CELLULAR RESPONSES TO DENTAL EXTRACELLULAR MATRIX MOLECULES

By

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ABSTRACT

Dental pulp contains mesenchymal stem cells (MSCs) similar to those present within bone marrow. Several factors are postulated to contribute to the signalling involved in regulating these cells. This project aimed to investigate the role of pulp and dentine extracellular matrix (pECM/dECM) in the regulation of pulp cell behaviour during health and disease. pECM/dECM molecules were extracted using 10% EDTA, pH7.2 followed by 0.5M-NaCl, pH11.7 and 0.1M tartaric acid, pH2.0, respectively containing protease inhibitors. Proteomic analysis demonstrated the complexity of the ECM extractions. pECM-coated cultureware reduced pulp cell proliferation rates and increased stem cell marker expression compared with controls. Pulp cells exhibited multipotential capacity, with pECM-coated culture surfaces enhancing differentiation activity. pECM and dECM promoted pulp cell migration through an active rho dependent pathway and the chemotactic effects of these ECM molecules were enhanced following acidic/proteolytic degradation. Recruited cells exhibited increased stem cell marker expression. dECM and pECM possessed demonstrable bacteriostatic activity against three anaerobic bacteria associated with dental disease. Dental pulp cells were shown to be viable and capable of secreting mineral when encapsulated within a pECM/alginate scaffold and exposed to dECM molecules. Dental ECMs play important roles in regulating cellular and tissue responses during health and disease.

Dedicated to the Memory of Maurice Conley

"Gramps bought my first science textbook"

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List of Abbreviations:

α -MEM	Alpha modified minimum essential medium
aP2	Adipocyte protein 2
ADM	Adrenomedullin
ALP	Alkaline phosphatase
AMP	Antimicrobial peptide
A.S.	Ammonium sulphate
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BMSC	Bone marrow stem cell
BrdU	Bromodeoxyuridine
CGRP	Calcitonin gene-related peptide
Coll-Iα	Collagen type I alpha
DCPA	Dicalcium phosphate anhydrous
dECM	Dentine extracellular matrix
DMEM	Dulbecco's modified minimum essential media
DMP-1	Dentine matrix protein 1
DPP	Dentine phosphoprotein
DSP	Dentine sialoprotein
DPSC	Dental pulp stem cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FCS	Foetal calf serum

GAG	Glycosaminoglycan
HB-EGF	Heparin-binding epidermal growth factor
HCA	High content cell analysis
IPS	Induced pluripotent stem
LDH	Lactate dehydrogenase
LNGFR	Low-affinity nerve-growth-factor receptor
LPA	Lysophosphatidic acid
LPL	Lipoprotein lipase
MMP	Matrix metalloprotease
MSC	Mesenchymal stem cell
MTA	Mineral trioxide aggregate
MEPE	Matrix extracellular phosphoglycoprotein
MicroCT	Micro-computed tomography
NCP	Non-collagenous protein
NGF	Nerve growth factor
NKA	Neurokinin A
NPY	Neuropeptide Y
OA	Osteoadherin
OC	Osteocalcin
ON	Osteonectin
OP	Osteopontin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF-B	Platelet-derived growth factor B subunit

PDLSC	Periodontal ligament stem cell
pECM	Pulpal ECM
PPAR2	Peroxisome proliferator-activated receptor -2
ROS	Reactive oxygen species
RT	Reverse transcription
S1P	Sphingosine-1-phosphate
SCAP	Stem cells from apical papilla
SEM	Scanning electron microscopy
SHED	Stem cells from human exfoliated deciduous teeth
SIBLING	Small integrin-binding ligand, N-linked glycoprotein
SP	Substance P
TCP	Tricalcium phosphate
TGF-β	Transforming growth factor β
TTCP	Tetracalcium phosphate
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal polypeptide
VWF	vonWillebrand factor

1.0 INTRODUCTION

1.1 Regenerative Dental Therapies

Dental caries, also known as tooth decay, is a bacterial associated infection that causes the demineralisation of enamel and dentine and subsequent pulpal tissue damage, ultimately leading to tooth loss (Katehashi *et al*, 1965). Dental caries is reported to be one of the most prevalent infectious diseases in the world (Cooper et al, 2010). Current dental treatments are relatively crude, using a range of dental restorative materials to provide a physical barrier restoring the anatomical structure of the tooth, often with high failure rates (Mjör and Gordan, 2002; Manhart *et al*, 2004). As a result, there is currently much research into developing regenerative cellular based dental treatments, in either scaffold or scaffold-free methods (Zheng et al, 2011; Hu et al, 2006; Yu et al, 2006 ; Nakao et al, 2007; Young et al, 2002; Duailibi et al, 2008; Xu et al, 2008; Zhang et al, 2006).

The extracellular matrix (ECM) is connective tissue, made up of matrix proteins and basement membrane providing structural support to cells (Section 1.4.2). The ECM and its components have been shown to play vital roles in cellular regulation during the wound healing of many tissues (Section 1.2), and therefore are likely to have regulatory effects within dental tissues. A scaffold based cellular therapy uses a scaffold material in an attempt to recreate the role of the ECM, whilst scaffold-free cellular therapies use cell populations that once implanted will ultimately be responsive to ECM signalling. ECM signalling has been shown to have a major influence on the regulation of cells (Section 1.2.1.4), and therefore a deeper insight into how ECM signalling can influence cell behaviour may be vital to progressing regenerative dental treatments.

1.2 The Role of ECM during Wound Healing

1.2.1. Cellular Processes involved in Wound Healing

Following wound injury, a healing response is initiated involving resident and migratory cell populations, extracellular matrix (ECM) molecules and the action of soluble signalling molecules (Velnar *et al*, 2009). The wound healing response consists of a progressive series of events that occur with the purpose of restoring the integrity of the tissue. These events include four highly integrated and overlapping phases that can broadly be characterised as haemostasis, inflammation, proliferation and tissue remodelling (Gosain and DiPietro, 2004). Interactions between cells and the ECM environment play an important role in cellular regulation during these wound healing processes (Alford and Hankenson, 2006; Bornstein and Sage, 2002; Schultz *et al*, 2011; Wight and Potter-Perigo, 2011).

1.2.1.1 The Role of ECM during Haemostasis

The first phase of wound healing involves haemostasis. This starts immediately after the wound event and involves vascular constriction and fibrin clot formation (Pool, 1977). These processes aim to prevent exsanguination and protect the vascular system so that the tissue or organ may continue to function despite the wound. As well as maintaining tissue and organ function, a temporary fibrin matrix or scaffold in which the longer term healing of the wound can occur is established during haemostasis (Jespersen, 1988).

Damage to blood vessels exposes ECM components to the circulating platelets and interactions between these cells and the ECM components, particularly von Willebrand factor (VWF), collagen I, collagen VI, fibronectin, thrombospondin, and laminin, lead to thrombus formation (Rivera *et al*, 2009). These interactions occur via specific cell surface binding sites leading to the activation of downstream signalling pathways, important for haemostasis. Platelets bind to collagen via β1 and β3 integrins and glycoprotein VI immunoglobulin, leading to platelet degranulation and the release of chemokines (Nieswandt *et al*, 2009; Rivera *et al*, 2009). Following collagen binding, fibrinogen and VWF bind to activated $\alpha_{IIb}\beta_3$ integrins leading to platelet-platelet connections and the formation of a thrombus (Rivera *et al*, 2009). Platelet behaviour during haemostasis is tightly regulated by the binding of ECM proteins highlighting the critical role these ECM proteins play during this early stage of wound healing.

1.2.1.2 The Role of ECM during Inflammation

As bleeding is brought under control, an inflammatory phase is initiated. The majority of wounds have some degree of microbial infection and the purpose of this phase is to establish an immune barrier against the invading microorganisms by the sequential infiltration of neutrophils, macrophages and lymphocytes (Gosain and DiPietro, 2004; Campos *et al*, 2008; Broughton *et al*, 2006).

During the inflammatory stage of wound healing, specific immunocompetent cells use phagocytosis to remove pathogens and tissue debris from the environment. Monocyte binding

to ECM proteins such as fibronectin, laminin, and serum amyloid P component will enhance phagocytosis via both IgG Fc receptors and complement receptors (Brown and Goodwin, 1988). Enhanced phagocytosis will lead to increased degradation of ECM components. The ECM binding of monocytes can trigger the activation of growth factors, such as platelet-derived growth factor B subunit (PDGF-B) and transforming growth factor β (TGF-β), inducing monocyte differentiation into macrophages (Shaw et al, 1990; Haskill *et al*, 1988).

During the inflammatory phase, macrophages remove apoptotic neutrophils, preventing the release of toxic proteases and reactive oxygen species (ROS) from dying cells that could exacerbate tissue injury (Fadok *et al*, 1998). The phagocytosis of apoptotic neutrophils by macrophages is dependent on macrophage binding to ECM proteins via β2 integrins (Sindrilaru *et al*, 2009). The inflammatory stage involves immunocompetent cells establishing an immune barrier against invading microorganisms, with the differentiation and phagocytosis activities of these cells tightly regulated by ECM interactions showing another important role for ECM proteins during wound healing.

1.2.1.3 The Role of ECM during Migration and Proliferation

Once haemostasis has occurred and an immune barrier has been successfully established, the tissue response shifts from defence towards repair during the subsequent migratory and proliferative phase. This phase is characterised by the proliferation of and matrix deposition by recruited cells present at the wound site (Clark, 1993; Guo and DiPietro, 2010; Velnar *et al*, 2009).

The cells and processes involved in this phase of wound healing vary between tissues. In the repair of soft tissues, such as skin, the behaviour of fibroblast and endothelial cells is significantly influenced by the ECM. The migration of fibroblast and endothelial cells into the wound space is induced in response to growth factors that can be directly or indirectly bound to fibrin within the ECM (Clark, 2003). Indeed, fibrin directly binds to thrombin (Pospisil *et al*, 2003), basic fibroblast growth factor (bFGF) (Sahni *et al*, 1998), PDGF and TGF-β (Clark, 2003). The ability of fibrin to bind to glycosaminoglycans (GAGs), such as heparin (Sahni *et al*, 1998), results in indirect interactions with GAG-bound growth factors such as heparin-binding epidermal growth factor (HB-EGF), PDGF and vascular endothelial growth factor (VEGF) (Clark, 2003). ECM-bound as well as soluble growth factors induce cell migration from surrounding tissue. This migration is enabled by the inclusion of important adhesive proteins, such as fibronectin and vitronectin, within the fibrin matrix that interact with migratory cells via β1, β2 and β5 integrins (Greiling and Clark, 1997).

Fibroblast binding to fibronectin stimulates the production of ECM components including proteoglycans, hyaluronic acid and collagens. These alter the mechanical properties of the wound environment and play an important role in host cell functions (Schultz et al, 2011). Newly deposited proteoglycans play a role in the interactions between the ECM components facilitating processes, such as collagen fibrillogenesis (Clark, 1993). Hyaluronic acid plays a role in cell and growth factor attachment and increases cell motility (Clark, 1993). Collagen binds to fibroblasts via β1 integrin receptors, stimulating the production of matrix metalloproteases (MMPs) that proteolytically degrade existing ECM components, permitting cell migration (Steffensen *et al*, 2001).

The proliferative phase of bone fracture healing has been termed the reparative phase (Mann and Payne, 1989). This phase is characterised initially by fibrocartilage formation, followed by primary bone formation (Gerstenfeld et al, 2003). During these processes, chondrocytes and fibroblasts are integral to fibrocartilage formation, whilst osteoprogenitors and osteoblasts are central to primary bone formation. The functional activities of the cells involved in bone fracture repair are strongly influenced by the ECM, especially matricellular proteins. Matricellular proteins are a class of proteins within the ECM that serve as biological mediators of cell function by interacting directly with the cells or indirectly through modulating the activity of growth factors, proteases and other ECM proteins (Alford and Hankenson, 2006). An example of a matricellular protein is osteopontin, which is deposited in the matrix as it is mineralised (Perrien *et al,* 2002). The role of osteopontin in modulating the formation of new bone during fracture repair occurs through multiple mechanisms inducing osteoblast differentiation and new matrix secretion (Alford and Hankenson, 2006).

The influence of ECM on cells during this phase of wound healing is not restricted to only molecular interactions that occur between cells and their environment at the cell-ECM interface but also biomechanical forces at this interface. Biomechanical forces are exerted on cells by integrin binding to ECM components, generating tensional forces that alter cell shape (Ingber, 1993), which can subsequently influence functional activities, such as growth, proliferation, differentiation and motility (Ghosh and Ingber, 2007). The degradation of the ECM due to injury alters the forces exerted on the cell, subsequently altering cell morphology. This change in shape consequently influences cell proliferation and migration during this phase of wound healing (Ghosh and Ingber, 2007).

1.2.1.4 The Role of ECM during Wound Remodelling

The final phase of wound healing is known as the remodelling phase and this can last for several years. This phase is characterised by the rearrangement of the wound ECM to give an architecture which resembles that of normal tissue physiologically (Guo and Dipietro, 2010). As with all of the previous phases of wound healing, ECM plays a key role in the regulation of cellular activity within this phase. During the remodelling phase of cutaneous wound healing, fibroblasts bound to ECM components such as fibronectin differentiate into myofibroblasts in the presence of extracellular TGF-β1 (Wipff *et al*, 2007). Myofibroblasts interact with collagens and growth factors to contract the wound (Singer and Clark, 1999), as well as depositing type I collagen to replace early matrix broken down by the action of MMPs (Witte and Barbul, 1997).

During the remodelling phase of bone fracture healing, osteoclasts resorb bone so that secondary bone formation can occur (Gerstenfeld et al, 2003). This process is heavily modulated by peptide growth factors, such as bone morphogenetic proteins (BMPs). BMPs are a unique subfamily within the TGF-β superfamily that can influence many events during wound healing, such as migration of progenitor cells, proliferation of mesenchymal cells, differentiation to chondrogenic or osteogenic lineages, vascular invasion and remodelling of bone (Reddi, 1992; Reddi and Cunningham, 1993; Onishi *et al*, 1998). Matrix-bound BMPs play a key role in regulation of osteoclast differentiation and maturation (Onishi *et al*, 1998). Indeed, BMP-2 and BMP-4 have been shown to play a crucial role in stimulating the bone-resorbing activity of mature osteoclasts required for the remodelling of the wound matrix (Kaneko *et al*, 2000). As mesenchymal stem cells (MSCs) from dental pulp and bone marrow

have been shown to be controlled by many similar factors it is possible that many of these factors that contribute to bone wound healing may also contribute to dental wound healing (Huang *et al*, 2009).

The regulation of wound healing processes can be viewed as a dynamic reciprocal relationship between the cells and their ECM microenvironment. These interactions are depicted below in Figure 1.1 (Schultz *et al*, 2010).

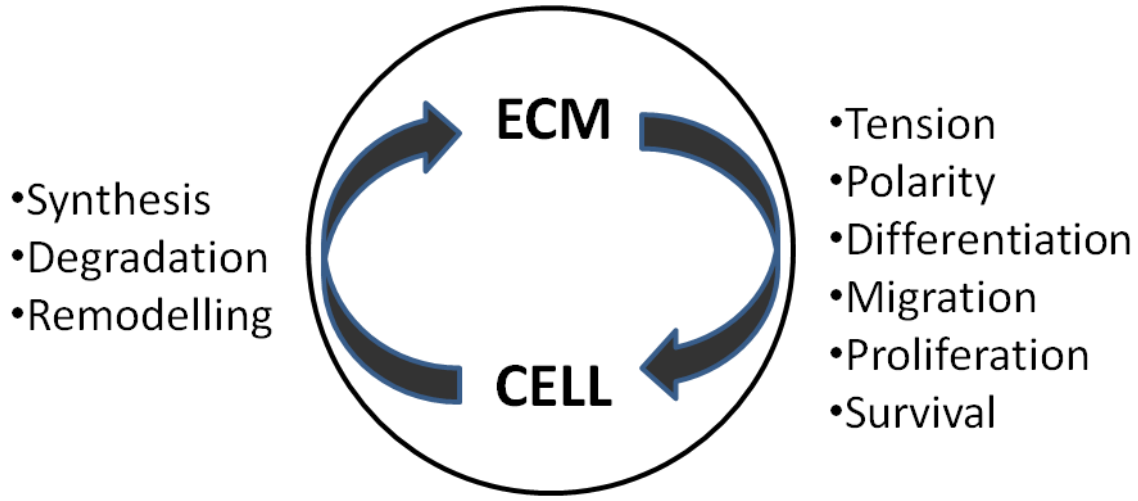


Figure 1.1 Diagrammatic representation of the dynamic reciprocity between cells and ECM. Cells synthesise ECM, which can become degraded and remodelled during the wound healing process. Changes in ECM regulate cell tension, polarity, differentiation, migration, proliferation and survival. As a result, this change in cellular activity influences ECM synthesis, which in turn will influence cellular activity. Reproduced from Schultz *et al* (2010).

1.2.1.5 The Role of Stem Cells during Wound Healing

Cell types involved in repair processes vary between tissues. Postnatal stem cells provide a precursor population for differentiation into the tissue specific phenotype necessary during wound healing. The action of stem cells in wound healing is an area of increasing research attention (Guo and DiPietro, 2010). For example in skin, within its complex architecture there are a variety of cells of both ectodermal and mesodermal origins, derived from corresponding progenitor/stem populations (Cha and Falanga, 2007). Bone marrow derived cells, such as mesenchymal stem cells, haematopoietic stem cells and endothelial progenitor cells have all been shown to contribute in some capacity to skin repair during wound healing (Wu *et al*, 2007; Rea *et al*, 2009). Populations of circulating bone marrow derived cells, from haematopoietic and mesenchymal lineages contribute to the ECM production within the wound matrix (Li *et al*, 2006; Spaeth *et al*, 2009; Schultz *et al*, 2010). In addition epidermal stem cells from the bulge area of hair follicles and from the basal layer of epidermis give rise to migratory keratinocytes during wound healing (Cha and Falanga, 2007).

For bone repair, the osteoprogenitors required for bone fracture healing processes most likely come from a pool or niche of postnatal stem cells. However, the exact source of these osteoprogenitors remains ambiguous with several potential sources postulated to contribute (Schindeler *et al*, 2008). Potential sources of osteoprogenitors include the periosteum, (Malizos and Papatheodorou, 2005; Hutmacher and Sittinger, 2003), and the bone marrow (Baksh et al, 2004; Colnot et al, 2006). Indeed it has been suggested that multiple sources contribute to the osteoprogenitor supply during wound healing (Schindeler *et al*, 2008) and these include circulating populations within the vasculature (Collett and Canfield, 2005;

Eghbali-Fatourechi *et al*, 2005), and resident populations within surrounding local tissues (Rumi *et al*, 2005).

1.2.2 Dentine/ Pulp Development and Regeneration

It is important to have an understanding of the cellular events that take place during tooth development, repair and regenerative processes as future regenerative dental therapies may attempt to mimic natural cellular regulation. The stages of tooth development are described here and illustrated with a schematic drawing of life cycle of the tooth showing contribution from neural crest cells (Figure 1.2).

1.2.2.1 Neural Crest Cells

Neural crest cells are migratory multipotential stem cells that give rise to various cell and tissue types including the development of dentine, pulp and periodontium (Chai *et al*, 2000). Neural crest cells separate from the neuroectoderm as the neural tube forms during embryonic development. These cells migrate from the neural tube to other regions within the embryo where differentiation occurs. The behaviour of these cells is heavily influenced by interactions with released signalling molecules. Examples of factors that influence neural crest cell behaviour include BMPs that have been shown to induce neurogenesis and smooth muscle differentiation (Shah *et al*, 1996). Neural crest cells play an important role in the development of the head region and are involved in the formation of the cranial sensory ganglia and most of the connective tissue of the head (Ten Cate, 2003). Data indicating that neural crest cells are involved in tooth development were shown by the detection of labelled crest cells in the

dental mesenchyme (Lumsden, 1988; Imai *et al*, 1996). Another study used a two component genetic construct to follow the migration and differentiation of neural crest cells, demonstrating their involvement in the formation of condensed dental mesenchyme, dental papilla, odontoblasts, dentine matrix, pulp, cementum, periodontal ligaments, chondrocytes in Meckel's cartilage, mandible, the articulating disc of temporomandibular joint and branchial arch nerve ganglia (Chai *et al*, 2000). The contribution of the neural crest cells to the developed pulp was found to be predominantly within the odontoblast layer and the peripheral areas of the pulp, with only small contribution to the pulp core. This relatively comprehensive study also demonstrated that there was a dynamic distribution of cranial neural crest cells and non-cranial neural crest-derived cells present during tooth and mandibular morphogenesis.

As development proceeds, blood vessels increasingly penetrate the dental papilla, leading to a complex network of pulp capillaries (Takahashi, 1985). This complex network is most likely responsible for the delivery of immunocompetent cells to the pulp tissue (Jontell *et al*, 1987; Jontell *et al*, 1988). The developing pulp therefore contains a mixture of resident mesenchymal cells already present at the development site (1st branchial arch) where the dental lamina and tooth buds develop, as well as those derived from the migrated cranial crest cells and migrated vascular derived cells, due to the mixed cellular origins this tissue is known as the Ectomesenchyme (Goldberg *et al*, 2004).

1.2.2.2 Ecto-mesenchymal Cells

Tooth development occurs at the first branchial arch, where localised thickening of dental epithelium occurs (Thesleff *et al*, 1997; Thesleff and Tummers, 2008). Odontogenic signals

passing from the epithelium to mesenchyme regulate odontogenesis during this initial stage. Epithelial signals that have been indentified include FGF8, BMP4, Shh and Wnt10b, which control the expression of ecto-mesenchymal transcription factors (Yen *et al*, 2008). The epithelial bud continues to proliferate at a slower rate than the ectomesenchyme, whilst ectomesenchyme cellular density increases adjacent to the bud in a process known as condensation of the ectomesenchyme. The tooth bud grows larger and the epithelium folds giving the tooth germ the appearance of an epithelial cap sitting on a ball of ectomesenchyme. From this point, it is possible to distinguish more clearly between its different components. The cap-like structure is the epithelial outgrowth, called the enamel organ. The ball-like structure is condensed ectomesenchymal cells that will form the dentine and the pulp; this is called the dental papilla. Surrounding condensed ectomesenchymal cells form the supporting periodontal structures of the tooth; this is the dental follicle (Ten Cate, 2003).

1.2.2.3 The Tooth Germ

Morphological and functional changes occur within the tooth germ to give rise to the distinct dental cell populations of a developed tooth. With continuing tooth development, the cap stage tooth germ merges into the bell stage when the enamel organ comprises of four distinct cell layers: the inner enamel epithelium, outer enamel epithelium, stellate reticulum and stratum intermedium. The inner and outer epithelia are continuous with each other and where these meet at the cervical loop, continued growth of the bi-layered epithelium will map out the tooth root following crown development. Continued proliferation of the inner epithelium during crown development allows for growth in size of the tooth germ, but as morpho-differentiation ensues in the late bell stage, local arrest of inner epithelial cell proliferation

leads to epithelial folding and determination of crown morphology. Prior to dental hard tissue formation, the inner epithelial cells differentiate into enamel secreting ameloblasts while the ectomesenchymal cells of the dental papilla adjacent to the inner enamel epithelium, differentiate into dentine secreting odontoblasts. Reciprocal epithelial-mesenchymal interactions are important for signalling many of these events during tooth development. The dental papilla becomes the dental pulp once fully developed and contains fibroblasts, blood vessels, nerves, lymphatic ducts as well as odontoblast cells. The dental follicle gives rise to the cementoblasts, osteoblasts and fibroblasts. These three cell types respectively produce the cementum, bone and periodontal ligament fibres that form the support structures of the tooth (Yen *et al*, 2008).

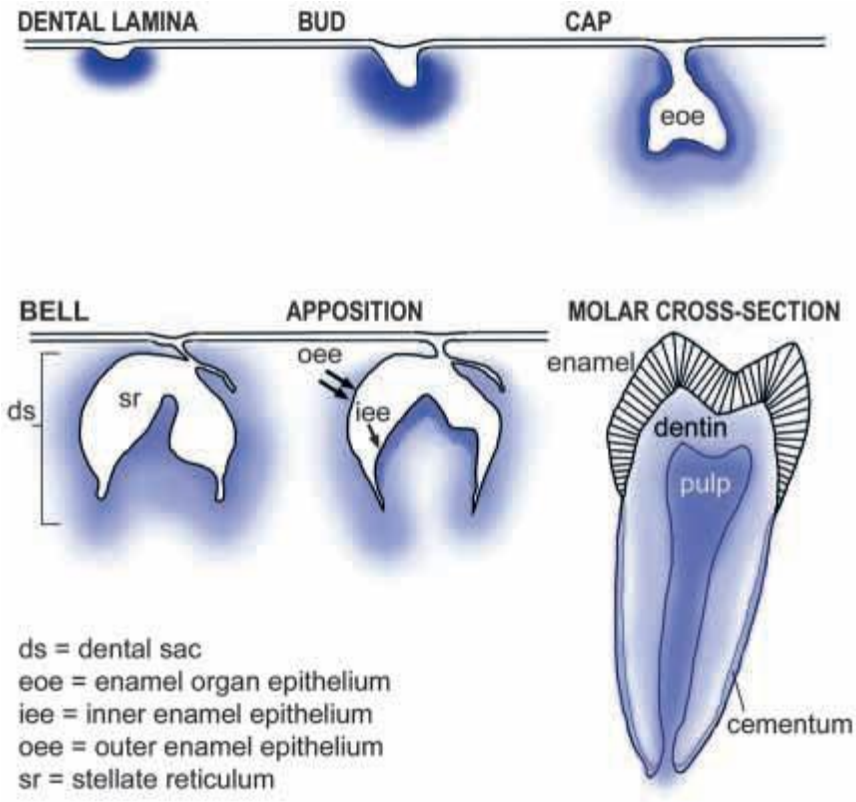


Figure 1.2 Schematic drawing of life cycle of the tooth showing contribution from neural crest cells (blue). Reproduced from Chai *et al*, 2000.

1.2.2.4 Odontoblast Differentiation and Dentinogenesis

Odontoblasts are the cells responsible for the formation and maintenance of dentine and thus, understanding the development and characteristics of these cells may provide an insight into tooth regeneration. The differentiation of ectomesenchymal cells in the dental papilla to odontoblasts is initiated by the influence of the inner dental epithelium and its associated basement membrane. Epithelial-mesenchymal signals have been shown to induce odontoblast differentiation for tooth formation (Kollar *et al*, 1980). The mechanism for this odontoblast differentiation involves the action of epithelial cell secreted growth factors, with those of the TGF-β family reportedly playing a significant role (Ruch *et al*, 1995; Thesleff and Vaahtokari, 1992).

Odontoblasts are post-mitotic cells that are generally present for the duration of the tooth's lifespan and demonstrate four distinct morphologies during their cellular life cycle. These morphologies were first identified using electron microscopy and are described as pre-, young-, old- and short-odontoblasts (Takuma *et al*, 1971). These different morphologies reflect the functional activity of the odontoblasts and are now referred to as pre-, secretory, transitional and aged (Couve, 1986; Romagnoli *et al*, 1990; Couve and Schmachtenberg, 2011). Pre-odontoblasts are cells that develop the intracellular requirements for protein synthesis and secretion. Secretory odontoblasts develop from the pre-odontoblasts and are responsible for the active production of primary dentine. Transitional odontoblasts undergo an autophagic process, degrading some intracellular organelles resulting in the odontoblasts changing towards a less active cell type with limited secretory activity. The final stage in the life cycle is a resting odontoblast characterised by smaller cells that have very few secretory

granules and much reduced secretory activity. Odontoblasts are post-mitotic cells and therefore, the replacement of cells lost through injury to the tissue requires recruitment of appropriate progenitor cells and their subsequent differentiation to an odontoblast-like cell phenotype (Smith *et al*, 1990), which can participate in reparative dentinogenesis (Section 1.2.2.3).

