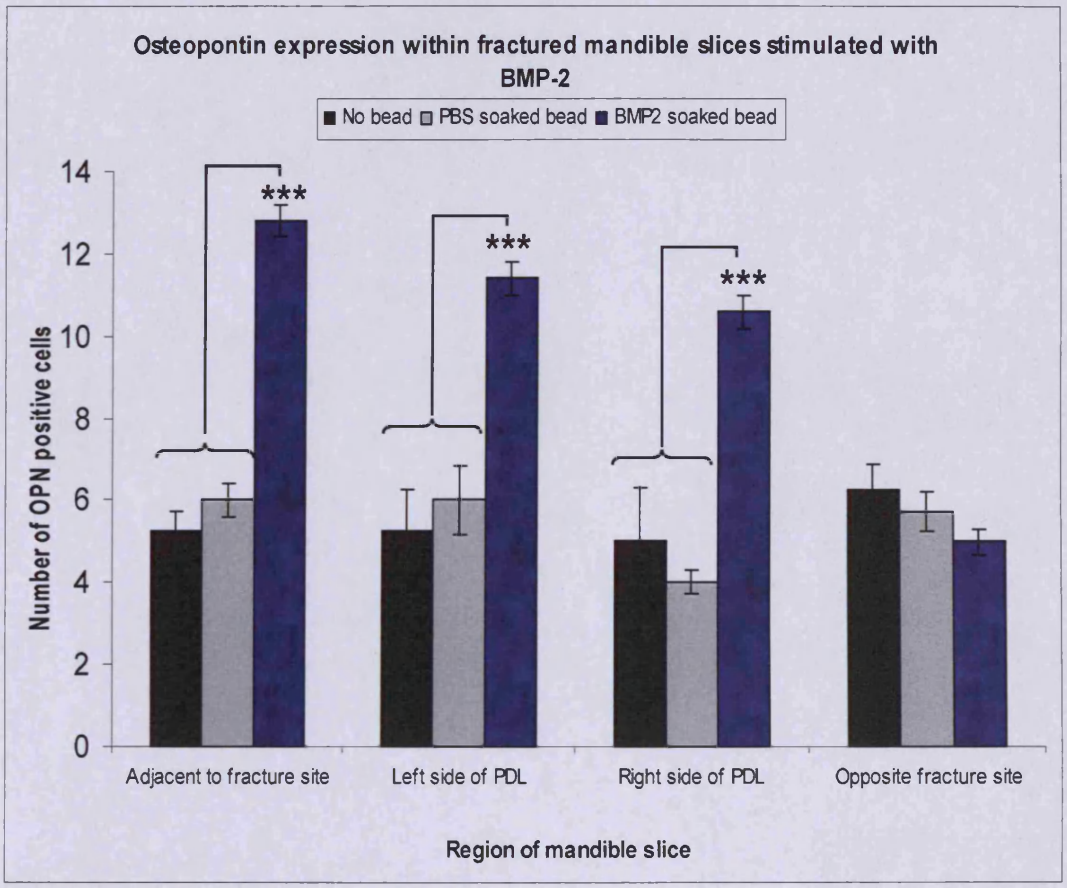

Figure 3.12: Number of osteopontin positive cells within various 100μm² areas of periodontal ligament in fractured mandible slices stimulated with BMP-2, compared with control slices cultured in the presence of bead alone, or in the absence of bead.

*** $p<0.001$

3.3.2.3 Bone sialoprotein immunolocalisation

Localisation of a second bone marker, bone sialoprotein, demonstrated a similar expression pattern to osteopontin, with increased expression observed in TGF-β1 and BMP-2 stimulated mandible slices, within cells of the PDL closely associated with the site of stimulation (Figures 3.13A and 3.14A). Expression again appeared to be reduced within the PDL of control mandible slices cultured with either bead alone (Figures 3.13B and 3.14B) or in the absence of bead (Figures 3.13C and 3.14C), although this could again be due to a decrease in cell number rather than actual expression. Statistical analysis demonstrated that there was a significant increase in the number of BSP positive cells in mandible cultures stimulated with TGF-β1, within 100μm^2 areas of PDL adjacent to the site of stimulation, and also 100μm either side ($p<0.01$) (Figure 3.15). The increase within BMP-2 stimulated slices was also significant, within 100μm^2 areas of PDL adjacent to the site of stimulation, and also 100μm either side ($p<0.001$) (Figure 3.16). Control counts from a mandible region opposite the site of stimulation did not reveal any significant changes in BSP expression within cultures stimulated with either TGF-β1 or BMP-2. BMP-2 treatment appeared to stimulate a greater increase in BSP immunopositivity when compared with TGF-β1 stimulated mandible slices, in a similar way to osteopontin expression, but this was again not shown to be significant. Negative controls (as prepared in 3.3.2.1) showed only the methyl green counterstain with no non-specific staining (Figure 3.13D and 3.14D).

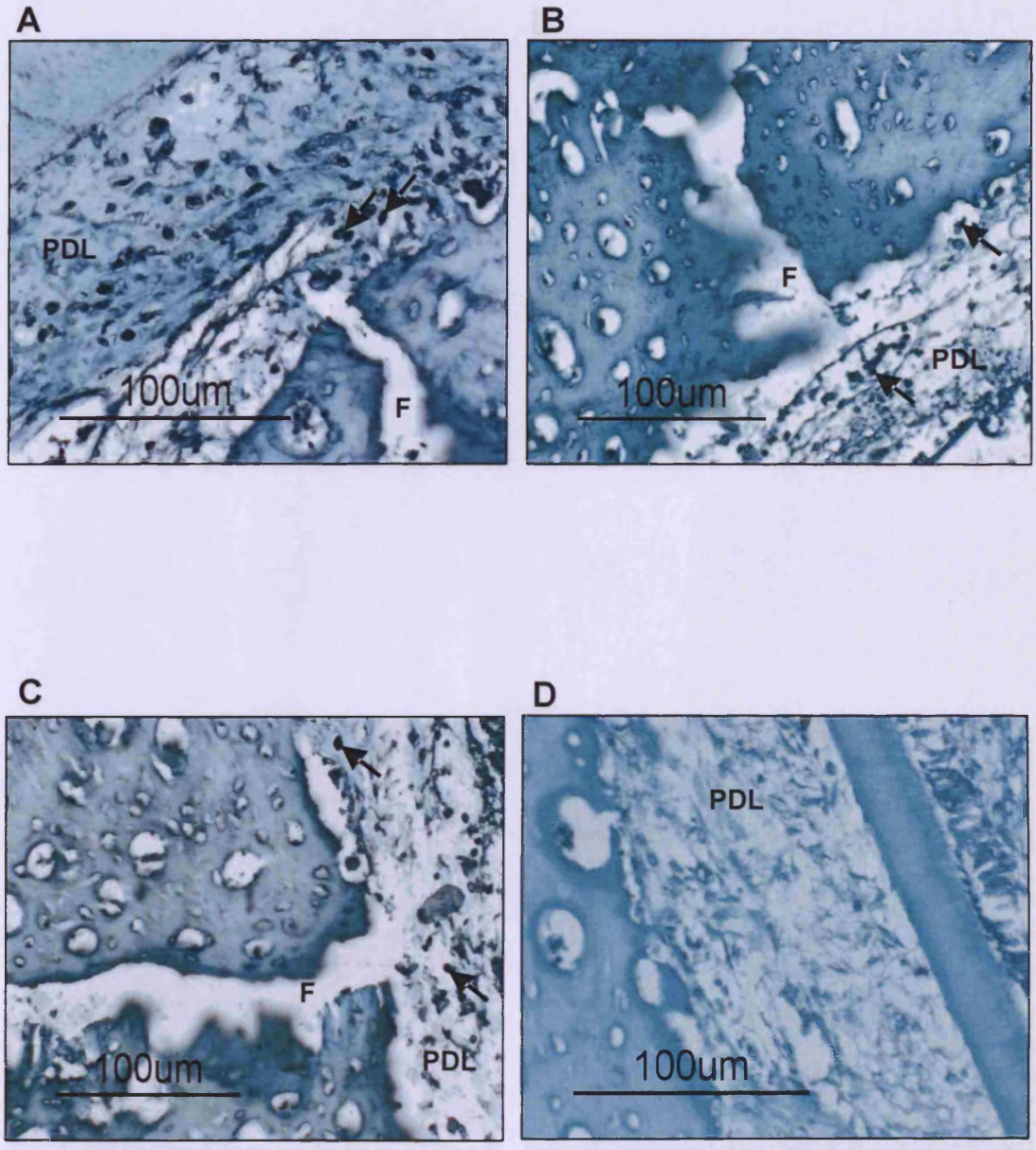

Figure 3.13: Mandible slices immunohistochemically stained for expression of bone sialoprotein (examples of BSP immunopositivity indicated by arrows). Increased cellular expression can be observed within (A) cells of the periodontal ligament (PDL) in mandible slices stimulated with TGF-β1, when compared with (B) control mandible slices cultured with bead alone or (C) control slices cultured in the absence of bead. (D) Negative controls using a non-immune IgG exhibits only the methyl green counterstain.

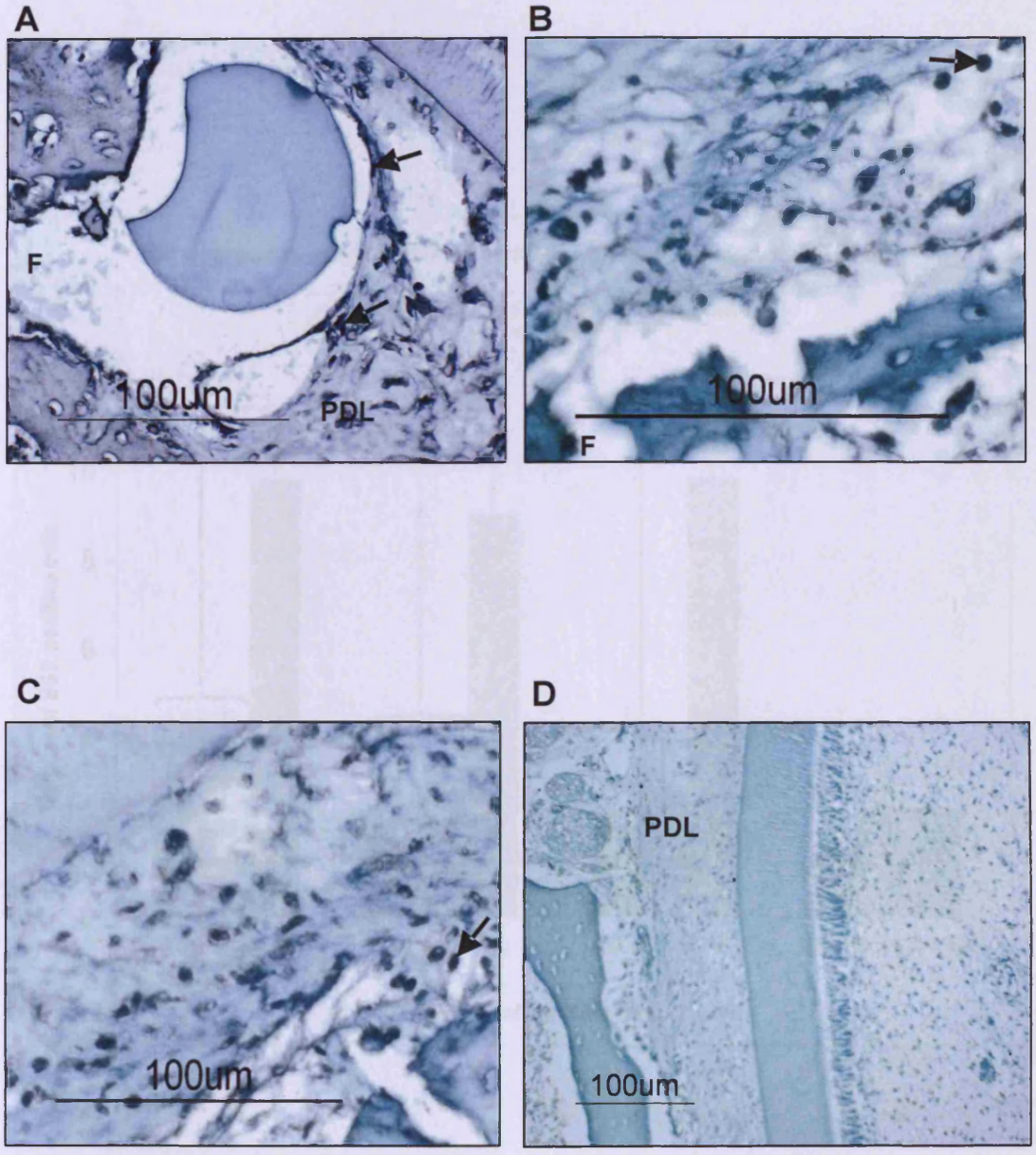

Figure 3.14: Mandible slices immunohistochemically stained for expression of bone sialoprotein. Increased cellular expression (examples indicated by arrows) can be observed within (A) cells of the periodontal ligament (PDL) in mandible slices stimulated with BMP-2, when compared with (B) control mandible slices cultured with bead alone or (C) control slices cultured in the absence of bead. (D) Negative controls using a non-immune IgG exhibits only the methyl green counterstain.

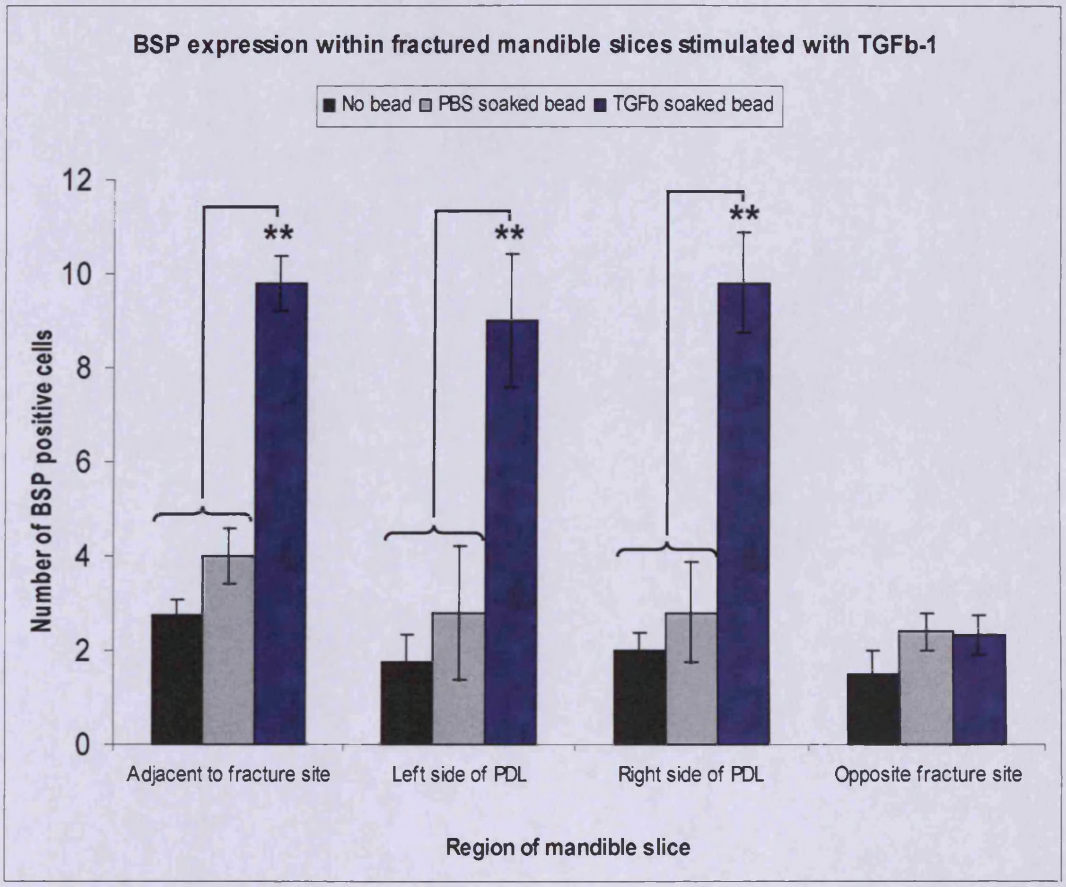

Figure 3.15: Number of bone sialoprotein positive cells within various 100μm² areas of periodontal ligament in fractured mandible slices stimulated with TGF-β1, compared with control slices cultured in the presence of bead alone.

** $p<0.01$

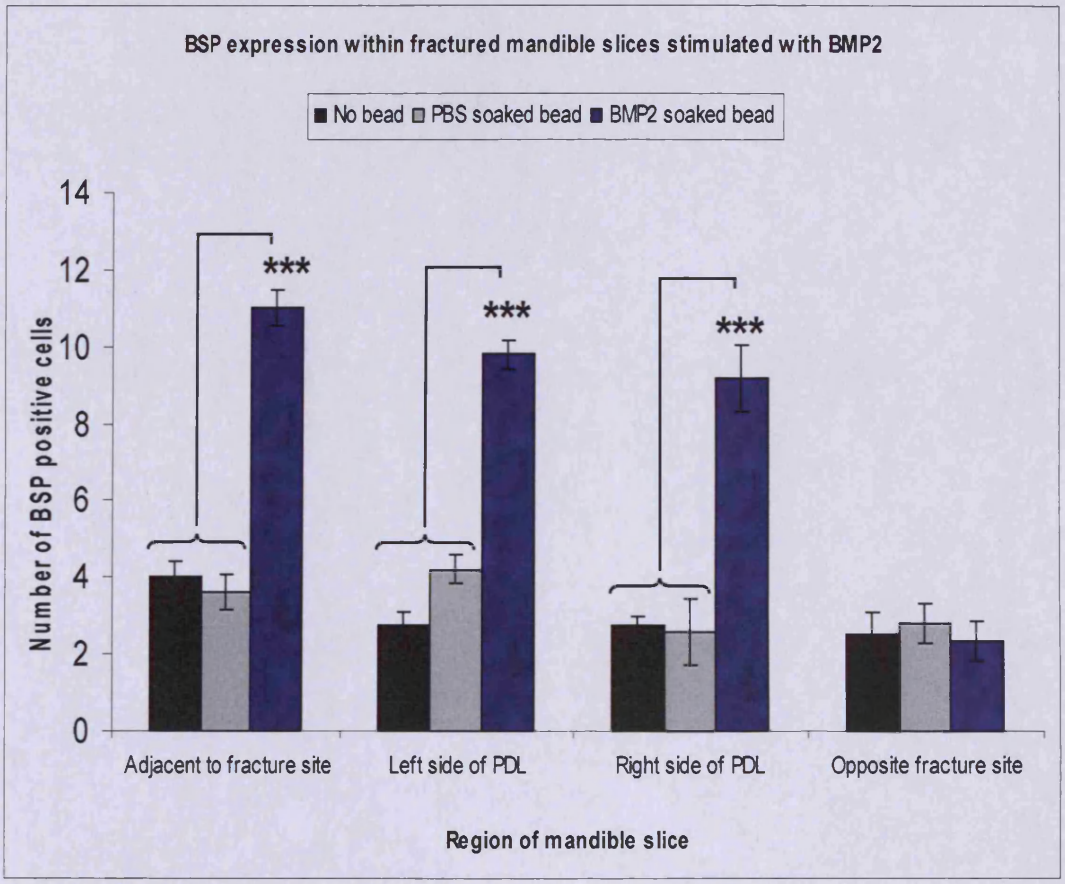

Figure 3.16: Number of BSP positive cells within various 100μm² areas of periodontal ligament in fractured mandible slices stimulated with BMP-2, compared with control slices cultured in the presence of bead alone, or in the absence of bead.