Once the odontoblasts differentiate during the late bell stage of tooth development, secretion of the unmineralised organic matrix of pre-dentine begins. This organic matrix consists of type I collagen and associated non-collagenous ECM components. The odontoblasts are also responsible for the mineralisation of pre-dentine. Odontoblasts mineralise the organic matrix during dentine formation by the release of membrane bound vesicles near the dental basement membrane, which provide a localised environment in which crystal nucleation can occur (Stratmann *et al*, 1996}. Within these vesicles, crystal growth occurs and eventually the crystals rupture the membrane-bound vesicles and fuse with adjacent crystals in the ECM. The need for matrix vesicles to achieve mineralisation of this early matrix reflects the fact that at this stage, the terminal differentiation of odontoblasts is incomplete and they are unable to secrete specific matrix molecules involved in mineral nucleation. Once fully differentiated, the odontoblasts secrete a matrix containing many proteins, including two proteins that are characteristically associated with, but not unique to, dentine; dentine phosphoprotein (DPP) and dentine sialoprotein (DSP) (Butler, 1998). This matrix is capable of mineral nucleation, which becomes the primary mode of mineralisation throughout the remainder of dentinogenesis. As the odontoblasts secrete the organic matrix of primary dentine, they retreat towards the centre of the pulp leaving their single cytoplasmic process embedded in the newly secreted dentine. This process gives rise to the tubular structure of dentine (Yen *et al*, 2008).

The secretion of primary dentine occurs at a rate of approximately 4μm per day during tooth development until the completion of root formation (Kawasaki *et al*, 1980).

Secondary dentine is deposited throughout life by the same odontoblasts that formed the primary dentine and shows tubular continuity with the primary dentine, although is secreted at a much slower rate of approximately 0.5μm per day (Stanley *et al*, 1959; Baume, 1980).

1.2.2.5 Tertiary Dentinogenesis

Tertiary dentinogenesis occurs after tooth formation is completed and describes the secretion of dentine in response to external influences (Kuttler, 1959). Tertiary dentinogenesis can be further classified into either reactionary or reparative, due to differences in the biological events that take place during its formation (Smith *et al*, 1995). Reactionary dentine is a tertiary dentine matrix secreted by surviving post-mitotic odontoblasts in response to a relatively mild stimulus. Reparative dentine is a tertiary dentine matrix secreted by a new generation of odontoblast-like cells in response to a more severe stimulus following the death of the original post-mitotic odontoblasts responsible for primary and physiological secondary dentine secretion (Smith *et al*, 1995).

Reactionary dentinogenesis occurs in response to more mild stimuli, which cause existing primary odontoblasts to increase their matrix secretion, leading to the formation of a reactionary tertiary dentine (Smith *et al*, 1995). The activity of specific growth factors released from the dentine is thought to be one mechanism of odontoblast stimulation during reactionary dentinogenesis (Smith *et al*, 1995; Smith *et al*, 1994).

Reparative dentinogenesis occurs after death of primary odontoblasts with the stages represented in Figure 1.3 below (Baume, 1980; Smith *et al*, 1990; Smith *et al*, 1995; Lesot *et al*, 1994). An intense stimulus, such as localised tissue damage caused by severe deep caries (Stage 1), causes primary odontoblast death (Stage 2). Following odontoblast death, a precursor cell population within the pulp migrates and differentiates into a new generation of odontoblast-like cells (Stage 3). These newly formed cells secrete new matrix for tissue repair (Stage 4). Understanding the regulation of the events of reparative dentinogenesis may be useful for the potential recapitulation of physiological dentinogenic events using stem cells for regenerative dental treatments.

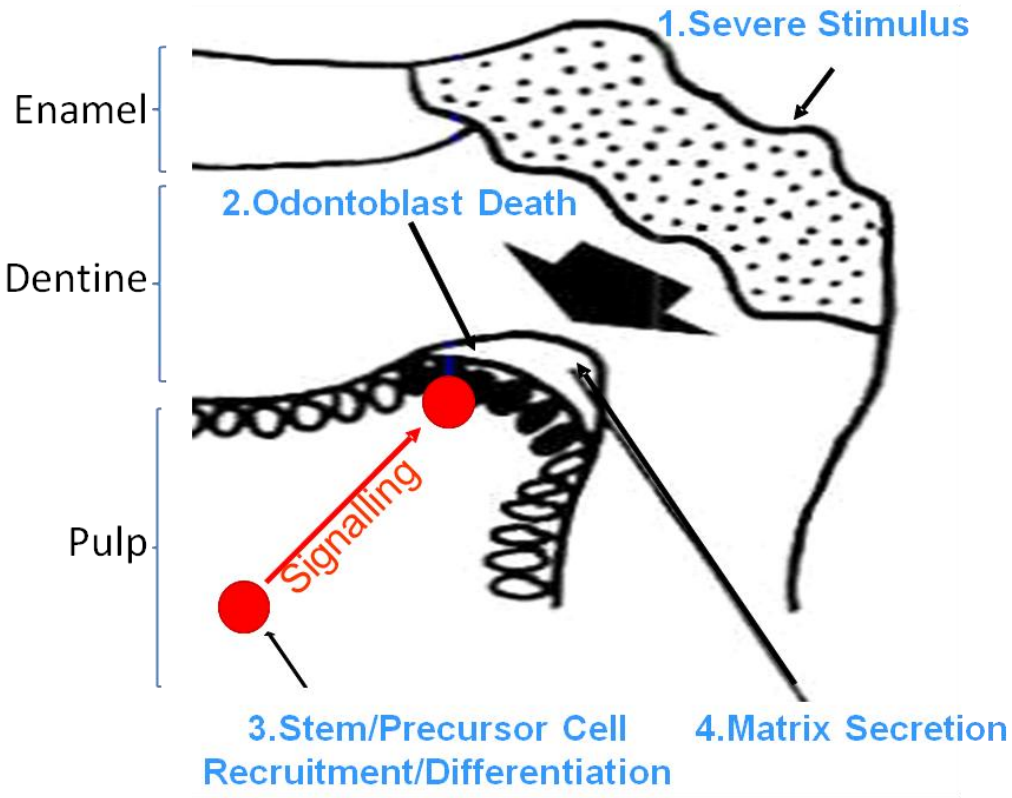


Figure 1.3 Illustration showing the stages of tertiary dentine formation during reparative dentinogenesis. Modified from Smith et al (1995).

1.2.2.6 Progenitor Cells and Signalling involved in Reparative Dentinogenesis

Questions still remain with regards to the progenitor cells involved in reparative dentinogenesis. There are several potential pulpal cell populations from which the odontoblasts involved in reparative dentinogenesis may arise. The cell-rich layer of Höhl adjacent to the odontoblasts contains undifferentiated mesenchymal cells that may be one progenitor source of odontoblast-like cells (Cotton, 1968). These cells have experienced the same developmental history as the primary odontoblast cell population with the exception of the final signalling step for terminal differentiation. Other pulpal cells, such as undifferentiated mesenchymal cells, perivascular cells (pericytes) and fibroblasts have also been suggested as progenitor cell sources (Ruch, 1998; Senzaki, 1980). The clonogenic and highly proliferative cell population from enzymatically disaggregated adult dental pulp, termed dental pulp stem cells (DPSCs), represents a potential source of the progenitors (Gronthos *et al*, 2000). It may be one or a number of these different cell populations that differentiate into odontoblast-like cells for reparative dentinogenesis. Indeed, recently it has been shown that during tooth repair, both a pericyte-derived mesenchymal cell and an additional source of cells with MSC-like properties that have been recruited toward area of tissue damage, contribute to new odontoblast cell formation (Feng *et al*, 2010)

As well as the source of these progenitor cells, the signalling pathways involved in the recruitment and differentiation of these cells also remains to be fully elucidated. The signalling involved in reparative dentinogenesis is complex with different factors that may influence the recruitment and differentiation of stem and progenitor cells. The recruitment of progenitor cells to form a new generation of odontoblast-like cells occurs following a strong

damage stimulus, such as severe caries, that causes the death of the original odontoblasts. A recent study suggested that the recruitment and differentiation of progenitor cells involved the action of BMPs released from the demineralised matrix following odontoblast apoptosis (Mitsiadis *et al,* 2008). Cell apoptosis will release many factors and intracellular components that may contribute to the overall signalling pathways involved in dental repair.

Another factor that will influence the dental repair signalling pathways is the infiltration of inflammatory cells to the site of tissue damage (Rutherford *et al,* 2000). Dendritic cells, T-lymphocytes, macrophages, neutrophils and B-lymphocytes accumulate in the pulp during an inflammatory response to invading carious microorganisms (Baume, 1970; Izumi *et al*, 1995; Goldberg *et al,* 2008). These inflammatory and immune cells will release cytokines and growth factors that will contribute to the complex signalling events taking place within the pulp. Examples of the molecules involved include the expression of the growth factor TGF-β1 (de Saint-Vis *et al*, 1998) and the antimicrobial chemokine CCL20 (Marcet *et al*, 2007) by dendritic cells.

The influence of dentine matrix molecules on dental derived cells, such as odontoblasts and DPSCs, has been another area of interest. Current evidence supports the idea that these molecules have a regulatory role within the tooth. Specific molecules that are members of the TGF-β superfamily, including TGF-β1 and BMP-2, have been shown to promote odontoblast differentiation (Begue-Kirn *et al*, 1994; Begue-Kirn *et al*, 1992). As well as specific molecules, dentine matrix extracts containing a variety of bioactive molecules from dentine have been investigated. Isolated ethylenediaminetetraacetic acid (EDTA)–soluble dentine matrix components have been shown to induce odontoblast differentiation and tubular matrix

secretion *in vivo* (Smith *et al* 1990; Tziafas *et al*, 1995), as well as stimulating the differentiation and mineralisation of human DPSCs *in vitro* (Liu *et al*, 2005). Although EDTA gives relatively high yields of TGF-β extraction from dentine, other extractants will also release this molecule. Saline, citric acid, hypochlorite, mineral trioxide aggregate (MTA), and calcium hydroxide have all been shown to be capable of releasing TGF-β and other bioactive molecules from dentine (Zhao *et al*, 2000; Tomson *et al*, 2007) highlighting the potential action of cavity treatments during tooth restoration. Another family of bioactive molecules within the dentine matrix that have been found to have an influence on pulp-derived cells is the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family. Dentonin is a 23-amino-acid peptide derived from matrix extracellular phosphoglycoprotein (MEPE), a SIBLING molecule, that has been found to enhance DPSC proliferation (Liu *et al*, 2004; Six *et al*, 2007) and recruitment (Six *et al*, 2007). Other bioactive ECM SIBLING molecules, such as bone sialoprotein, dentine sialoprotein, dentine phosphoprotein, and dentine matrix protein-1 have also been implicated in signalling the formation of reparative dentine (Goldberg *et al*, 2006). Notably, different concentrations of these same dentine matrix molecules has already been shown to induce differing cellular effects including differentiation, angiogenesis and apoptosis (He *et al*, 2005).

To date, research into the effects of dentine matrix molecules has focused on molecules, either individually or within a mixture, that are in an intact and undegraded state. During severe caries, (a common stimulus of reparative dentinogenesis), the matrix molecules of dentine and pulp may be degraded due to the action of acids and enzymes. Acids, produced by carious micro-organisms, demineralise the inorganic components, and enhance the proteolytic susceptibility of organic components of the dentine matrix (Armstrong, 1958; Hojo *et al*,

1994; Katz *et al*, 1987; Goldberg and Keil, 1989). Carious dentine has been shown to contain several different types of organic acids secreted by invading microorganism; lactic, acetic, propionic, i-butyric, n-butyric, i-valeric, n-valeric, i-caproic and n-caproic acids (Hojo *et al*, 1991). Of these acids, lactic, acetic and propionic were found to be the most abundant and will therefore be the most physiologically relevant acids in recreating the breakdown of dental ECM (Hojo *et al*, 1994).

Enzymes also play an important role in the degradation of the components of the organic dentine matrix. Carious bacteria secrete proteases that cause proteolytic breakdown of dentine ECM components (Armstrong, 1958; Larmas, 1972). Host-derived proteases MMPs, may also play a role in dentine matrix breakdown following demineralisation (Tjaderhane *et al*, 1998; Larmas, 2001). These MMPs can be derived from odontoblasts, pulpal tissue and immune cells and will likely be activated by the pH changes that occur during acidic carious demineralisation leading to the breakdown of matrix molecules (Dayan *et al*, 1983; Larmas, 2001; Tjaderhane *et al*, 2001). Enzymatically-derived ECM breakdown products may therefore play a role in the signalling involved in the wound environment. Recreating MMP-derived degradation products from dental ECM may provide a physiologically relevant model to study functional activities.

The specific interactions that occur between ECM molecules and cell surface receptors mean these molecules play a dynamic role in signalling. The smaller degradation molecules or peptides from ECM have been suggested as more adapted to fit the role of signalling molecules (Goldberg *et al*, 2009), as they can bind to appropriate cell surface receptors whereby they activate specific signalling cascades (Veis, 2003). For example it has been

shown that Dentonin, a peptide derived from MEPE, a member of the SIBLING family, stimulates the commitment, proliferation, and differentiation of osteoblast-like progenitors (Six *et al*, 2007). Therefore, the signalling environment present in the tooth during reparative dentinogenesis is likely extremely complex, represented in Figure 1.4 below. Odontoblast factors and intracellular components released by apoptosis (Factor 1), inflammatory cytokines and growth factors (Factor 2), and dentine and pulp matrix derived molecules and the acidic and enzymatic breakdown products of these matrix molecules (Factor 3) may all contribute.

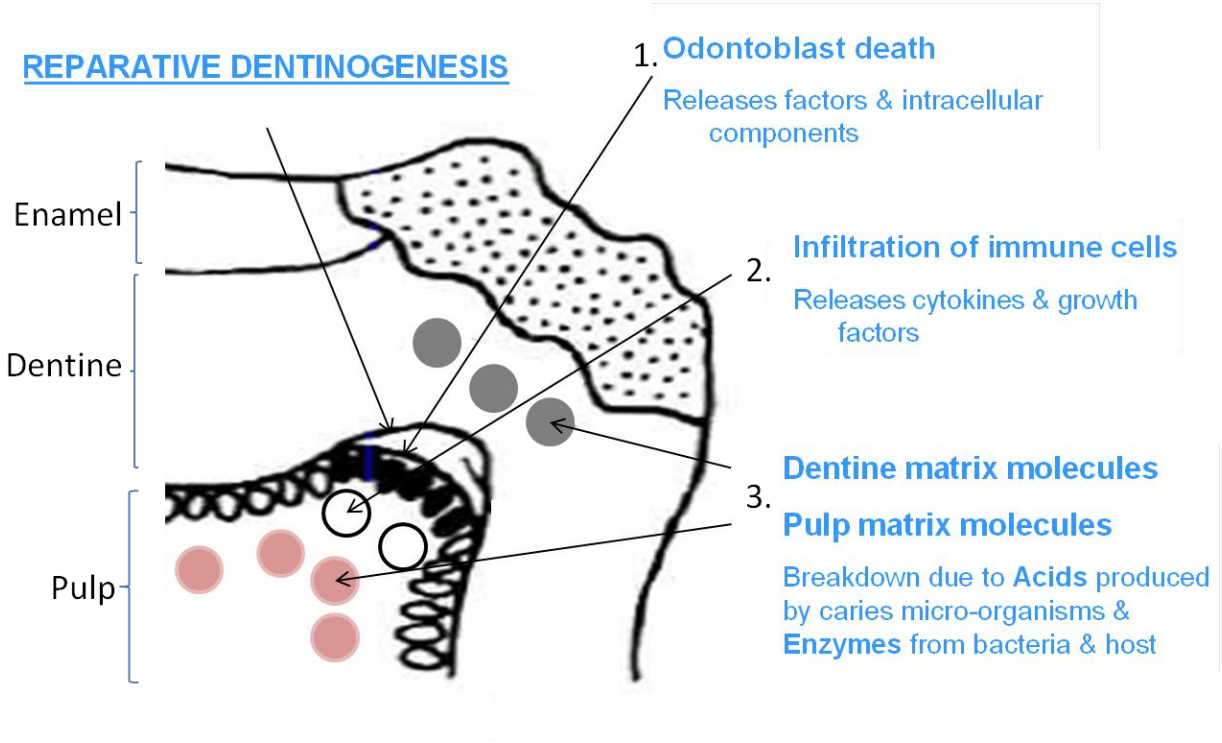


Figure 1.4 Illustration showing the potential factors involved in signalling during reparative dentinogenesis. Modified from Smith et al (1995).

1.3 Stem Cells

1.3.1 Embryonic Stem Cells

Embryonic stem cells are derived from the inner cell mass of the early embryo (76 hours after copulation plug formation) and are capable of unlimited, undifferentiated proliferation *in vitro* (Martin, 1981; Evans and Kaufman, 1981). These cells can be characterised by three essential properties; derivation from the pre-implantation or peri-implantation embryo, prolonged undifferentiated proliferation, and stable developmental potential to form cells of all three embryonic germ layers, even after prolonged culture (Thomson and Marshall, 1998). These properties make embryonic stem cells useful in developmental biology, drug discovery, and transplantation medicine (Thomson et al, 1998). The culture of embryonic stem cells often relies on a cell feeder layer to maintain stem cell charcteristics, however recent studies have shown that some ECM components may be used instead (Kobel and Lutolf, 2010; Rodin *et al*, 2010; Melkourmian *et al*, 2010; Klim *et al*, 2010; Villa-Diaz *et al*, 2010).

1.3.2 Induced Pluripotent Stem Cells

Induced pluripotent stem (IPS) cells are currently attracting significant interest in the stem cell biology field. IPS cells are developed adult cells reprogrammed into less committed pluripotent cells. This reprogramming can be performed by the transduction of specific genes using retroviral vectors to produce different types of IPS. The most commonly used factors (Oct-3/4, Sox2, c-myc and Klf4) were initially identified by screening 24 pre-selected factors (Takahashi *et al*, 2006) and have been used by different groups to produce IPS cells from

adults cells such as fibroblasts (Takahashi *et al*, 2006; Okita *et al*, 2007; Wernig *et al*, 2007). Following the identification of these factors, another group confirmed a partially overlapping group of factor (Oct 3/4, Sox2, Nanog and Lin28) that could also be used to produce IPS cells (Yu *et al*, 2007). IPS cells have been shown to possess developmental potential close to that of embryonic stem cells, as evaluated by teratoma and chimera formation (Takahashi *et al*, 2006; Wernig *et al*, 2007).

However, the reprogramming of the somatic fibroblast cells used for the production of these cell types was very inefficient. It has been suggested that the use of less committed cell populations, such as haematopoietic stem cells, may provide a starting population that is more susceptible to reprogramming (Rizzino, 2007). Dental pulp tissue may contain several populations of stem and progenitor cells and because of this has been investigated as a potential tissue source to improve reprogramming efficiency. Several dental tissue derived mesenchymal-like stem populations have been shown to have improved reprogramming efficiency compared with more mature somatic cells (Yan *et al*, 2010**)**. As well as inefficiency with reprogramming (typically less than 1%), these techniques involve the use of viral vectors that have specific biosafety concerns such as genomic integration, immunogenic responses and regulated gene expression (Maguire-Zeiss and Federoff, 2004). As such, there is a need to develop a method of producing induced pluripotent stem cells by efficient chemical or biological signals that do not incorporate into the cells' DNA. Methods of culture that improve the stem characteristics of a population are being investigated to help progress this area of research.

1.3.3 Post-natal Stem Cells

1.3.3.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a non-haematopoietic stromal cell population present in most connective tissues of the body and are capable of differentiation into cells such as osteoblasts, chondrocytes and adipocytes (Augello *et al*, 2010). This definition relies solely on the analysis of *in vitro* culture-expanded cell populations and despite several decades of investigation, the location and function of MSCs within their *in vivo* environment is not well defined (Augello *et al*, 2010). This is mainly due to the lack of specific markers allowing their unambiguous identification (Bianco *et al*., 2008; Jones and McGonagle, 2008; Morikawa *et al*., 2009). Furthermore, it is possible that MSC phenotype and potentiality may be altered by the transfer from the *in vivo* to the *in vitro* environment. During the *ex vivo* manipulation of MSCs, marker expression changes following the removal of the cells from their natural environment (Jones *et al*., 2002). Isolation and maintenance of MSCs in a more physiologically relevant environment may provide a better insight into their characteristics.

Although MSCs are not pluripotent, their multipotential differentiation capacity makes them useful candidates for regenerative medicine strategies. However, advances in any therapeutic MSC use are slowed by lack of understanding of *in-vivo* MSC properties within their tissues and niches (Augello *et al*, 2010).

1.3.3.2 Dental Stem Cells

Several different populations of stem cells have been reported within dental pulp. A clonogenic and highly proliferative cell population from enzymatically disaggregated adult dental pulp has been isolated (Gronthos *et al*, 2000). These cells were termed dental pulp stem cells (DPSCs) and were shown to have the ability to regenerate an *in vivo* dentine-pulp-like complex that was composed of mineralised matrix with some tubules lined with odontoblasts, and fibrous pulp-like tissue containing blood vessels (Gronthos *et al*, 2000). DPSCs were also later shown to have self-renewal capacity and ability to differentiate along multiple lineages (Gronthos *et al*, 2002). Exfoliated human deciduous teeth have also been found to contain a population of multipotent stem cells (Miura *et al*, 2003) and these cells were termed stem cells from human exfoliated deciduous teeth (SHED). A third population of mesenchymal stem cells have been identified in the root apical papilla of human teeth (Sonoyama *et al*, 2006); these were termed stem cells from apical papilla (SCAP). Clonogenic cells with postnatal stem cell characteristics have been isolated from periodontal ligament by enzymatic disaggregation (Seo *et al*, 2004) and these cells were termed periodontal ligament stem cells (PDLSCs). Each of the dental derived stem cell like populations is very heterogeneous in nature and there may be overlap in the populations. Comparisons between the specific dental derived stem cell populations as well as comparisons to stem cells from other tissue will be discussed below.

1.3.3.2.1 The Isolation of Dental Stem Cells

The presence of post-natal stem cells in several tissues has led to investigations assessing the potential of these cells for use in regenerative therapies. However, despite these investigations there is still no well defined protocol for the isolation of post-natal stem cells. This is due to a lack of mesenchymal stem cell specific markers and the low frequency (<1%) of MSCs within the adults tissues. Methods for the isolation of post-natal stem cells from any tissue have to overcome these problems.

The simplest method for isolating post-natal stem cells is by their ability to adhere to tissue culture polystyrene surfaces. MSCs can be isolated by polystyrene adherence as first shown using bone marrow (Friedenstein *et al*, 1976). The ability of mesenchymal stem cells to adhere to the polystyrene culture surface, which many other cell types do not, allowed isolation of a population containing stem cells (Friedenstein *et al*, 1976). However, this isolation technique does not produce a homogeneous population as some other cells types will also adhere to the culture surface. Although this method does not give a pure population, it can still be used as an initial isolation step to give populations of cells capable of behaving with some stem cell-like characteristics. MSCs have demonstrated an increased adhesion to surfaces coated with proteins such as fibronectin, type I collagen, type II collagen, vitronectin and poly-l-lysine (Tsuchiya *et al*, 2001). Cells from the dental pulp have also been shown to adhere to laminin via integrins (Zhu *et al*, 1998). The use of ECM protein coated substrates may be useful in improving the isolation of DPSCs or in encouraging DPSCs to adhere to different substrates.

DPSCs and SHED were first isolated and cultured from pulp tissue following its digestion with collagenase type I and dispase (Gronthos *et al*, 2000, Miura *et al*, 2003). The cell populations obtained were found to be clonogenic and highly proliferative (Gronthos *et al*, 2000). Single-colony-derived DSPC strains were found to show different rates of odontogenesis (Gronthos *et al*, 2002) suggesting that cells isolated in this manner were heterogeneous, within a mixed population of progenitor/stem cells.

Without the use of cell specific markers for selection, obtaining pure stem cell populations is unlikely. Therefore, cell sorting or enrichment techniques, such as fluorescence-activated cell sorting (FACs) and magnetic activated cell sorting (MACs) have been employed. FACs has been used to successfully isolate haematopoietic stem cells (Jamieson *et al*, 2004) and embryonic stem cells (Reddy *et al*, 1992). Magnetic and flow cytometry-based methods have also been used to improve the isolation of MSCs from human bone marrow (Zannettino *et al*, 2007). FACs has been used to sort cells that express the STRO-1 antigen to enrich human DPSC populations (Yang *et al*, 2007a; Yang *et al*, 2007b). STRO-1 positive cells from human dental pulp were able to differentiate towards an odontoblast phenotype, whilst STRO-1 negative cells exhibited a fibroblast phenotype (Yang *et al*, 2007a). The STRO-1 positive sorted pulp cells also demonstrated a defined multilineage potential and were more homogeneous in nature (Yang *et al*, 2007b). These data suggests that STRO-1 may be a suitable antigen for the isolation of more homogenous stem cell populations from human dental pulp. Other markers have been used to isolate more homogeneous populations of stem cells from dental pulp. Notably, the expression of c-kit and CD34 has been used to isolate a population of stromal stem cells of neural crest origin from dental pulp (Laino *et al*, 2005). Flow cytometry has been used to isolate side populations from within dental pulp cells to

produce populations with enriched stem cell characteristics with self-renewal capabilities over a long proliferatve lifespan and multi-lineage potential (Iohara *et al*, 2006). Two sub-fractions of the dental pulp side populations, ($CD31^-/CD146^-$ and $CD105^+$ cells) with enhanced angiogenic and neurogenic potential have demonstrated improved pulp regenerative characteristics compared with unsorted populations (Nakashima *et al*, 2009). MACs has also been used to isolate a stem cell population from rat embryonic mandibular processes using low-affinity nerve-growth-factor receptor (LNGFR) as a positive marker (Zhang *et al*, 2006a). These combined data demonstrate that cell selection approaches can be used to produce more homogeneous populations with stem cell characteristics. There remains a question as to what is the optimal method for isolating stem cell populations from tissue sources. In addition evidence suggests that the dental pulp may contain several different populations of stem cells (Sonoyama *et al*, 2006; Gronthos *et al*, 2000; Miura *et al*, 2003) further adding to the complexity.

1.3.3.2.2 Comparing Stem Cells from Dental Pulp and other Sources

Stem cells from dental pulp show promising characteristics for potential use in regenerative therapies. However, it is unclear as to whether these cells isolated from dental pulp can be utilised more successfully in dental regenerative treatments compared with stem cells from other sources. The similarities and differences in the characteristics of these individual cell populations will be discussed below.

For use in dental regenerative therapies, a key requirement is the ability of the stem cells to differentiate into odontoblast-like cells and form dentine matrix. DPSCs have been shown to

be capable of differentiating to form tooth-like structures with the dentine matrix exhibiting some tubular features when transplanted xenogenically with hydroxyapatite-tricalcium phosphate powder (Gronthos *et al*, 2000). *In vitro* evidence has shown that DPSCs can differentiate into odontoblast-like cells (Shi *et al*, 2001) and produce mineralised deposits when exposed to EDTA–soluble dentine matrix components and a mineralization supplement (Liu *et al*, 2005).

Stem cells from other sources have also been shown to be capable of differentiation into dental cells. c-kit enriched bone-marrow-derived cells have the ability to differentiate into ameloblast-like cells (Hu *et al*, 2006) while bone marrow-derived mixed populations have been shown capable of replacing embryonic mesenchymal cells in a tooth germ, developing into a complete tooth structure following exposure to odontogenic signals (Ohazama *et al*, 2004). These data suggests that if a suitable source of cells were identified to replace the embryonic oral epithelium, non-dental cells could be used to create a tooth germ *in vitro* for use in tooth replacement.