*** $p<0.001$

3.4 DISCUSSION

This study has demonstrated the responsiveness of the *ex vivo* fractured mandible model to exogenously applied growth factors. Both TGF-β1 and BMP-2, key players in bone repair, were capable of affecting cellular behaviour within the model, influencing proliferation, migration, and / or differentiation. Treatment of mandible slices with either growth factor stimulated an aggregation of cells within the PDL adjacent to the site of stimulation, and an increase in both cell number and PCNA immunopositivity within this area. The increase in cell number observed after stimulation with TGF-β1 or BMP-2 could be due to an increase in cell migration towards the site of stimulation; TGF-β1 in particular is known to act as a chemoattractant for osteogenic cells (Lucas 1989). Alternatively, as the PCNA data may suggest, the growth factors may be initiating intracellular signalling cascades to increase cellular proliferation, thus increasing cell numbers (Centrella et al. 1994; Jueren et al. 1999). This increase in cell number and PCNA immunopositivity was closely associated with the agarose bead, as demonstrated in Figures 3.2A and 3.6A, with aggregation of cells, and increased numbers of PCNA immunopositive cells, observed around the edge of the bead. It could be hypothesised that this was simply an edge effect due to the bead being present, but slices cultured in the presence of bead alone (as demonstrated in figure 3.2C) did not show a similar aggregation of cells associated with the bead. This observation was reproducible within each mandible slice. Results suggest that the effects observed are due to the effects of the growth factor, rather than just the

physical presence of the bead itself. Comparisons between TGF-β1 stimulated slices and BMP-2 stimulated slices exposed some differences in both cell number and PCNA immunopositivity, with TGF-β1 appearing to elicit a greater response than BMP-2. Adjacent to the fracture site, an average number of 97 ± 2 cells were counted within TGF-β1 stimulated slices, an increase to the 89 ± 3 cells counted within BMP-2 stimulated slices, with similar differences also observed 100μm^2 either side of the fracture site. Similar differences in PCNA immunopositivity were also observed, with an average increase of 3 PCNA positive cells per 100μm^2 area observed in TGF-β1 stimulated cultures when compared with BMP-2 stimulated cultures, both adjacent to the site of fracture and 100μm either side. Although this difference was not shown to be significant by statistical analysis, it may still indicate a difference in the cellular effects induced by the two growth factors. TGF-β1 is known to be a potent stimulator of both migration and proliferation of mesenchymal stem cells and osteoprogenitors in bone repair (Barnes et al. 1999), and while BMP-2 has also been shown to stimulate proliferation of osteoprogenitors (Shui-bing et al. 2009; Yamaguchi et al. 1991), its main function is to drive the differentiation of these cells in repair processes (Hughes et al. 2006; Lieberman et al. 2002). This may explain the increase in cell numbers and PCNA immunopositivity observed in TGF-β1 stimulated mandible slices compared with slices stimulated with BMP-2. Studies using TGF-β1 in an *ex vivo* tooth slice culture system (Sloan et al. 1998) demonstrated similar results to those presented here, with exogenously applied TGF-β1 having mitogenic effects on subodontoblast cells within the dentine-pulp complex (Dobie et al. 2002; Sloan and Smith 1999).

To assess the effects of TGF-β1 and BMP-2 on the behaviour of the cells within the mandible model, the expression of two bone matrix proteins were investigated: osteopontin, and bone sialoprotein. Expression patterns were similar for both markers, with TGF-β1 and BMP-2 increasing immunopositivity within PDL cells adjacent to the site of stimulation, as well as 100μm either side, probably due to the growth factors diffusing through the tissue from the beads placed within the fracture site. Importantly, no increase in immunopositive cells could be detected within PDL areas opposite to the site of stimulation, or within control slices cultured in the presence of bead alone, indicating that the observed results on cell behaviour were due to the presence of the growth factors, rather than the presence of the bead itself. This data correlates with previous studies demonstrating that TGF-β1 and BMP-2 can upregulate expression of both osteopontin and bone sialoprotein *in vitro* (Hullinger et al. 2001; Lecanda et al. 1997; Ogata et al. 1997), although within the mandible model it is not yet elucidated whether the increase in immunopositivity is due to the growth factors increasing expression of the proteins within target cells, or due to an increased number of cells present within the site of stimulation, thus increasing the immunopositivity observed. Future work could aim to further investigate the cause of this increase in immunopositivity, perhaps using laser capture microdissection to isolate PDL cells from within the mandible tissue. Osteopontin and bone sialoprotein gene levels could then be analysed in the presence or absence of growth factors, to elucidate if these molecules are causing increased expression.

Comparison of immunopositive cell numbers between slices treated with TGF-β1 and BMP-2 indicated an increase in the number of cells positive for both osteopontin and BSP within the PDL of slices stimulated with BMP-2; this increase was not proved to be significant with statistical analysis, but this may be due to the small numbers of immunopositive cells present, so any changes observed may not necessarily be statistically significant. The increase, even though not significant, may still be indicative that within the mandible model BMP-2 is more capable of stimulating osteoblast differentiation than TGF-β1. This hypothesis correlates with published data, since although TGF-β1 is also thought to be involved in stimulating some early stages of osteoblast differentiation (Bostrom and Asnis 1998; Janssens et al. 2005), it is the BMPs which are the most potent stimulators of osteoblast differentiation, with a unique ability to independently induce ectopic bone formation (Cheng et al. 2003a; Katagiri and Takahashi 2002; Phimphilai et al. 2006; Wozney and Rosen 1998).

This study has demonstrated that the *ex vivo* mandible culture model is suitable for investigating the role of specific growth factors in repair processes in bone. The results obtained were reproducible, in terms of both histology and immunohistochemical characterisation, and similar patterns and results were observed within the mandible slices during each experiment. The data obtained from this study correlates with the hypothesis that TGF-β1 and BMP-2 work synergistically together, with TGF-β1 being involved in the recruitment and proliferation of mesenchymal stem cells and osteoprogenitors, and BMP-2 driving the differentiation of these cells down

an osteoblastic lineage. Although only a small selection of proliferation / differentiation markers have been investigated, future work should investigate a larger group of markers, especially downstream markers of TGF-β1 and BMP-2, such as the signalling transducing Smad proteins (Miyazawa et al. 2002), transcription factors such as Runx2, Msx2, and Dlx5, and other proteins such as osteocalcin (Bostrom and Asnis 1998; Janssens et al. 2005; Matsubara et al. 2008; Phimphilai et al. 2006). Expression of TGF-β1 and BMP-2 receptors, known to alter at different stages of osteoblast differentiation and bone repair (Centrella et al. 1995; Onishi et al. 1998; Yamashita et al. 1996), should also be investigated to allow for full validation of the *ex vivo* model, of its responsiveness and functionality.

This study has investigated the effects of exogenous growth factors, added to the model alone; the basis behind growth factor therapies currently in practice (Boden et al. 2000; Friedlaender et al. 2001; Govender et al. 2002; Vaccaro et al. 2003). The reproducibility of the results obtained from the model may make it a valuable tool for testing future novel therapeutics, such as bioactive materials, for clinical use. However, the synergy apparent between these various bioactive factors means that investigation of the many endogenous growth factors present *in vivo* within the bone matrix itself may be essential in advancing these therapies.

CHAPTER 4

GROWTH FACTOR EXPOSURE FROM BONE SURFACES BY CHEMICAL TREATMENT MODALITIES

4.1 INTRODUCTION

The extracellular matrix of bone is known to contain stores of endogenous growth factors bound within it (Frolik et al. 1988; Hauschka et al. 1986; Taipale and Keski-Oja 1997). Release of these molecules following bone injury or trauma allows presentation of these factors to target cells, and subsequent cellular signalling to initiate reparative processes at these sites, leading to optimal repair and regeneration of the bone (Ramirez and Rifkin 2003; Schonherr and Hausser 2000). Interactions between growth factors and the ECM can be mediated by proteins such as collagen (Reddi 2000), binding protein antagonists such as noggin and chordin in the case of the BMPs (Reddi 2001), or by proteoglycans; macromolecules comprised of at least one glycosaminoglycan (GAG) chain attached to a core protein. Cytokines and growth factors can interact with either the GAG moieties of the proteoglycans, or their core proteins, and different cytokines bind to different proteoglycans (Baker et al. 2009; Bernfield et al. 1999; Ruoslahti 1989; Ruoslahti and Yamaguchi 1991). For example, it has been widely shown that the small leucine-rich proteoglycans decorin and biglycan can bind TGF-β1 with high affinity, and that this interaction is mediated via the core protein (Hildebrand et al. 1994; Imai et al. 1997; Yamaguchi et al. 1990). Such

immobilisation of growth factors within the bone matrix provides an accessible reservoir of molecules crucial in repair processes, and may also protect the growth factors from degradation (Ruoslahti and Yamaguchi 1991).

Release of matrix-bound growth factors, via osteoclastic resorption in response to bone injury, results in the presence of a 'cocktail' of bioactive molecules at the site of trauma (Dallas et al. 2002). This enables the growth factors to work synergistically, activating a complex network of signalling pathways and stimulating optimal repair processes within the damaged bone (Hughes et al. 2006; Lieberman et al. 2002). This *in vivo* situation contrasts to current clinical therapies utilising bioactive growth factors, where treatments usually contain only a single growth factor (Gautschi et al. 2007). These growth factor therapies face problems due to the massive milligram doses required to elicit a therapeutic response in humans, doses which are in stark contrast to the natural *in vivo* situation (Reddi 1998). Future clinical treatments may benefit from utilising the array of growth factors already residing *in vivo* within the bone matrix, rather than addition of exogenous growth factors. There are several chemical treatments that have been demonstrated to solubilise growth factors such as TGF-β1 from within the mineralised matrix of dentine, including acids, ethylenediaminetetraacetic acid (EDTA), and calcium hydroxide (Graham et al. 2006; Zhao et al. 2000). Acids, including EDTA, solubilise growth factors by matrix demineralisation, by local matrix dissolution or by chelating calcium ions present within the mineralised matrix (De-Deus et al. 2008). It is, however, unclear whether this simply releases bound growth factors, or if a more complex mechanism is

responsible for the release of the matrix-bound bioactive molecules. Calcium hydroxide appears to solubilise growth factors via a unique mechanism, since other alkaline agents have not previously been reported to solubilise matrix components from dentine, and the proteins released after matrix dissolution with calcium hydroxide differ from those released with EDTA (Graham et al. 2006). This may suggest that interaction of growth factors and proteins with the ECM is more complex than simple mineral binding, although currently the mechanism for growth factor solubilisation by calcium hydroxide is unknown.

This thesis chapter aims to further investigate the actions of these chemical treatment modalities, by developing an *in vitro* bone slab model to analyse the release of growth factors from within bone matrix. Chemical treatments will include EDTA, citric acid, and calcium hydroxide, known to release growth factors as mentioned above. Slabs will also be treated with sodium hydroxide, an alkaline not previously shown to release bioactive molecules, to compare with the alkaline calcium hydroxide. In particular, the release of TGF-β1 will be analysed, since this growth factor plays a pivotal role in bone repair, stimulating migration and proliferation of osteoprogenitors (Barnes et al. 1999; Bostrom and Asnis 1998; Janssens et al. 2005). Reduced serum levels of TGF-β1 have also been demonstrated in patients exhibiting delayed bone healing (Zimmermann et al. 2005). Release of TGF-β1 from the matrix may therefore be of clinical relevance in the treatment of impaired bone healing. By developing and utilising this model, the aim is also to analyse the effects of the released bioactive molecules on bone marrow stromal cell behaviour, in terms of both proliferation and differentiation.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of tissue culture reagents

Washing Medium

Washing medium of alpha-modified Minimum Essential Medium (α-MEM) (Invitrogen, UK) was prepared containing 10% concentration of a penicillin / streptomycin / amphotericin solution (containing 10,000 units of penicillin, 10mg/ml of streptomycin, and 25µg/ml amphotericin B) (Invitrogen, UK).

Culture Medium

Mineralising culture medium of α-MEM (Invitrogen, UK) was prepared containing 15% heat inactivated FCS (Invitrogen, UK), 1% concentration of a penicillin / streptomycin / amphotericin solution (containing 10,000 units of penicillin, 10mg/ml of streptomycin, and 25µg/ml amphotericin B) (Invitrogen, UK), 10mM β-glycerophosphate (Invitrogen, UK), 10nM dexamethasone (Sigma Chemical Co, UK), and 50µg/ml ascorbic acid (Invitrogen, UK).

4.2.2 Preparation and chemical treatment of bone slabs

Bone slabs were prepared from the femurs of 28 day old male Wistar rats, freshly sacrificed by CO_2 asphyxiation. Femurs were dissected and cut in half, longitudinally. The marrow was removed from the marrow cavity and the remaining bone was then cut into 2mm slabs using a sterile scalpel. The femur bone slabs were incubated in 1mg/ml collagenase / dispase (Sigma

Chemical Co, UK) for 30 min to enzymatically disrupt cellular bonds, washed thoroughly in 70% ethanol and PBS, and freeze-thawed three times to remove all residual cells from the bone surface. Bone slabs were either left untreated, or treated with chemical agents known to solubilise growth factors from the bone matrix; 17% EDTA (pH 7.2), 0.02M calcium hydroxide (pH 11.7, saturated solution), 0.2M sodium hydroxide (pH 11.7), 10% citric acid (pH 3), or as a negative control, PBS (pH 7.4). Bone slabs were treated for 5 min, 10 min, or 15 min, gently rinsed in distilled water, and air-fixed for 48 hrs, in sterile conditions. A total of six bone slabs were used per treatment, for each time point.

4.2.3 Immunogold labelling of released TGF-β1 from bone surfaces

Treated femur bone slabs were immunostained for TGF-β1 using an immunogold procedure with scanning electron microscope (SEM) imaging (de Harven et al. 1984; Zhao et al. 2000). After fixation, bone slabs were blocked for 1 hr with 5% goat serum and 10% BSA in TBS, followed by a 15 min buffer wash (1% goat serum, 1% BSA, 0.1% Tween 20 in TBS). Bone slabs were then incubated with the primary antibody, a polyclonal rabbit anti-human TGF-β1 antibody (Santa Cruz Biotech) diluted 1:100 in buffer, for 2 hrs. Following incubation with primary antibody, bone slabs were washed twice in buffer, and incubated with a gold-labelled goat anti-rabbit secondary antibody (British Biocell International, particle diameter 30nm, protein 3.6μg/ml) diluted 1:100, for 1 hr. As a negative control, staining was inhibited by preincubation with a 10-fold excess of specific blocking peptide (Santa

Cruz Biotech). Following a further two washes in buffer and two washes in distilled water, bone slabs were incubated with silver enhancing agent (British Biocell International) for 15 min. All bone slabs were then immediately washed in distilled water for 2 x 15 min, air dried for 24 hrs, and examined under an EBT1 scanning electron microscope (SEMTech Ltd, Derbyshire, UK) at a magnification of x 550. The number of gold particles were automatically counted within 20μm^2 areas of bone per slab, within five random fields of view, using Image ProPlus software. A total of 6 bone slabs were used for each time point, for each treatment. Standard errors of the mean were calculated, and mean values were analysed using one-way ANOVA and Tukey's post hoc test. Differences in TGF-β1 release were analysed between the time points of each treatment, as well as between different chemical treatment types.

4.2.4 Isolation and culture of bone marrow stromal cells

Femurs were dissected from 28 day old male Wistar rats, freshly sacrificed by CO_2 asphyxiation, soft tissue removed with a sterile scalpel blade, and placed into sterile washing medium. Bone marrow stromal cells were collected from each femur by inserting a 21 gauge syringe needle containing 15ml of culture media into the marrow cavity and flushing out the contents into a T75 culture flask (Greiner Bio-One). Cells were cultured at 37°C, in an atmosphere of 5% CO_2 in air, in a humidified incubator, for 5 days. After five days culture media was removed and the cells incubated with 5ml trypsin-EDTA (Invitrogen, UK) at 37°C for 5 min to detach cells from their monolayer.

Cells were then resuspended in 1ml culture medium, counted using a haemocytometer, and seeded onto individual bone slabs in a 96-well tissue culture plate (Greiner Bio-One) at a density of $1x10^4$ cells/cm^2. Bone slabs were either pre-treated with EDTA for 15 min, calcium hydroxide for 5 min, as described in 4.2.2, or were left untreated as a negative control. Cells were cultured at 37°C, in an atmosphere of 5% CO_2 in air, for 7 days, with media changes every 48 hrs.

4.2.5 Visualisation of cellular cytoskeleton

After 7 days of culture, media was removed from the bone slabs and the cells were fixed in 2% paraformaldehyde for 30 min. Cells were then washed in TBS for 3 x 10 min. To visualise the actin cytoskeleton with phalloidin, cells were permeabilised in 3% Triton-X 100 for 30 min. To prevent non-specific binding, cells were blocked with 1% BSA for 1 hr. Cells were incubated for 2 hrs at 4°C in the dark with 0.2ml of phalloidin-FITC (diluted 1:16) (Sigma Chemical Co, UK), followed by a 1 hr incubation at room temperature in the dark with the nuclear counterstain bisbenzimide (Sigma Chemical Co, UK). Following a final wash in 0.5% Tween 20 in TBS, bone slabs were removed from the 96-well plate and mounted onto glass microscope slides. Cells were viewed under an Olympus AX70 fluorescence microscope and photographed with a Nikon DXM digital camera and ACT-1 imaging software. A total of five bone slabs were used for each treatment. Cell numbers were automatically counted within 200μm^2 areas of bone stained with phalloidin, using Image ProPlus software. 200μm^2 areas within five random fields of view were

counted per slab, and standard errors of the mean calculated. Mean values were analysed using one-way ANOVA and Tukey's post hoc test. Each treatment type was compared with untreated bone slabs, to statistically analyse differences in cell numbers in response to each chemical treatment. Comparisons were also made between the different treatment types, to statistically analyse any cell number differences between chemical treatment types.

4.2.6 Immunocytochemical characterisation of cells

After 7 days of culture, media was removed from the bone slabs and the cells were fixed in 2% paraformaldehyde for 30 min. Cells were then washed in TBS for 3 x 10 min. To prevent non-specific binding, cells were blocked with 1% BSA for 1 hr, then incubated overnight at room temperature with 0.2ml of the primary antibody for osteopontin (clone LF-124) (Fisher et al. 1995) or alkaline phosphatase (Santa Cruz Biotech), diluted appropriately in 1% BSA in TBS (Table 4.1). For negative controls, a nonimmunogenic control antibody (IgG isotype control; Sigma Chemical Co, UK) was used instead of the primary, along with a primary exclusion.

Following incubation with primary antibody, the cells were washed in 0.5% Tween 20 in TBS for 3 x 10 min and incubated in the presence of 0.2ml of the appropriate secondary antibody (Table 4.1), diluted in 1% BSA in TBS. A nuclear counterstain, bisbenzimide (Sigma Chemical Co), was included with the secondary antibody at a dilution of 1:50. Cells were incubated at room

temperature for 1 hr in the dark. Following a final wash in 0.5% Tween 20 in TBS, bone slabs were removed from the 96-well plate and mounted onto glass microscope slides. Cells were viewed under an Olympus AX70 fluorescence microscope and photographed with a Nikon DXM digital camera and ACT-1 imaging software. Image ProPlus software was used to automatically count the number of cell nuclei (stained blue by bisbenzimide), as well as the number of cells expressing either alkaline phosphatase or osteopontin (stained green with FITC), within five random 200μm^2 fields of view per bone slab. This enabled a percentage of positive cells to be calculated. A total number of 5 bone slabs were cultured for each treatment, for each marker.

Primary antibody	**Source of primary antibody**	**Primary antibody source / species reactivity**	**Primary antibody dilution**	**Secondary antibody**	**Secondary antibody dilution**
Osteo-pontin	Larry Fisher (Fisher et al. 1995)	Monoclonal mouse anti-human	1:50	Goat anti-mouse IgG FITC-conjugated	1:50
Alkaline Phos-phatase	Santa Cruz Biotech	Polyclonal rabbit anti-human	1:50	Goat anti-rabbit IgG FITC-conjugated	1:50

Table 4.1. Immunocytochemistry antibodies.

4.3 RESULTS

4.3.1 Immunogold labelling of released TGF-β1 from bone surfaces

4.3.1.1 EDTA treated bone surfaces

SEM examination of bone surfaces treated with 17% EDTA (pH 7.2) for 5 min (Figure 4.1A) and 10 min (Figure 4.1B) yielded few TGF-β1 molecules, with an average of 6 ± 1.3 immunogold particles counted per 20μm^2 area of bone surface for each treatment time (Figure 4.5). However, after extension of the treatment time to 15 min (Figure 4.1C), the number of released TGF-β1 molecules increased to an average of 27 ± 4.4 immunogold particles per 20μm^2 area of bone surface (Figure 4.5). Statistical analysis showed this increase to be highly significant ($p<0.001$). Negative control bone surfaces treated with PBS (Figure 4.1D) showed negligible TGF-β1 after all treatment times (Figure 4.5), while negative controls incubated with an excess of specific peptide to TGF-β1 showed no immunogold labelling (Figure 4.1E).

4.3.1.2 Calcium hydroxide treated bone surfaces

SEM examination of bone surfaces treated with 0.02M calcium hydroxide (pH 11.7) revealed a pattern of TGF-β1 release different from that observed with EDTA treatment. A treatment time of 5 min (Figure 4.2A) released an average number of 39 ± 8.6 TGF-β1 labelled immunogold particles per 20μm^2 area of bone surface (Figure 4.5). Subsequent treatment times of 10

min (Figure 4.2B) and 15 min (Figure 4.2C) revealed a decline in TGF-β1, with an average of 11 ± 1.6 and 6 ± 0.9 immunogold particles counted per 20μm^2 area of bone surface respectively (Figure 4.5). Statistical analysis showed the number of immunogold particles to be significantly higher after a treatment time of 5 min, when compared to either 10 min or 15 min treatments ($p<0.001$). The maximum number of released TGF-β1 particles after calcium hydroxide treatment (39 ± 8.6) was larger than the maximum released after EDTA treatment (27 ± 4.4). Negligible TGF-β1 was observed after all treatment times on negative control bone surfaces treated with PBS (Figures 4.2D and 4.5). Negative controls incubated with an excess of specific peptide to TGF-β1 showed no immunogold labelling (Figure 4.2E).

4.3.1.3 Citric acid treated bone surfaces

SEM examination of bone surfaces treated with 10% citric acid (pH 3) yielded a similar pattern of immunogold labelling to bone surfaces treated with EDTA, with release of TGF-β1 increasing with treatment time. Treatment times of 5 min (Figure 4.3A) and 10 min (Figure 4.3B) yielded average immunogold counts of 11 ± 1.5 and 13 ± 0.8 particles per 20μm^2 area of bone surface, respectively. Extending the treatment time to 15 min (Figure 4.3C) increased the average number of gold particles per 20μm^2 area of bone surface to 23 ± 0.9 (Figure 4.5), although this increase was not shown to be significant ($p>0.05$). The increase after 15 min was, however, significant when compared with PBS treated control surfaces ($p<0.05$) (Figure 4.3D), which showed negligible TGF-β1 after all treatment times (Figure 4.5). Comparison

between citric acid and EDTA revealed a higher number of released immunogold particles after EDTA treatment; 27 ± 4.4 particles compared to 23 ± 0.9. No immunogold labelling was observed on negative controls incubated with an excess of specific peptide to TGF-β1 (Figure 4.3E).

4.3.1.4 Sodium hydroxide treated bone surfaces

Treatment of bone surfaces with 0.2M sodium hydroxide (pH 11.7) produced a pattern of TGF-β1 immunogold labelling different from all other treatment types, including calcium hydroxide. Treatment with sodium hydroxide for 5 min (Figure 4.4A) yielded an average number of 12 ± 2.2 immunogold particles per 20μm^2 area of bone surface, which was increased to 21 ± 3.1 particles after extension of the treatment time to 10 min (Figure 4.4B). Further extension of the treatment time to 15 min led to a decrease in immunogold particles, with an average of 17 ± 1.4 particles counted per 20um^2 area of bone surface (Figure 4.4C). The increase in immunogold particles after 10 min treatment was not statistically significant when compared to 5 min or 15 min treatment times ($p>0.05$), but it was significantly higher than PBS treated control surfaces ($p<0.05$) (Figure 4.4D), which showed negligible TGF-β1 after all treatment times (Figure 4.5). The maximum number of TGF-β1 immunogold particles released after 10 min sodium hydroxide treatment (21 ± 3.1) was significantly lower than the maximum number released after 5 min calcium hydroxide treatment (39 ± 8.6) ($p<0.05$). Negative controls which were incubated with an excess of specific peptide to TGF-β1 showed no immunogold labelling (Figure 4.4E).

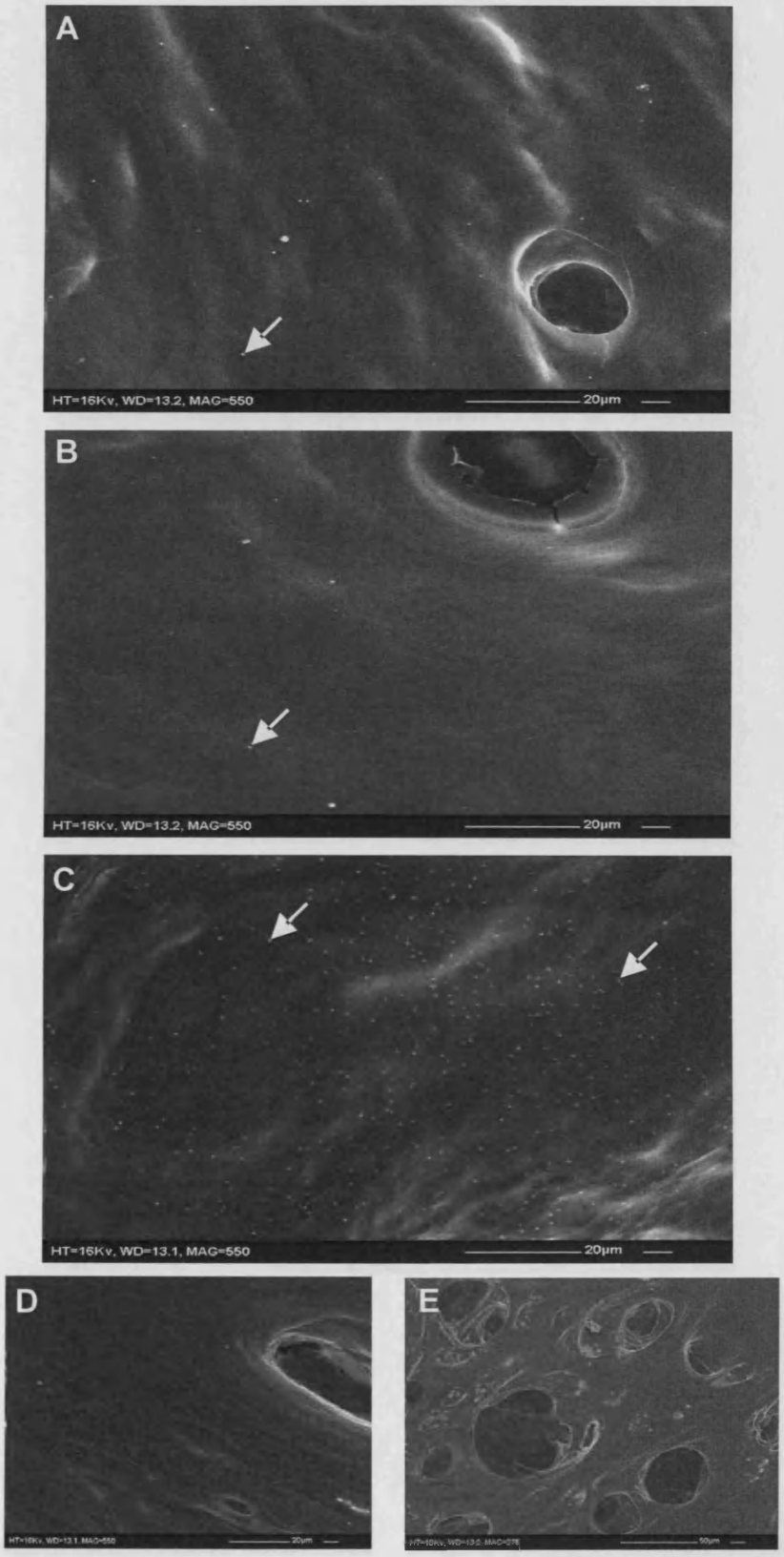

Figure 4.1: Immunogold labelling of TGF-β1 (as indicated by arrows) released from bone surfaces treated with 17% EDTA for (A) 5 min, (B) 10 min, or (C) 15 min. Negative controls were treated with (D) PBS or (E) incubated with a 10-fold excess of specific peptide to block TGF-β1 staining.

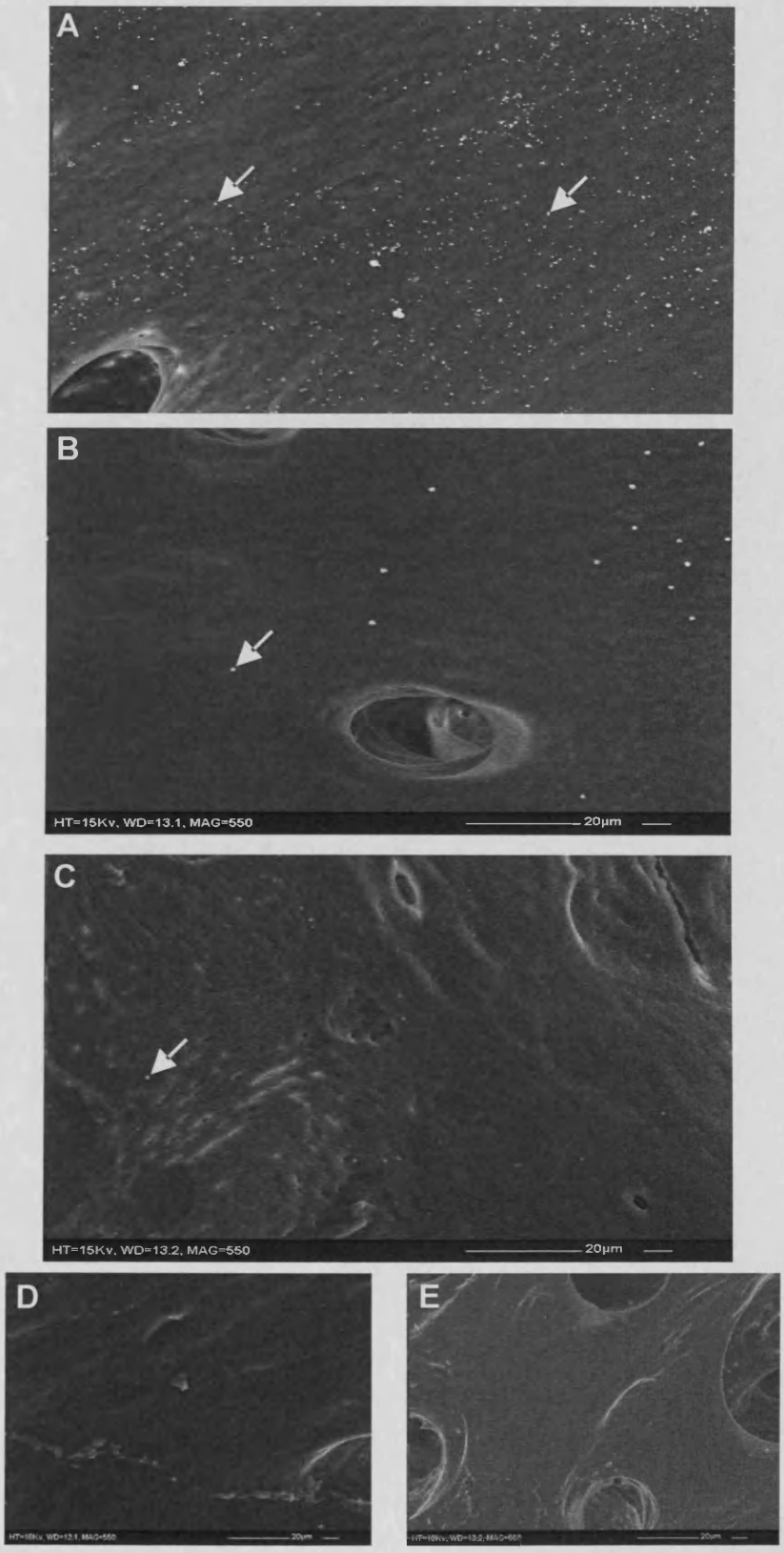

Figure 4.2: Immunogold labelling of TGF-β1 (indicated by arrows) released from bone surfaces treated with 0.02M $Ca(OH)_2$ for (A) 5 min, (B) 10 min, or (C) 15 min. Negative controls were treated with (D) PBS or (E) incubated with a 10-fold excess of specific peptide to block TGF-β1 staining.

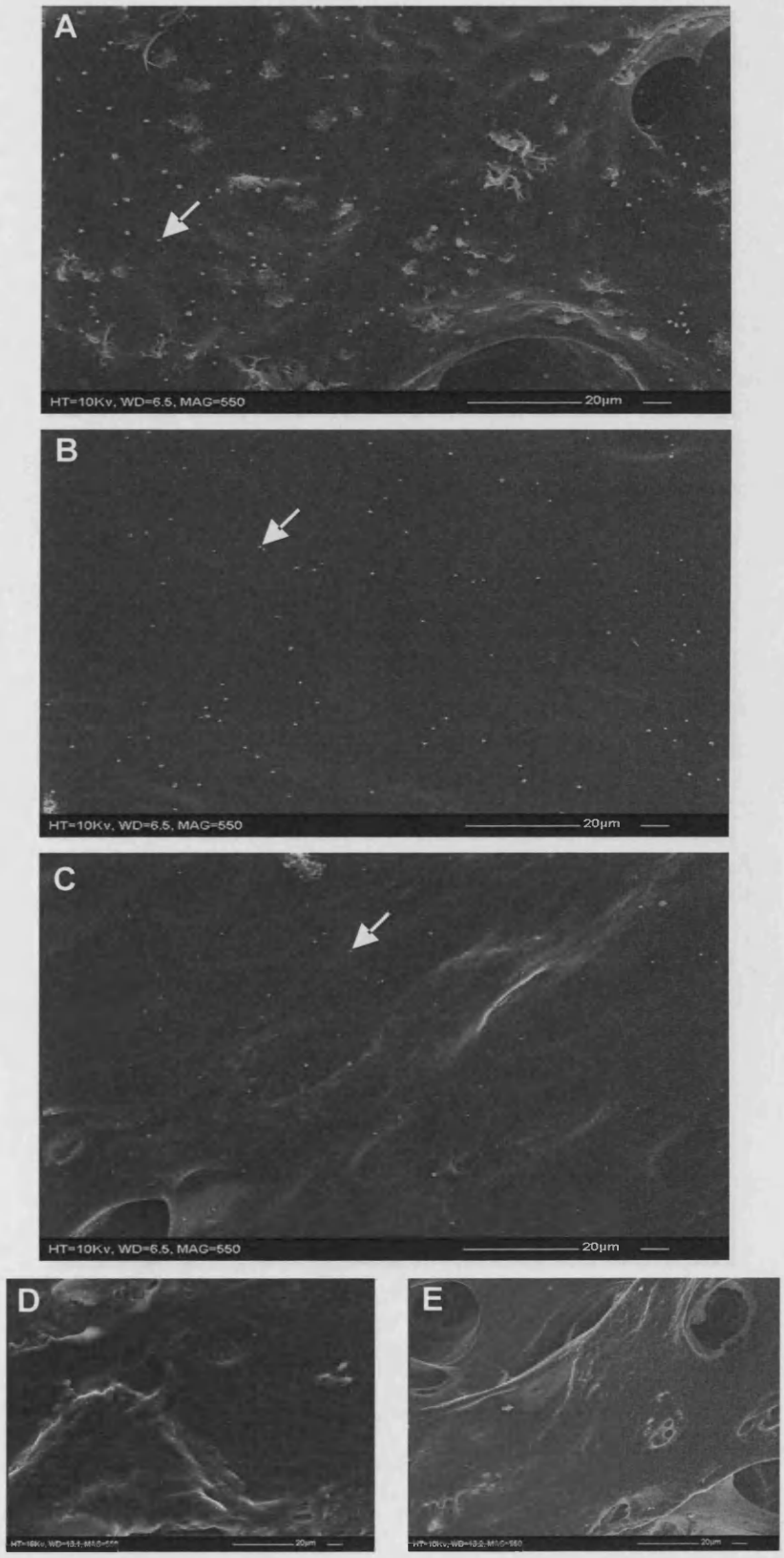

Figure 4.3: Immunogold labelling of TGF-β1 (indicated by arrows) released from bone surfaces treated with 10% citric acid for (A) 5 min, (B) 10 min, or (C) 15 min. Negative controls were treated with (D) PBS or (E) incubated with a 10-fold excess of specific peptide to block TGF-β1 staining.