Stem cells from dental and non-dental sources both offer potential for use in dental regenerative therapies. However, it is the differences between the cells that may affect their potential uses. Bone marrow stem cells (BMSCs) have been compared with DPSCs and it has been found that these cells have similar protein and gene expression profiles *in vitro* and their functional activity may be regulated by similar factors (Gronthos *et al*, 2000; Shi *et al*, 2001). However, the same study also found significant differences in proliferative ability and developmental potential between these cell types *in vitro*. DPSCs showed a higher proliferation rate than BMSCs, but did not produce calcified nodules throughout the adherent

cell layer unlike the BMSCs (Gronthos *et al*, 2000). Differences were seen in differentiation activity and development potential, with BMSCs demonstrating the greatest osteogenic potential (Gronthos *et al*, 2000; Batouli *et al*, 2003), DPSCs demonstrating relative lower chondrogenic potential, and both DPSCs and SCAP demonstrating lower rates of adipogenesis in comparison with BMSCs (Zhang *et al*, 2006; Sonoyama *et al*, 2008). The neurogenic potential of dental stem cells may be greater than that of BMSCs, possibly due to their neural crest origin (Huang *et al*, 2009). DPSCs and BMSCs are controlled by many similar factors and have a similar protein expression profile, but differ significantly in proliferative activity and differentiation potentials *in vitro*, as well as in their ability to develop into distinct tissues representative of the micro-environments from which they were derived *in vivo* (Huang *et al*, 2009).

DPSCs also differ from other stem cell populations derived from the dental pulp tissue. SHED have been found to be distinct from DPSCs as they exhibit a higher proliferation rate and increased cell-population doubling time (Miura *et al*, 2003). The same study also found that SHED could not form a dentine-pulp-like complex, unlike DPSCs. However, SHED cells seeded with or without human endothelial cells in a biodegradable scaffold within an *in vivo* tooth slice model were found to produce a highly vascularised connective tissue with a similar architectural and histological structure to that of natural dental pulp (Cordeiro *et al*, 2008). SCAP have been found to form a typical dentine structure when transplanted into immunocompromised mice with a HA/TCP carrier (Sonoyama *et al*, 2006). This study also demonstrated that SCAP had greater population doubling times, tissue regenerative capacity and number of STRO-1 positive cells compared with DPSCs. Building up a profile of the differing characteristics between postnatal stem cells is improving our knowledge of the cells

for use in regenerative therapies. However, the heterogeneous nature of the dental pulp stem cell populations and the unclear relationship between these distinct or overlapping populations means there is still much uncertainty in current understanding of dental pulp cell populations.

1.3.3.2.3 Dental Tissue Engineering

The majority of research on how different biomaterial interactions can alter stem cell behaviour has been performed on cells from non-dental origin. However, dental stem cells have been used in combination with biomaterial surfaces in some investigations. A recent study demonstrated that tooth formation *in vivo* could be achieved by seeding dissociated tooth cells on a scaffold material and implanting the constructs into the omentum of immunocompromised rats (Sumita *et al*, 2006). This study reported that a collagen sponge scaffold allowed tooth production with a higher degree of success than using a poly-glycolic acid fibre scaffold. The tissue arrangement of the teeth formed from collagen scaffolds has been demonstrated to be improved by the sequential seeding of the scaffold with epithelial and mesenchymal cells (Honda *et al*, 2007). Another study has also demonstrated that it is possible to produce pulp-like connective tissue by seeding SHED and human endothelial cells on a biodegradable poly-L-lactic acid scaffold in an *in vivo* tooth slice model (Cordeiro *et al*, 2008). Combined these studies provide evidence that there is the potential for dental cells to be used in combinations with biomaterials for dental regeneration.

Three key elements of a traditional tissue engineering approach are stem cells, morphogens, and a scaffold of extracellular matrix (Nakashima *et al*, 2005). The scaffold provides a physiochemical and biological three-dimensional micro-environment for cell growth,

differentiation, promotion of adhesion and migration. The morphogens are extracellular signals within the scaffold able to induce or accelerate biological reparative responses of the stem cells included within the scaffold. One group of potential morphogens of interest for use in dental tissue engineering may be the bioactive molecules obtained from the dentine matrix. The effects that these molecules can have on DPSCs have been discussed above (Section 1.1.2.6) and highlight how these molecules may have application in tissue engineering. Controlling the effects of these molecules on stem cell behaviour will be vital for successful use within tissue engineering scaffolds. The ECM in pulp reportedly plays a significant role in the regulation of stem cell behaviour (Burness and Sipkins, 2010). It has been suggested that synthetic ECM may provide the optimal scaffold for reparative dentinogenesis (Bohl *et al*, 1998). Hydrogels, such as alginate, have been used to deliver growth factors to the pulp providing a matrix to enhance dentine regeneration (Dobie *et al*, 2002). Peptide-amphiphile (PA) hydrogel scaffolds containing osteogenic supplement have also been shown to promote the proliferation and differentiation of encapsulated SHED and DPSC cells (Galler *et al*, 2008). SHED cells were shown to form soft tissues, whilst DPSCs were shown to deposit more mineralised hard tissues (Galler *et al*, 2008). Potentially a system using SHED and DPSCs in combination with PA hydrogels may produce both soft and mineralised tissue for dental regeneration. Other 3D scaffolds have been assessed for use with dental pulp stem cells to determine potential for use in tissue regeneration. Spongeous collagen, porous ceramic and fibrous titanium mesh were assessed with DPSCs, and all showed deposition of mineralised ECM with DSPP positive expression (Zhang *et al*, 2006b). However, it should be noted that the tissue formed on these three scaffold materials was more connective tissue-like, as opposed to dentine tissue-like, showing there is the potential for improved tissue specificity in such applications. DMP1 has been used as a morphogen in a collagen scaffold to successfully

induce the differentiation of DPSC in a rat model of pulp capping (Almushayt *et al,* 2006). TGF-β1 has also been used as a morphogen in a 3D collagen scaffold (Nie *et al,* 2006) and it was found that it induced more odontoblast-like characteristics than in pulp cells cultured in the same scaffold composition, but lacking the morphogen. These studies highlight the significant influence morphogens, such as bioactive molecules from within the dentine matrix, can have on cells utilised for tissue engineering applications. Investigating the influence of dentine matrix molecules and other morphogens on dental pulp cells in the encapsulated 3D culture methods may provide further insight into the behaviour of DPSCs in 3D, useful for future tissue engineering applications.

There is the potential for DPSCs to be used with scaffold materials to create an artificial tooth germ for tooth formation in adults. There is also the possibility of using DPSCs in combination with biomaterials to create implants capable of responding to signalling, either from within the tooth or from the scaffold, in order to differentiate and form new dentine aiding tooth repair. However, both these methods rely on *in vivo* transplantation to develop the tooth construct, and to date, both methods are unable to generate tooth constructs that mimic the exact shape and size of a natural tooth (Zheng *et al,* 2011). Despite this, investigating the responses of DPSCs within different environments may provide information useful for future tissue engineering strategies.

1.4 Stem Cell Niches

1.4.1 Niche Environments

For stem cells, a niche is defined as an environment that allows the persistence and quiescence of stem cells, as well as enabling the production of a more mature progeny (Schofield, 1978). The environment within a stem cell niche is thought to be critical for cell survival and regulation, with specific cellular signals within the niche directly influencing both proliferation and differentiation (Burness and Sipkins, 2010). Many adult tissues reportedly contain stem cell niche microenvironments important for the regulation and survival of these postnatal stem cells (da Silva Meirelles *et al*, 2006).

In brain, neural stem cells have been reported to reside in specialised niche environments within the sub-ventricular zone of the lateral ventricle and in the sub-granular zone of the hippocampus (Doetsch, 2003; Palmer *et al*, 2000). Three distinct stem cell niches exist within the epidermis; the interfillicular epidermis, the sebaceous gland and the hair follicles (Burness and Sipkins, 2010). Bone marrow niche environments contain haematopoietic and mesenchymal stem cell populations. The bone marrow contains at least two distinct stem cell niches, the osteoblastic/endosteal niche and the vascular niche (Yin and Li, 2006). The numerous reports of stem cell-like populations within the dental pulp indicate that it is likely niche environments exist within pulp tissue. A DPSC niche in human dental pulp was suggested to be present in the perivascular and perineural sheath regions, identified using STRO-1, CD146, and pericyte-associated antigen as markers of the DPSCs (Shi and Gronthos, 2003). This implies that dental pulp stem cells and BMSCs both have at least one common

niche environment in the perivascular region, leading to speculation that this niche environment may exist throughout the whole body to give a perivascular MSC niche (da Silva Meirelles *et al*, 2006). MSCs may be derived from the vasculature and respond to local tissue specific signals or they be derived from local mesenchymal tissues and later migrate to the vasculature, it is also possible that it is a combination of the two mechanisms. Regardless of location, these niche environments will provide important signals for the regulation of stem cell self-renewal, proliferation, differentiation, mobilization, and homing (Yin and Li, 2006). The ECM will contribute significantly to these biochemical and biomechanical signals provided by niche environments. Therefore, investigation into the effects of ECM on stem cells during health and at wound sites may provide a useful insight into stem cell regulation.

1.4.2 The Dental Pulp and Pulp ECM

Dental pulp is a loose connective tissue, rich in ground substance and containing relatively fewer fibres compared with denser connective tissues. The structural organisation of pulp tissue can be divided into four layers.The outer most layer of the pulp comprises a **single layer of odontoblasts** that line the periphery of the pulp chamber, with their cell bodies within the pulp and cytoplasmic extensions extending into the dentine matrix. Other tissue elements, such as a terminal capillary network, nerve fibres passing between the odontoblasts and dendritic cells, are present within this layer. Components such as collagen fibres, proteoglycans and fibronectin have also been found to be present between the odontoblasts in this layer. These components make up fibrous structures during the first stages of mantle formation, known as von Korff fibres (Hargreaves and Goodis, 2002).

The next layer located inwardly is the **cell-free zone**, containing unmyelinated nerve fibres, blood capillaries and the processes of fibroblasts. Beneath this layer is the **cell-rich zone (layer of Höhl)** with a much higher density of cells and other structures, e.g. fibroblasts, undifferentiated mesenchymal cells, macrophages, lymphocytes, blood capillaries, and nerves (Hargreaves and Goodis, 2002).

The deepest layer of the pulp is the central connective tissue mass known as the **pulp proper or pulp core**. This layer contains structures such as larger blood vessels, nerves and collagen fibre bundles. Fibroblasts are the most abundant cell population in this layer, but undifferentiated mesenchymal cells and defence cells, such as macrophages, are also present (Hargreaves and Goodis, 2002).

The ECM of the pulp is the environment in which the DPSCs reside. Pulp ECM contains a relatively large amount of collagen, with type I collagen constituting 56.4%, type III 41% and type V 2% of the collagen within the pulp (Shuttleworth *et al*, 1978; Tsuzaki *et al*, 1990). The presence of type VI collagen has also been reported (Shuttleworth *et al*, 1992). Non-collagenous proteins such as fibronectin, tenascin, osteonectin and osteopontin have been shown to be present in the ECM of dental pulp (Martinez *et al*, 2004). Proteoglycans are another important component of dental pulp ECM and characterisation indicates that dental pulp contains the larger PG1 and smaller PG2 proteoglycans (Pearson *et al*, 1986). More recently, the proteoglycans versican, hyaluronan and link protein have been shown to form an aggregate within the dental pulp accounting for the majority of the proteoglycans present (Shibata *et al*, 2000}. In human adults, the GAGs, chondroitin sulphates, account for 60%, dermatan sulphate 34% and hyaluronic acid for 2% of the proteoglycans in the pulp

(Goldberg *et al*, 1995). The profile of the phospholipids in the dental pulp of several species has also been described, and it has been found that a similar profile exists between species (Ellingson *et al*, 1975). The eight common phospholipids that were found in bovine, rat and rabbit dental pulp included phosphatidylcholine, lysophosphatidylcholine, sphingomyelin, phosphatidylserine, phosphatidylinositol, ethanolamine phosphoglycerides, phosphatidic acid and cardiolipin (Ellingson *et al*, 1975). This profile of phospholipids is distinctive to the ECM of dental pulp.

1. 5 Investigating Cellular Responses to Dental ECM Molecules

The current trend of dental research is attempting to move away from the traditional material based treatments towards more advanced cellular based regenerative therapies. For these treatments to be utilised successfully, a deeper understanding of cellular regulation in the *in vivo* environment, as well as regulation following *in vitro* isolation is vital.

During severe caries stem cells within the pulp are involved in a natural repair process known as reparative dentinogenesis (Section 1.2.2.5). However, the signalling pathways involved in the recruitment and differentiation of the cells involved in this process remains to be fully elucidated. Further investigation into the regulation of pulp cells during natural repair processes may provide a useful insight for the potential recapitulation of these processes in future regenerative therapies.

Gronthos et al, 2000, reported that a clonogenic and highly proliferative stem cell population could be isolated from dental pulp (Section 1.3.3.2). Since isolation, this cell population is the

focus of much interest as a candidate population for future regenerative dental therapies (Section 1.3.3.2.2). Understanding how these cells can be influenced *in vitro* will be also useful in the progression of future regenerative therapies.

1.6 Project Aims

This project aims to investigate the role of pulp and dentine ECM in the regulation of dental pulp cell behaviour.

It is hypothesised that pulp ECM provides a specialised environment that plays a key role in the regulation of its stem cell niches. This study therefore investigates the influence of isolated pulp ECM components on primary pulp cell cultures. Analysis of growth and proliferation, together with stem cell marker expression and differentiation capacity, will be performed to determine whether the pulp ECM environment contributes to the maintenance of a stem cell niche (Figure 1.5.A).

It is hypothesised that the matrix molecules within the pulp and dentine will also contribute to the signalling involved in the recruitment and differentiation of pulp cells during reparative processes such as reparative dentinogenesis. This study will investigate whether pulp and dentine matrix molecules can influence pulp cell recruitment (Figure 1.5.C) and whether the differentiation characteristics of DPSCs can be influenced by the ECM environment (Figure 1.5.D). As these molecules may be degraded by the action of carious bacterially-derived acids and enzymes, this study will also investigate whether any change in the functional activity ECM is seen following physiologically relevant degradation conditions.

As well as influencing pulp cells during reparative processes, it is hypothesisd that matrix molecules will also play a role in innate immunity. ECM from other tissues has been shown to possess antimicrobial activity therefore, dental ECMs may possess similar activity (Sarikaya *et al*, 2002). This characteristic will slow the progression of bacterial invasion during dental disease. The present study will therefore investigate whether pulp and dentine matrix molecules can influence the growth and survival of bacteria associated with dental disease (Figure 1.5.B).

Investigation of dental pulp populations in an environment that is more physiologically relevant will provide a better understanding of their behavioural properties and therefore, provide further insight into how these cells may be used in regenerative medicine strategies. This study will also aim to investigate how dental pulp cells can be used to mimic physiologically relevant ECM environments for *in vitro* dental tissue engineering.

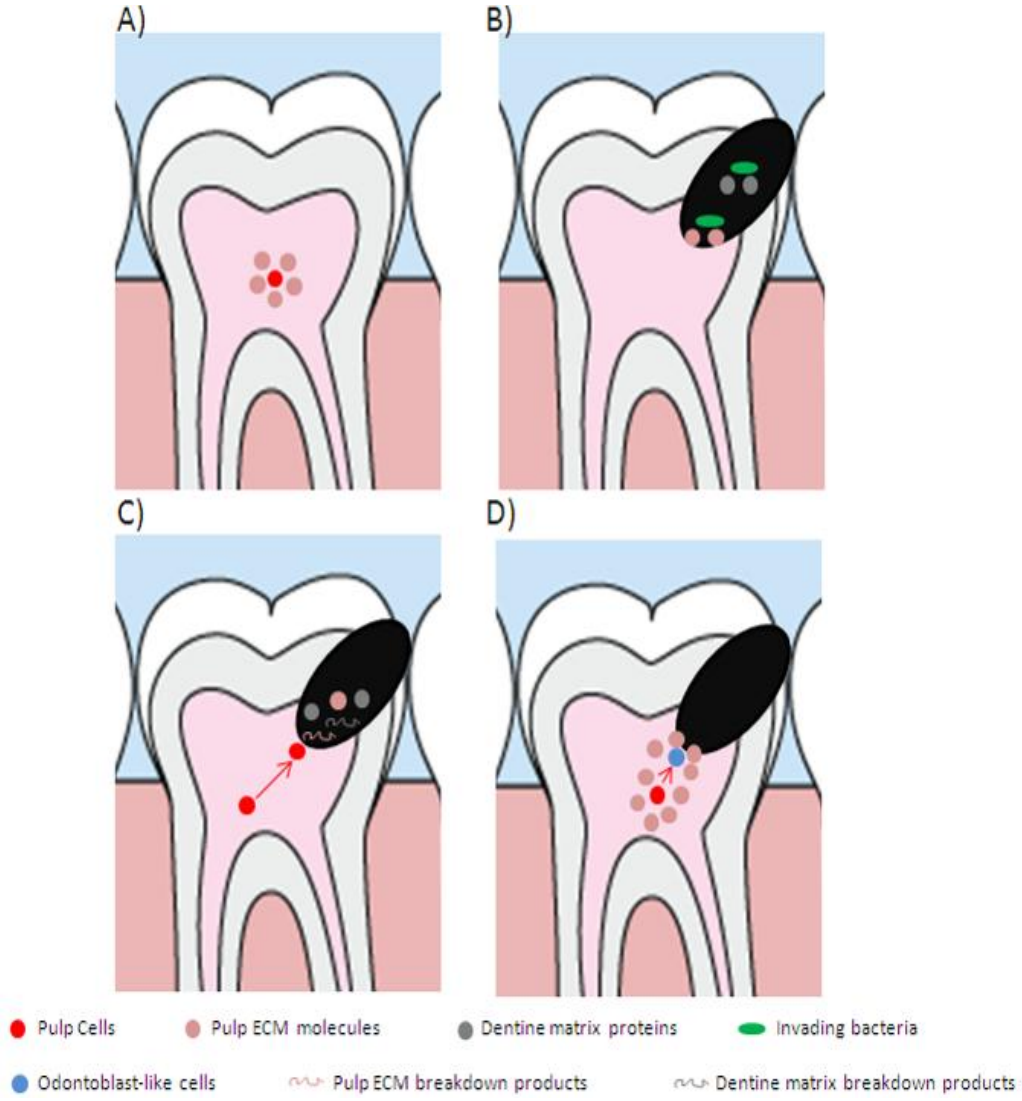


Figure 1.5 Illustration showing project aims investigating the; A) influence of pulp ECM environment on pulp cells, B) antibacterial activity of pulp and dentine matrix molecules, C) influence of pulp and dentine matrix molecules and their breakdown products on the recruitment of pulp cells, D) the influence of pulp ECM environment on the differentiation of pulp cells.

2.0 MATERIALS AND METHODS

2.1 Animal Tissue Isolation

2.1.1 Isolation of Whole Bovine Incisor Teeth

Fresh bovine tissue was used for the isolation of large yields of pECM. Bovine mandibular incisor teeth were obtained from freshly killed 1-2 month old male Holstein Friesian calves. Bovine heads from a local abattoir (Bates' Wholesale Butchers, Birmingham, UK) were rapidly transported after slaughter to the laboratories at Birmingham School of Dentistry for tooth extraction. A scalpel incision was made between each mandibular incisor deep into the periodontium to loosen attachment and non-toothed forceps were used to extract each incisor from the jaw. Any remaining soft tissue was dissected from the crown or root surfaces of the extracted teeth. The teeth were maintained in sterile αMEM medium prior to pulp tissue isolation.

2.1.2 Isolation of Bovine Incisor Pulpal Tissue

The pulpal cavity was exposed by cutting away a longitudinal slice of root dentine using a scalpel. The pulpal tissue was excavated from the cavity and placed onto a glass slide using forceps and a scalpel, under aseptic conditions. After washing in sterile PBS, the pulpal tissue was then used for the extraction of ECM (Method 2.2.1).

2.1.3 Isolation of Whole Rodent Incisor Teeth

Rodent tissue was used for the isolation of primary dental pulp cells. Maxillary and mandibular incisor teeth were dissected from 100-120g Wistar Hannover rats that had been freshly killed by cervical dislocation (Aston University, Pharmaceutical Sciences Animal House, Birmingham, UK). In a laminar flow hood, non-toothed forceps and scalpels were used to carefully remove the surrounding hard and soft supporting tissues to enable removal of the incisors from the oral cavity. The teeth were then placed in sterile αMEM medium prior to removal of pulpal tissue (Method 2.1.4).

2.1.4 Isolation of Rodent Pulpal Tissue

Rodent incisors were washed with PBS and transferred to a sterile 90mm petri dish. A scalpel was used to cut away a longitudinal slice of root dentine to expose the pulpal cavity. The pulpal tissue was then excavated from the cavity and placed onto a glass slide (Fisher Scientific, UK). After washing in sterile PBS, the tissue was then processed for primary rodent pulp cell culture (Method 2.4.2.1).

2.1.5 Isolation of Rodent Femora

Rodent tissue was used for the isolation of primary bone marrow cells. The femora from 100-120g Wistar Hannover rats were dissected out using sterile scissors and scalpel, with care taken to ensure that no external tissues contacted the femoral bones during removal. This procedure was performed by cutting and pinning back the skin around the legs to expose the

internal tissues. Soft tissues were dissected away from the two femoral bones, which were then removed and placed in a sterile Universal container containing 20ml of αMEM medium. The femoral bones were processed for primary bone marrow cell isolation and culture (Method 2.4.2.3).

2.2 Preparation of Extracellular Matrix (ECM) Components

2.2.1 Extraction of Pulpal ECM (pECM)

Bovine incisor pulp tissue was mechanically dissected, using a sterile scalpel, into pieces approximately $0.5mm^3$ – $1mm^3$ in size on a sterile glass slide and then transferred into a sterile Eppendorf® tube. 1ml of ice cold 0.5M NaCl (Sigma-Aldrich, UK) extraction solution (pH 11.7) containing the protease inhibitors 25mM EDTA (Sigma-Aldrich, UK), 1mM phenylmethylsulphonyl fluoride (Sigma-Aldrich, UK) and 5mM *N*-ethylmaleimide (Sigma-Aldrich, UK) and 1.5mM sodium azide (BDH Laboratory Supplies, UK), a bacteriostatic agent, were included in each eppendorf tube (Bellon *et al*, 1988). Dissected tissue was then homogenised using an Ultra-Turrax T8 homogeniser (IKA, Labortechnik, Germany) for 1 minute on ice and subsequently left for 24 hours at 4^oC with gentle agitation, to disaggregate the tissue to expose a larger surface area to the extraction solution. Following centrifugation at 2100 g for 5 minutes using an Eppendorf 5804R centrifuge (Eppendorf, UK), the supernatant was removed and the tissue pellet was re-suspended in 1ml fresh 0.5M NaCl extraction solution (Sigma-Aldrich, UK). This process of re-suspending, homogenising, stirring and centrifugation was repeated 3 times and the supernatants were pooled. The remaining pulp tissue was re-suspended in 1ml ice cold 0.1M tartaric acid (Hopkin & Williams Ltd, UK)

solution (pH 2.0) and subjected to the same homogenisation, stirring and centrifugation protocol as described for the NaCl solution above to obtain two tartaric acid supernatants, which were subsequently pooled (Bellon *et al*, 1988).

2.2.2 Dialysis of ECM extracts

The NaCl and tartaric acid soluble ECM extracts were exhaustively dialysed against dH_2O within 19mm diameter dialysis tubing (Scientific Laboratory Supplies, UK) for 2 weeks at 4^oC with daily changes of dH_2O, allowing the replacement of extraction chemicals with dH_2O.

2.2.3 Lyophilisation

Following 2 weeks of dialysis, the dialysates were transferred to round bottomed flasks and shell frozen under liquid nitrogen prior to lyophilisation for 24 hours using an Edwards 4K Modulyo freeze dryer. The resultant lyophilised ECM extracts were recovered and stored at -20^oC prior to use.

2.2.4 Preparation of Dentine Extracellular Matrix (dECM)

Human tissue was used for the isolation of dECM. An EDTA-soluble human dentine matrix preparation (Smith & Leaver, 1979) in a lyophilised form was kindly provided by Gay Smith (University of Birmingham, UK). This ECM preparation was solubilised in PBS immediately prior to use.

2.2.5 Degradation of ECM Extracts

2.2.5.1 Acidic Degradation of ECM

ECM extracts were dissolved in 10ml of either 0.1M acetic acid (BDH Laboratory Supplies, UK) or 0.1M lactic acid (Sigma-Aldrich, UK) at a concentration of 0.1μg/ml and were incubated at 37ºC in 5% CO_2 in air for 48 hours to allow degradation by acidic action. These preparations were used for 1D-polyacrylamide gel electrophoresis (Method 2.3.1) and chemotaxis assays (Method 2.8).

2.2.5.2 Enzymatic Degradation of ECM Extracts

ECM extracts were dissolved in 10ml of sterile dH_2O at a concentration of 0.1μg/ml and incubated at 37^{o}C in 5% CO_2 in air for 48 hours to allow auto-degradation by enzymatic action to occur, as described by Smith (1984). These preparations were used for 1D-polyacrylamide gel electrophoresis (Method 2.3.1) and chemotaxis assays (Method 2.8).

2.2.6 Ammonium Sulphate Salt Fractionation of ECM Extracts

Salt fractionation of ECM extracts was performed by protein precipitation using increasing concentrations of ammonium sulphate from 30% - 90% saturation to separate the molecules by molecular charge. Lyophilised ECM extracts were dissolved in 10ml ice cold sterile PBS at a concentration of 2mg/ml and ammonium sulphate (Fisher Scientific, UK) added to reach a saturation of 30% (1.76g). Following vortex mixing for 2 minutes and gentle agitation on an

orbital mixer (Denley, UK) for 1 hour at room temperature, the sample was centrifuged at 2100 g for 15 minutes and further ammonium sulphate added to the supernatant to 50% saturation. This process was further repeated to 70% and 90% ammonium sulphate saturations and the four precipitated fraction pellets were washed and dialysed exhaustively against distilled water prior to lyophilisation as described above (Method sections 2.2.2.and 2.2.3).

2.3 Characterisation of ECM Components

2.3.1 Electrophoretic Separation of ECM Components

2.3.1.1 1D-Polyacrylamide Gel Electrophoresis

An ECM solution was prepared by adding 0.5mg of lyophilised powder (Method 2.3) to 10µl 4x lithium dodecyl sulphate (LDS) buffer (Invitrogen NP0007) and 26µl deionised water. This solution was incubated at 105^{o}C in a block heater (Grant Instruments, UK) for 2 minutes to denature the proteins. 4µl of NuPAGE reducing agent (Invitrogen NP0004) was added to samples and the solution was incubated at 105^{o}C for a further 10 minute denaturing period. A molecular weight marker ladder solution was prepared containing 10µl Mark12 MW standard containing 12 polypeptides markers in the range of 2.5 kDa to 200 kDa (Invitrogen LC5677), 5µl 4xLDS buffer (Invitrogen NP0007) and 5µl deionised water.

A NuPAGE 10% Bis-Tris 1.0mm x 10 well gel (Invitrogen NP0301BOX) was inserted into a running tray and submerged in NuPAGE 2-(N-morpholino) ethanesulphonic acid (MES) sodium dodecyl sulphate (SDS) 20X Running Buffer (Invitrogen NP0002) containing 0.5ml

NuPAGE Antioxidant (Invitrogen NP0005). The wells in the gel were loaded with a set volume up to 10µl for either molecular weight ladder or test solution. Gels were electrophoresed at 200V for 35 minutes. After this running time, the gel was removed from the tray and placed in a clean glass dish to enable silver staining of proteins according to the SilverXpress Silver staining kit (Invitrogen LC6100) protocol as described below.