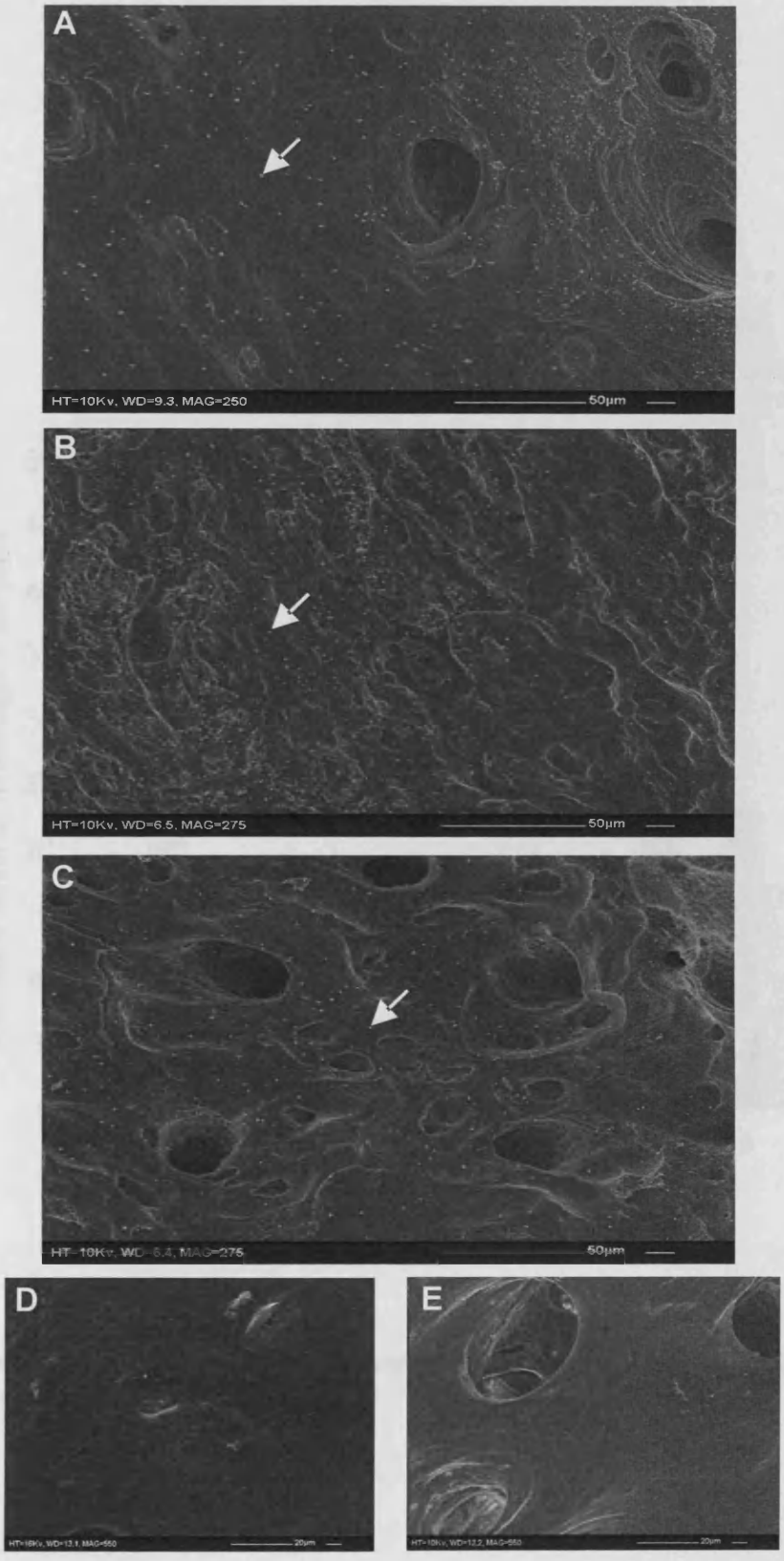

Figure 4.4: Immunogold labelling of TGF-β1 (indicated by arrows) released from bone surfaces treated with 0.2M NaOH for (A) 5 min, (B) 10 min, or (C) 15 min. Negative controls were treated with (D) PBS or (E) incubated with a 10-fold excess of specific peptide to block TGF-β1 staining.

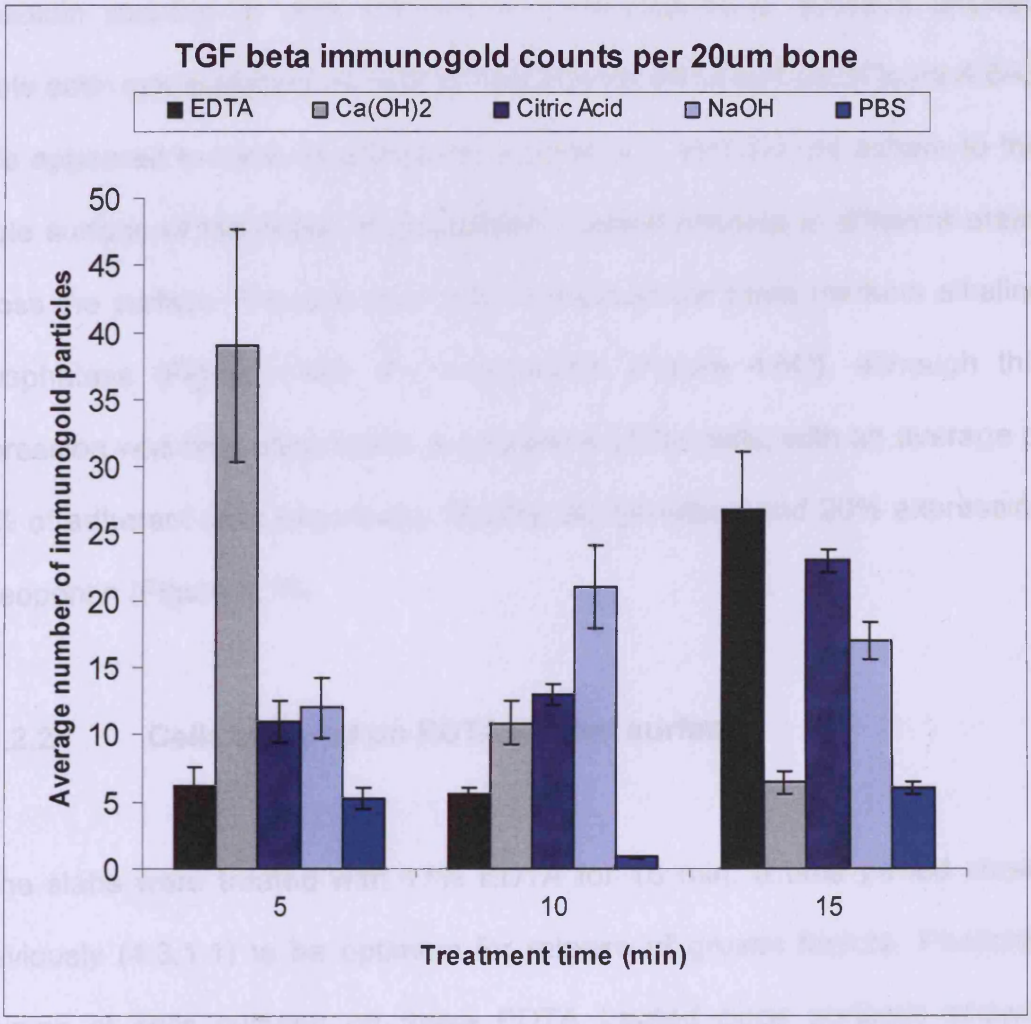

Figure 4.5: Number of TGF-β1 labelled immunogold particles per 20μm² area of treated bone surface.

4.3.2 Immunocytochemical characterisation of cultured cells

4.3.2.1 Cells cultured on untreated surfaces

Phalloidin staining of cells cultured on untreated bone surfaces showed visible actin cytoskeletons of cells adhered to the bone surface (Figure 4.6A). Cells appeared to have an elongated morphology, and did not adhere to the whole surface of the bone, but appeared in small clusters in different areas across the surface. The adherent cells expressed the bone markers alkaline phosphatase (Figure 4.6B) and osteopontin (Figure 4.6C), although this expression was only observed in a proportion of the cells, with an average of 22% of adherent cells expressing alkaline phosphatase and 20% expressing osteopontin (Figure 4.10).

4.3.2.2 Cells cultured on EDTA treated surfaces

Bone slabs were treated with 17% EDTA for 15 min, a time period shown previously (4.3.1.1) to be optimum for release of growth factors. Phalloidin staining of cells cultured on these EDTA treated bone surfaces showed visible actin cytoskeletons of cells adhered to the bone surface (Figure 4.7A). In contrast to untreated bone surface, cells had a more cuboidal morphology and appeared to adhere across the entire surface of the bone slab, rather than in discrete areas. Cell numbers appeared to be higher on EDTA treated surfaces when compared with untreated surfaces, which was confirmed with automated cell counting. The average number of cells on EDTA treated bone

was calculated to be 34 ± 7.5 cells per 200μm^2 area, a significant increase from the average number of cells on untreated bone, calculated as 9 ± 0.5 cells per 200μm^2 area of bone ($p<0.01$) (Figure 4.9). There appeared to be a higher number of cells expressing the bone markers alkaline phosphatase and osteopontin on EDTA treated bone slabs (Figures 4.7A and B) when compared with untreated bone slabs, with an average of 81% of adherent cells expressing alkaline phosphatase and 72% expressing osteopontin (Figure 4.10). Whether this increase was due to increased cellular expression or rather an increase in cell numbers remains to be elucidated.

4.3.2.3 Cells cultured on calcium hydroxide treated surfaces

Bone slabs were treated with 0.02M calcium hydroxide for 5 min, a time period shown previously (4.3.1.2) to be optimum for release of growth factors. Phalloidin staining showed cells with visible actin cytoskeletons adhered across the surface of these calcium hydroxide treated bone slabs (Figure 4.8A). Adherence of cells to the calcium hydroxide treated bone slabs, and their morphology, was very similar to those cultured on EDTA treated surfaces, with cells having a more cuboidal morphology than those cultured on untreated surfaces and adhering across the entire bone surface. Cell numbers again appeared increased when compared to cells cultured on untreated bone surfaces, which was confirmed with automated cell counting. The average number of cells adhered to calcium hydroxide treated bone surfaces was calculated to be 29 ± 3.7 cells per 200μm^2 area of bone, a significant increase from the 9 ± 0.5 cells per 200μm^2 area adhered to

untreated bone ($p<0.05$) (Figure 4.9). Statistical analysis indicated that there was no significant difference in cell numbers adhered to calcium hydroxide treated bone surfaces and EDTA treated surfaces ($p>0.05$). Similarly to EDTA treatment, a higher proportion of cells appeared to be expressing alkaline phosphatase and osteopontin within calcium hydroxide treated bone slabs (Figures 4.7B and C) when compared with untreated bone slabs, with an average of 80% of adherent cells expressing alkaline phosphatase and 75% expressing osteopontin (Figure 4.10). Again this could be due to an increase of cell number rather than an actual increase in protein expression.

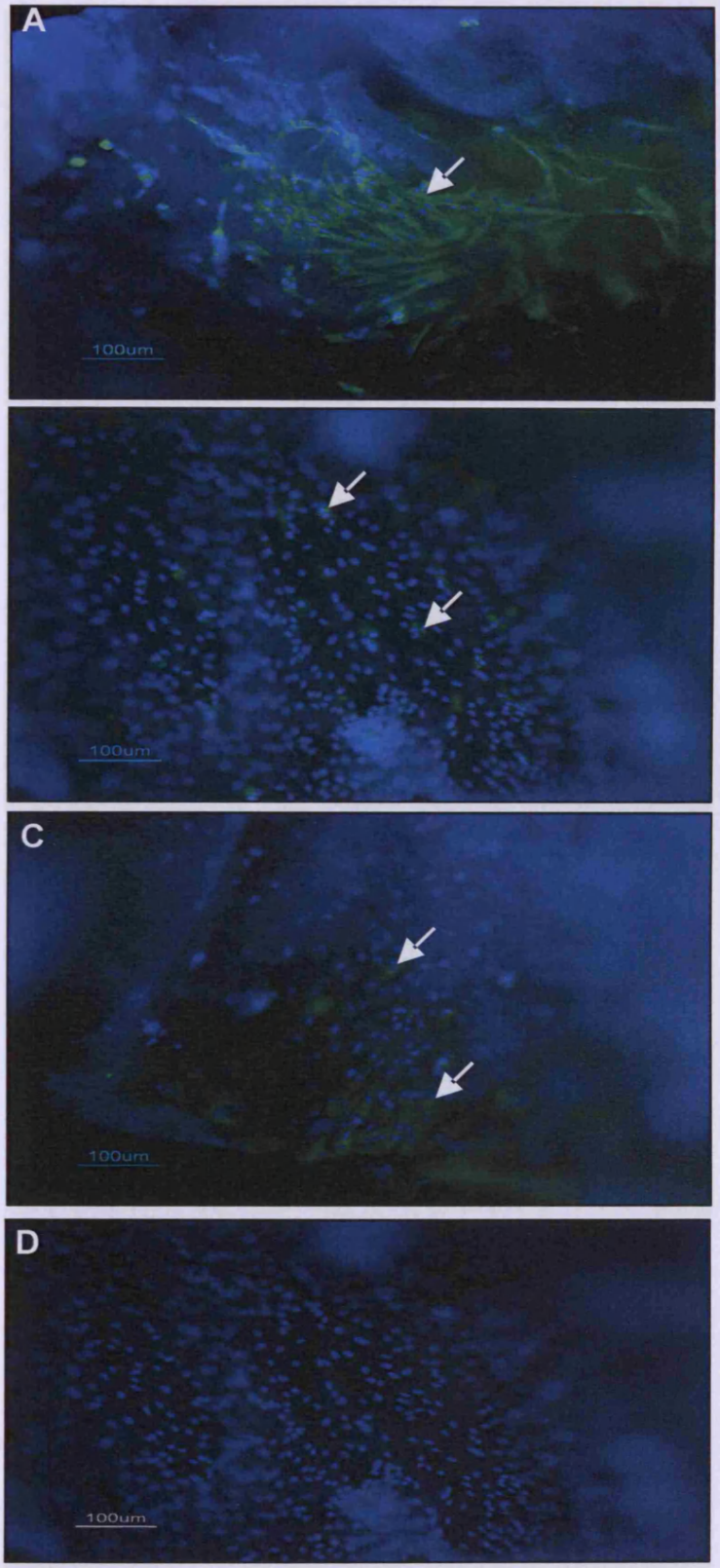

Figure 4.6: Immunocytochemical characterisation of cells cultured on untreated bone slabs. (A) Phalloidin staining demonstrated adherence of cells to the bone. (B) Adherent cells expressed the bone markers alkaline phosphatase and (C) osteopontin (examples indicated by arrows). (D) Negative controls using a non-immune IgG showed only the nuclear counterstain.

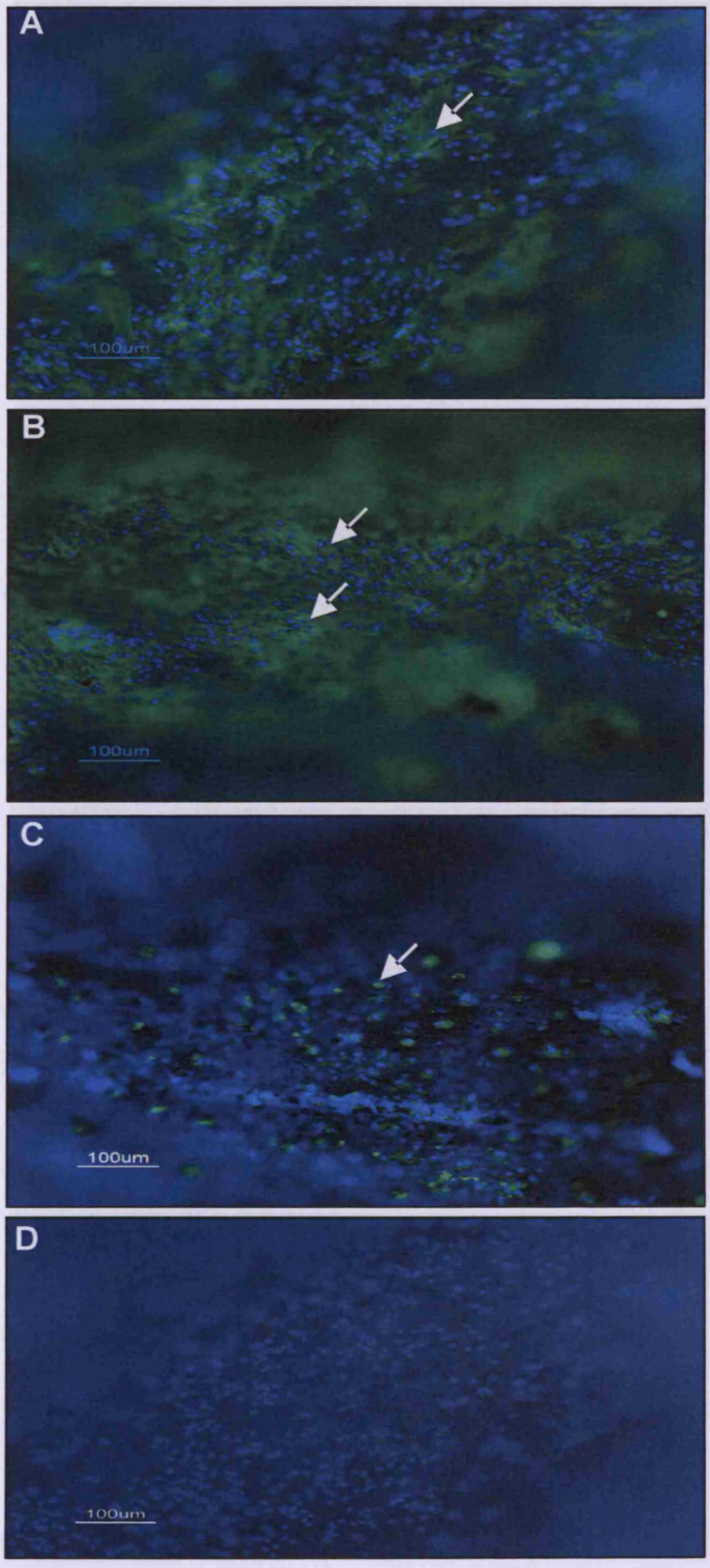

Figure 4.7: Immunocytochemical characterisation of cells cultured on bone slabs treated with 17% EDTA. (A) Phalloidin staining demonstrated increased adherence of cells to the treated bone and a more cuboidal morphology. (B) Adherent cells expressed the bone markers alkaline phosphatase and (C) osteopontin (examples indicated by arrows). (D) Negative controls using a non-immune IgG showed only the nuclear counterstain.