The gel was initially submerged in 210ml of a fixing solution, which contained 90ml ultrapure water (Sigma, UK), 100ml methanol (VWR, UK) and 20ml acetic acid (BDH Laboratory Supplies, UK), for 20 minutes. Subsequently, this fixing solution was decanted and replaced with two changes of 105ml sensitising solution, containing 52.5ml ultra pure water, 50ml methanol and 2.5ml sensitiser (Invitrogen LC6100) for 10 minutes each. The sensitiser solution was then decanted and the gel was washed twice with 100ml ultra pure water for 5 minutes each. The gel was then exposed to 100ml of the staining solution, containing 5ml stain A, 5ml stain B (Invitrogen LC6100) and 90ml ultra pure water for 25 minutes. The staining solution was then decanted and the gel was washed twice with 100ml ultra pure water for 5 minutes each. The gel was then submerged in 100ml developing solution containing 95ml ultra pure water and 5ml developer (Invitrogen LC6100) for between 3-15 minutes. During this time, the glass dish containing the gel was placed on a HAN250 A3 studio lightbox (Hancocks Co, UK) and images were captured as the bands developed and became visible. To halt the developing reaction, 10ml Stopper solution (Invitrogen LC6100) was added to the solution in the glass dish.

2.3.1.2 Agilent Protein Chip Electrophoretic Analysis

Protein chip electrophoresis uses protein chips that contain an interconnected set of gel microchannels that sieve proteins by size during electrophoresis. ECM extracts were subjected to 1D-electrophoresis using the reagents and protocol for the Agilent protein 230 kit (Agilent Technologies Inc, Germany). Protein solutions were prepared by dissolving ECM extracts in 1% (w/v) SDS (Sigma-Aldrich, UK) at a concentration of 1mg/ml. A mixture consisting of 4µl protein solution and 2µl 1M dithiothreitol denaturing solution was incubated at 100˚C for 5 minutes then, 84µl deionised water was added. A protein chip containing gel-dye mix and destaining solution was loaded with 6µl protein sample and 6µl molecular weight marker ladder per lane, respectively. The chip was placed in the Agilent 2100 Bioanalyser where electrophoresis was performed using an at 120v for 40 minutes to separate proteins on the basis of their molecular weights. Data were subsequently analysed with the image analysis software Bioanalyser (Agilent Technologies Inc, Germany), which identifies the molecular weights of the bands present.

2.3.2 Dye Binding Assay for Total Non-Collagenous Protein

This method was applied to enable the quantification of non-collagenous protein (NCP) within the pECM preparation using the protein-dye binding properties of Coomassie Brilliant Blue G-250 (Bradford, 1976). The Bradford reagent was prepared by dissolving 20mg Coomassie Brilliant Blue G-250 (Sigma-Aldrich, UK) in 10ml of 95% (v/v) aqueous methanol, which was then added to 20ml of 85% (v/v) phosphoric acid. 170ml of dH_2O was added to produce a final volume of 200ml reagent. This solution was filtered through

Whatman filter paper (Whatman International Ltd, UK) to remove undissolved dye prior to use.

1ml of the prepared Bradford reagent was added to a micro-cuvette, then 100µl dH_2O was added and the absorbance of this solution was obtained at 595nm as a reference blank in a Philips UV/VIS Spectrometer. A standard curve was generated by adding 100µl of bovine serum albumin (BSA) (Sigma-Aldrich, UK) to give final concentrations ranging between 0-10µg/ml. A test protein sample was generated by dissolving 1mg ECM powder in 1ml dH_2O. The NCP content of the ECM was determined by adding 100µl of sample to 1ml Bradford reagent in a micro-cuvette, mixing thoroughly, leaving 5 minutes to ensure thorough binding had occurred and then measuring the absorbance at 595nm in a Philips UV/VIS Spectrometer. The absorbance of the sample was plotted on a standard curve (Figure 2.1) to determine the concentration of NCP.

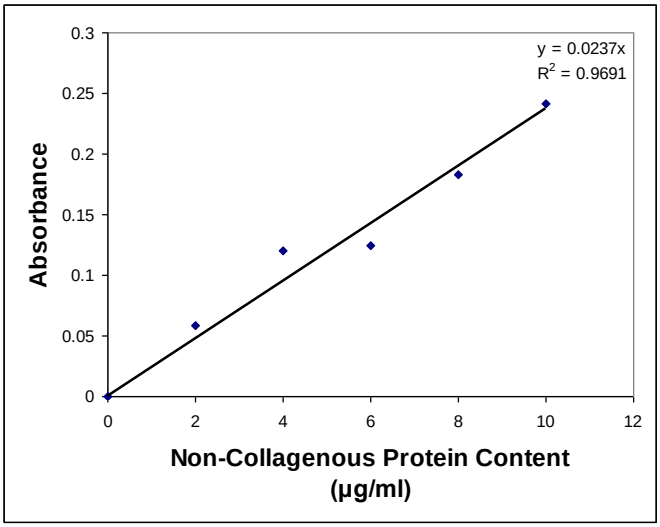


Figure 2.1 Standard curve showing absorbancies obtained at 595nm for a range of non-collagenous protein concentrations. The equation for the line and the R^2 value are shown on the graph, demonstrating an acceptable value of lineage variance above 0.9.

2.3.3 Dye Binding Assay for Glycosaminoglycan Quantification

To enable the quantification of glycosaminoglycans (GAGs), the GAG-dye binding of dimethyl methylene blue method was applied (Farndale *et al*, 1986). The Farndale reagent was prepared by dissolving 4mg dimethyl methylene blue (Hopkin & Williams Ltd, UK), 0.75g glycine (BDH Laboratory Supplies, UK) and 0.584g sodium chloride (Sigma-Aldrich, UK) in 250ml dH_2O. This solution was filtered through Whatman filter paper (Whatman International Ltd, UK) to remove undissolved dye.

1ml of the Farndale reagent was added to 100µl dH_2O in a micro-cuvette and the absorbance of this was measured at 525nm in a Philips UV/VIS Spectrometer. The value obtained was used as the reference blank. A standard curve was produced by adding 100µl of the GAG chain standard, chondroitin-4-sulphate from bovine trachea (Sigma-Aldrich, UK), in 0.5M acetic acid (BDH Laboratory Supplies, UK) giving a final concentration ranging between 0-5µg/ml. A standard test protein sample was generated by dissolving 1mg ECM powder in 1ml dH_2O. The GAG content of the ECM was determined by adding 100µl of sample to 1ml Farndale reagent in a micro-cuvette, mixing thoroughly and then the absorbance was measured immediately at 525nm. The absorbance of the sample was plotted on the standard curve, depicted in Figure 2.2, to determine the concentration of GAGs.

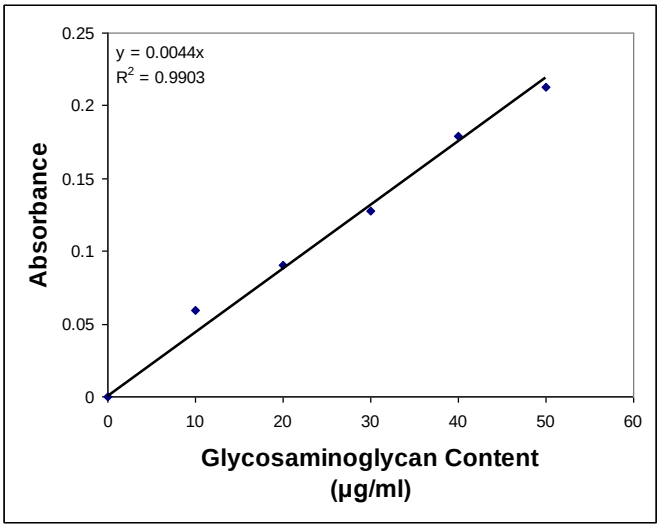


Figure 2.2 Standard curve showing absorbancies obtained at 525nm for a range of glycosaminoglycan concentrations. The equation for the line and the R^2 value are shown on the graph demonstrating an acceptable value of lineage variance above 0.9.

2.3.4 Picro-Sirius Red Assay for Collagen Quantification

The method applied enabled the quantification of soluble collagens using the selective collagen binding properties of Sirius Red in saturated picric acid (Tullberg-Reinert and Jundt, 1999). The dye solution was prepared by dissolving 0.1g Sirius Red F3B (BDH Laboratory Supplies, UK) in 100ml saturated aqueous picric acid solution (Hopkin & Williams Ltd, UK).

Collagen standards were prepared by dissolving acid-soluble rat tail collagen type I (Sigma-Aldrich, UK) in 0.5M acetic acid in a range of 0-2000µg/ml. A standard test protein sample was prepared by dissolving 1mg ECM extract powder in 1ml dH_2O. 100µl of test/blank/standards were added into separate 1.5ml Eppendorf® tubes with 1ml dye solution and mixed gently at room temperature for 30 minutes. The samples were centrifuged at 2100g for 5 minutes, and the supernatant was removed without disturbing the collagen pellet. 1ml of

0.1M HCl (BDH Laboratory Supplies, UK) was added to the pellets in each tube, centrifuged at 2100g for 5 minutes, and then removed to ensure no unbound dye remained. 1ml of 0.5M NaOH was then added to each tube and vortexed vigorously to release the bound dye. The solutions were then transferred to a 96-well plate (Corning, UK) and the absorbance determined using a 490-570nm filter on an ELX800 Universal Microplate reader (Bio-tex Instruments, USA). The absorbance of the test protein samples was plotted against the collagen standard curve (Figure 2.3) to determine the quantity of collagen in the ECM extracts.

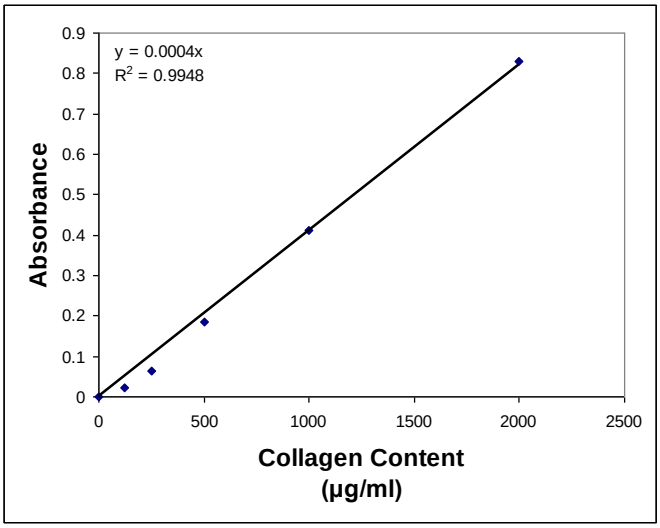


Figure 2.3 Standard curve showing absorbancies determined using a 490-570nm filter for a range of collagen concentrations. The equation for the line and the R^2 value are shown on the graph, demonstrating an acceptable value of lineage variance above 0.9.

2.3.5 Scanning Electron Microscopy (SEM)

SEM was performed on ECM coated culture polystyrene surfaces (Method 2.4.3.1) and standard/control culture polystyrene surfaces. Prior to analyses, surfaces were exposed to 2.5% glutaraldehyde (Agar Scientific, UK) in a 0.1M sodium cacodylate buffer (Fisher Scientific, UK) for 30 minutes for fixation to occur. Surfaces were dehydrated by sequential 10 minute treatments in increasing concentrations (v/v) of ethanol (Sigma-Aldrich, UK) (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%) followed by exposure to hexamethyl-disilazane overnight in a fume cupboard where evaporation resulted in the final drying of samples (Sigma-Aldrich, UK). Culture surfaces were then cut into samples of approximately $1cm^2$ using wire cutters. Conductive Acheson electrodag (Agar Scientific, UK) was used as an adhesive to attach the samples to aluminium SEM stubs (Agar Scientific, UK). The attached samples were sputter coated with a fine layer of gold under vacuum using an Emitech K550X sputter coater for 2 minutes at 25 milliamps. Secondary electron photomicrographs were obtained of the samples using an accelerating voltage of 10kV using a Joel JSM-840A scanning electron microscope at x100, x1000 and x4000 magnifications.

2.3.6 Proteomic Analysis

Extracted pulp ECM (Method 2.2) was separated using 1D-polyacrylamide gel electrophoresis (Method 2.3.1) and subjected to the proteomic analysis steps shown in Figure 2.4. Initially, the entire gel lane was dissected into two halves and then minced into $1mm^2$ pieces. The two minced aliquots were placed in separate Eppendorf® tubes and transported to Oxford Proteomics (University of Oxford, UK) where the gel pieces were digested in 0.25%

(w/v) trypsin, 1mM EDTA·4Na (Gibco, UK) and processed using an LCMS Orbitrap mass spectrometer (Thermo Scientific). Data were analysed using the Central Proteomics Facilities Pipeline (CPFP) (University of Oxford, UK) to determine the identities of the proteins present in the sample.

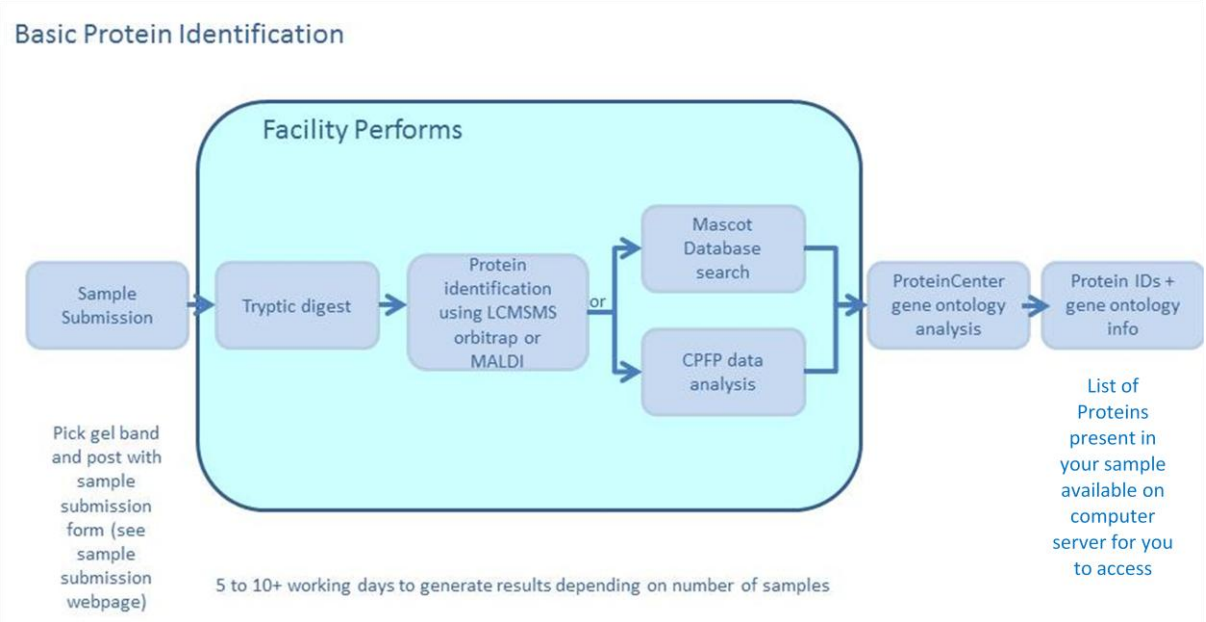


Figure 2.4 Flow diagram showing the mass spectrometry processing and bioinformatic analysis of the ECM sample performed at Oxford University Central Proteomics Facility. Reproduced from Oxford Proteomics website: www.proteomics.ox.ac.uk/index.htm.

2.4 Cell Culture

2.4.1 Cell Culture Media and Reagents

2.4.1.1 Primary Cell Culture Medium

Alpha modified minimum essential medium (α–MEM) (Biosera, UK), containing 2mM L-glutamine (Sigma-Aldrich, UK) supplemented with 1% penicillin / streptomycin (100 units/ml of penicillin with 100μg/ml streptomycin) (Sigma-Aldrich, UK) and 10% foetal calf serum (Biosera, UK) under aseptic conditions, was used for the culture of all primary cells.

2.4.1.2 Cell Line Culture Medium

Dulbecco's modified minimum essential media (DMEM) (Biosera, UK), containing 2mM L-glutamine (Sigma-Aldrich, UK) supplemented with 1% penicillin / streptomycin (100 units/ml of penicillin with 100μg/ml streptomycin) (Sigma-Aldrich, UK) and 10% foetal calf serum (Biosera, UK) under aseptic conditions was used in the culture of all cell lines.

2.4.1.3 Sterile Phosphate Buffered Saline (PBS)

Phosphate buffered saline was prepared by dissolving 7.8g NaCl (Sigma-Aldrich, UK), 1.5g K_2HPO_4 (Sigma-Aldrich, UK) and 0.2g KH_2PO_4 (Sigma-Aldrich, UK) in 1 litre of distilled water and adjusted to pH. 7.5. This solution was autoclaved at 121^{o}C to sterilise prior to use in any cell culture procedure.

2.4.2 Cell Isolation and Preparation

2.4.2.1 Primary Pulpal Cell Preparation by Enzymatic Digestion

Single cell pulp populations were obtained from rodent tissue according to enzymatic disaggregation techniques (Patel *et al*, 2009). Rodent pulpal tissue (Method 1.4) was minced on a glass slide into pieces approximately $1mm^2$ using a sterile scalpel under aseptic conditions. The tissue was then transferred into a 10ml Falcon® tube containing 2ml of 0.25% (w/v) trypsin, 1mM EDTA·4Na (Gibco, UK) and this was incubated at 37^oC for 30 minutes with gentle agitation to aid the digestion process. Subsequently, an equal volume of supplemented α–MEM was added to prevent any further enzymatic action.

The suspension containing the enzymatically released cells and the remaining pulp tissue was then passed through a cell strainer with a pore size of 70μm (BD Flacon, Belgium) into a 50ml Falcon® tube (VWR, UK). This cell suspension in the 50ml Falcon® tube was then centrifuged at 900g for 3 minutes to pellet the cells. The supernatant was removed and the pellet was re-suspended in 1ml α–MEM to provide primary pulpal cells for use in subsequent experiments.

2.4.2.2 MDPC-23 Cell Preparation

The spontaneously immortalized odontoblast-like MDPC-23 cell line derived from fetal mouse molar papillae (Hanks *et al*, 1998) were kindly donated by Prof. Jacques Nör

(University of Michigan, USA) and used as comparison to the less differentiated dental pulp cells. Cryovials of these cells were revived from storage in liquid nitrogen by incubating at 37^{o}C for ~2 minutes, adding 1ml fresh DMEM, centrifuging at 900g for 3 minutes to pellet the cells, then re-suspending the pellet in 5ml fresh DMEM. The 5ml cell suspension was then seeded into a 25cm^{2} culture flask and allowed to initially adhere and proliferate over a period of several days until approximately 80% confluent.

2.4.2.3 Primary Bone Marrow Cell Isolation

Under aseptic conditions, the femoral bones (Method 1.5) were dissected at the epiphyseal plate with a scalpel to expose the marrow cavity. A sterile 10ml syringe (Appleton Woods, UK) attached to a 19G needle 1.1 x 40mm (Appleton Woods, UK) was used to flush 10ml supplemented αMEM through the cavity into a Universal container. This cell suspension was then passed through a cell strainer with a pore size of 70μm (BD Flacon, Belgium) into a 50ml Falcon® tube (VWR, UK) to obtain a single cell population. A viable cell count of the filtered suspension was performed using 0.4% trypan blue solution (Sigma-Aldrich, UK) and an improved Neubauer haemocytometer (Hawksley, UK).

2.4.2.4 C3 Transferase Dosing of Pulp Cells

Primary isolated dental pulp cells (Method 2.4.2.1) were dosed with primary cell culture medium (Method 2.4.1.1) containing 1μg/ml cell permeable C3 transferase (Cytoskeleton Inc, USA) for 2 hours, a dose reported to selectively inactivate the GTPases rhoA, rhoB and rhoC

(Bement *et al*, 2005; Mammoto *et al*, 2004). Dosed cells were used in transwell chemotaxis assays (Method 2.8.1) and LDH cytotoxicity assays (Method 2.10).

2.4.3 Cell Culture on ECM

2.4.3.1 Preparation of ECM Coated Surfaces

Pulpal ECM (Method 2.2) was dissolved in sterile PBS under aseptic conditions to a concentration of 1mg/ml. 1ml of this ECM solution was pipetted onto 35mm^2 culture dish (Sarstedt, UK) surfaces to provide complete coverage. As control 1ml of PBS was pipetted onto separate 35mm^2 culture dishes (Sarstedt, UK). Dishes were incubated at 4^{o}C for 24 hours to allow ECM protein adsorption as previously reported (Dimilla *et al*, 1992). After this time, excess solution was removed and the surfaces were washed 3 times with sterile PBS to remove unbound protein. Surfaces were used immediately after preparation in cell culture techniques.

2.4.3.2 Measurement of Cell Adhesion and Proliferation

Cells ($2x10^4$) were seeded on ECM coated and PBS control conditioned 35mm^2 polystyrene culture surfaces (Sarstedt, UK) with six dishes used for each technical replicate. After 24 hours incubation at 37^{o}C in 5% CO_2 in air, non-adherent cells were removed by gently washing the plates with PBS and subsequently, adherent cells were detached by treatment with 0.25% (w/v) Trypsin, 1mM EDTA·4Na (Gibco, UK) for 5 minutes. Viable cells were counted with addition of 0.4% trypan blue solution (Sigma-Aldrich, UK) and an improved

Neubauer haemocytometer (Hawksley, UK) to measure initial cell adhesion. Cell growth was assessed by cell counting after 4-days culture. Images were captured using a Nikon TE-DH100w camera attached to a Nikon Eclipse TE300 microscope (Nikon, UK) to monitor cell morphology.

2.4.3.3 High Content Cell Analysis (HCA)

Primary rodent pulp cells (Method 2.4.2.1) were seeded in black 96-well tissue culture grade flat bottom plates (Corning, UK) at a density of $5x10^3$ cells in 200µl primary culture medium (Method 2.4.1.1). After 24 hours incubation at 37^oC in 5% CO_2 in air, cells were gently washed with PBS and fixed with 200µl 10% paraformaldehyde (VWR, UK) for 30 minutes. Fixed cells were washed twice with PBS and then stored in 200µl PBS. A sealing film (Jencons, UK) was used to cover the wells in the plate to prevent dessication during transport to Imagen Biotech (Manchester, UK) for staining and analysis.

For HCA, plates were washed with PBS and then each well was incubated with 100µl Oct4 primary antibody (Abcam, UK) diluted 1:100 in 0.1M phosphate buffer pH7.8 with 0.1% bovine serum albumin (BSA) for 1 hour at room temperature. Cells were washed 3 times in wash buffer and then incubated with a goat anti-rabbit IgG secondary antibody conjugated to an Alexa Fluor® 488 fluorescent label (Invitrogen, UK) for 1 hour. Cells were washed with PBS prior to analysis.

Cells were analysed using an automated fluorescent imaging microscope, an ArrayScan High Content Screening (HCS) Imaging Cytometer (Cellomics, UK). The software used for the

data acquisition and analysis was ArrayScan II Data Acquisition and Data Viewer 3.0 (Cellomics, UK). This software determined the number of cells in each well and allowed quantification of the expression of Oct4 as a percentage of the population.

2.5 Molecular Biology Techniques

2.5.1 RNA Isolation

RNA isolation was performed using the QIAGEN RNeasy minikit (Qiagen, UK). Media was removed from cell cultures and a PBS wash was performed to ensure removal of any remaining medium. The cells were lysed using 350µl of 1% (v/v) β-mercaptoethanol in RLT kit lysis buffer. 350µl of 70% (v/v) ethanol was then added to the lysate and the total volume was vortex mixed prior to application to an RNeasy mini-column assembly, which drained into a 2ml collection tube. The assembly was centrifuged for 15 seconds at 8,000 g and the eluate discarded. 700µl of kit wash buffer was added to the column followed by centrifugation for 15 seconds at 8000g. The RNeasy column with bound RNA was transferred to a new 2ml collection tube and 500µl of ethanol kit buffer was added. This was centrifuged for 15 seconds at 8000g. The eluate was discarded and another 500µl of ethanol kit buffer was added to the assembly and this was centrifuged for 2 minutes at 8000g. To ensure that no residual ethanol remained on the membrane, the column was centrifuged for another 1 minute at 11000g. The RNeasy column was transferred to a 1.5ml flat bottom collection tube (Appleton Woods, UK) and 30µl molecular grade RNAse-free water was added to the column membrane and this was then centrifuged for 1 minute at 8000g to collect the RNA.

2.5.2 RNA Analysis

To determine the concentration of the isolated RNA solution, the absorbance of a sample solution at 260nm was obtained using an Eppendorf Biophotometer (Eppendorf, UK). RNA was also electrophoresed on a 1% (w/v) agarose gel to confirm its presence and integrity. An agarose gel was prepared by boiling a 1% (w/v) agarose (WebScientific, UK) solution in 70ml of 1x tris(hydroxymethyl)-aminomethane (TAE) buffer (Qiagen, UK), cooling to 60°C prior to adding 3µl of 10,000X SYBR gold nucleic acid staining solution (Invitrogen, UK), then allowing the mixture to cool fully in a gel tray with well forming combs inserted. 1µl of the RNA solution was mixed with 3µl RNAse-free water and 1µl 10X loading buffer (Catalogue number G190A, Promega, UK); this was then loaded into a well of the 1% agarose gel and electrophoresed at 120V for ~30 minutes. The gel was then transferred to a G:Box gel documentation and analysis unit (Syngene, UK) where the presence of 18s and 28s bands was examined with UV illumination for confirmation of RNA quality, an example of which is provided in Figure 2.5.

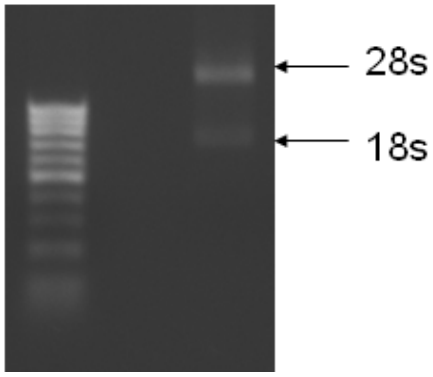


Figure 2.5 Image showing RNA electrophoresed on a 1% agarose gel with the 28s and 18s RNA bands visible. 4µl PCR Ranger 100bp DNA ladder (Norgen, UK), also shown as DNA standard.

2.5.3 Reverse Transcription (RT) of RNA

The QIAGEN Omniscript RT kit (Qiagen, UK) was used to reverse transcribe the isolated RNA into cDNA. 1.5-2µg of the RNA was diluted in a total volume of 12µl of sterile molecular biology grade water (BDH Laboratory Supplies, UK). 2µl of 10µM oligo-dT primer (Ambion, UK) was added to the 12µl RNA solution and this mixture was incubated at 80^{o}C for 10 minutes and then quenched on ice for 5 minutes. This approach enabled more efficient reverse transcription by minimising RNA secondary structures, therefore improving its accessibility. 2µl reverse transcriptase buffer, 2µl deoxynucleosidetriphosphates (dNTP) mix, 1µl RNase inhibitor (Promega, UK) and 1µl Omniscript reverse transcriptase were then added to the mixture. This 20µl of solution was vortex mixed and then incubated at 37^{o}C for 1 hour to allow the reverse transcription reaction to occur, forming single stranded cDNA. The solution was heated at 95^{o}C for 5 minutes to stop the reaction.