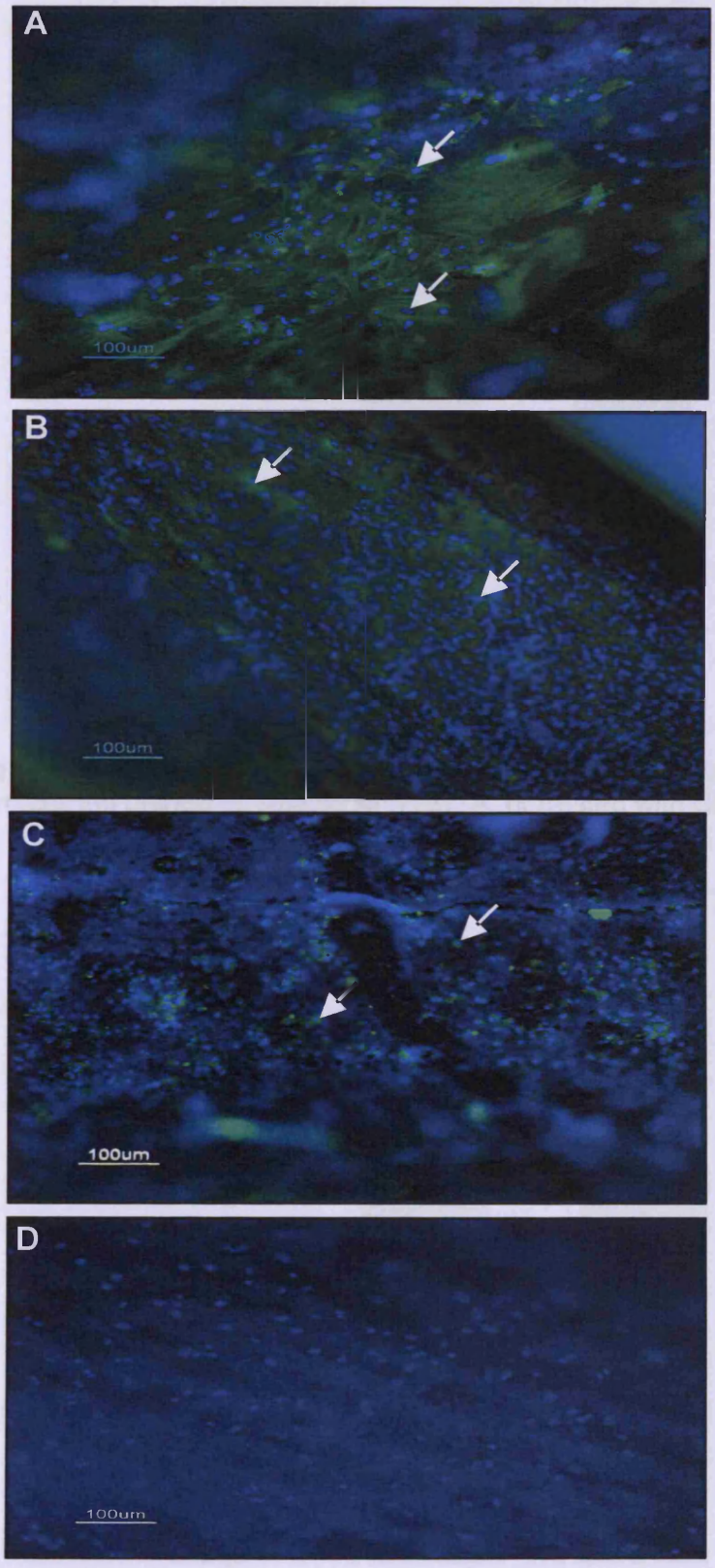

Figure 4.8: Immunocytochemical characterisation of cells cultured on bone slabs treated with 0.02M $Ca(OH)_2$. (A) Phalloidin staining demonstrated increased adherence of cells to the treated bone and a more cuboidal morphology. (B) Adherent cells expressed the bone markers alkaline phosphatase and (C) osteopontin (examples indicated by arrows). (D) Negative controls using a non-immune IgG showed only the nuclear counterstain.

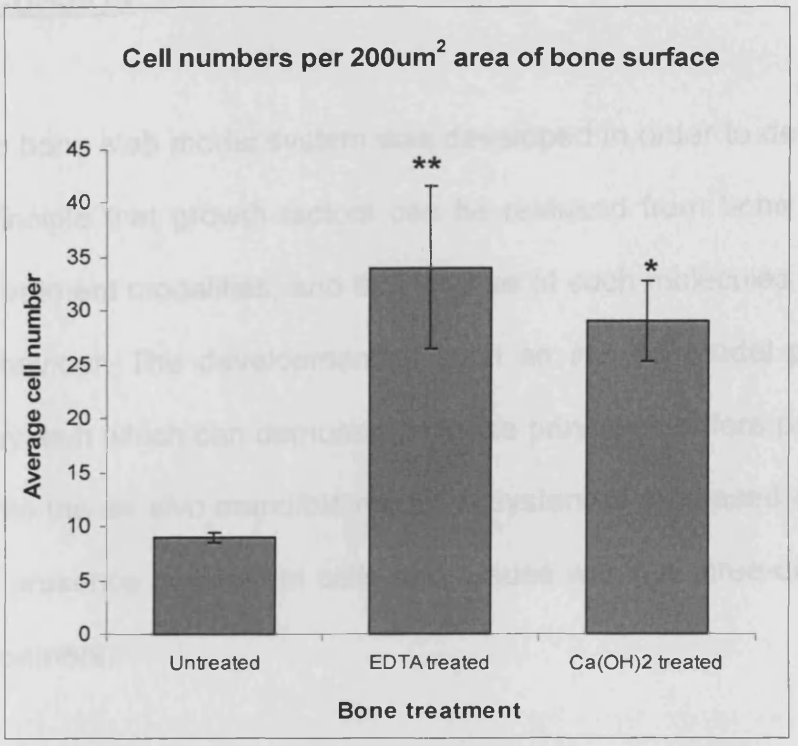

Figure 4.9: Average number of phalloidin stained cells cultured on bone surfaces left untreated, treated with EDTA, or treated with $Ca(OH)_2$.

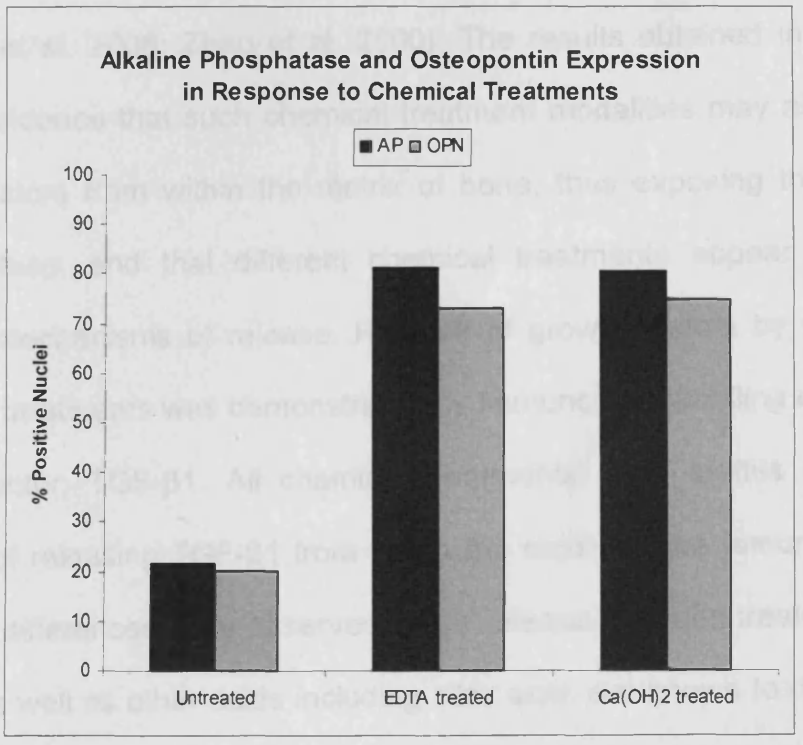

Figure 4.10: Average % positive cell nuclei for alkaline phosphatase or osteopontin expression on bone surfaces left untreated, treated with EDTA, or treated with $Ca(OH)_2$.

4.4 DISCUSSION

This *in vitro* bone slab model system was developed in order to demonstrate proof of principle that growth factors can be released from bone matrix by chemical treatment modalities, and that release of such molecules can affect cellular behaviour. The development of such an *in vitro* model provides a simplified system which can demonstrate these principles before progressing the work into the *ex vivo* mandible model, a system of increased complexity due to the presence of different cells and tissues within a three-dimensional microenvironment.

Release of growth factors by chemical treatment modalities has been shown extensively in dentine, in particular using EDTA or calcium hydroxide (Graham et al. 2006; Zhao et al. 2000). The results obtained in this study provide evidence that such chemical treatment modalities may also release growth factors from within the matrix of bone, thus exposing them on the bone surface, and that different chemical treatments appear to employ different mechanisms of release. Release of growth factors by the varying chemical treatments was demonstrated by immunogold labelling of one such growth factor, TGF-β1. All chemical treatments used in this study were capable of releasing TGF-β1 from within the matrix of the femur bone slab, although differences were observed in the release between treatment types. EDTA, as well as other acids including citric acid, are known to demineralise both bone and dentine matrix (De-Deus et al. 2008; Walsh and Christiansen 1995; Yamaguchi et al. 1996). They accomplish this by either local

dissolution of mineralised matrix, releasing calcium ions, or by acting as chelating agents capable of binding and sequestering calcium ions within the mineralised matrix, thus reducing their activity (De-Deus et al. 2008; Dorozhkin 1997; O'Connell et al. 2000; Yamaguchi et al. 1996). Both of these treatments displayed a similar pattern of TGF-β1 solubilisation, with the majority of solubilisation occurring only after a treatment time of 15 min. There were a small number of TGF-β1 particles released after 5 min and 10 min treatment, which may indicate an initial gradual release by the chemicals. However, the number of particles released after these earlier treatment times were similar to those observed from PBS treated control slabs, and may therefore be residual TGF-β1 particles that have remained on the bone surface, rather than particles released by the treatments. The similar solubilisation patterns observed with EDTA and citric acid treatment suggests that both chemicals release growth factors by a similar mechanism. EDTA did however release increased quantities of TGF-β1 from the bone when compared with citric acid, suggesting that although the mechanism of release is similar for both chemicals, EDTA may be more efficient at solubilising the matrix. This may be due to EDTA being a stronger acid, having pKa values of 1.99, 2.67, 6.16 and 10.26 (Inoue et al. 1995), compared to higher pKa values of 3.13, 4.76 and 6.40 for citric acid (Silva et al. 2009), which may confer a greater ability for matrix dissolution to EDTA. It could also be due to EDTA being a more potent chelating agent, due to the presence of six binding sites within the chemical that may associate with calcium ions, sequestering them and reducing their activity (Barnett and Uchtman 1979). It is also possible that citric acid, at a 10% solution (far more concentrated than

the 10mM citric acid used to activate recombinant TGF-β1 used previously in section 3.2.1) with a pH of 3, is capable of denaturing proteins (including TGF-β1), whereas EDTA which is optimum at a physiological pH of 7.2, may not have such an effect. This is supported by evidence from previous studies demonstrating that EDTA is capable of releasing bioactive molecules from dentine. Graham and colleagues (Graham et al. 2006) demonstrated that application of EDTA-extracted dentine matrix proteins to an odontoblast-like cell line induced TGF-β1 expression in a dose-dependent manner, while Tomson and colleagues (Tomson et al. 2007) used ELISA methodology to demonstrate the presence of active TGF-β1 in dentine matrix proteins extracted with EDTA. These studies suggest that EDTA treatment, at least in part, is not denaturing the growth factors it releases. This hypothesis is also supported in our study, since EDTA treatment (and subsequent growth factor release) is capable of eliciting effects on cells cultured upon such treated surfaces. If time had permitted, and perhaps an experiment to consider for future work, it may have been constructive to culture bone marrow stromal cells on citric acid treated surfaces, to determine if growth factors released with this chemical treatment were capable of affecting cellular behaviour. It may be hypothesised that if citric acid denatured growth factors, thus releasing fewer bioactive molecules, there may be a decrease in the cellular effects observed with this treatment compared with EDTA.

The TGF-β1 release observed with EDTA and citric acid was in contrast to the pattern of release observed with calcium hydroxide treatment, which solubilised the greatest quantity of TGF-β1 after a treatment time of only 5

min, with very little release observed with extended treatment times. Interestingly, treatment of the bone slabs with sodium hydroxide, an alkaline with the same pH as calcium hydroxide, showed a pattern of release that differed from all other treatment types, including calcium hydroxide. Solubilisation of growth factors from the mineralised matrix of bone or dentine by alkaline agents other than calcium hydroxide has not previously been reported, but the results displayed in this study demonstrate that sodium hydroxide is capable of releasing TGF-β1 from within the matrix of bone. The majority of TGF-β1 was released after a 10 min treatment time with sodium hydroxide, although the quantity released was significantly lower than the maximum quantity released with calcium hydroxide after a treatment time of 5 min ($p<0.05$). As yet, the mechanism for growth factor solubilisation by alkaline treatments such as calcium hydroxide remains elusive. One possible hypothesis is that the alkaline agents alter the charge of growth factors within the mineralised matrix of the bone, since an amino acid placed in alkaline conditions will favour its negatively charged anion form. This may alter the interaction of the growth factor with the matrix, facilitating release of the protein and exposure on the bone surface. Alternatively, it may be that the alkaline reagents affect the matrix proteins that keep the growth factors within their bound state, again perhaps by influencing their charge, by the disassociated molecules of the base binding or interacting with these proteins. Both calcium hydroxide and sodium hydroxide are strong bases, used within this study at the same pH of 11.2, although sodium hydroxide is a slightly stronger base with a lower pKb value of 0.2, compared to values of 2.43 and 1.4 for calcium hydroxide. Thus the differences observed in the

release of TGF-β1 by these two chemicals may be attributed to their composition. Calcium hydroxide has two hydroxyl groups available for interaction with matrix proteins and / or growth factors, affecting the charge of such proteins and thus possibly affecting the release of growth factors from the matrix. Sodium hydroxide, however, has only a single hydroxyl group, so may therefore interact to a lesser degree with sequestered growth factors or matrix proteins, as it may have a reduced capacity to alter the protein charge. Thus it may be hypothesised that calcium hydroxide has a greater efficacy for altering protein charge and thus growth factor release. An alternative hypothesis to alkaline chemicals facilitating actual release of growth factors may be that interaction of these treatments with matrix proteins could be having an effect on the antigens of the growth factors, making the antibody-recognised epitope more accessible and thus increasing the number of immunogold particles observed. However, this study has also shown that cellular behaviour is significantly affected by calcium hydroxide treatment of bone slabs, suggesting that bioactive growth factors are being released by this treatment, rather than simply the antigenicity of the growth factors being affected.

Release of growth factors from within the mineralised matrix of bone by chemical treatment modalities can only be considered clinically relevant if the molecules released are active, and thus capable of affecting cell behaviour at a site of bone injury. Culture of primary bone marrow stromal cells on treated and untreated bone slab surfaces provided a model system to investigate cell growth and differentiation in response to such chemical treatments. The bone

marrow contains a crucial store of mesenchymal stem cells and osteoprogenitor cells that are capable of migrating towards a site of bone injury, responding to growth factors and signals once there which stimulate differentiation of these cells down the osteoblastic lineage (Bielby et al. 2007). Cultured cells were first stained with phalloidin, to visualise the actin cytoskeleton of the cells and their adherence to the bone slabs, to determine if chemical treatments were affecting the morphology, growth or adhesion of cells to the bone slabs. Cells cultured on untreated bone slabs did not adhere to the whole surface of the bone, but were present in small clusters in different areas across the surface, and had a more elongated morphology, more like that of a fibroblast cell. This correlates with previous studies which have demonstrated such a fibroblast-like morphology of bone marrow stromal cells when cultured on plastic surfaces for up to 7 days of culture (Ciapetti et al. 2006; ter Brugge and Jansen 2002), suggesting that untreated bone slabs may have similar characteristics to smooth surfaces in terms of their effects on cell morphology. Once the bone slabs had been treated however, with either EDTA (for 15 min) or calcium hydroxide (for 5 min), the cells appeared to adhere across the whole surface of the bone slab, and cell numbers were significantly increased when compared with untreated bone slabs. Cells also appeared more cuboidal than those cultured on untreated surfaces, with morphology more resembling mature cells of the osteoblast lineage. For EDTA treatment, one hypothesis for the increase in cell numbers and change in morphology may be the alteration of surface topography. It is well documented that surface topography can markedly affect the behaviour of osteoblast progenitors, with rougher surfaces facilitating attachment of cells,

as well as their differentiation down the osteoblast lineage (Anselme 2000; Boyan et al. 1998; Boyan et al. 1996; Deligianni et al. 2001b; Hatano et al. 1999). It is possible that treatment of the bone slabs with EDTA alters the surface topography; it has already been demonstrated in dentine that EDTA treatment can remove the smear layer and roughen the dentine surface (Buchalla et al. 2007; Eldeniz et al. 2005; Zhao et al. 2000). Thus EDTA may be having a similar effect on the bone slabs, roughening their surface by demineralising the matrix and facilitating the adhesion of bone marrow stromal cells (Deligianni et al. 2001b). There is conflicting evidence regarding the effects of surface topography on cellular proliferation, but some previous studies have shown an increase in proliferation of bone marrow stromal cells and osteoblasts cultured upon roughened surfaces (Deligianni et al. 2001a; Kamal et al. 2001; Kim et al. 2007; Naoki et al. 2008). In conjunction with EDTA treatment releasing growth factors that may increase cellular proliferation, including the FGFs (Mansukhani et al. 2000), IGFs (Hughes et al. 2006), and TGF-β1 (Barnes et al. 1999), it is possible that EDTA treatment is both facilitating adhesion of cells onto the bone surface, and increasing their proliferation, thus increasing cell numbers.

Calcium hydroxide, which also significantly increased cell numbers, is unlikely to alter surface topography as EDTA treatment may do, as it is not involved in surface demineralisation, but instead may alter the charge of proteins and growth factors within the bone matrix. This may cause an increase in the hydrophillicity of the bone surface, which previous studies have shown to increase proliferation and differentiation of bone marrow

stromal cells (Anselme 2000; Kim et al. 2007; Naoki et al. 2008; Wall et al. 2009). Thus alteration of hydrophillicity, together with the released growth factors mentioned above, may explain the increased cell numbers observed upon bone slabs after treatment with calcium hydroxide.