2.5.4 Concentration and Purification of cDNA

480µl of sterile molecular biology grade water (BDH Laboratory Supplies, UK) was added to the 20µl of synthesized cDNA and the 500µl of mixture was transferred to a Microcon YM-30 spin basket assembly (Millipore, UK). This was centrifuged at 8000g for 6.5 minutes and the eluate was discarded. 550µl of sterile molecular biology grade water was then added to the spin basket assembly and centrifuged at 8000 g for 7 minutes and the eluate discarded. The fluid volume remaining on the surface of the filter membrane was measured using a sterile pipette. If the volume was greater than 50µl, the spin basket was centrifuged further for up to 2 minutes to further concentrate the cDNA solution. Once the volume was less than 50µl, the

spin basket was inverted in a collection tube and centrifuged at 1250g for 3 minutes. An estimate of the concentration of the cDNA was obtained by measuring the absorbance of a sample at 260nm in an Eppendorf Biophotometer (Eppendorf, UK).

2.5.5 Semi-quantitative Reverse Transcription (RT) Polymerase Chain Reaction (PCR)

The PCR was established in a 0.2ml PCR tube (Appleton Woods, UK) on ice to prevent degradation of the sample and minimise mis-priming. The PCR mix contained 12.5µl 2xREDTaq Ready mix (Sigma-Aldrich, UK), 1µl of 1µM forward primer (Invitrogen, UK- sequences in Table 2.1), 1µl of 1µM reverse primer (Invitrogen, UK- sequences in Table 2.1), and 12.5µl of molecular biology grade water (BDH Laboratory Supplies, UK) containing 50-100ng of cDNA. These components were mixed thoroughly and placed in a GeneAmp 2700 PCR thermocycler (Applied Biosystems, UK). The amplification parameters were optimised for individual primers, details of which are provided in Table 2.5.5. The common cycling parameters were an initial denaturation step of 94^{o}C for 5 minutes, followed by 1 minute cycles of 94^{o}C denaturation for 20 seconds, annealing of primers at 60/60.5^{o}C for 20 seconds, extension at 68^{o}C for 20 seconds with the number of cycles adjusted according to the optimal number for the specific assay. After the cycling, there was a final extension at 72^{o}C for 10 minutes followed by cooling at 4^{o}C.

Primer	Primer Sequence (5' → 3')	Annealing Temperature (°C)	Cycle Number
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	F-CCCATCACCATCTTCCAGGAGC R-CCAGTGAGCTTCCCGTTCAGC	60.5	24
Dentine sialophosphoprotein (DSPP)	F-TGC ATT TTG AAG TGT CTC GC R-CCT CCT GTC TTG GTG TGG TT	60.5	30
Dentine matrix protein 1 (DMP-1)	F-CGG CTG GTG GTC TCT CTA AG R-CAT CAC TGT GGT GGT CCT TG	60.5	30
Oesteopontin (ON)	F-AAG CCT GAC CCA TCT CAG AA R-GCA ACT GGG ATG ACC TTG AT	60.5	27
Oesteocalcin (OC)	F-TCC GCT AGC TCG TCA CAA TTG G R-CCT GAC TGC ATT CTG CCT CTC T	60.5	27
Osetonectin (ON)	F-AAA CAT GGC AAG GTG TGT GA R-AGG TGA CCA GGA CGT TTT TG	60.5	23
Osteoadherin (OA)	F-AAC CTT AGC CAC AAC AAA ATT AA R-TTG CTT CAG TTT GTT ATG TCC	60.5	27
Nestin	F-CAT TTA GAT GCT CCC CAG GA R-AAT CCC CAT CTA CCC CAC TC	60.5	23
Collagen type I	F-GGGCAAGACAGTCATCGAAT R-TTGGTTTTTGGTCACGTTCA	60	25
Alkaline Phosphatase	F-CTC CGG ATC CTG ACA AAG AA R-ACG TGG GGG ATG TAG TTC TG	60.5	25
Adipocyte protein 2 (aP2)	F-TGGAAACTCGTCTCCAGTGA R-GCTCATGCCCTTTCGTAAAC	60.5	25
Lipoprotein lipase (LPL)	F-GTCACCAGCATCCCCATTAT R-TTCCGGATAAAACGTTCTCG	60.5	25
Proliferator-activated receptor-2 (PPAR2)	F-CTGGCCTCCCTGATGAATAA R-GCACGTGCTCTGTGACAATC	60.5	25
Nanog	F-TATCGTTTTGAGGGGTGAGG R-CAGCTGGCACTGGTTTATCA	605	30
Sex determining region Y (Sox-2)	F-ATACAAGGGAATTGGGAGGG R-AAACCCAGCAAGAACCCTTT	60.5	25
Lin28	F-TTTCTTGTTTCCCCCAAATG R-AGAGGGGCTGGTTGTAAGGT	60.5	25
Krueppel-like factor 4 (Klf4)	F-ATCATGGTCAAGTTCCCAGC R-ACCAAGCACCATCGTTTAGG	60.5	25
C-myc	F-CTTACTGAGGAAACGGCGAG R-GCCCTATGTACACCGGAAGA	60.5	25
Stem cell antigen-1 (Sca-1)	F-AGCTCTTTGATCTGCCGTGT R-CTGCAGGCAATCCAATTTTT	60.5	26
CD90 (Thy-1)	F- AACCAGAACCTTCGACTGGA R-AGGAAGGAGAGGGAAAGCAG	60.5	26
Vimentin	F-AGATCGATGTGGACGTTTCC R-GCAGGTCCTGGTATTCACG	60.5	23
CD44	F-TGG GTT TAC CCA GCT GAA TC R-CTT GCG AAA GCA TCA ACA AA	60.5	23
Stem cell factor (SCF)	F- CCTTTGAGCAGAGGAAGCAA R-TCAAGGTGCAGGTTGCACTA	60.5	26

Table 2.1 Sequences and amplification parameters for rat primer sequences used in PCR. Rodent primer sequences were designed using Primer Blast programme; http://www.ncbi.nlm.nih.gov/tools/primer-blast/. The identities of the genes that the designed sequences coded for were confirmed by searching NCBI website for matching sequences. Oligonucleotides were purchased from Invitrogen, UK.

2.5.6 Gel Electrophoresis

Agarose gels were prepared by boiling a 1.5% (w/v) agarose (WebScientific, UK) solution in 70ml of 1xTAE buffer (Qiagen, UK) in a Samsung M1714 microwave for 2 minutes at 850w, cooling to 60°C before adding 3µl of ethidium bromide (Helena Bioscience, UK) nucleic acid stain, then allowing the mixture to cool and set in a gel tray with well forming combs inserted. 5 µl of the PCR mixtures were loaded into the wells of the gel prior to electrophoresis at 120V for 30-45 minutes. The gel was then transferred to a G:Box gel documentation and analysis unit (Syngene, UK) where images of gels illuminated with UV light were obtained with GeneSnap software (Syngene, UK) and analysed using GeneTools image analysis software (Syngene, UK) giving semi-quantitative values of band intensities.

2.6 Microbiological Techniques

2.6.1 Culture of Bacteria

Anaerobic bacteria were kindly provided by the Forsyth Institute, Boston and were originally purchased from the American Type Culture Collection (ATCC). The in-house identification of the bacteria was verified by colony morphology, gram staining, catalase activity and PCR amplification of species specific genes (Palmer, 2010). The reference system used for this identification was Bergey's Manual of Determinative Bacteriology (Holt 1994). Tryptone soya agar plates (Oxoid, UK) containing 5% (v/v) horse blood (Oxoid, UK) were innoculated with *Streptococcus mutans* (ATCC no. 25175) *Streptococcus oralis* (ATCC no. 35037) and *Enterococcus faecalis* (ATCC no. 29212) and grown in a miniMACS anaerobic workstation

(Don Whitley Scientific Limited, UK) at 37°C, with re-plating required for all organisms every 7 days.

2.6.2 Antimicrobial Assays

Individual colonies of cultured bacteria were used to inoculate 10ml of tryptone soya broth (Oxoid, UK) and were placed in a miniMACS anaerobic workstation (Don Whitley Scientific Limited, UK) at 37°C with gentle agitation for bacterial growth to occur. Following growth, bacterial cell number was determined by measuring the optical density of an aliquot of the solution at 600nm in a Jenway 6300 spectrophotometer. Non-inoculated samples of tryptone soya broth (Oxoid, UK) were used to correct for media changes in absorbance and the 600nm values used to determine bacterial growth were applied and had been previously determined by the Forsyth Institute, Boston. The bacterial suspensions were then diluted to $2x10^5$ cells/ml and 100µl of suspension added to 96-well plates (Corning, UK) containing various concentrations (0.1-100µg/ml) of ECM extracts in 100µl tryptone soya broth. A negative control containing tryptone soya broth alone and antibiotics (Sigma-Aldrich, UK-Aldrich, UK) of 0.5units/ml penicillin and 0.5µg/ml streptomycin within the broth were used. For each technical replicate 8 wells were used. Bacterial growth in each well was recorded after a 24 hour period by assessment of turbidity measured at 570nm using an ELX800 Universal Microplate reader (Bio-tex Instruments Inc).

To determine whether the antimicrobial effects of ECM preparations were bacteriostatic or bactericidal, after the initial 24 hours growth in test solutions, the bacteria were isolated by centrifugation and re-suspended in fresh tryptone soya broth in the absence of the ECM

extracts or antibiotic preparations. Following a further 24 hours growth, bacteria were quantified as described above.

2.7 Lineage Induction of Pulpal Cells

Primary pulpal cells ($2x10^4$) (Method 2.4.2.1) were seeded on ECM coated and PBS control conditioned $35mm^2$ polystyrene culture surfaces (Sarstedt, UK) (Method 2.4.3.1) and incubated at 37^oC in 5% CO_2 in air. For each technical replicate 6 dishes were used. Control cultures were grown in primary culture medium (Method 2.4.1.1) and test cultures were induced with primary culture medium containing differentiation supplements as listed below (Methods 2.7.3.1, 2.7.2.1 and 2.7.1.1). Cultures were grown for up to 3 week time periods with media changes every second day.

2.7.1 Osteogenic Differentiation

2.7.1.1 Osteogenic Medium

α–MEM (Biosera, UK), containing 2mM L-glutamine (Sigma-Aldrich, UK) was supplemented with 1% penicillin / streptomycin (100 units/ml of penicillin with 100μg/ml streptomycin) (Sigma-Aldrich, UK) and 10% foetal calf serum (Biosera, UK), as well as the key osteogenic medium constituents 10^{-7}M dexamethasone (Sigma-Aldrich, UK), 10mM β-glycerophosphate (Sigma-Aldrich, UK), and 50μg/ml ascorbic acid (Sigma-Aldrich, UK) (Gronthos *et al*, 1994; Zhang *et al*, 2006).

2.7.1.2 Alizarin Red Staining for Mineralisation Analysis

A stock solution of 40mM alizarin red (BDH Laboratory Supplies, UK) was adjusted to pH4.2 with 10mM acetic acid (BDH Laboratory Supplies, UK) (Gregory *et al*, 2004). Staining was performed by fixing cells in 10% paraformaldehyde (VWR, UK) for 30 minutes, washing cultures with PBS then adding 1ml alizarin red solution to each culture for 20 minutes with gentle agitation. Excess stain was removed by gently washing with excess PBS solution. Images of the stained dishes were captured photographically using a Nikon TE-DH100w camera attached to a Nikon Eclipse TE300 microscope (Nikon, UK) and the cultures were de-stained in 10mM acetic acid for 30 minutes. Subsequently, a 25cm cell scraper (Sarstedt, UK) was used to ensure complete detachment of cells from the surface and the alizarin red / cell mixture was transferred to an Eppendorf® tube. This mixture was vigorously vortexed for 30 seconds and then heated to 85^{o}C for 10 minutes to ensure all alizarin red stain was dissolved. The mixture was cooled and the pH adjusted to 4.2 using 10% (w/v) ammonium hydroxide (Sigma-Aldrich, UK) to prevent pH influencing absorbance readings. The mixtures were transferred to a 96-well plate (Corning, UK) and optical densities were determined at 405nm in an ELX800 Universal Microplate reader (Bio-tex Instruments Inc) and compared with a standard curve obtained by measuring dilutions of the 40mM alizarin red stock solution at 405nm (Figure 2.6) to determine the concentrations of the solutions.

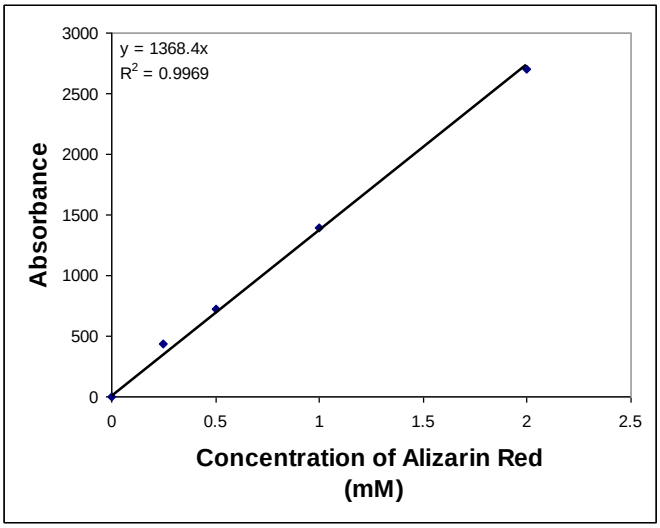


Figure 2.6 Standard curve showing absorbancies determined at 405nm for a range of alizarin red concentrations. The equation for the line and the R^2 value are shown on the graph, demonstrating an acceptable value of lineage variance above 0.9.

2.7.2 Adipogenic Differentiation

2.7.2.1 Adipogenic Medium

Alpha modified minimum essential medium (α–MEM) (Biosera, UK), containing 2mM (0.584g/l) of L-glutamine (Sigma-Aldrich, UK) was supplemented with 1% penicillin / streptomycin (100 units/ml of penicillin with 100μg/ml streptomycin) (Sigma-Aldrich, UK) and 10% foetal calf serum (Biosera, UK), as well as the key adipogenic medium constituents 0.5μM hydrocortisone (Sigma-Aldrich, UK), 60μM indomethacin (Sigma-Aldrich, UK), and 0.5mM 1-methyl-3-isobutylxantine (Sigma-Aldrich, UK) (Gronthos *et al*, 2002; Zhang *et al*, 2008).

2.7.2.2 Oil Red O Staining for Adipogenesis

A stock solution of oil red O was prepared by dissolving 0.5g of oil red O (VWR, UK) in 200ml propan-2-ol (VWR, UK), heating at 56^{o}C for 1 hour and then cooling to room temperature (Zhang *et al*, 2008; Choi *et al*, 2005). A working solution was prepared by adding three parts stock solution to two parts dH_2O and filtering immediately prior to use to remove any un-dissolved stain. The oil red O staining was performed by fixing the cells in 10% paraformaldehyde (VWR, UK) for 30 minutes, washing with 60% propan-2-ol (VWR, UK), adding the working solution of oil red O for 10 minutes, washing with 60% propan-2-ol and finally washing several times with tap water. Photographic images of the stained dishes were obtained using a Nikon TE-DH100w camera attached to a Nikon Eclipse TE300 microscope (Nikon, UK). The cultures were de-stained in 100% propan-2-ol and the optical densities of the solutions obtained from the de-staining were determined at 570nm in an ELX800 Universal Microplate reader (Bio-tex Instruments, USA) and plotted against a standard curve obtained by measuring dilutions of the oil red O stock solution at 570nm (Figure 2.7).

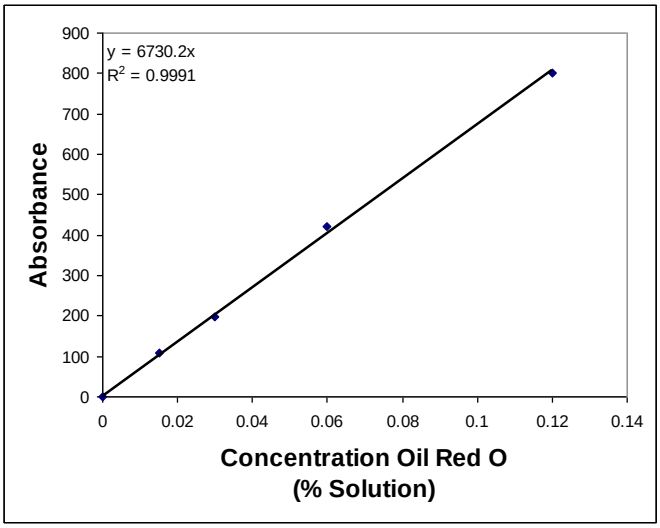


Figure 2.7 Standard curve showing absorbancies determined at 570nm for a range of oil red O concentrations. The equation for the line and the R^2 value are shown on the graph, demonstrating an acceptable value of lineage variance above 0.9.

2.7.3 Odontogenic Medium

Alpha modified minimum essential medium (α–MEM) (Biosera, UK), containing 2mM (0.584g/l) of L-glutamine (Sigma-Aldrich, UK) was supplemented with 1% penicillin / streptomycin (100 units/ml of penicillin with 100μg/ml streptomycin) (Sigma-Aldrich, UK) and 10% foetal calf serum (Biosera, UK), as well as the key odontogenic medium constituents 10mM β-glycerophosphate (Sigma-Aldrich, UK), 50μg/ml ascorbic acid (Sigma-Aldrich, UK), and lyophilised 1μg/ml EDTA-soluble human dentine matrix preparation (Method 2.2.4) (Smith *et al*, 1990; Couble *et al*, 2000).

2.8 Chemotaxis Assays

2.8.1 Chemotaxis Transwell Assay

30µl of potential chemoattractant solutions (detailed below) were pipetted into the wells of a 96-well micro-chemotaxis plate 8µm-pore size (Neuro Probe, UK) (Beattie *et al*, 2009; Reing *et al*, 2009; Cheng *et al*, 2010). For each technical replicate 8 wells were used. Primary bone marrow cells ($3x10^4$) (Method 2.4.2.3), enzymatically isolated rat primary pulp cells ($3x10^4$) (Method 2.4.2.1), or odontoblast-like MDPC-23 cells ($3x10^4$) (Method 2.4.2.2) were seeded in wells of the upper chamber on the 8µm-pore membrane (Neuro Probe, UK) in 25µl unsupplemented medium. The micro-chemotaxis plate was incubated at 37^oC in 5% CO_2 in air for 3 hours to allow migration to occur. Cells on the surface of the membrane were removed together with the membrane and 1mg/ml calcein AM (Biotium Inc, UK) was added to each well at a final dilution ratio of 1:500 for 30 minutes to label cells prior to luminescence measurement with a Twinkle LB 970 Luminometer (Berthold Technologies, UK). Readings were converted to actual cell numbers using standard curves of known cell numbers as provided in Figure 2.8.

Potential chemoattractants examined in these experiments included:

- A negative control of unsupplemented medium.
- A positive control of medium containing 1-10% FCS.
- Pulp (Method 2.2.3) and dentine (Method 2.2.4) ECM extracts at a concentration of 0.1µg/ml medium.

- Acid breakdown products of pulp and dentine ECM extracts (Method 2.2.5.1) at a concentration of 0.1μg/ml medium.
- Enzymatic breakdown products of pulp and dentine ECM extracts (Method 2.2.5.2) at a concentration of 0.1μg/ml medium.

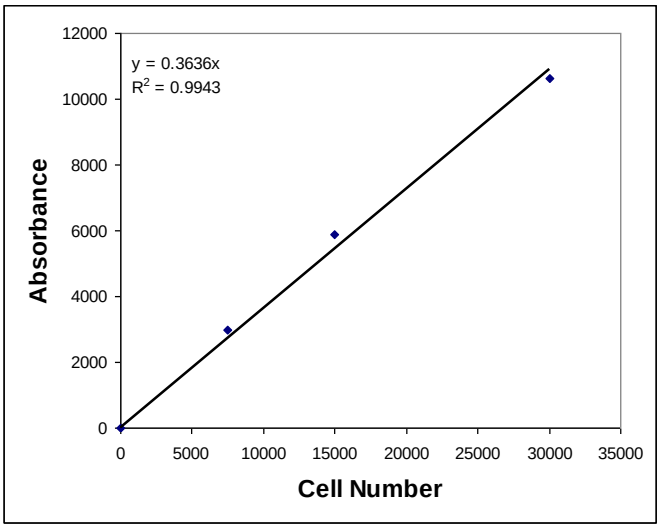


Figure 2.8 Standard curve showing absorbancies for a range of cell numbers labelled with 1mg/ml calcein AM (Biotium Inc, UK) and measured using a Twinkle LB 970 Luminometer. The equation for the line and the R^2 value for are shown on the graph, demonstrating an acceptable value of lineage variance above 0.9.

2.8.2 Agarose Spot Cell Migration Assay

2.8.2.1 Cell Migration into Agarose Spots

A 0.75% (w/v) agarose solution (WebScientific, UK) was heated to boiling for 2 minutes in a Samsung M1714 microwave for 2 minutes at 850w to dissolve the agarose and aliquots of 90μl were cooled to 40^{o}C on a block heater (Grant Instruments, UK). 10μl of sterile PBS

containing ECM (1µg/ml) was added to the 90µl aliquot to give an agarose solution with a final concentration of 0.1µg/ml ECM molecules. Alternatively, 10µl of sterile PBS (containing no ECM) was added as a negative control or 10µl FCS was added and used as a positive control for chemotaxis. 10µl of the 100µl agarose solution was pipetted onto polystyrene cell culture surfaces (35mm dishes or 24 well plates) and allowed to cool and solidify at 4^{o}C for 5 minutes to form a solid agarose spot. 8 agarose spots were analysed for each technical replicate. 50,000 primary rodent pulp cells (Method 2.4.2.1) were seeded in 1ml αMEM onto the culture surface around the spots and incubated at 37^{o}C in 5% CO_2 in air for 24 hours to allow adhesion and migration to occur (Wiggins and Rappoport, 2010). The total number of cells that migrated into each spot was counted under visualisation with a Nikon Eclipse TE300 microscope (Nikon, UK), with over 50% of the cell body having to be inside the spot perimeter to be classed as migrated. Images were captured containing the perimeter of spots enabling cell migration to be assessed. Fields at the perimeters of the agarose spots were also visualised using a Zeiss Axiovert 200 time-lapse video microscope. Images were recorded every 10 minutes and converted into AVI videos files and analysed using image-J software (National Institutes of Health, USA).

2.8.2.2 Tribometric Testing of Agarose Spots

The strength of agarose gel adhesion to the polystyrene culture surface was tested using a custom built tribometer (Longshore Systems Engineering, UK), the design of which is provided in Figure 2.9. 0.75% (w/v) agarose gels with and without addition of 1µg/ml dECM were prepared in 1cm^2 moulds on polystyrene surfaces. A mounted teflon probe was aligned with the surface of the polystyrene with application of a normal load of 1N. The probe was

brought into contact with the agarose gel, moving at a constant speed of 0.5mm/s. The resulting force acting between the probe and gel was measured by the maximum deflection of the probe holder against a load cell and was used to determine the force required to break the agarose spot adhesion with the polystyrene.

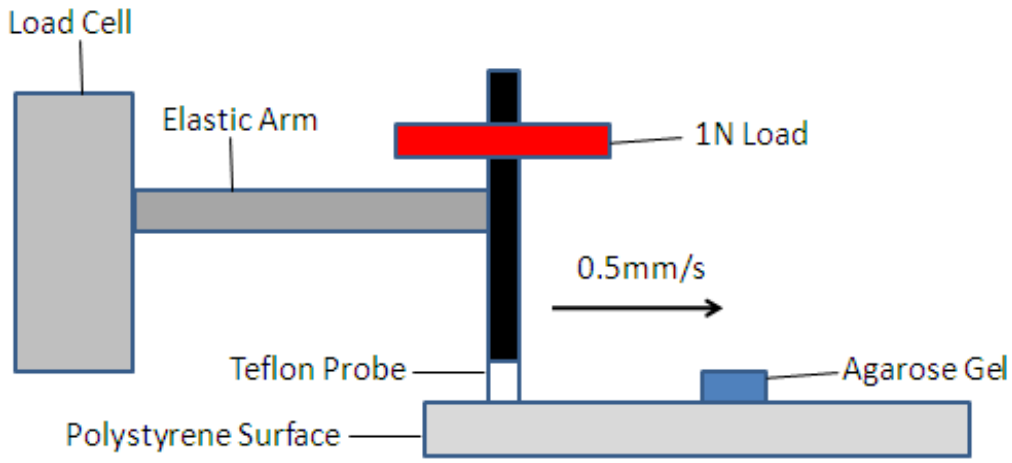


Figure 2.9 Diagrammatic representation of tribometer set up for testing strength of agarose gel adhesion to polystyrene surface.

2.9 Bromodeoxyuridine (BrdU) Proliferation Assay

Primary rodent pulp cells ($2x10^4$) (Method 2.4.2.1) were seeded on ECM coated and PBS control conditioned $35mm^2$ polystyrene culture surfaces (Sarstedt, UK) (Method 2.4.3.1). 6 dishes were used for each technical replicate. After 1 week of growth at 37^oC in 5% CO_2 with regular changes of medium, the dishes were treated using the reagents and protocol provide with the 5-bromo-2-deoxy-uridine labelling and detection kit II (Roche Applied Sciences, Germany). Medium was removed from the culture and replaced with 500µl BrdU labelling medium containing BrdU labelling reagent at 1:1000 dilution in sterile medium. After 60

minutes incubation at 37°C in 5% CO_2, the BrdU labelling medium was removed and the dish washed 3 times in 1ml PBS. The cells were then fixed for 20 minutes at room temperature with 1ml ethanol fixative (30% absolute ethanol / 70% 50mM glycine). All the liquid was removed from the wells and the dishes placed at -20°C for 30 minutes. After PBS washing, 700µl anti-BrdU (kit reagent) was added to each dish for 30 minutes at 37°C in 5% CO_2, then removed and surfaces were PBS washed again. 700µl AP conjugate (kit reagent) was then added to each dish for 30 minutes and incubated at 37°C in 5% CO_2. Dishes were washed with PBS again before 700µl colour substrate (kit reagent) was added to each dish an incubated for 30 minutes at room temperature. Finally, dishes were PBS washed and examined using a Nikon Eclipse TE300 microscope (Nikon, UK). Proliferating cells incorporate BrdU into cellular DNA and this can be detected by positive antibody staining. A graticule was used to count positive and negative cells in fields of view and averaged to determine the percentage of the cell population in the proliferative phase.

2.10 Lactate Dehydrogenase (LDH) Cytotoxicity Assay

To assess whether the antimicrobial activity of the ECM preparations represented a general cytotoxic effect, LDH release from pulpal cells exposed to ECM extracts was determined colorimetrically using a kit (Roche Applied Science, UK). LDH is commonly used marker of cytotoxicity as it is a stable enzyme present in all cells that is released when the plasma membrane is damaged. Enzymatically isolated rodent primary pulp cells ($5x10^3$) were seeded in 200µl α–MEM in 96-well plates (Corning, UK) and cultured for 16 hours at 37°C in 5% CO_2 to enable cell adherence. Cells were then washed with PBS and exposed to concentrations of ECM ranging between 1-100µg/ml in 200µl α–MEM for 48 hours. A

negative control of α−MEM alone was used and a positive control represented addition of 5µl lysis buffer (kit reagent) to cultures containing α−MEM 30 minutes prior to the end of the 48 hour incubation period. Following culture, 100µl medium from each well was removed and added to 100µl dye/catalysis solution (kit reagent) and incubated at room temperature with gentle agitation on an orbital mixer (Denley, UK) for 30 minutes. After this time period, 50µl stop solution (kit reagent) was added and optical densities were measured at a wavelength of 490nm with an ELX800 Universal Microplate reader (Bio-Tex Instruments INC).

2.11 Alginate Gel Encapsulation

2.11.1 Encapsulation and Release of Cells

Low viscosity sodium-alginate (Sigma-Aldrich, UK) was prepared at 1, 3 and 5% w/v in PBS/αMEM (1:1) to provide medium within the gels, and autoclaved at 121°C prior to use. Primary rodent pulp cells (Method 2.4.2.1) were slowly dispersed by pipetting throughout the alginate at a density of $5x10^5$cells/ml. The alginate/pulp cell dispersion was added dropwise from a 1ml Gilson pipette into 35mm^2 culture dishes (Sarstedt, UK) containing 100mM $CaCl_2$ and incubated at 37˚C for 1 hour to form cross-linked spheres (Hunt *et al*, 2009; Hunt *et al*, 2010). Spheres were then washed 3 times in αMEM and re-suspended in control or lineage inductive media (Method 2.7).

Cells were released from alginate gels by incubation in 100mM tri-sodium citrate (Sigma-Aldrich, UK) for 1 hour at 37˚C with 5% CO_2 to break calcium cross-links. Released cells were then pelleted by centrifugation at 2,100 g for 3 minutes prior to re-suspension in culture

medium and seeding in 35mm^2 culture dishes (Sarstedt, UK). After 24 hours, adherent cells were detached by treatment with 0.25% (w/v) Trypsin, 1mM EDTA·4Na (Gibco, UK) for 5 minutes. Numbers were determined using the counting method described in Method 2.4.3.2.

2.11.2 Analysis of Alginate Gels

Encapsulated cells were fixed within alginate gels by submerging them in 10% v/v paraformaldehyde (VWR, UK) for 30 minutes. Fixed gels were placed in histological processing cassettes and progressively dehydrated by increasing concentrations of alcohol for 15 minutes each (35% (v/v), 50%, 70%, 95% and 3 changes of 100%). Gels were then submerged in 2 changes of xylene (VWR, UK) for 15 minutes each. Dehydrated gels were submerged in 2 changes of paraffin wax (Sakura, UK) for 30 minutes each. Gels were then embedded in paraffin wax (Sakura, UK) and stored at room temperature. Wax embedded samples were cooled to 5˚C and 5µm sections cut using a microtome (Leica RM 2035). Sections were floated onto glass slides and heated to 60˚C for 1 hour to aid adhesion. Wax was removed from the slides by immersion in xylene (VWR, UK) and gels were stained with haematoxylin and eosin (H&E) solution (Surgipath Europe Ltd, UK). Coverslips were then mounted on the slides using drops of XAM (BDH Laboratory Supplies, UK).

2.11.3 Micro-Computed Tomography (MicroCT) Analysis

Alginate gels were scanned using a Skyscan 1172 MicroCT system (e2v Technologies, UK). The entire alginate gel was scanned at 80kV, 100µA at an isotropic resolution of 4µm with a

camera exposure time of 200ms, a rotation step of 0.3°, frame averaging of 4 and omission of an X-ray filter. Files were reconstructed using NRecon 1.6.2 software (SkyScan, e2v technologies, UK). A hydroxyapatite mineral phantom composed of tetracalcium phosphate (TTCP) / dicalcium phosphate anhydrous (DCPA) powder and an α-tricalcium phosphate (α - TCP) powder (Hofmann *et al*, 2007) was scanned at the same time with the same settings. Using NRecon 1.6.2 software (SkyScan, e2v technologies, UK) the intensity values of the pixels from the phantom were used as a threshold level and pixels with lower values were removed from the scanned gel images. Images were threshold adjusted using a hydroxyapatite phantom. The approach resulted in images with only material of a density similar to that of hydroxyapatite present.

2.12 Statistical Analysis

Data were expressed as means $\pm$ standard deviation. Statistical analysis of data was performed using paired student T-tests with Microsoft Excel software (Microsoft Corporation, USA) or one-way ANOVA with SPSS software (SPSS Inc, USA) with $p<0.05$ deemed as statistically significant from control.

3.0 PREPARATION OF PULP ECM

Pulp ECM (pECM) components were isolated for various functional studies within the present project to determine the cellular influences of these molecules. Whilst guanidinium chloride has been previously used for isolation of ECM components (Waddington *et al,* 2004; Hall 1996; Rahemtulla *et al,* 1984), its denaturing effects (Camilloni *et al,* 2008; Lau *et al*, 1998) are not appropriate to enable subsequent robust biological studies of function. A sequential tartaric acid and sodium chloride extraction (Bellon *et al*, 1988) was utilised to overcome such issues. This chapter investigates the reproducibility of this extraction procedure for pECM was investigated together with the analysis of its composition.

3.1 Characterisation of Extracted pECM Components

Three replicate preparations of pECM were isolated using a tartaric acid and sodium chloride extraction procedure described in Section 2.2.1 (Bellon *et al*, 1988) and these were characterised for their composition. Colourimetric techniques utilised the protein-dye binding properties of Coomassie Brilliant Blue G-250 (Bradford, 1976), the GAG-dye binding properties of dimethyl methylene blue (Farndale *et al*, 1986), and the collagen binding properties of Sirius red in saturated picric acid (Tullberg-Reinert and Jundt, 1999) to confirm the presence of the reported three major constituents of pECM from three replicate extractions (Table 3.1). These components were found in similar proportions in each extraction with the relative concentrations of the components always identifying a trend of NCPs > collagen > GAGs. The relative proportions of the NCPs in the three extracts were 56%, 60% and 59%, the proportions of GAGs were 5%, 5% and 5%, and the proportions of collagen within the extracts were 40%, 35% and 36%. 1D-polyacrylamide gel electrophoresis (PAGE) analysis demonstrated that the three separate extracts of the ECM exhibited similar profiles of proteins (Figure 3.1). These profiles appeared to contain a relatively large number of proteins ranging in size from 3.5kDa to >200kDa and appeared relatively consistent between replicate extracts, with distinct regions of high protein abundance (e.g. 55-66kDa) observed in all extractions. Proteomic mass spectrometry of pECM extracts was performed using an LCMSMS Orbitrap mass spectrometer (Thermo Scientific, UK) and identified the presence of 119 proteins by cross-referencing the molecular weights of detected peptides with the Central Proteomics Facilities Pipeline database (Table 3.2). Many proteins previously reported to be present within the pECM including collagen type I, collagen type III, decorin, tenascin, versican core protein, hyaluronan and proteoglycan link protein 1 and fibronectin were detected

(Shuttleworth *et al*, 1978; Tsuzaki *et al*, 1990; Martinez *et al*, 2004). Combined, data indicated that the extraction method used in this study was successful in deriving consistent preparations of ECM from pulp.

Extract	NCP (µg/mg ECM)	GAGs (µg/mg ECM)	Collagen (µg/mg ECM)
ECM 1	200.42	16.14	142.22
ECM 2	235.02	19.55	135.55
ECM 3	121.52	11.4	72.99

Table 3.1 Colourimetric analysis of pECM extracts showing quantification of non-collagenous protein (NCP), glycosaminoglycans (GAGs) and soluble collagen content in three separate pECM extractions, demonstrating that all three extractions contained the three main components of pECM in comparable proportions.

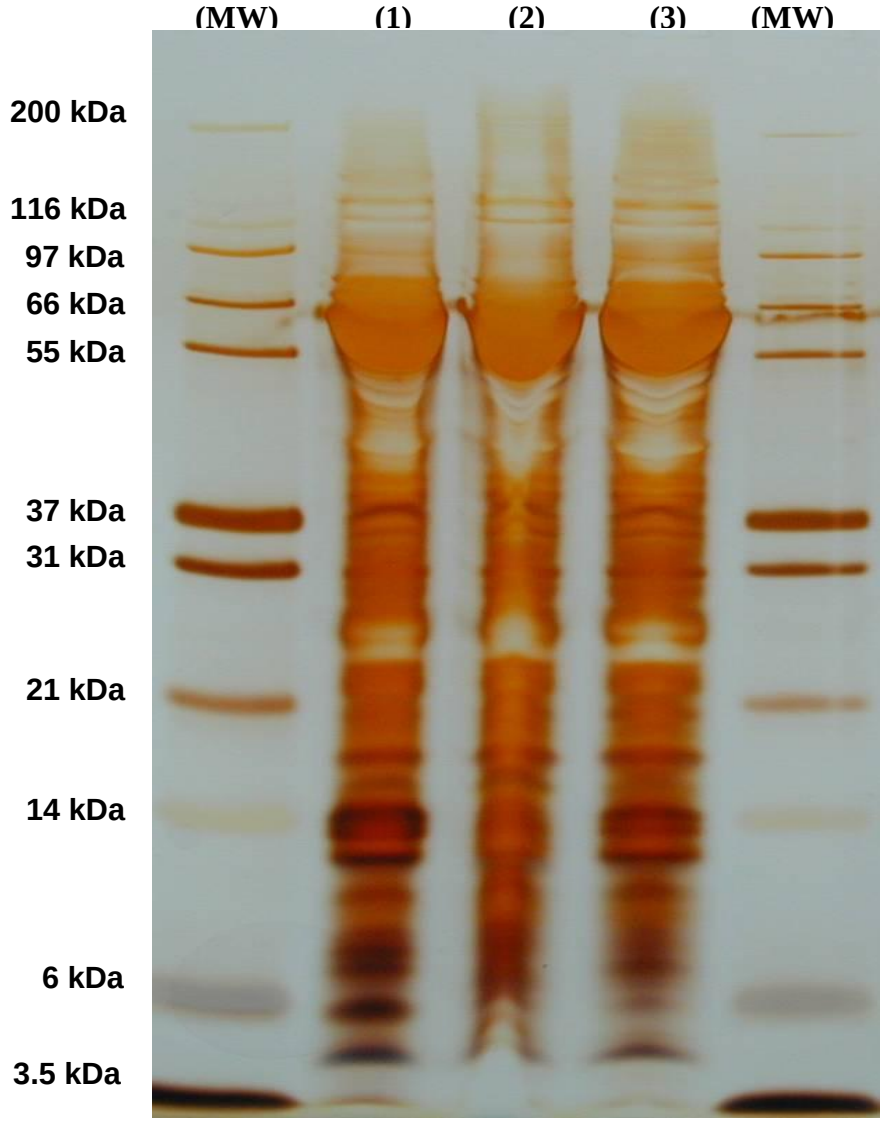


Figure 3.1 Image of 1D-PAGE analysis of the three replicate extracts of pECM (1, 2 and 3). 0.125mg of pECM loaded in a NuPAGE 10% Bis-Tris gel, and visualised using SilverXpress Silver staining kit. 10µl of Mark12 molecular weight (MW) ladder was also loaded as a weight reference. Image confirms the presence of a large number of proteins over a range of sizes and shows the reproducibility of the extraction procedure to produce extracts with similar profile.

A)

Score	Mass (Da)	Name
3666	71244	Serum albumin
2731	79870	Serotransferrin
74	80002	Lactotransferrin
40	81772	Melanotransferrin
1171	168953	Alpha-2-macroglobulin
729	42052	Actin, cytoplasmic 1
381	42381	Actin, aortic smooth muscle
535	40889	Hyaluronan and proteoglycan link protein 1
379	53933	Fibrinogen beta chain
346	67484	Fibrinogen alpha chain
344	46396	Serpin A3-4
337	46322	Serpin A3-2
281	46482	Serpin A3-5
184	47140	Serpin A3-7
293	47639	Alpha-enolase
237	188675	Complement C3
235	39212	Annexin A1
234	38882	Annexin A2
224	50935	Tubulin alpha-1D chain
224	371757	Versican core protein
223	29637	Carbonic anhydrase 3
219	36124	Annexin A5
207	54091	Alpha-1B-glycoprotein
204	53752	Vimentin
184	46314	Pigment epithelium-derived factor
182	80966	Gelsolin
173	30258	Apolipoprotein A-I
167	52974	Hemopexin
139	39193	Alpha-2-HS-glycoprotein
139	50435	Elongation factor 1-alpha 1
138	71082	Heat shock cognate 71 kDa protein
55	70699	Heat shock 70 kDa protein 1-like
136	50839	Fibrinogen gamma-B chain
125	16023	Hemoglobin subunit beta
118	16001	Hemoglobin subunit beta
122	25078	Trypsin
115	40289	Decorin
110	76202	Annexin A6
106	54904	Vitamin D-binding protein
106	50095	Tubulin beta-5 chain
105	22423	Peroxiredoxin-1
101	52653	Uridine 5'-monophosphate
99	22359	Ras-related protein Rab-1B

Score	Mass (Da)	Name
96	23464	Rho GDP-dissociation inhibitor 1
93	39074	Lumican
86	36073	Glyceraldehyde-3-phosphate dehydrogenase
79	63059	Glucose-6-phosphate isomerise
79	18792	Cofilin-1
77	101620	Inter-alpha-trypsin inhibitor heavy chain H4
75	19810	Adenine phosphoribosyltransferase
70	43434	Fetuin-B
68	36985	L-lactate dehydrogenase B chain
66	31817	Myristoylated alanine-rich C-kinase substrate
65	52827	Antithrombin-III
61	10359	Histone H1.1
59	29096	Carbonic anhydrase 2
59	83445	Heat shock protein HSP 90-alpha
58	15219	Profilin-1
56	28620	Tropomyosin alpha-4 chain
49	69930	Kininogen-1
47	28406	14-3-3 protein gamma
46	62638	Dihydropyrimidinase-related protein 2
45	28948	Phosphoglycerate mutase 1
42	24579	GTP-binding nuclear protein Ran
40	129499	Collagen alpha-2(I) chain
39	39538	Beta-2-glycoprotein 1
37	12112	Ig kappa chain V region GOM
34	51503	Septin-8
34	6214	Mitochondrial import receptor subunit TOM7 homolog
31	56616	Pyruvate kinase isozyme R
28	105012	Inter-alpha-trypsin inhibitor heavy chain H2
27	23453	Alpha-1-acid glycoprotein
26	79617	Solute carrier family 15 member 1
25	76997	cGMP-dependent protein kinase 1
25	66570	Cholinesterase
25	146136	Myosin-VI
24	49576	Bystin
22	73826	Protein THEMIS
22	84164	Complement C2
20	105319	Alpha-actinin-4
19	55666	Fascin-2

B)

Score	Mass (Da)	Name
1611	168953	Alpha-2-macroglobulin
1465	71244	Serum albumin
331	57847	Keratin, type I cytoskeletal 10
246	54986	Keratin, type I cytoskeletal 10
46	76992	Keratin, type I cytoskeletal 9
299	188675	Complement C3
266	63922	Keratin, type II cytoskeletal 1
250	64812	Keratin, type II cytoskeletal 2 epidermal
136	63069	Keratin, type II cytoskeletal 5
107	58027	Keratin, type II cytoskeletal 79
201	25078	Trypsin
177	79870	Serotransferrin
169	139880	Collagen alpha-1(I) chain
155	46322	Serpin A3-2
109	46396	Serpin A3-4
130	120020	Ceruloplasmin
126	40289	Decorin
120	371757	Versican core protein
98	144958	Complement factor H
87	129499	Collagen alpha-2(I) chain
87	227583	Myosin-9
80	16242	Hemoglobin subunit beta
73	42064	Actin, cytoplasmic 1
71	196922	Tenascin
58	93708	Collagen alpha-1(III) chain
53	275519	Fibronectin
50	193234	Clathrin heavy chain 1
36	39074	Lumican
34	112947	Protein FAM65B
34	26173	Alpha-S2-casein
30	39193	Alpha-2-HS-glycoprotein
23	12112	Ig kappa chain V region GOM
23	82462	Sulfate transporter
23	35448	Lambda-crystallin homolog;
21	55906	Cytochrome P450 2B4
18	89509	Putative RNA exonuclease NEF-sp
17	14083	Histone H2A type 1

Table 3.2 Table shows the names of the proteins identified within pECM extract using proteomic mass spectrometry analysis with the molecular weight in Daltons shown and a protein score for each. 0.125mg of pECM was loaded on a NuPAGE 10% Bis-Tris gel and separated by 1D-PAGE and the lane split into two samples (A and B) at the median plane for separate mass spectrometry analysis. Protein scores indicate the likelihood that the observed match between the experimental data and the database sequence. Analysis identified 119 proteins within the extract, 90 of these had a protein score >45 (not underlined) indicating a statistical significance (P=<0.05).

3.2 Degradation Analysis of ECM

The pECM and dECM preparations used in this study were subjected to acidic and enzymatic degradation to attempt to reproduce disease relevant extracts. ECM extracts were dissolved in sterile dH_2O at a concentration of 0.1μg/ml and incubated at 37^oC in 5% CO_2 in air for 48 hours to allow partial auto-degradation by innate proteolytic enzymatic action to occur as previously described by Smith and Smith,1984. ECM extracts were dissolved in either 0.1M acetic (BDH Laboratory Supplies, UK) or 0.1M lactic acid (Sigma-Aldrich, UK) at a concentration of 0.1μg/ml and were incubated at 37^oC in 5% CO_2 in air for 48 hours to allow partial degradation by acidic action to replicate plaque bacterial acid activity.

Gel electrophoresis analysis demonstrated a differential protein profile for pECM following degradation treatments (Figure 3.2). Notably in the untreated pECM there was relatively intense staining indicating a large number of proteins, this was reduced following both acidic and enzymatic treatments. In addition to the numerous minor changes to the intensities of specific protein bands, there were also several major visible changes. Following enzymatic treatment, there was a region of intense protein banding at approximately 6-8kDa, which was not present prior to treatment of the pECM. Following acidic treatment, protein profiles at low molecular weight regions (<15kDa) were no longer apparent (Figure 3.3). These differences indicated that the degradation conditions influenced the protein profile and composition of the pECM and that proteolysis had occurred.

The staining of the dECM protein profile was not as intense as the pECM with relatively few bands distinguishable (Figure 3.2). It was therefore not possible to visually determine whether the degradation treatments had an influence on the dECM protein profile. Degraded dECM extracts were used in later cell migration assays, along with pECM breakdown extracts, to determine if any change in functional activity was detectable following physiologically relevant degradation.

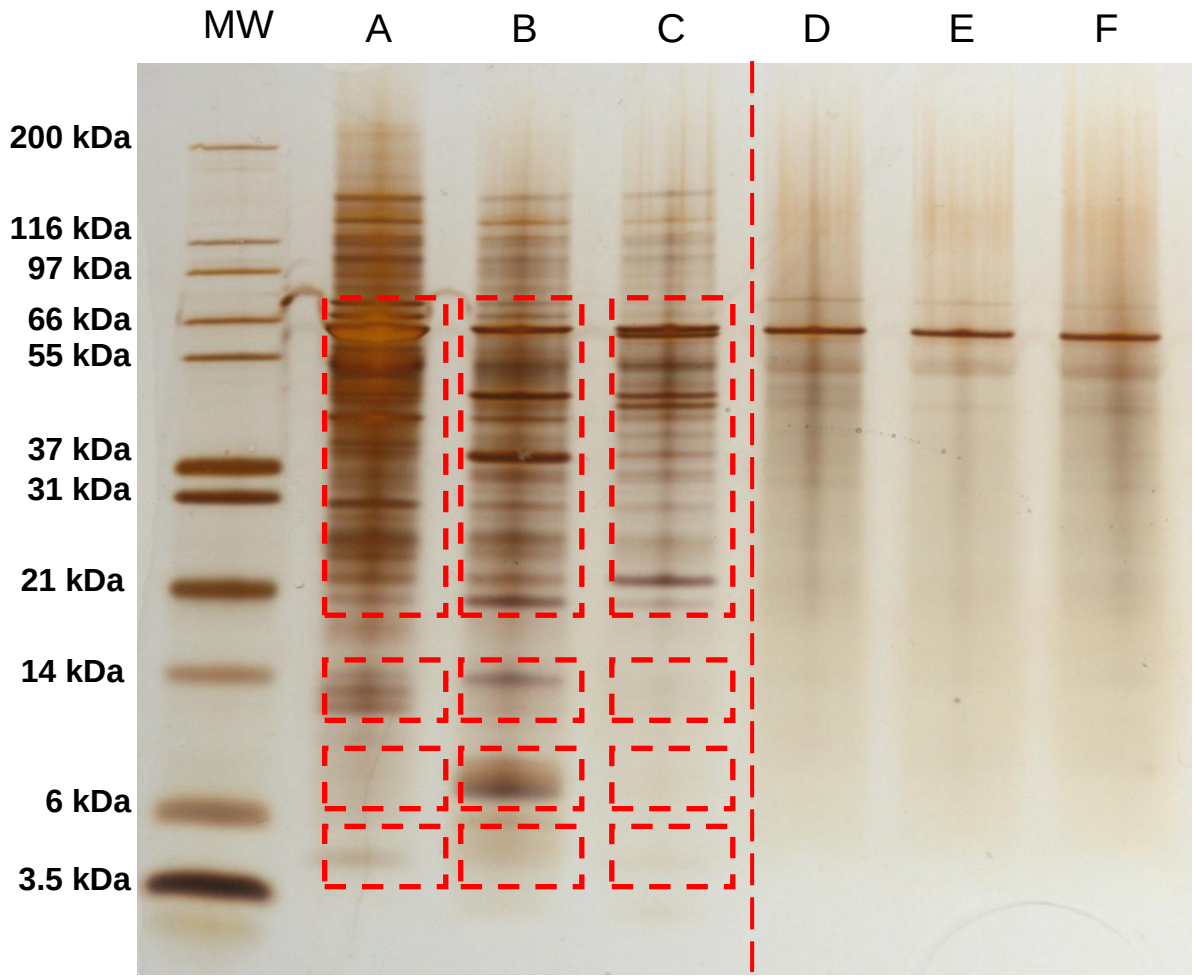


Figure 3.2 1D-PAGE analysis of A) untreated pECM, B) enzymatic autodegraded pECM, C) acetic acid autodegraded pECM, D) untreated dECM, E) enzymatic autodegraded dECM, F) acetic acid degraded dECM. 0.125mg of each ECM condition was loaded on a NuPAGE 10% Bis-Tris gel, and visualised using SilverXpress Silver staining kit. 10µl of Mark12 molecular weight (MW) ladder was also loaded as a reference. Image shows changes in the protein profile of pECM following enzymatic and acidic treatments (indicated by red boxes). The staining of the dECM was not as intense as the pECM with relatively fewer bands distinguishable, indicating less proteins present within the sample.

3.3 Ammonium Sulphate (A.S.) Fractionation of pECM

To investigate the functional activities of the different components within pECM, ammonium sulphate charge fractionation was performed. This approach separates the components by charge to produce fractions with differing compositions which can then be analysed for functional activity.

1D-PAGE analysis demonstrated a differential protein profile of pECM following ammonium sulphate fractionation (Figure 3.3). The intensity of specific protein banding differed between fractions and red boxes within the figure highlight areas where specific proteins have been separated between the fractions providing different profiles. Further analysis of fractions by Agilent protein chip PAGE confirmed a differential profile of proteins, with the most abundant proteins in each fraction present in differing proportions and at differing molecular weights (Table 3.3). The proportion of the most abundant proteins changed between each fraction with a 63.4kDa protein making up 63.8% of the 30% A.S. fraction, a 69.6kDa protein making up 72.4% of the 50% A.S. fraction, a 69.2kDa protein making up 92.2% of the 70% A.S. fraction and a 71.5kDa protein making up 88.7% of the 90% A.S. fraction. Of the most abundant proteins in the samples, there were bands unique to specific fractions; with proteins of 23.5, 40.6, 55.4 and 78.2kDa only detected in the 30% A.S. fraction, proteins of 29.6, 45.5 and 131.5kDa only detected in the 50% A.S. fraction, proteins of 14.7, 38.5 and 27.9kDa only detected in the 70% A.S. fraction, and proteins of 140.1 and 142kDa only detected in the 90% A.S. fraction. These protein profile data indicated successful charge separation of specific pECM components by ammonium sulphate fractionation. These fractions were used in later pulp cell migration and antimicrobial assays to study their functional activity.

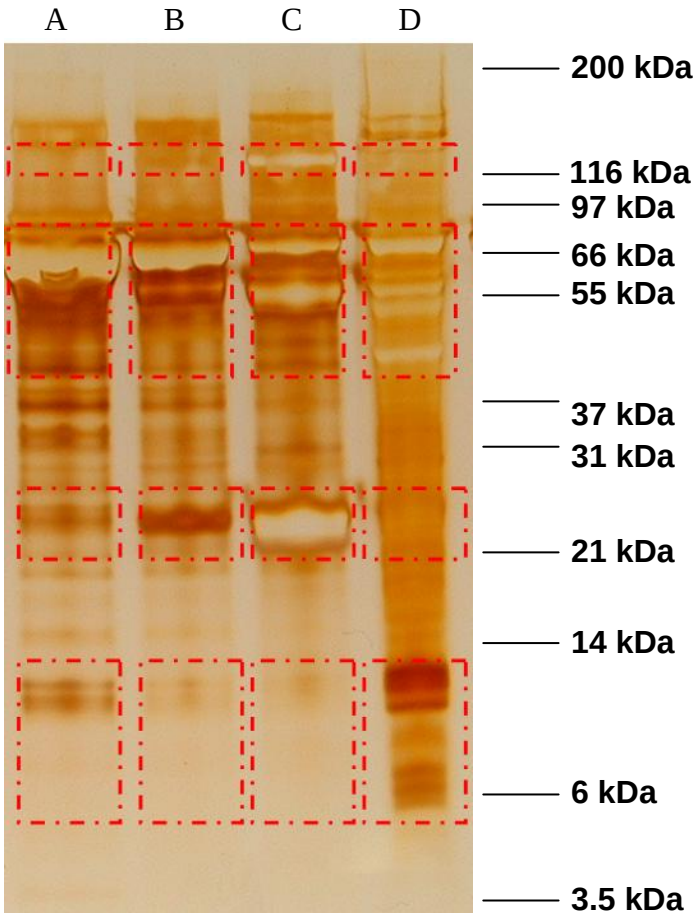


Figure 3.3 1D-PAGE analysis of A) precipitate formed by 90% saturated ammonium sulphate solution, B) precipitate formed by 70% saturated ammonium sulphate solution, C) precipitate formed by 50% saturated ammonium sulphate solution, and D) precipitate formed by 30% saturated ammonium sulphate solution. 0.125mg of each pECM fraction loaded on a NuPAGE 10% Bis-Tris gel, and visualised using SilverXpress Silver staining kit. Image shows ammonium sulphate fractionation of pECM produced fractions with differences in protein banding (indicated by red boxes).

30 % Ammonium Sulphate Fraction		50 % Ammonium Sulphate Fraction		70 % Ammonium Sulphate Fraction		90 % Ammonium Sulphate Fraction	
Size [kDa]	**% Total**	**Size [kDa]**	**% Total**	**Size [kDa]**	**% Total**	**Size [kDa]**	**% Total**
23.5	8	29.6	14.2	14.7	1.1	46	0.4
40.6	9.1	45.5	0.9	22.1	0.5	50.1	0.9
55.4	2.4	69.6	72.4	27.9	1.1	71.5	88.7
63.4	63.8	84	3.9	38.5	0.3	85.6	8.8
70.8	11.8	131.5	8.6	48.6	0.3	140.1	0.3
78.2	4.9			69.2	92.2	142.8	0.9
				84.4	4.5		

Table 3.3 Data indicating molecular weights and percentage abundance of proteins present in fractions as determined by Agilent protein chip PAGE. I) unfractionated ECM, II) precipitate formed by 30% saturated ammonium sulphate solution III) precipitate formed by 50% saturated ammonium sulphate solution, IV) precipitate formed by 70% saturated ammonium sulphate solution, V) precipitate formed by 90% saturated ammonium sulphate solution. Data show that ammonium sulphate fractionation produced pECM fractions with different protein profiles.