Cells cultured on the bone slabs were also stained for two markers of osteoblast differentiation, osteopontin and alkaline phosphatase, to investigate the differentiation state of the adherent cells. Cells adherent to untreated bone slabs did express both osteopontin and alkaline phosphatase, although the expression was observed in only a proportion of the cells, with 22% expressing alkaline phosphatase and 20% expressing osteopontin. This observation would perhaps be expected, due to the culture of the cells in a supplemented media including dexamethasone that promotes differentiation down an osteoblastic lineage. However, treatment of the bone surfaces with either EDTA or calcium hydroxide appeared to further stimulate expression of these bone markers when compared with untreated slabs, with an apparent increase in the number of cells expressing osteopontin and alkaline phosphatase: 81% of cells were positive for alkaline phosphatase and 72% positive for osteopontin after treatment with EDTA, while 80% of cells were positive for alkaline phosphatase and 75% positive for osteopontin after calcium hydroxide treatment. This observed increase may be due to the chemical treatments stimulating an increase in cellular expression of the proteins, or may be due to the increased number of cells already positive for alkaline phosphatase and osteopontin adhering to the treated bone surfaces (Hatano et al. 1999; Schwartz et al. 1999). Growth factors released by both

EDTA and calcium hydroxide, such as the BMPs (Cheng et al. 2003a; Wozney 1992) and the IGFs (Celil and Campbell 2005), may stimulate osteoblast differentiation of the bone marrow stromal cells. As mentioned previously, EDTA alteration of surface topography may also influence the differentiation of the cultured bone marrow stromal cells, with previous studies demonstrating an increase in alkaline phosphatase and osteocalcin on rougher surfaces (Deligianni et al. 2001a; Hatano et al. 1999), while calcium hydroxide may affect differentiation via its effects on hydrophillicity (Anselme 2000; Wall et al. 2009).

The results presented in this study appear to demonstrate that growth factors can be released from within the mineralised matrix of bone, and that these factors can actively influence the behaviour of cells cultured on the bone surface. The data presented here may suggest that development of future clinical therapies for treating cases of impaired bone repair may benefit from utilising chemical treatments to release bioactive growth factors already residing *in vivo* within the matrix of the bone, rather than adding in exogenous growth factors. In this way, a 'cocktail' of bioactive molecules may be released into the site of injury, enabling these factors to work synergistically, and ultimately stimulating optimal repair processes within the injured bone.

CHAPTER 5

STIMULATION OF THE *EX VIVO* FRACTURED MANDIBLE MODEL WITH CHEMICALLY RELEASED ENDOGENOUS GROWTH FACTORS

5.1 INTRODUCTION

The previous chapter demonstrated that the bone matrix contains many endogenous growth factors that can be released *in vitro* by chemical treatment modalities, including EDTA and calcium hydroxide. It also demonstrated that release of these bioactive growth factors *in vitro* may affect cellular behaviour, by influencing proliferation and differentiation of osteoprogenitor cells. Following this demonstration of proof of principle within an *in vitro* bone slab cell culture system, the aim of this chapter was to develop this further into an *ex vivo* system, to examine the effects of these growth factor-releasing chemical treatments on the cells residing with the *ex vivo* mandible model. While the *in vitro* cell culture system developed in the previous chapter was a useful model to demonstrate growth factor release, and to gain some insight into the release mechanisms of the various chemical treatments, the culture of a single cell type does not represent the many interactions that occur between cell and tissue types during bone repair *in vivo* (Arnold 1987; Hughes et al. 2006; Schindeler et al. 2008). The *ex vivo* model contains a range of different cell and tissue types cultured *in situ*, in a three-dimensional environment as opposed to the monolayer of *in vitro* cell culture, and therefore allows examination of the effects of the chemical

treatments in a situation far more representative of an *in vivo* situation. The presence of the fracture site within the model allows the chemical treatments to be tested in a situation representative of bone repair *in vivo*, without the complicating systemic influences, and the ethical and cost implications associated with *in vivo* studies.

This study may also be compared with the previous results acquired from treatment of the mandible model with TGF-β1 and BMP-2, to determine if releasing a 'cocktail' of endogenous growth factors from within the bone matrix elicits a greater response than adding single exogenous growth factors. As mentioned previously, application of single exogenous bioactive factors is the basis behind current growth factor clinical therapies (Friedlaender et al. 2001; Gautschi et al. 2007; Govender et al. 2002), but the limited success that these treatments have had may be addressed by utilising endogenous growth factors already bound within the matrix of bone. Previous studies have demonstrated that the use of combinations of different growth factors can improve both wound healing (Lynch et al. 1989; Lynch et al. 1987) and bone regeneration (Meraw et al. 2000). The possibility of releasing combinations of endogenous growth factors within an *in vivo* situation may therefore provide a promising alternative clinical treatment for bone repair. In dentistry applications calcium hydroxide-based materials such as Dycal® are widely used to drive release of growth factors from dentine matrix, facilitating formation of a dentine bridge to repair the dentine-pulp complex after injury (Graham et al. 2006; Hörsted-Bindslev et al. 2003; Tziafas et al. 2000). Emdogain, a purified enamel matrix protein product, is

also used in clinical periodontal treatment to aid tissue regeneration, increasing growth factor release and inducing formation of cementum and alveolar bone (He et al. 2004; Nakamura et al. 2001). Expansion of such dental-based treatments, or development of novel treatments that can facilitate growth factor release, for use in bone injury therapeutics may overcome some of the problems associated with current growth factor therapies using exogenously added bioactive molecules. Development of the *ex vivo* mandible organ culture system may provide an ideal model system with which to test such therapeutics.

5.2 MATERIALS AND METHODS

5.2.1 Preparation and chemical treatment of fractured mandible slices

Fractured mandible slices were prepared from 28 day old male Wistar rats immediately before chemical treatment (as described in 2.5.1). Prior to embedding in semi-solid agar medium, 10µl of either 17% EDTA (pH 7.2), 0.02M calcium hydroxide (pH 11.7, saturated solution), or a control solution of PBS (pH 7.4) was applied to the fracture site with a 2mm microbrush (Microbrush International) under a dissecting microscope (Bausch and Lomb). The microbrush was used to ensure that each chemical treatment was applied to the whole surface of the fracture site, in the same amount of 10µl. Slices were treated for 15 min (EDTA), or 5 min (calcium hydroxide and PBS), and slices were partially submerged in media during treatment times, so that the slice remained hydrated but the chemical treatment did not wash away from the site of application at the fracture site. After each treatment time, slices were gently washed in PBS for 3 x 2 min to remove the chemical treatment, and transferred into Trowel type cultures (as described in 2.2.2) for 7 days.

5.2.2 Histological examination

After culture, fractured mandible slices stimulated with EDTA / calcium hydroxide (plus respective control slices) were prepared for histological H&E staining (as described in section 2.2.3). A total of 5 mandible slices were

cultured for each treatment type and each control. Cell numbers were automatically counted within randomly selected 100μm^2 areas of the PDL using Image ProPlus software. Cells were counted within the fracture site itself, within 100μm^2 of the PDL adjacent to the site of fracture, and within 100μm^2 areas of the PDL to the left and right of the fracture site. A control count was also taken within a 100μm^2 area of the PDL at a mandible region opposite the site of stimulation. Standard errors of the mean were calculated, and values analysed using one-way ANOVA and Tukey's post hoc test, to analyse differences between treatment types and controls.

5.2.3 Immunohistochemistry

Fractured mandible slices stimulated with EDTA / calcium hydroxide, plus respective control slices, were stained immunohistochemically for expression of the proliferation marker PCNA, and the bone markers osteopontin and bone sialoprotein, as described in sections 2.2.6 and 3.2.4. A total of 5 mandible slices were cultured for each treatment type and control, for each marker. Cells positive for each of the markers were automatically counted within randomly selected 100μm^2 areas of the PDL, both adjacent to the site of fracture, and to the left and right of the fracture site, using Image ProPlus software. A control count was also taken within a 100μm^2 area of the PDL at a mandible region opposite the site of stimulation. Standard errors of the mean were calculated, and mean values were analysed using one-way ANOVA and Tukey's post hoc test, to analyse differences between treatment types and controls.

5.3 RESULTS

5.3.1 Histology of pre-treated mandible slices

Mandible slices pre-treated with either EDTA (Figure 5.1A) or calcium hydroxide (Figure 5.1B) and cultured for 7 days demonstrated an increase in the number of PDL cells close to the site of fracture and stimulation, when compared with areas opposite the stimulation site (Figure 5.1C) or with control PBS treated slices (Figure 5.1D). Automated cell counts (Figure 5.2) showed this increase in cell number to be significant in EDTA and calcium hydroxide treated slices, both adjacent to the site of fracture ($p<0.001$), and 100μm either side ($p<0.01$). No differences were observed in cell number between mandible slices pre-treated with EDTA and slices pre-treated with calcium hydroxide.

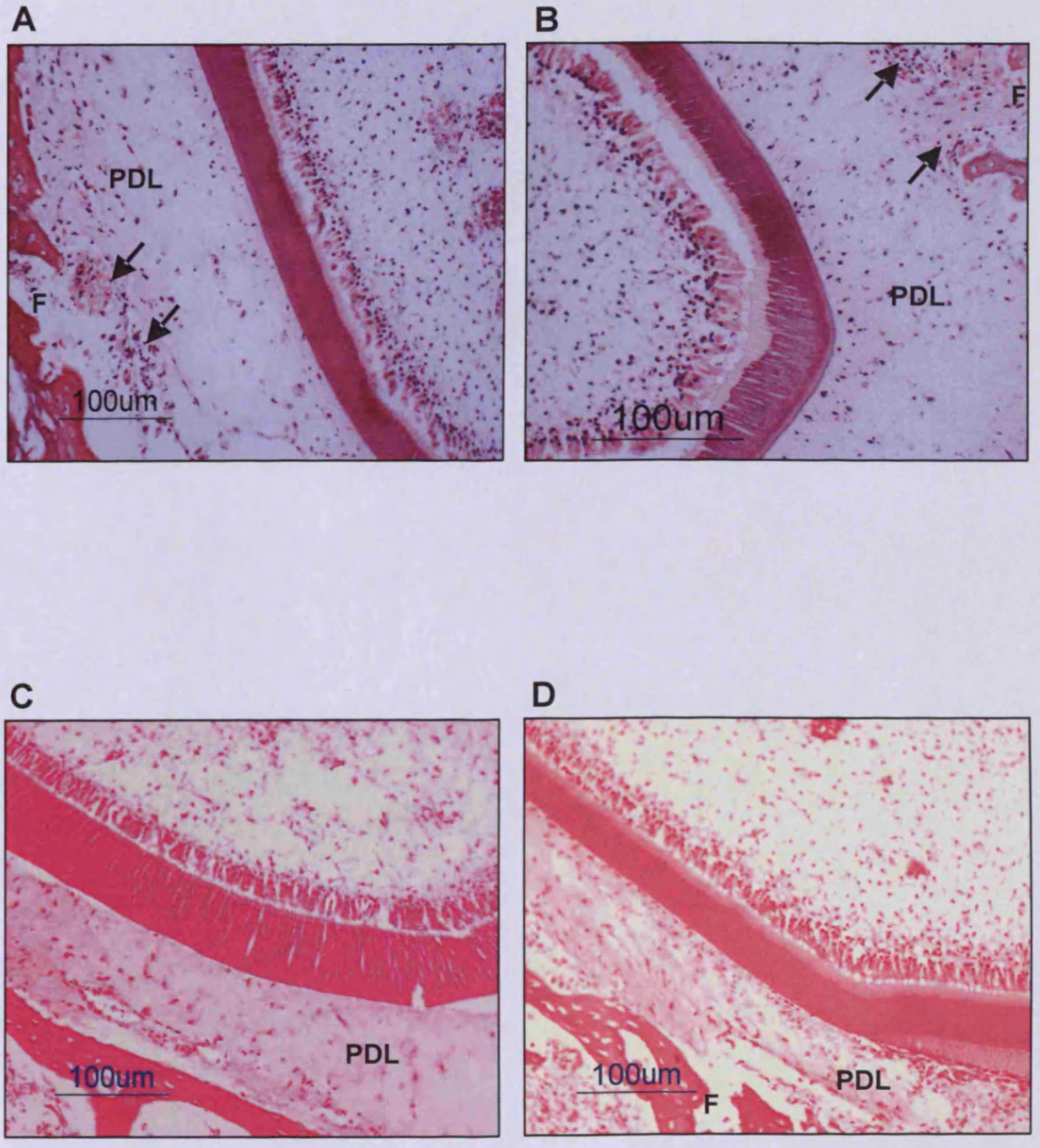

Figure 5.1: Fractured mandible slices cultured for 7 days following pre-treatment with (A) 17% EDTA or (B) 0.02M $Ca(OH)_2$. Aggregation of cells within the periodontal ligament (PDL) can be observed within close proximity to the site of fracture (F) after both treatments. Similar aggregation cannot be observed within (C) regions of PDL at the opposite side of stimulation, or (D) in control slices treated with PBS.

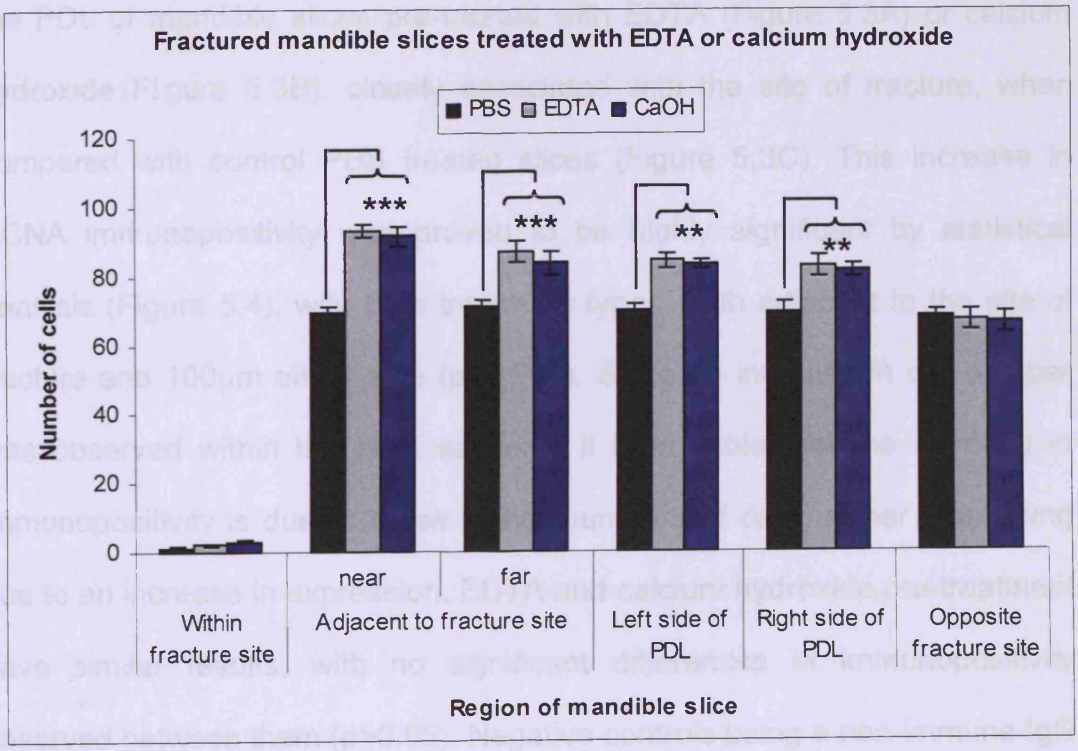

Figure 5.2: Average cell numbers within various 100μm² areas of periodontal ligament in fractured mandible slices treated with EDTA or $Ca(OH)_2$, compared with control slices treated with PBS.

** $p<0.01$ *** $p<0.001$

5.3.2 Immunohistochemical characterisation of pre-treated mandible slices

5.3.2.1 PCNA immunolocalisation

An increase in the number of PCNA positive cells could be observed within the PDL of mandible slices pre-treated with EDTA (Figure 5.3A) or calcium hydroxide (Figure 5.3B), closely associated with the site of fracture, when compared with control PBS treated slices (Figure 5.3C). This increase in PCNA immunopositivity was proved to be highly significant by statistical analysis (Figure 5.4), with both treatment types, both adjacent to the site of fracture and 100μm either side ($p<0.001$). Since an increase in cell number was observed within the H&E sections, it is possible that the increase in immunopositivity is due to these higher numbers of cells, rather than being due to an increase in expression. EDTA and calcium hydroxide pre-treatment gave similar results, with no significant differences in immunopositivity observed between them ($p>0.05$). Negative controls using a non-immune IgG showed no immunoreactivity (Figure 5.3D).

5.3.2.2 Osteopontin immunolocalisation

Pre-treatment of mandible slices with EDTA (Figure 5.5A) or calcium hydroxide (Figure 5.5B) resulted in an increase in cells immunopositive for osteopontin within the PDL in close proximity to the site of fracture, compared with control slices treated with PBS (Figure 5.5C). Statistical analysis of

automated cell counts (Figure 5.6) proved this increase to be significant in slices treated with both chemicals, adjacent to the site of treatment ($p<0.001$), and 100μm either side. EDTA pre-treatment did appear to have a greater effect than calcium hydroxide, with a higher number of osteopontin positive cells observed adjacent to the site of fracture, although this increase was not shown to be significant ($p>0.05$). No immunoreactivity was observed within negative controls where the primary antibody was replaced with a non-immune IgG (Figure 5.5D).

5.3.2.3 Bone sialoprotein immunolocalisation

The expression pattern of bone sialoprotein was similar to that of osteopontin, with an increase in the number of immunopositive cells observed in the PDL of mandible slices pre-treated with EDTA (Figure 5.7A) or calcium hydroxide (Figure 5.7B) when compared with PBS control slices (Figure 5.7C). Statistical analysis of automated counts (Figure 5.8) proved the increase observed within both EDTA and calcium hydroxide pre-treated slices to be significant, both adjacent to the site of treatment ($p<0.001$) and 100μm either side ($p<0.001$). Adjacent to the site of fracture, EDTA appeared to induce a greater response than calcium hydroxide, with a slightly higher number of bone sialoprotein positive cells observed in EDTA pre-treated mandible slices, but this difference was not shown to be significant ($p>0.05$). Negative controls replaced the primary antibody with a non-immune IgG and showed no immunoreactivity (Figure 5.7D).