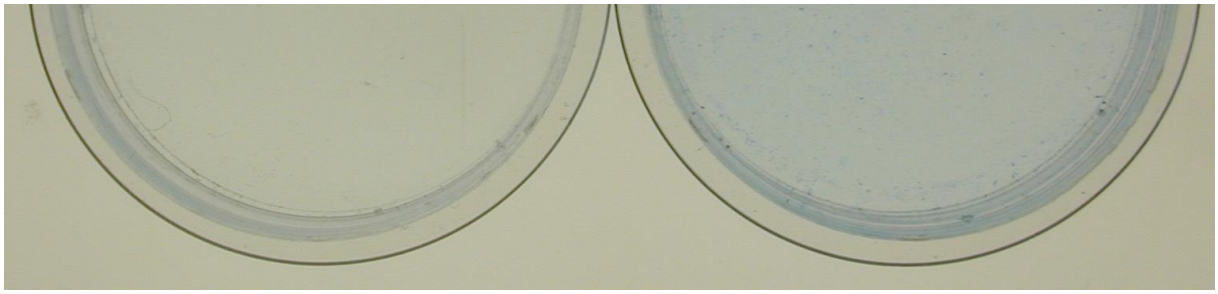
3.4 ECM Coating of Culture Surfaces

Extracted ECM proteins were used to coat culture surfaces to attempt to mimic the *in vivo* pulp environment using a previously reported procedure to produce culture surfaces with adsorbed ECM proteins (Dimilla *et al*, 1992). Surfaces were subsequently assessed for the presence of ECM components using Coomassie blue staining and SEM imaging.

Following pECM treatment, cell culture surfaces stained positive with Coomassie blue (Figure 3.4A) which indicated that direct protein adsorption had occurred on these surfaces. SEM images of cell culture surfaces showed differences in surface topography following pECM treatment (Figure 3.4B) which further supported the Coomassie blue staining analysis. Combining these data supported previous studies showing a direct protein adsorption method could produce culture surfaces with bound ECM proteins (Dimilla *et al*, 1992). This approach was used in further studies to create surfaces containing bound ECM proteins to attempt to mimic the *in vivo* pulp environment for the culture of primary pulp cells to determine whether a pECM environment could influence cell behaviour.

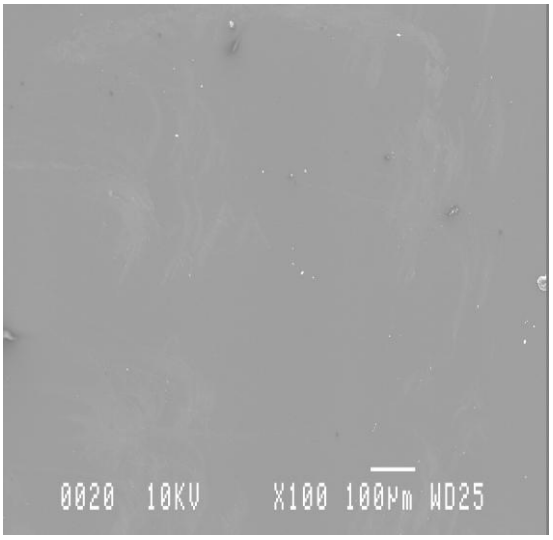
A)

i) ii)



B)

i) ii)



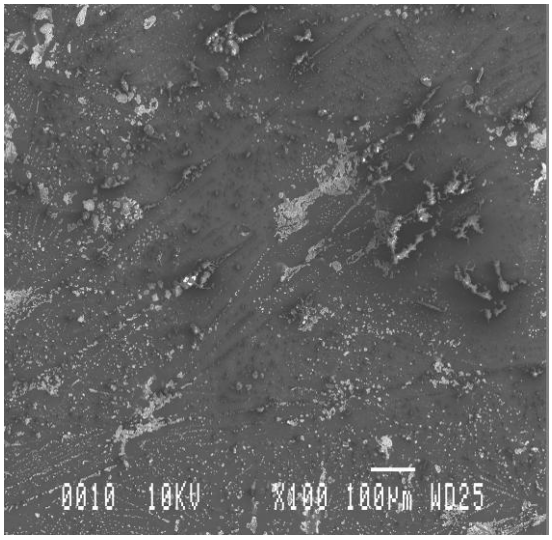


Figure 3.4 A) Coomassie blue staining of uncoated control polystyrene culture surfaces (i) and ECM treated test culture surfaces (ii). Positive staining of coated dishes shows successful protein adsorption resulting in an ECM coated cell culture environment. B) Secondary electron photomicrographs from SEM of untreated culture polystyrene surfaces (i) and ECM treated culture polystyrene (ii) at x100 magnification. Images show a change to the surface appearance following protein treatment indicating that protein treatment had likely resulted in coated surfaces.

4.0 THE EFFECTS OF THE PULP ECM ENVIRONMENT ON CULTURED DENTAL PULP CELLS

Individual ECM components and products containing whole ECM extracts are commercially available and partially enable the recapitulation of the physiological environment *in vitro* for cell culture analysis. These products are used to provide 2D or 3D cellular substrates and have reportedly had significant influences on cell adhesion, cellular interactions and resulting cellular phenotype (Sitterley, 2008). The ECM composition of different connective tissues has been shown to vary substantially with studies demonstrating pECM to have a unique profile of constituents (Shuttleworth *et al*, 1978; Tsuzaki *et al*, 1990; Martinez *et al*, 2004; Goldberg *et al*, 1995; Pearson *et al*, 1986; Ellingson *et al*, 1975). This unique composition of dental pulp ECM may be important in the specialisation of the tissue providing a niche environment for pulpal cells including resident stem cells, actively regulating the cellular phenotype of these cells.

Previously ECM components have been shown to influence stem cell behaviour. Indeed, fibronectin coatings have been demonstrated to enhance mesenchymal stem cell adhesion and osteogenic differentiation, whilst collagen coating of cell culture substrates appeared not to exert this effect (Sogo *et al*, 2007). Interestingly the culture of embryonic stem cells on different ECM conditions has been shown to influence stem cell growth and differentiation (Baharvand *et al*, 2005). However, to date no study has attempted to mimic the ECM environment of the pulp in order to assess its effect on cell activity or phenotype. Such an approach may provide important information with regards to the characteristics of the pulp ECM environment and how it affects the behaviour of stem and other cells within it. The

following investigations were performed to examine whether the pulps' molecular environment may influence cell behaviour and phenotype. Degradation of pECM was performed to determine if its' functional activity was influenced by molecular damage that may occur during disease environment.

4.1 Cell Adhesion, Growth and Morphological Analysis of Cultures on pECM Surfaces

The previous chapter demonstrated that a direct protein adsorption method could be used to create pECM coated cell culture surfaces (Figure 3.4). These surfaces were used in the following studies to examine whether pECM influenced cell behaviour of primary pulp cells, primary bone marrow cells, and a MDPC-23 odontoblast-like cell population.

Cell adhesion to both control and pECM coated surfaces was demonstrated (Figure 4.1). Analysis demonstrated that coating culture surfaces with pECM did not affect the number of cells that adhered by 4 hours (Figure 4.2.A). Notably, primary pulpal cells adhered to the surfaces in significantly higher numbers than the other two cell types with 79% of the seeded primary pulp cells adhering compared with 53% of primary bone marrow cells and 45% of the MDPC-23 population. Cell counts following 4 day culture demonstrated significantly lower cell numbers on pECM treated surfaces compared with control culture surfaces (Figure 4.2.B). This trend was observed in all three cell types; with the primary pulp cell population 57% lower than the control surface, the primary bone marrow cell population 32% lower than the control surface, and the MDPC-23 cell population 47% lower than the control surface. All three adherent cell populations on the pECM significantly increased compared with the initial number of cells that adhered. Indeed increases over the initial number of adherent cells by day 4 for the primary pulp cells were 347%, primary bone marrow cell were 658%, and the MDPC-23 cell population was 2420% due to cell proliferation. These data indicated that reduced cell numbers on pECM compared with control culture surfaces were likely due to slower proliferation rates as the cell number was still significantly increasing. Phase contrast images illustrated the relatively lower cell densities at 4 days growth on a pECM coated surface compared with the control cell culture surface (Figure 4.1). These images also

indicated changes in cell morphology in cultures grown on pECM coated surfaces for 4 days with these cells appearing more sparsely spread, slightly larger in size and with more cellular processes extended. Preliminary BrdU staining analysis demonstrated an 8% decrease in the percentage of the primary pulp cell population in a proliferative state when cultured for 4 days on pECM coated surface compared with control tissue culture polystyrene (Figures 4.3). These data supported the cell count data indicating that pECM decreased the proliferation rate of primary pulp cells compared with cell cultured on standard culture polystyrene.

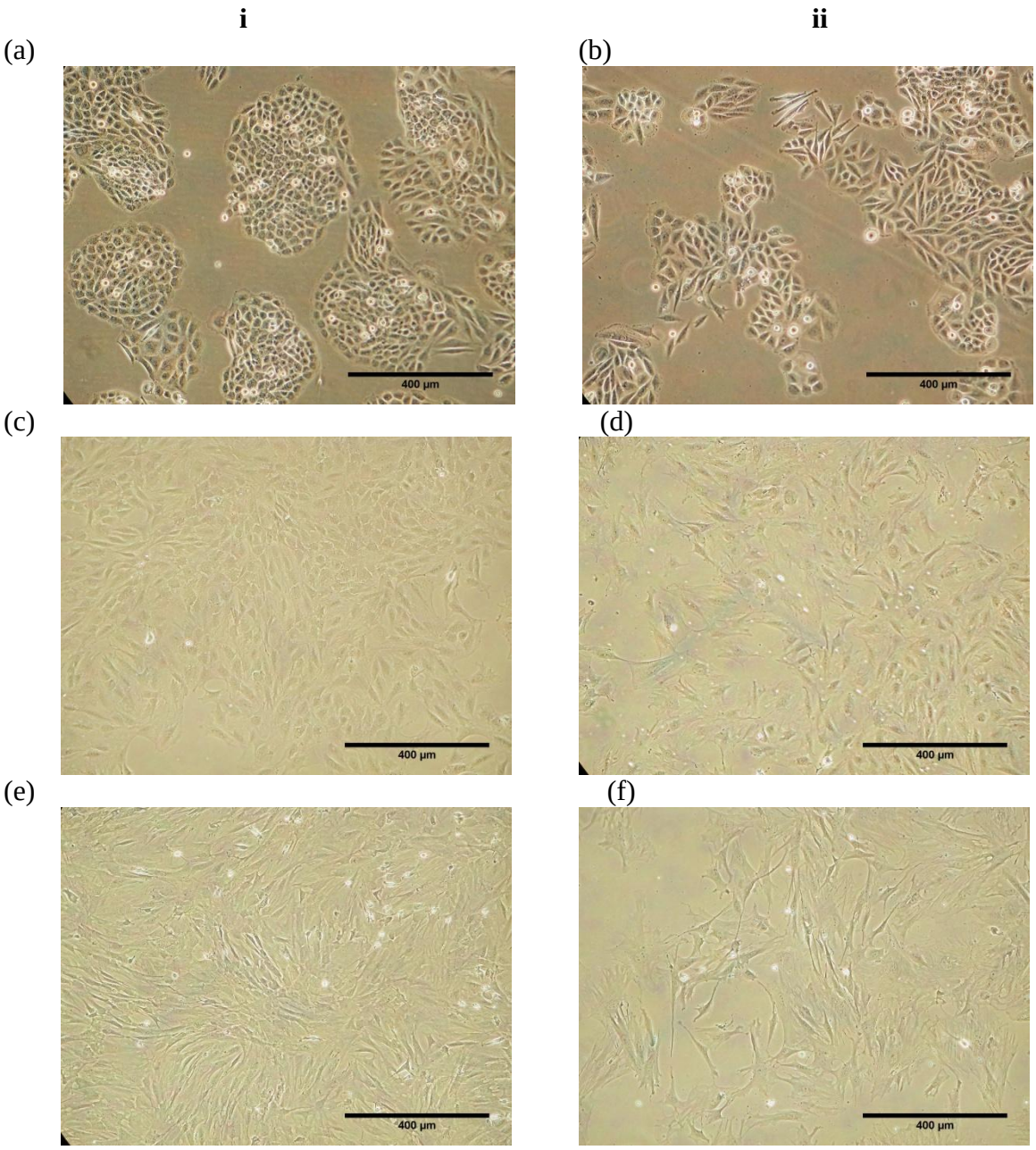


Figure 4.1 Phase contrast images of (a) MDPC-23 odontoblast-like cells on i) control culture surface, ii) pECM coated surface, (b) primary pulpal cells on i) control culture surface, (ii) pECM coated surface, (c) primary bone marrow cells on i) control culture surface and ii) pECM coated surface. Images show a decrease in cell densities after 4 days growth on a pECM coated surface compared with a control cell culture surface and indicate a change in morphology. Scale bars are shown.

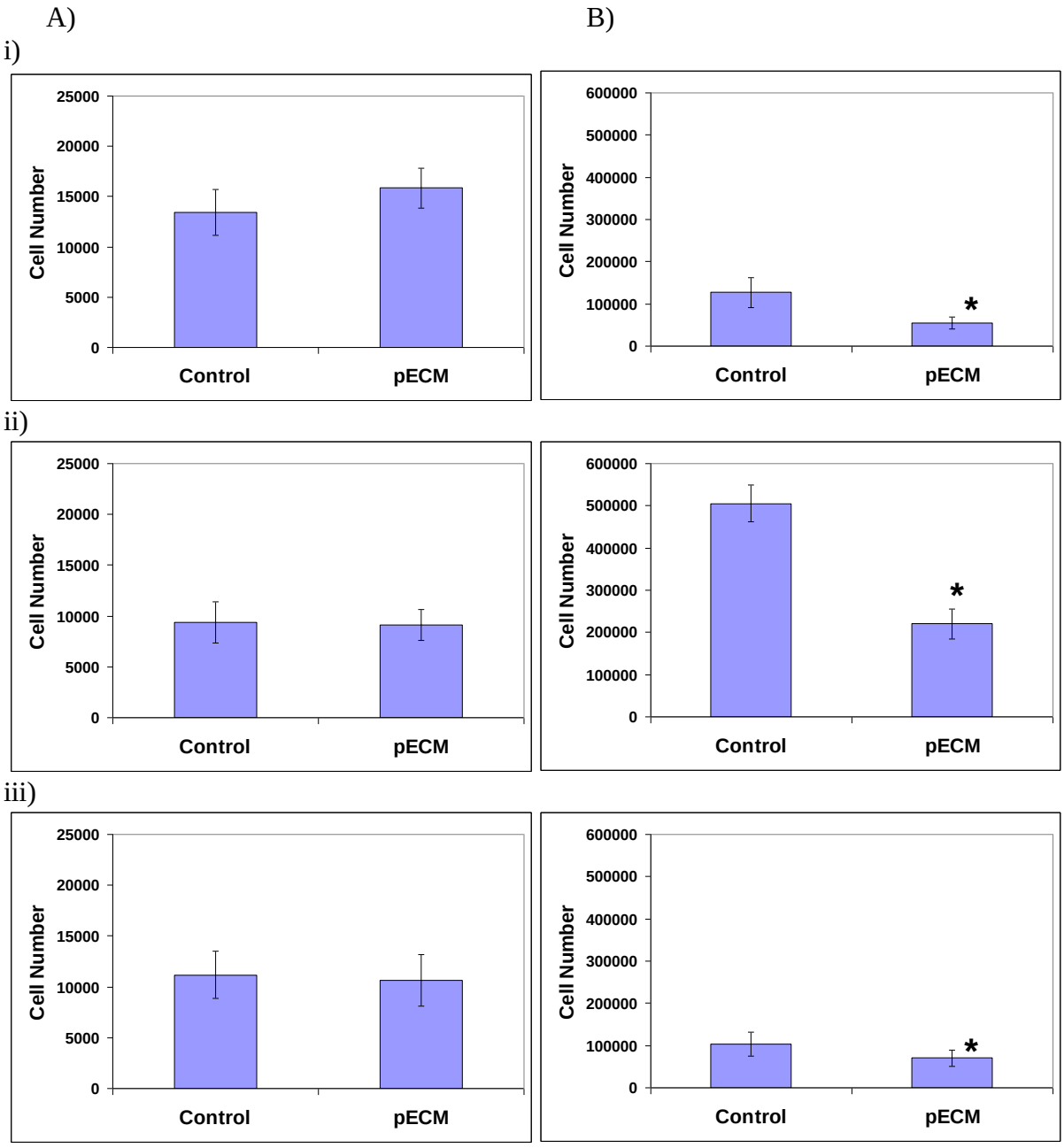
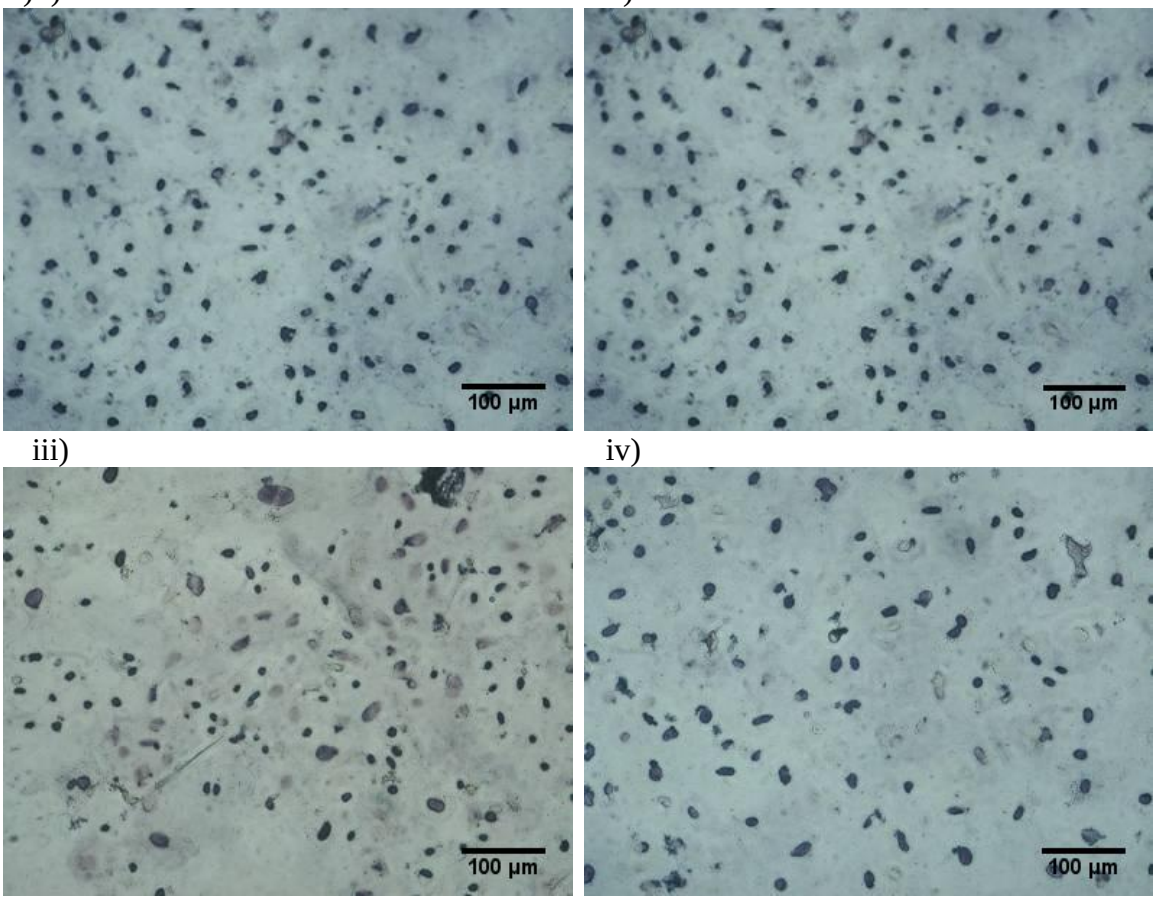


Figure 4.2 Data show the number of adherent (i) primary rodent dental pulp cells, (ii) MDPC-23 odontoblast-like cells and (iii) primary rodent bone marrow cells on control and pECM surfaces after (A) 24 hours culture and (B) 4 days culture. $2x10^4$cells were seeded on control and pECM coated surfaces. No statistical differences were found in cell adhesion after 24 hours. A statistically significant decrease ($p<0.05$) was detected in cell number in all three cell types when cultured on pECM surfaces by 4 days compared with the controls. N=12, standard deviation bars plotted, * = $P<0.01$.

A) i) ii)


100 µm
100 µm
100 µm
100 µm

iii) iv)

B)

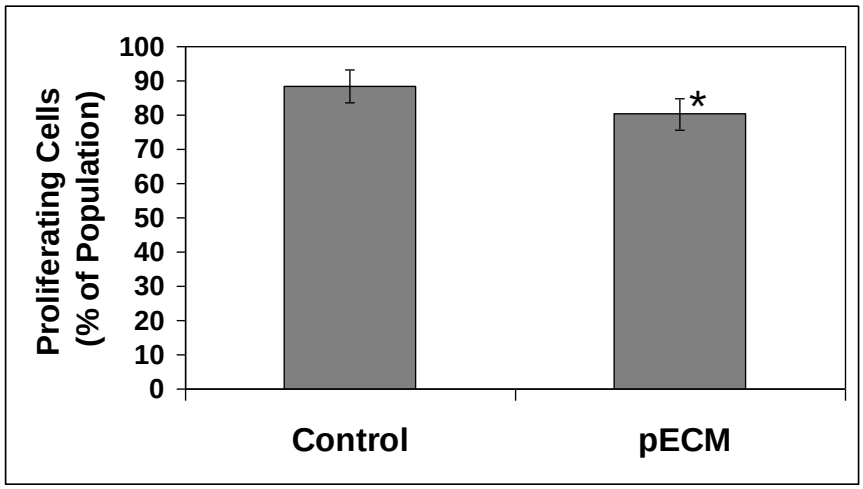

Proliferating Cells
(% of Population)
100
90
80
70
60
50
40
30
20
10
0
*
Control
pECM

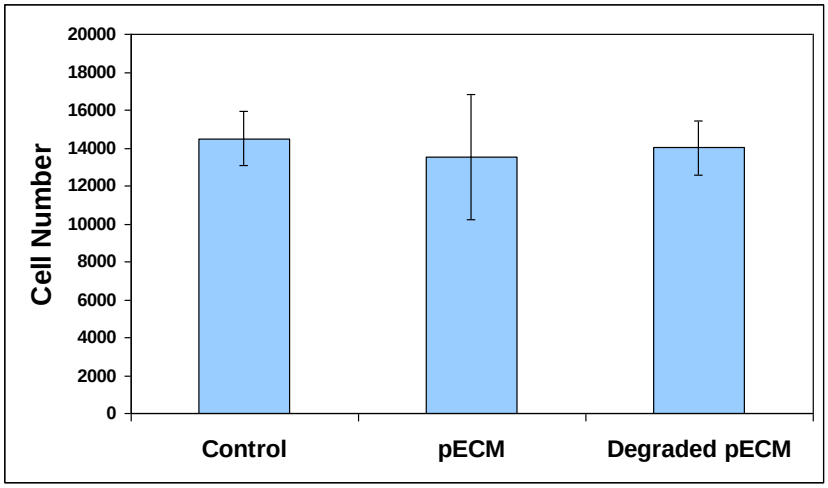
Figure 4.3 A) Photomicrographs of BrdU stained primary pulp cell populations following seven days culture on (i and ii) control culture polystyrene, and (iii and iv) pECM coated polystyrene. Images show actively proliferating cells (dark nuclei) and non-proliferating cells (light nuclei) in both culture conditions. B) Cell count data for the BrdU stained primary pulp populations. Cells were counted in 10 fields of view representing over 50% of the total stained area. Data showed a significant decrease in the percentage of cells actively proliferating in the pulp population cultured on pECM coated surfaces compared with control polystyrene surfaces. N=2, standard deviation bars plotted, * = $P<0.05$.

4.2 Cell Adhesion and Proliferation on Degraded pECM Surfaces

Partial auto-degradation of pECM was performed as described by Smith (1984), which was shown in the previous chapter to alter the protein profile of the pECM (Figure 3.4). Degraded pECM surfaces were used in this study to determine if enzymatic degradation, relevant to the *in vivo* disease environment, influenced functional activity.

No differences were detected in the number of cells that adhered to the uncoated control, pECM coated and degraded pECM coated surfaces after 24 hours (4.4.A). Again, primary pulp cells cultured on pECM coated surfaces for 4 days showed a statistically significant decrease in numbers compared with those on control tissue culture polystyrene (4.4.B). However, surfaces coated with pECM that were subject to enzymatic degradation did not show this significant decrease in cell proliferation although a slight trend towards a decrease in cell numbers was detected. This indicated a change in the functional activity of pECM following enzymatic breakdown that may have implications for the role of pECM within the *in vivo* disease environment.

A)



B)

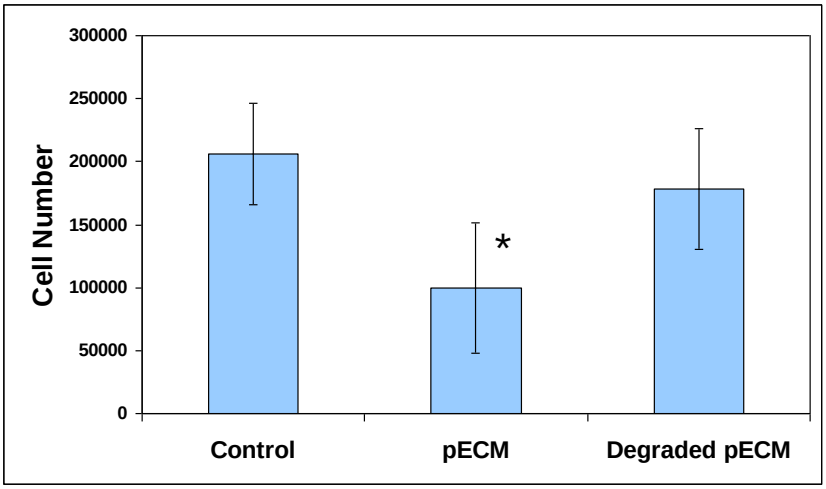


Figure 4.4 Primary pulp cell culture numbers after (A) 24 hours, and (B) 4 days culture on control, pECM and degraded pECM surfaces. No statistically significant differences were found in cell adhesion after 24 hours. A statistically significant decrease was seen in cell number on pECM surfaces after 4 days compared with the control and the degraded pECM surface. There was a minimal decrease in cell number on the degraded pECM compared with the control, however, this was not statistically significant. N=3, standard deviation bars plotted, * = $P<0.05$.

4.3 Gene and Protein Expression Analyses on ECM Surfaces

Gene expression analysis from gel images seen in Appendix 1, indicated increased expression levels of the dentinogenic markers (Dmp-1, Dspp) in the MDPC-23 odontoblast-like cells when cultured on pECM coated surfaces compared with control polystyrene surfaces (Figure 4.5). In contrast decreased expression of dentinogenic markers (Dmp-1, Dspp) was detected in primary pulpal cell populations when cultured on pECM coated surfaces compared with control polystyrene surfaces. Primary pulpal cells demonstrated increased expression levels of the pluripotent stem cell markers C-myc, Klf4, Lin28 and Sox2 when cultured on the pECM compared with control polystyrene surfaces. Primary pulpal cells also exhibited increased transcript levels of the mesenchymal and general stem cell markers Thy-1, Sca-1, CD44 and vimentin when cultured on pECM coated polystyrene surfaces compared with control polystyrene surfaces. The expression profile of the bone marrow cell population appeared more complex. These cells demonstrated relatively low levels of expression of the dentinogenic markers (Dmp-1, Dspp) compared with dental derived cell populations, and while the expression of several stem and mesenchymal markers were increased (C-myc, Klf4, Nanog, Sca-1, Vim and Cd44), others were relative lower in expression (Lin28, Thy-1, Sox2 and Scf) following culture on the pECM compared with control polystyrene surfaces.