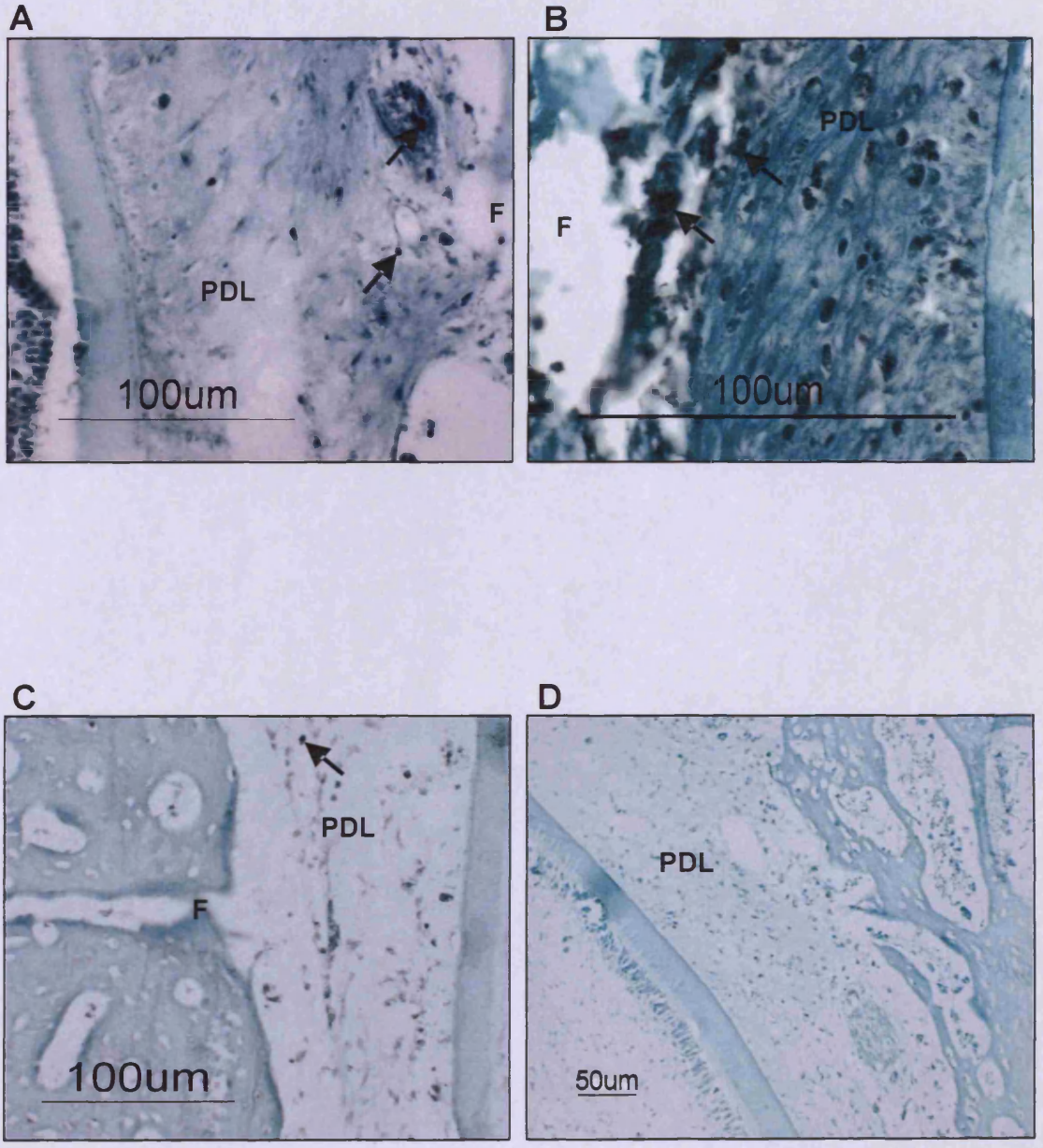

Figure 5.3: Fractured mandible slices immunohistochemically stained for expression of PCNA. Increased cellular expression (examples indicated by arrows) can be observed within cells of the periodontal ligament (PDL) in mandible slices treated with (A) EDTA or (B) $Ca(OH)_2$, when compared with (C) control mandible slices treated with PBS. (D) Negative controls using a non-immune IgG exhibits only the methyl green counterstain.

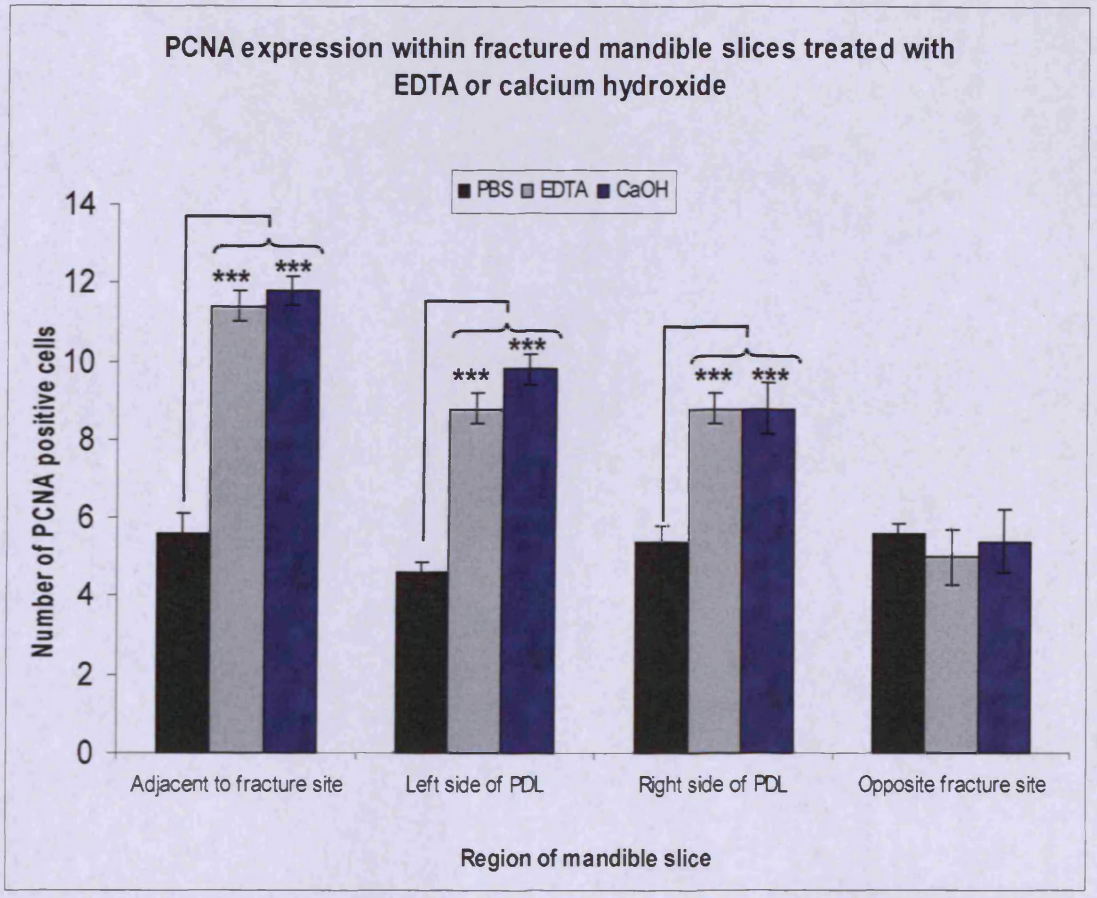

Figure 5.4: Number of PCNA positive cells within various 100µm² areas of periodontal ligament in fractured mandible slices treated with EDTA or $CA(OH)_2$, compared with control slices treated with PBS.

*** $p<0.001$

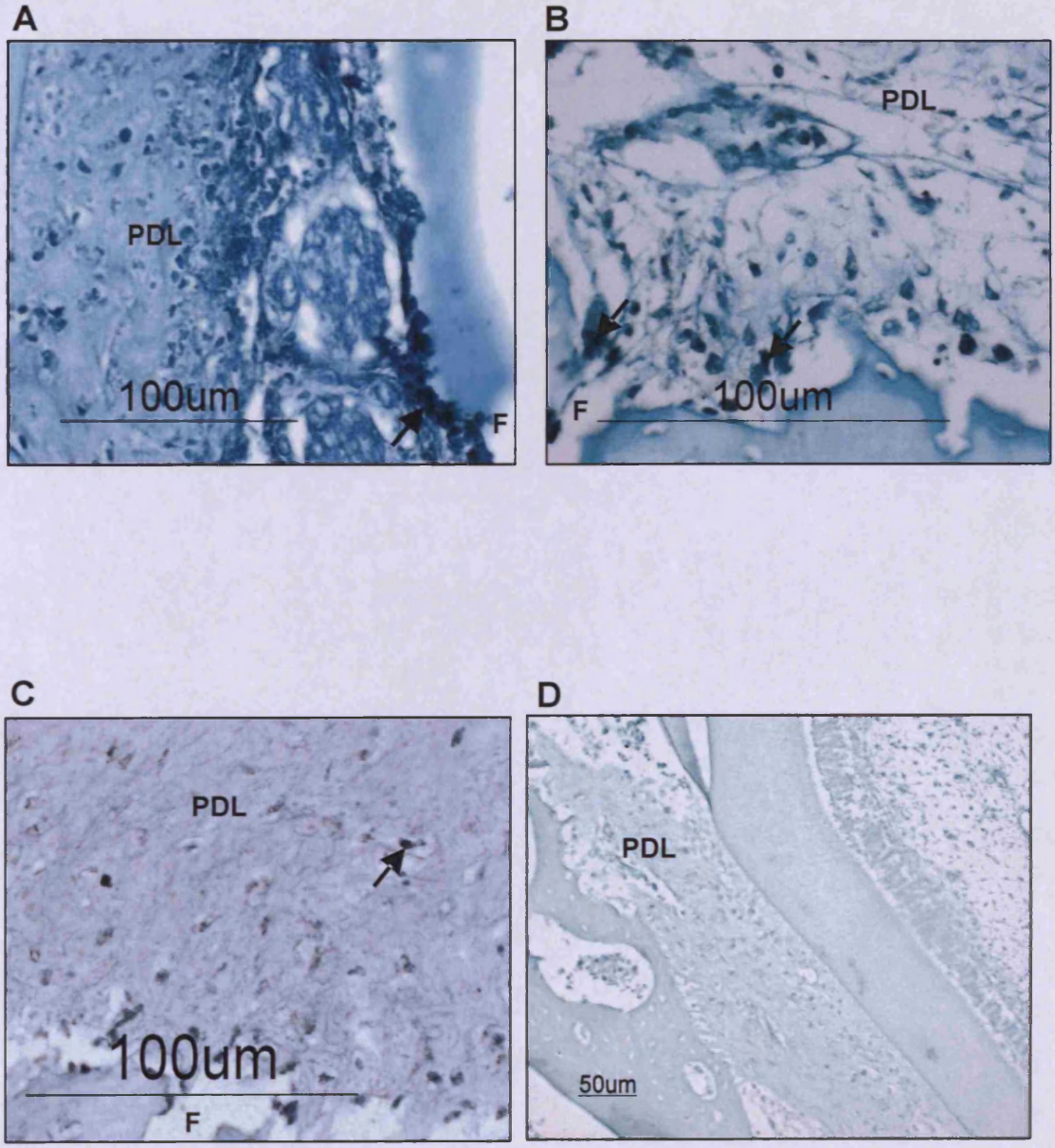

Figure 5.5: Mandible slices immunohistochemically stained for expression of osteopontin. Increased cellular expression (examples indicated by arrows) can be observed within cells of the periodontal ligament (PDL) adjacent to the fracture site (F) in mandible slices treated with (A) EDTA or (B) $Ca(OH)_2$ when compared with (C) control mandible slices treated with PBS. (D) Negative controls using a non-immune IgG exhibits only the methyl green counterstain.

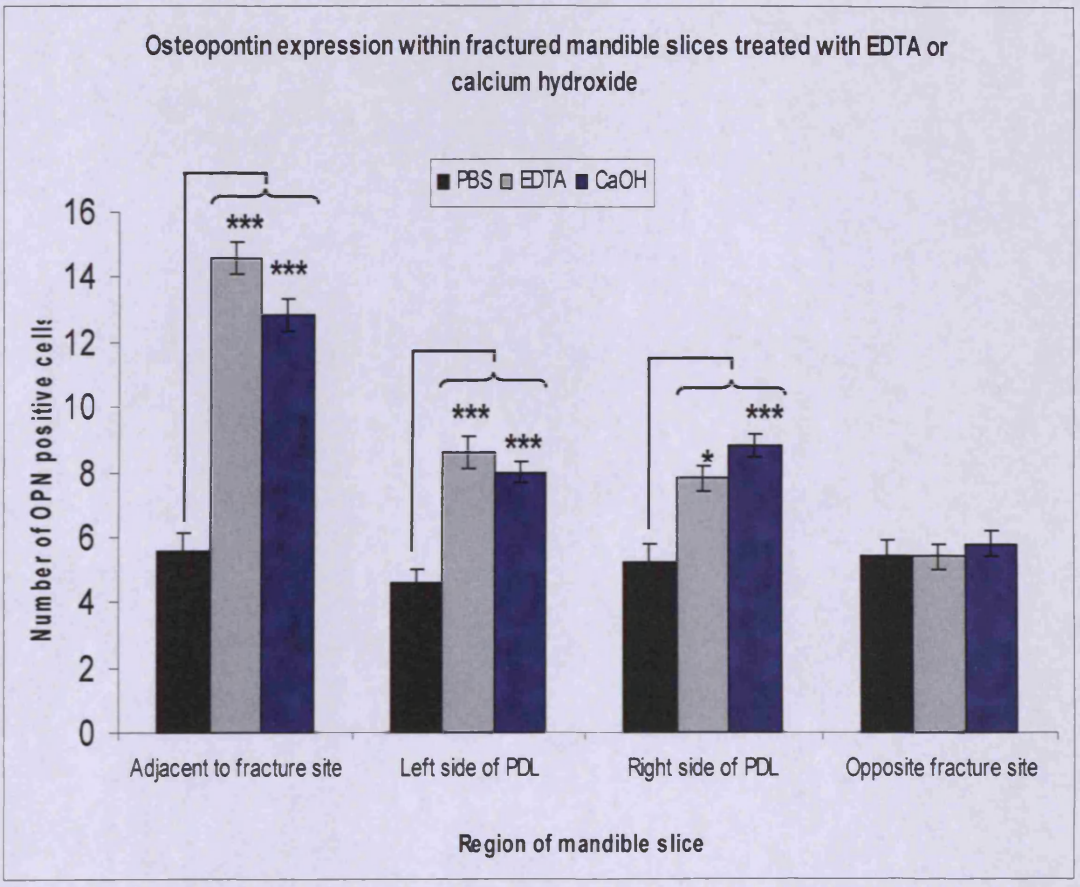

Figure 5.6: Number of osteopontin positive cells within various 100µm² areas of periodontal ligament in fractured mandible slices treated with EDTA or $Ca(OH)_2$, compared with control slices treated with PBS.

* $p<0.05$, *** $p<0.001$

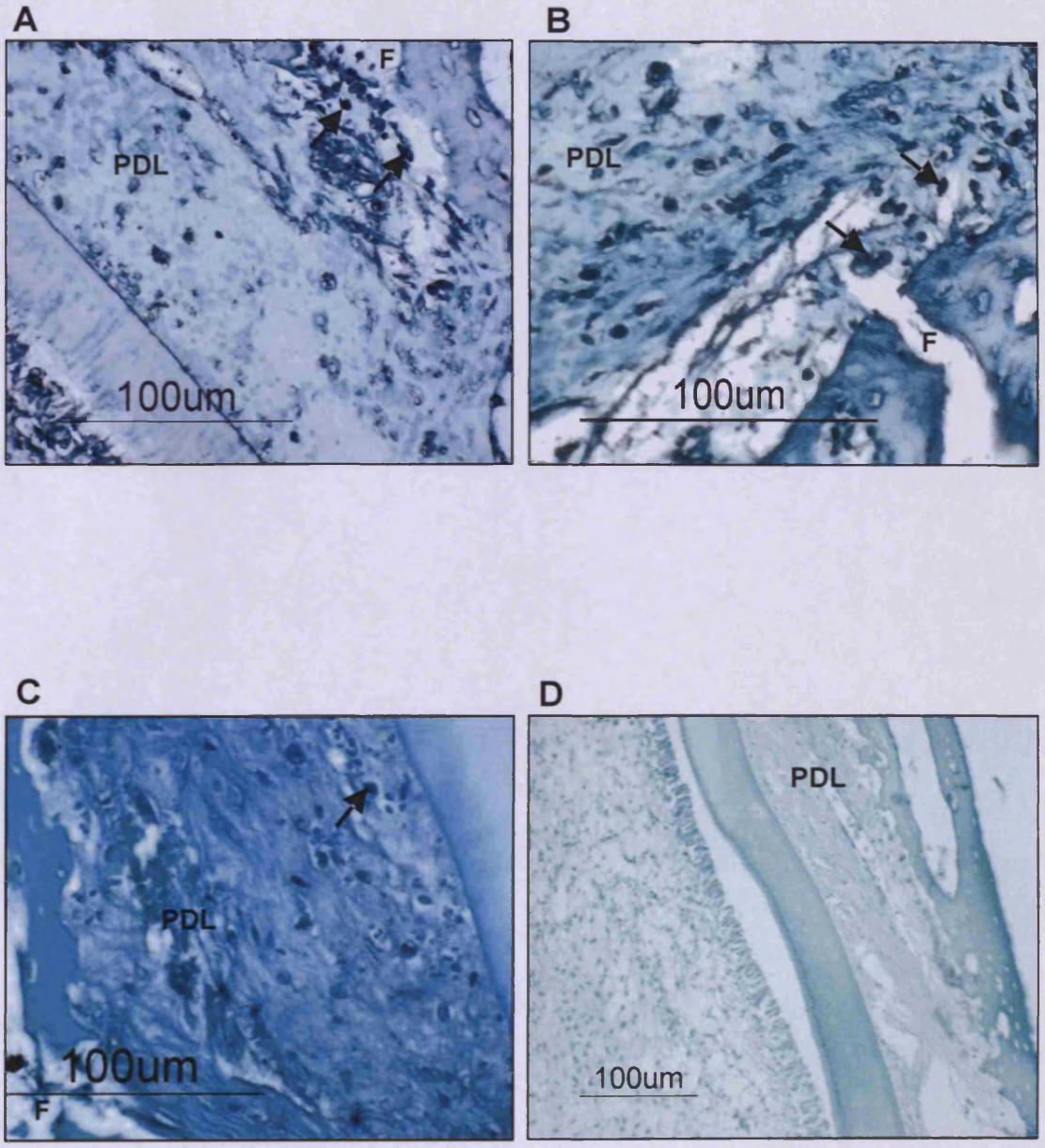

Figure 5.7: Mandible slices immunohistochemically stained for expression of bone sialoprotein. Increased cellular expression (examples indicated by arrows) can be observed within cells of the periodontal ligament (PDL) adjacent to the site of fracture (F) in mandible slices treated with (A) EDTA or (B) $Ca(OH)_2$ when compared with (C) control slices treated with PBS. (D) Negative controls using a non-immune IgG exhibits only the methyl green counterstain.

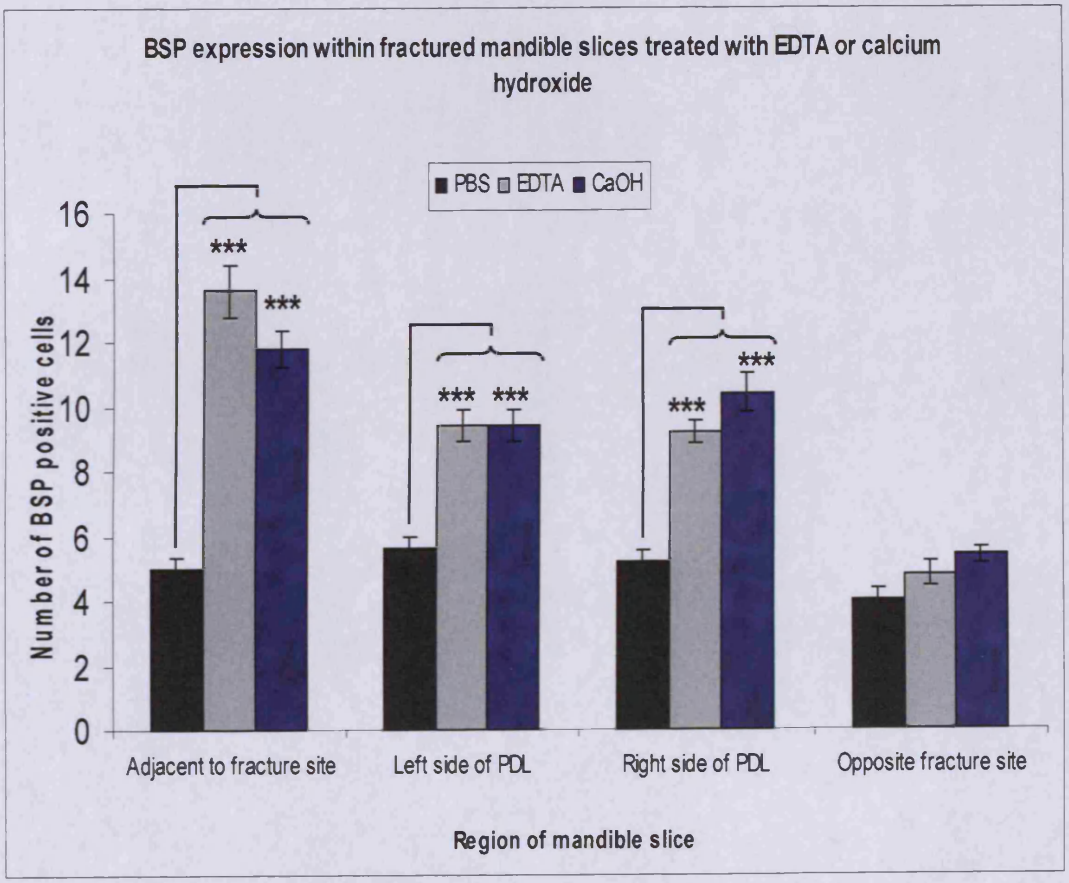

Figure 5.8: Number of bone sialoprotein positive cells within various 100μm² areas of periodontal ligament in fractured mandible slices treated with EDTA or $CA(OH)_2$ compared with PBS treated control slices.

*** $p<0.001$

5.4 DISCUSSION

The previous chapter of work demonstrated that both EDTA and calcium hydroxide were capable of releasing growth factors from within the matrix of bone, and influencing the behaviour of primary cells cultured on these surfaces. This study further developed this work, by demonstrating that release of growth factors in response to both EDTA and calcium hydroxide could influence cellular behaviour in an *ex vivo* mandible model system, a dynamic system more representative of an *in vivo* situation. Since both chemicals were applied as a pre-treatment, and were subsequently washed away from the mandible tissue, it is likely that the findings from this study are a result of the released growth factors initiating cellular signalling within the tissue, rather than a direct effect of the chemical treatment itself.

Both EDTA and calcium hydroxide pre-treatment significantly increased the number of cells within the PDL of mandible slices, adjacent to the site of fracture, and also 100μm either side, although to a lesser extent, probably due to diffusion of released growth factors through the tissue from the fracture site. The increase in cell numbers could be due to an increase in migration, or proliferation, or both processes. A number of different growth factors may be being released due to the chemical treatments (Finkelman et al. 1990; Graham et al. 2006; Zhao et al. 2000), so it is possible that they are functioning synergistically to influence cell behaviour (Bolander 1992; Bourque et al. 1993; Taipale and Keski-Oja 1997). Immunohistochemistry data showed that pre-treatment with both EDTA and calcium hydroxide

increased the number of PCNA immunopositive cells adjacent to the site of fracture, suggesting that proliferation was being influenced by the endogenous growth factors. There are a number of growth factors that are capable of driving such proliferation of osteoprogenitors, including the FGFs (Mansukhani et al. 2000), IGFs (Hughes et al. 2006), and TGF-β1; shown in the previous chapter to be released from the matrix after treatment with both EDTA and calcium hydroxide, and known to be involved in stimulating proliferation of mesenchymal stem cells and osteoprogenitors (Barnes et al. 1999; Bostrom and Asnis 1998; Centrella et al. 1994). Expression of two bone markers, osteopontin and bone sialoprotein, was also significantly increased in response to EDTA and calcium hydroxide pre-treatment, adjacent to the site of treatment and 100μm either side. This increase in the number of immunopositive cells could be due to migration of osteoblastic cells expressing these proteins towards the treatment site, or could also be due to the released growth factors inducing differentiation of cells residing within the PDL close to the site of fracture, increasing protein expression (Barnes et al. 1999; Lieberman et al. 2002). The evidence from the histology and cell counts, which show a significant increase in cell numbers in response to EDTA and calcium hydroxide pre-treatment, may suggest that the increase in immunopositivity for PCNA, osteopontin and bone sialoprotein is due to these increased cell numbers within the area. However, there was a difference in the expression of osteopontin and bone sialoprotein between mandible slices pre-treated with EDTA and calcium hydroxide, with a higher number of immunopositive cells observed within the PDL of EDTA pre-treated mandible slices. This difference was not shown to be significant by

statistical analysis, but this may be because of the small numbers of immunopositive cells that are present; any changes observed may not be large enough to necessarily be statistically significant. Despite the differences in immunopositivity not being significant, it may still indicate that the two treatment types have differential effects on cells within the mandible model, and may therefore suggest that it is not only an increase in cell numbers that is affecting immunopositivity, but that the growth factors released in response to chemical treatments are affecting cellular expression of proteins within the area. This hypothesis may also support the results found in the previous chapter, with EDTA and calcium hydroxide hypothesised to release growth factors by a different mechanism: EDTA by chelating calcium ions and thus demineralising the matrix, while calcium hydroxide may affect interactions of growth factors with the matrix by altering protein charge. It may also support the hypothesis that EDTA and calcium hydroxide release a different protein profile from bone matrix, as previously shown by Graham and colleagues (2006), which may affect the influence each treatment has on cellular behaviour; release of different combinations of growth factors or in different concentrations may have marked effects on the ability of the treatment to affect proliferation, migration and differentiation. For example, a higher concentration of released TGF-β1 may induce proliferation and migration of osteoprogenitor cells (Barnes et al. 1999), whereas higher concentrations of the BMPs may induce differentiation within these cells (Cheng et al. 2003a). Within the study by Graham and colleagues, EDTA was shown to be more efficient at solubilising growth factors than calcium hydroxide. This may explain the slightly higher cellular expression of osteopontin and bone

sialoprotein observed in response to EDTA within our study. Future work could further investigate the effects that these chemical treatments have on migration and differentiation, perhaps by utilising microinjection of labelled osteoprogenitors into the PDL of mandible slices (Taylor et al. 2008), enabling cells to be tracked and giving some indication of their migratory paths and differentiation states in response to the different chemical treatments.

Comparison of results observed in this chapter with those previously obtained from treatment with a single growth factor (either TGF-β1 or BMP-2) showed some interesting differences. Treatment with EDTA / calcium hydroxide and TGF-β1 / BMP-2 yielded similar results in terms of cell numbers and proliferation (PCNA expression). However, pre-treatment with EDTA and calcium hydroxide induced a greater increase in osteopontin and bone sialoprotein immunopositivity than treatment with either of the single growth factors ($p<0.001$). This may be due to the chemical treatments releasing a cocktail of growth factors from the matrix, enabling them to act in a synergistic manner and thus eliciting a greater cellular effect than addition of single exogenous growth factors (Lieberman et al. 2002; Meraw et al. 2000; Taipale and Keski-Oja 1997). The half-life of the released growth factors may also be different to the single exogenously added growth factors. The exogenous growth factors are activated before administration into the tissue, via citric acid. The endogenous growth factors released from the bone matrix may also be active, if bound to ECM components such as decorin and biglycan (Hildebrand et al. 1994). However it is also possible that growth

factors may be bound and subsequently released in a latent form, requiring activation by, for example, proteases within the tissue (Dallas et al. 2002; Janssens et al. 2005; Taipale et al. 1992; Todorovic et al. 2005). Since such proteases may remain within the *ex vivo* tissue during culture, it is possible that cleavage of growth factor from latent binding proteins could occur within the system. The treatments themselves may also participate in the cleavage and activation, since previous studies have demonstrated that both strong acids and strong alkalis can activate growth factors (Lawrence et al. 1985; Lyons et al. 1988). Latent TGF-β1 has been shown to have an extended half-life compared to active TGF-β1 (Wakefield et al. 1990), and therefore release of latent TGF-β1 by chemical treatments may explain the greater cellular effects seen in terms of osteopontin and bone sialoprotein expression. This difference in immunopositivity was only observed immediately adjacent to the site of fracture. Immunopositive counts taken from 100μm either side of the fracture site yielded no significant differences between slices pre-treated with chemicals and those treated with single exogenous growth factors. Interestingly, while treatment with TGF-β1 or BMP-2 yielded similar immunopositive counts both adjacent to the site of fracture and 100μm either side, chemically treated slices showed a decrease in immunopositivity further away from the fracture site. There may be several reasons for this observation. Firstly, it may be due to the method of delivery of the exogenous growth factors via agarose beads. The beads, once placed within the fracture site and cultured, allow diffusion of the growth factors from within the bead into the surrounding tissue. The placement of the beads within the fracture site itself enables slight protrusion of the beads into the PDL tissue, and may

therefore facilitate wider diffusion of soluble growth factors throughout the tissue, further away from the site of fracture. Such diffusion of growth factors from agarose beads has also been shown in previous studies, both in the tooth slice model (Sloan and Smith 1999), and in models investigating the role of growth factors in developmental processes (Dudas et al. 2006; Moore et al. 2002). It is also possible that the agarose beads may offer a certain degree of protection to the growth factors from degradation, keeping the growth factors within the bead itself until they diffuse out into the tissue. It may also be that the concentration of exogenous growth factors used in this study is higher than the concentration of endogenous growth factors that the chemical treatments can release from the matrix, allowing the effects to be elicited over a greater area. TGF-β1, for example, has been shown to be present *in vivo* in the ng/ml range (Finkelman et al. 1990), significantly lower than the μg/ml concentrations of exogenous growth factors required to elicit a response within this study. Taken together, these results may suggest that the chemical treatments elicit a greater response adjacent to the site of fracture in terms of osteoblast differentiation, due to the combination of synergistic growth factors they release (Meraw et al. 2000), as indicated by an increase in the osteopontin and bone sialoprotein bone markers. However, it appears that the agarose bead delivery method allows for greater diffusion of the bioactive molecules through the tissue, and may confer some degree of protection to the growth factors. It may therefore be beneficial for future clinical treatments to investigate either different delivery mechanisms of chemical treatments, or to look at a combination of chemical treatment with exogenous growth factors, to elicit the greatest cellular response.

CHAPTER 6

GENERAL DISCUSSION

The main aim of this thesis was to develop and validate an *ex vivo* rat mandible fracture model for investigation of specific cellular and molecular processes of bone repair, and to provide a functional model for testing novel therapeutic agents for clinical use. Proliferation, migration, activity, and differentiation of resident cell types within the model were investigated, including the responses of osteoblasts and their progeny, and the effect that growth factors have on the responses of these cells. Finally an *in vitro* bone slab cell culture model was developed to demonstrate release of bioactive growth factors from within the matrix of bone, and the effect of these molecules on primary bone marrow stromal cells.

Central to this thesis was the successful establishment of an *ex vivo* organ culture model, utilising excised slices of rat mandibular tissue, both intact and fractured, and subsequent validation of this model system, to establish cell behaviour within the cultured tissue. Between 8 and 10 mandible slices could be isolated per animal, enabling significant reduction of costs and ethical objections over the one animal, one experiment ethos often used for *in vivo* work, and thus central to the 3Rs aim of replacing, refining and reducing animal use in scientific research. Intact and fractured mandible slices were successfully cultured for up to 21 days, and tissues were shown to remain viable after this time period, with maintenance of tissue architecture and

cellular morphology. The fracturing process did not cause excessive cell or tissue damage, and viable populations of cells remained adjacent to the site of fracture, with which it would be possible to study early repair processes within the model system. Two different culture methods were utilised within this study; submerged base type cultures, and Trowel type cultures. While both methods are used widely in organ culture techniques, the results obtained from this study correlated with previous work suggesting that Trowel type culture systems promote viability and tissue morphology, probably due to an increased oxygen tension from culture at the liquid-gas interface, and optimum nutrient diffusion into all tissues areas from within the three dimensional agar medium (Bègue-Kirn et al. 1992; Nifuji and Noda 1999; Sloan et al. 1998). Resident cells within the mandible tissue slices were shown to be actively synthesising and secreting proteins, and cells of the osteoblast lineage were shown to survive throughout the culture period, essential for future development of the mandible model as an experimental system for investigating bone repair processes and for testing novel therapeutics. Fracturing of the mandible slices did not significantly alter cellular behaviour, suggesting that the mandible model may lack sufficient stimulus for initiating repair processes within the bone tissue, in response to the fracture alone. However, this thesis has demonstrated that the fractured mandible model can respond to stimulation with exogenous growth factors, or with chemical treatments to release endogenous growth factors from within the matrix, significantly altering the behaviour of cells resident within the model.

In order to develop the *ex vivo* mandible model as a viable system for testing novel therapeutics, it was essential to demonstrate the responsiveness and functionality of the model. Mandible slices were firstly stimulated with exogenous growth factors TGF-β1 and BMP-2. These were chosen due to their role as key players in bone repair processes, but this model could be used to investigate any of the wide range of bioactive molecules involved in these repair processes (Barnes et al. 1999; Cheng et al. 2003a; Janssens et al. 2005; Tsiridis et al. 2007). Both TGF-β1 and BMP-2 were capable of affecting the cells within the PDL of the mandible tissue, increasing migration / proliferation, and expression of bone matrix proteins osteopontin and bone sialoprotein. TGF-β1 however, appeared to have a greater effect on migration and proliferation, whereas BMP-2 appeared to have a greater effect on osteoblast differentiation, demonstrating that the *ex vivo* system is capable of responding to individual bioactive molecules in different ways. The experimental techniques used to establish the model enabled the generation of mandible slices of a consistent shape and size, and histology indicated that tissue architecture was consistently maintained within slices. The experimental results obtained from the model were also highly reproducible, with experiments performed on multiple slices yielding consistently similar results. This may make the system a valuable tool for both developing current therapeutics and testing future ones. These may include biomaterials (Benke et al. 2001; Neovius and Engstrand *In Press*) such as calcium phosphate bone cements used in dentistry and orthopaedic surgery (Bohner et al. 2005; Ooms et al. 2002), recombinant growth factor therapies (Luginbuehl et al. 2004; Malafaya et al. 2002), and novel drug compounds.

Application of such materials and compounds could be directly to the site of fracture, or, as in this study, could be applied using a bead delivery system. Alternatively, the materials and compounds could be applied to other areas of the mandible tissue, for example directly into the PDL.

Along with the *ex vivo* mandible model, this study also aimed to develop a second model system; an *in vitro* bone slab culture system, with which to analyse release of endogenous growth factors from within the bone matrix, and the effects these molecules can have on osteogenic cells. During *in vivo* bone repair, a cocktail of endogenous growth factors are released, synergistically eliciting optimal repair processes (Dallas et al. 2002; Hughes et al. 2006; Lieberman et al. 2002). It may therefore be beneficial for future clinical therapeutics to recapitulate this *in vivo* process of growth factor release. The simplified *in vitro* system developed was an ideal model to demonstrate these principles of growth factor release, before progression of this work into the more complex *ex vivo* mandible system. Using the bone slab culture model, this study demonstrated that chemical treatments such as EDTA and calcium hydroxide, widely known to solubilise growth factors from dentine matrix (Graham et al. 2006; Zhao et al. 2000), can also release growth factors from the matrix of bone. Another alkaline agent, sodium hydroxide, which has not previously been reported to solubilise growth factors, was also shown to facilitate TGF-β1 release. The differences in release kinetics between the chemical treatments suggested that the treatments may utilise different mechanisms to facilitate growth factor release. This study hypothesised that EDTA, together with citric acid, may

induce local dissolution of mineralised matrix, or act as chelating agents to bind calcium ions within the matrix, reducing their activity (De-Deus et al. 2008). The alkaline agents may alter protein charge, either the charge of the growth factors themselves, thus altering their interaction with the matrix, or the charge of the matrix proteins that keep the growth factors within their bound state. Differences in chemical composition between the alkaline agents may account for the differences in their release kinetics. Growth factors released by chemical treatments were shown to be capable of actively influencing the behaviour of osteogenic cells. Cell number was increased through either an increase in adhesion or proliferation, and cells appeared cuboidal, with a morphology more resembling mature cells of the osteoblast lineage. Expression of the bone markers osteopontin and alkaline phosphatase were also increased. When carried out in the *ex vivo* mandible model, treatment of the fracture site revealed similar results to the *in vitro* study, with both EDTA and calcium hydroxide pre-treatment increasing cell number, proliferation, and bone matrix protein expression. The release of a 'cocktail' of growth factors by these chemical treatments appeared to elicit different effects on cellular behaviour than those seen with single exogenously applied TGF-β1 or BMP-2. Chemical treatments appeared to have a greater effect on osteoblast differentiation adjacent to the site of fracture, suggesting that synergy between various growth factors may be important in eliciting optimal repair processes. However, the delivery of exogenous growth factors via agarose beads may help protect the growth factors from degradation, and appeared to allow greater diffusion of the bioactive molecules through the tissue. This may suggest that future clinical

therapies could benefit from investigating different delivery mechanisms of chemical treatments, or combining chemical treatments with exogenous growth factors, to elicit the greatest cellular response and stimulate optimal healing processes.

The *ex vivo* model, with cells and tissues cultured *in situ* in a three dimensional microenvironment, is a far more complex system than *in vitro* cell culture, and thus is more representative of an *in vivo* situation without the ethical and financial disadvantages associated with such experiments. Therefore the *ex vivo* model may represent a promising alternative to *in vivo* testing of novel clinical therapeutics, as well as an ideal system for answering specific questions about bone repair processes without the complex systemic influences found in an *in vivo* experimental system. The versatility of the model also enables further development for a range of uses, for example as a system to investigate the processes of bone loss associated with inflammatory bone diseases (Taylor et al. 2008), or to assess changes in bone remodelling caused by orthodontic forces. The model also allows for co-culture with other cell types, for example microinjection of cells into the mandible PDL, which will further widen the potential uses of the model. Microinjection of preosteoclasts or inflammatory cells, for example, would allow investigation of resorptive or inflammatory processes that would *in vivo* be facilitated via the vasculature. The development of this *ex vivo* mandible model may therefore be a useful tool in a wide range of investigations.

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