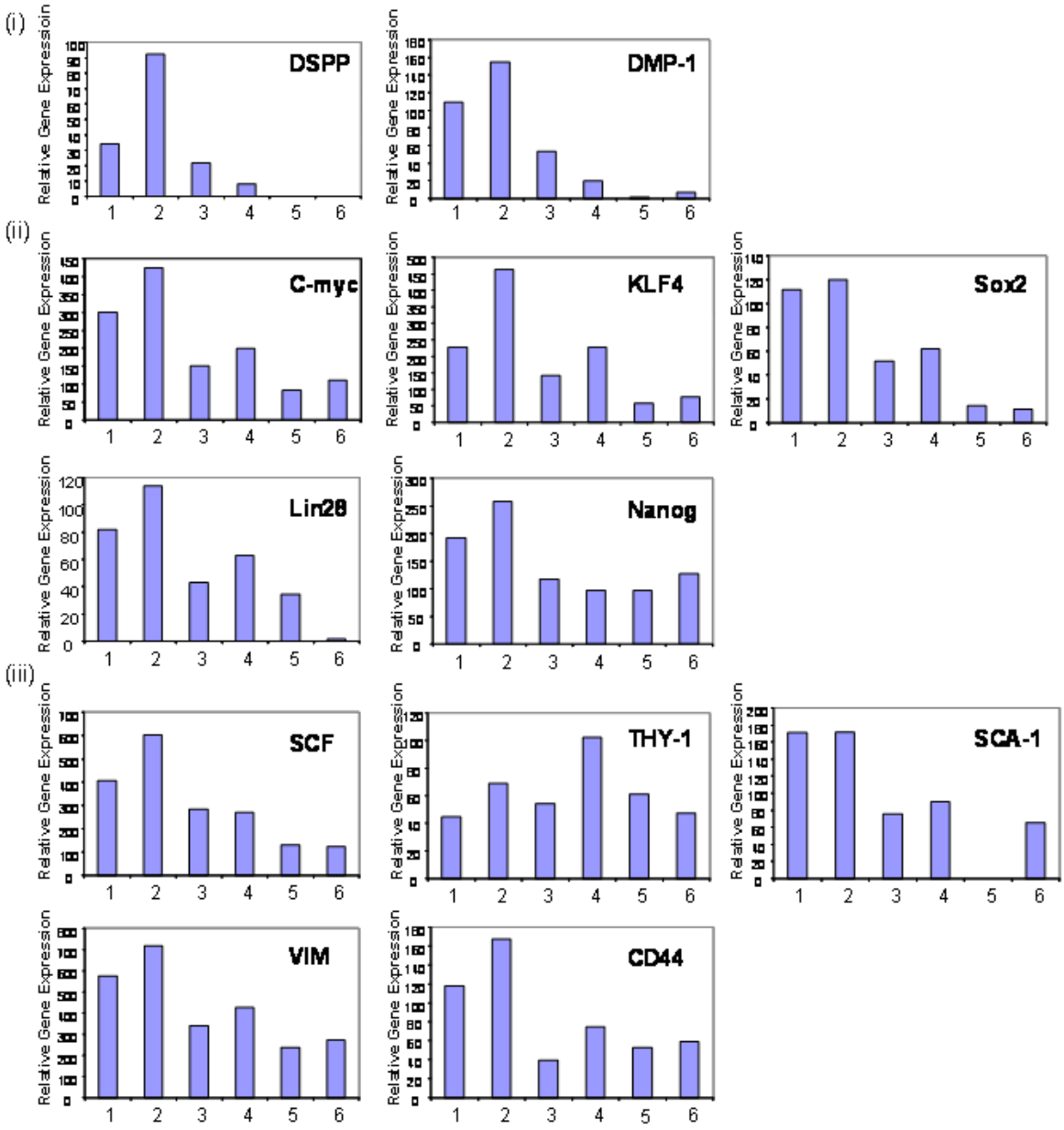
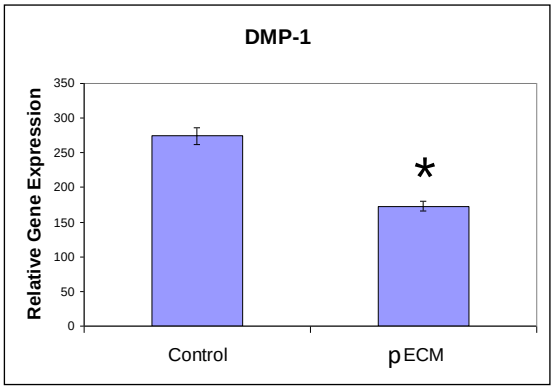


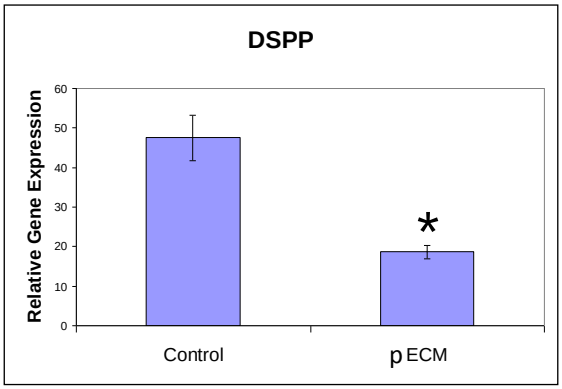
Figure 4.5 Densitometric analysis of gel images provided in Appendix 1. Intensities were normalised against GAPDH and plotted as relative gene expression for (i) dentinogenic markers, (ii) pluripotent stem cell markers, (iii) mesenchymal / general stem cell markers. Data show differential gene expression between 1= MDPC-23 cells on culture polystyrene, 2= MDPC-23 cells on pECM, 3= primary pulp cells on culture polystyrene, 4= primary pulp cells on pECM, 5= primary bone marrow cells on culture polystyrene, 6= primary bone marrow cells on pECM. (b) N=2. (Full names of gene abbreviations provided in Table 2.5.5).

More in-depth gene expression analysis focussed on primary pulp cells to confirm the gene expression changes seen in primary pulp cell populations when cultured on pECM compared with control culture polystyrene surfaces.

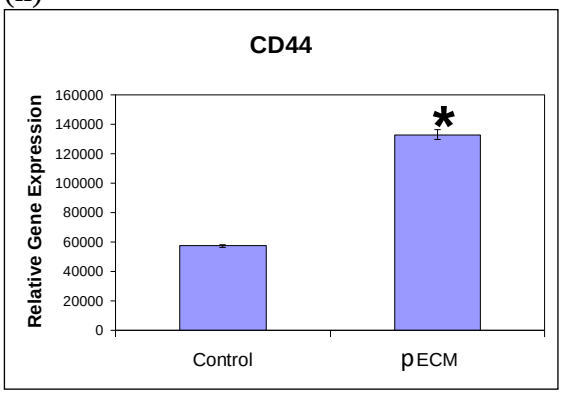
Data confirmed transcriptional changes in these cells when cultured in an environment containing pECM (Figure 4.6). Primary pulp cells demonstrated a statistically significant decrease in the expression of markers associated with dentinogenic differentiation (Dmp-1 and Dspp) when cultured on pECM compared with control polystyrene surfaces. An increase in the expression of mesenchymal / general stem cell markers (Vim, CD44, Sca-1, Scf and Thy-1), which was statistically significant for vim and cd44, was observed when primary pulp cells were cultured on pECM compared with control polystyrene surfaces. An increase in the expression of pluripotency markers (C-myc, Sox2, Lin28 and Klf4), which was statistically significant for Sox2, was detected when primary pulp cells were cultured on pECM compared with control polystyrene surfaces.

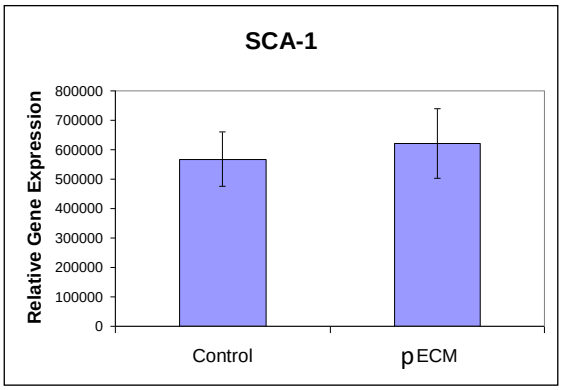
(i)

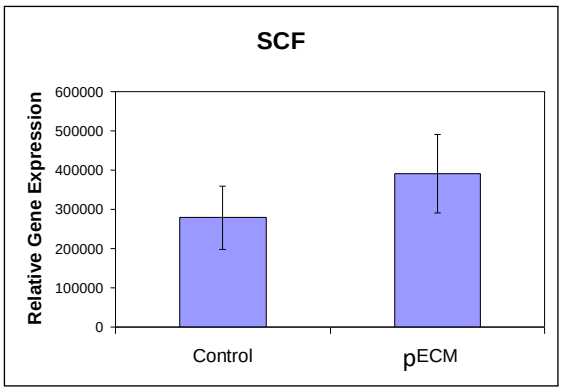

DMP-1
Relative Gene Expression
350
300
250
200
150
100
50
0
*
Control
pECM

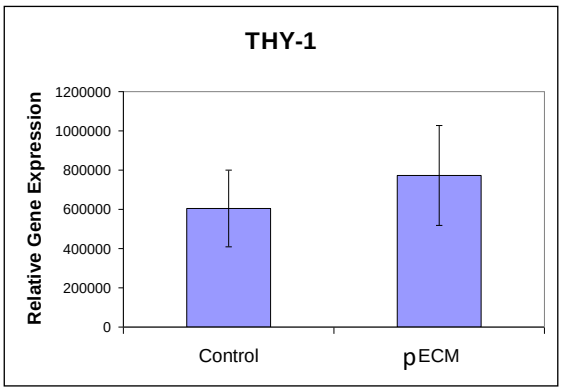

DSPP
Relative Gene Expression
60
50
40
30
20
10
0
*
Control
pECM

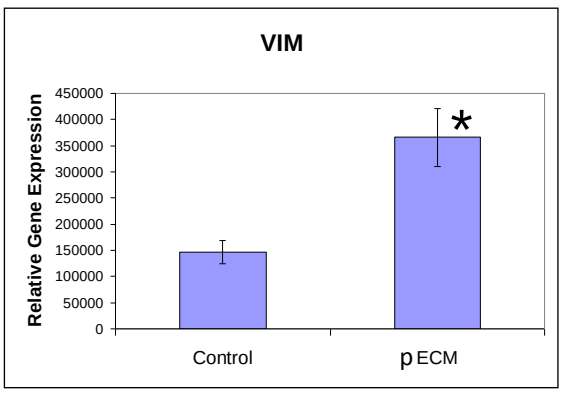
(ii)


CD44
Relative Gene Expression
160000
140000
120000
100000
80000
60000
40000
20000
0
*
Control
pECM


SCA-1
Relative Gene Expression
800000
700000
600000
500000
400000
300000
200000
100000
0
Control
pECM


SCF
Relative Gene Expression
600000
500000
400000
300000
200000
100000
0
Control
pECM


THY-1
Relative Gene Expression
1200000
1000000
800000
600000
400000
200000
0
Control
pECM


VIM
Relative Gene Expression
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
*
Control
pECM

(iii)

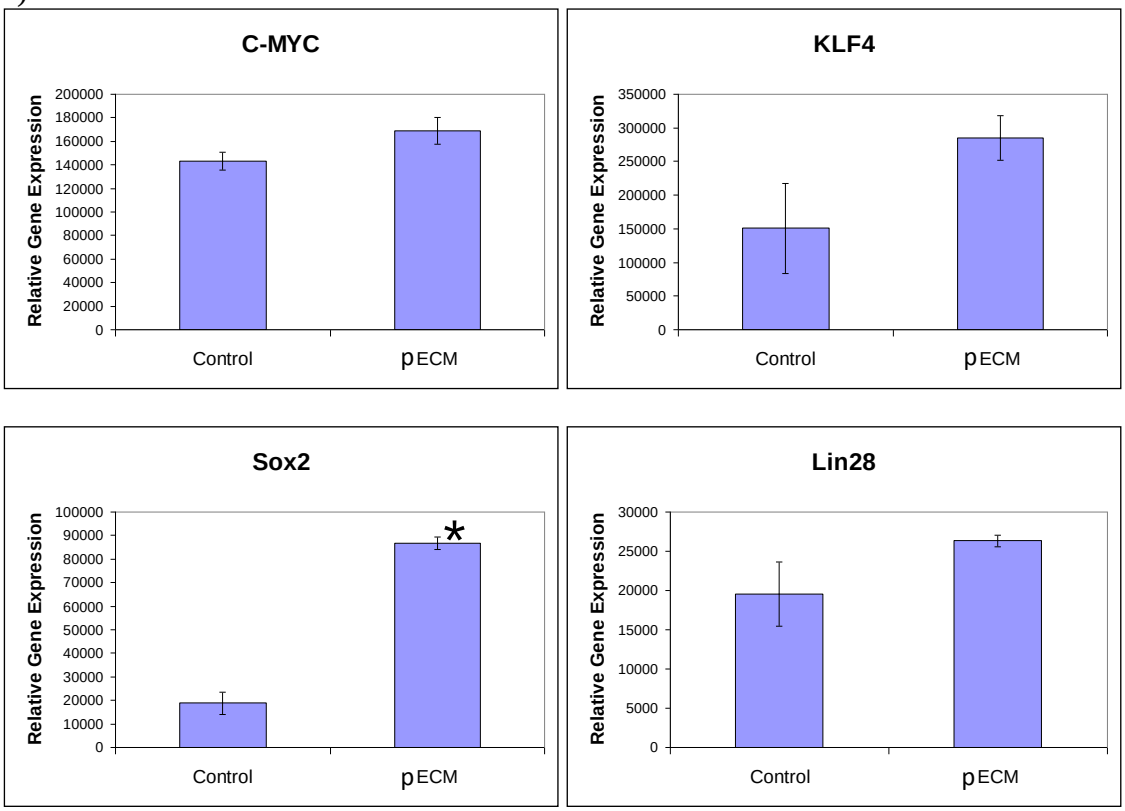


Figure 4.6 Densitometric analysis of PCR gel images (see Appendix 2). Intensities were normalised against GAPDH and plotted as relative gene expression. (i) dentinogenic markers, (ii) mesenchymal / general stem cell markers, (iii) pluripotent stem cell markers. Data showed there was a statistically significant decrease in primary rodent pulp cell population expression of the dentinogenic markers Dmp and Dspp when cultured on pECM. The expression of mesenchymal / general stem cell markers was increased when cultured on pECM. This increase was statistically significant for Cd44 and Vim. The expression of pluripotent stem cell markers was also increased when cultured on pECM. This increase was statistically significant for Sox2. Standard deviation bars plotted. * = $P<0.05$. N=3. Full names of gene abbreviations shown in (Table 2.5.5).

ArrayScan high content screening (HCS) imaging cytometer (Cellomics, UK) analysis was performed (Imagen Biotech, Manchester, UK) using primary pulp cells for the pluripotent stem cell marker Oct3/4. Data indicated that enzymatically obtained primary pulp cultures contained Oct3/4 positive cell populations (Figure 4.7). When cultured on standard control culture polystyrene surfaces for 4 days, the proportion of the population that stained positive was 19%. This proportion of positive cells increased to 55% in cultures grown on pECM coated surfaces for 4 days. This supported the gene expression data presented (Figure 4.6), that indicated that culture on pECM could enhance stem cell marker expression in primary pulp populations compared with culture on standard culture polystyrene.

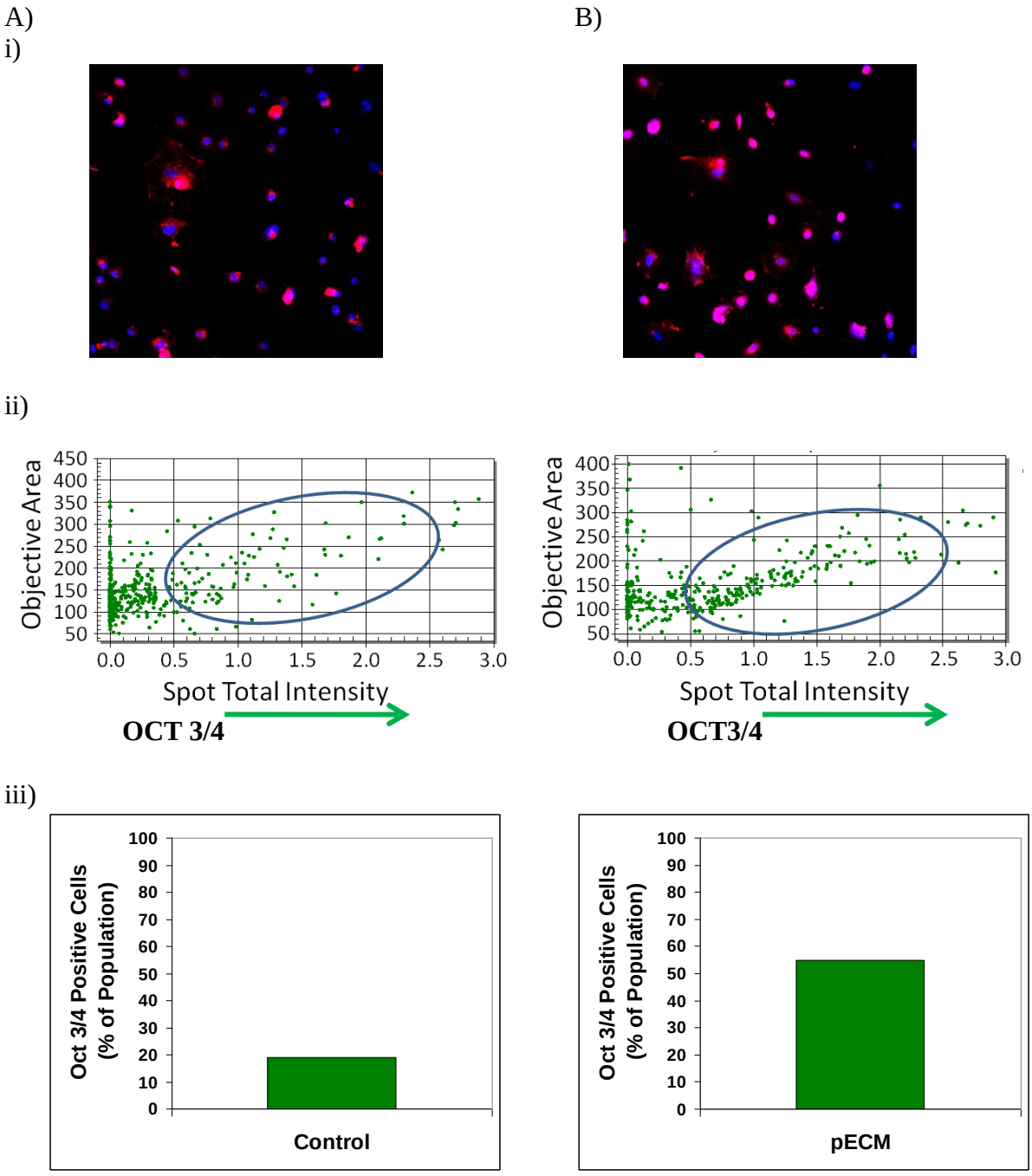


Figure 4.7 ArrayScan HCS imaging cytometer (Cellomics, UK) high content analysis of primary pulp cells stained for Oct3/4 after 4 days culture on (A) control culture polystyrene, (B) pECM. (i)= Representative image of the 10 images obtained for each well. (ii) Scatter plots of cells showing subpopulation information obtained from images. (iii) Graphical representation of scatter plot data showing the percentage of cells in the population that stained positive for Oct3/4. Data shows an increase in the percentage of the population staining positive for Oct3/4 after culture on pECM coated surfaces compared with control uncoated surfaces.

5.0 THE EFFECTS OF PULP AND DENTINE ECM COMPONENTS AND THEIR BREAKDOWN PRODUCTS ON DENTAL PULP CELL RECRUITMENT

The sequence of events for dental tissue regeneration involves stem/progenitor cell recruitment to the site of injury, induction of cellular differentiation and subsequent up-regulation of ECM secretion by these differentiated cells. However, the signalling involved in the recruitment of pulp cells during reparative dentinogenesis is not well defined with a range of different factors postulated to contribute. ECM molecules present within the dentine / pulp complex likely play a role in the signalling of stem/progenitor cell recruitment to the site of injury. Furthermore, the carious environment contains enzymes and acids derived from host cells and invading microorganisms, that partially degrade these ECM molecules releasing products which may possess enhanced bioactivity (Armstrong, 1958; Hojo *et al*, 1994; Katz *et al*, 1987; Goldberg and Keil, 1989).

The investigations described here were performed to determine whether pulp and dentine ECM preparations influenced pulp cell migration. ECM preparations were also subjected to physiologically relevant degradative conditions (Figure 3.4) to determine whether the relatively harsh carious environment influenced the activity of these molecules. To determine whether functional activity could be assigned to molecules with specific characteristics, extracts were separated on the basis of their charge prior to assay of their activity (Figure 3.5).

5.1 ECM Influences on Cell Migration

Transwell migration data indicated that dECM enhanced primary pulp cell migration compared with the negative control (Figure 5.1). Data illustrated the stimulatory effect on migration of dECM with a 29% increase in primary pulp cell migration induced by dECM compared with the negative control. Notably, the influence of dECM on cell migration was enhanced when subjected to acidic breakdown conditions compared with undegraded dECM. This increase was statistically significant ($P<0.05$) after acetic acid treatment of dECM compared with intact dECM (Figure 5.1).

In the transwell migration assay system, pECM also stimulated primary pulp cell migration compared with the negative control (Figure 5.2). Data showed that pECM induced a 43% increase in primary pulp cell migration compared with the negative control. The stimulatory effect of pECM on cell migration was enhanced when subjected to enzymatic action, although this increase was not statistically significant.

Ammonium sulphate precipitation of the pECM preparation was used to partially resolve the component molecules into differentially charged fractions to determine whether the stimulatory effect on migration was associated more with a specific charged grouping. The stimulatory effect on cell migration was observed to associate with all of the ammonium sulphate fractions obtained (Figure 5.3) suggesting that a range of molecules were responsible for the activity.

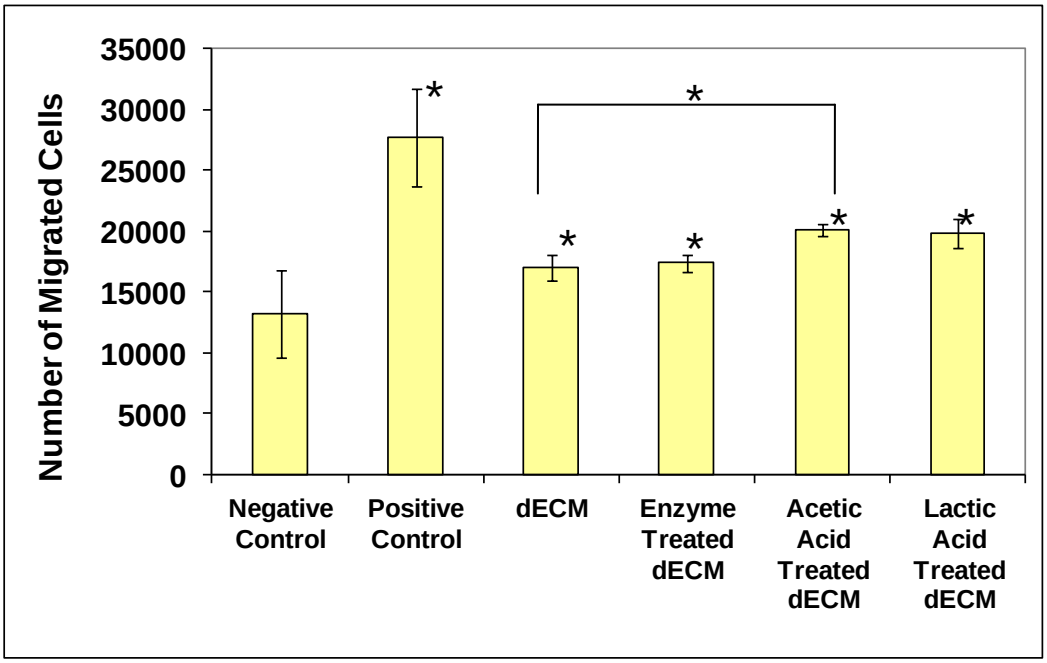


Figure 5.1 Transwell migration data showing pulp cell migration towards intact and partially degraded preparations of dECM. dECM and breakdown products had a statistically significant affect (*) on pulp cell migration compared with the negative control. Acid breakdown of dECM enhanced cell migration compared with intact dECM. The increase observed when dECM was degraded by acetic acid was also statistically significant compared with intact dECM. N=3, standard deviation bars plotted,* = P<0.05.

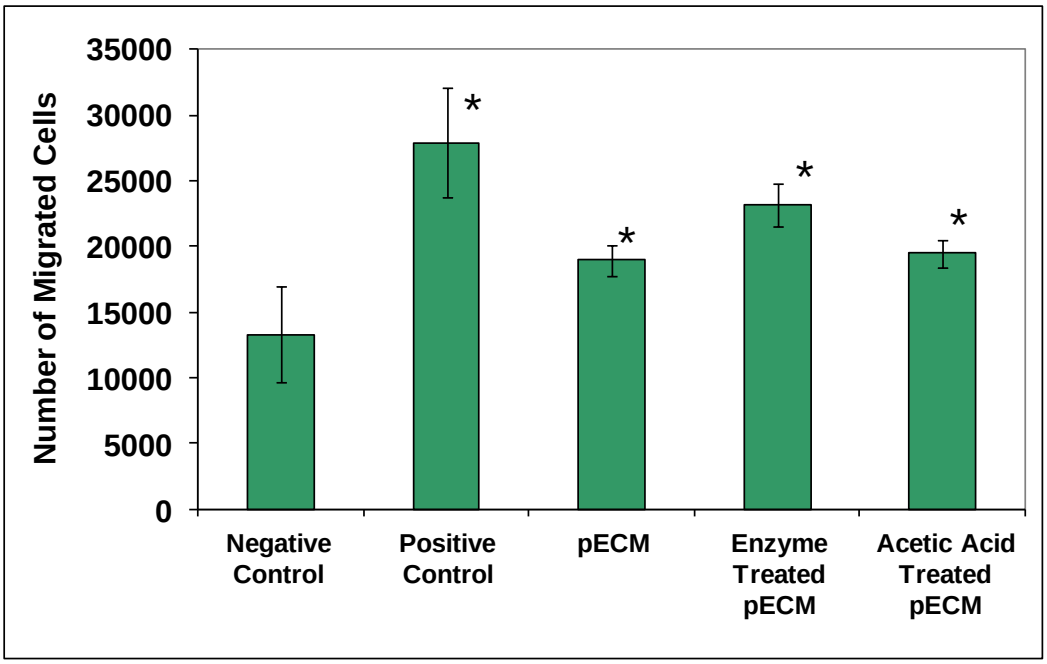


Figure 5.2 Transwell migration data showing pulp cell migration towards intact and degraded preparations of pECM. pECM and its breakdown products had a statistically significantly greater affect on pulp cell migration compared with the negative control. N=3, standard deviation bars plotted,* = P<0.05.

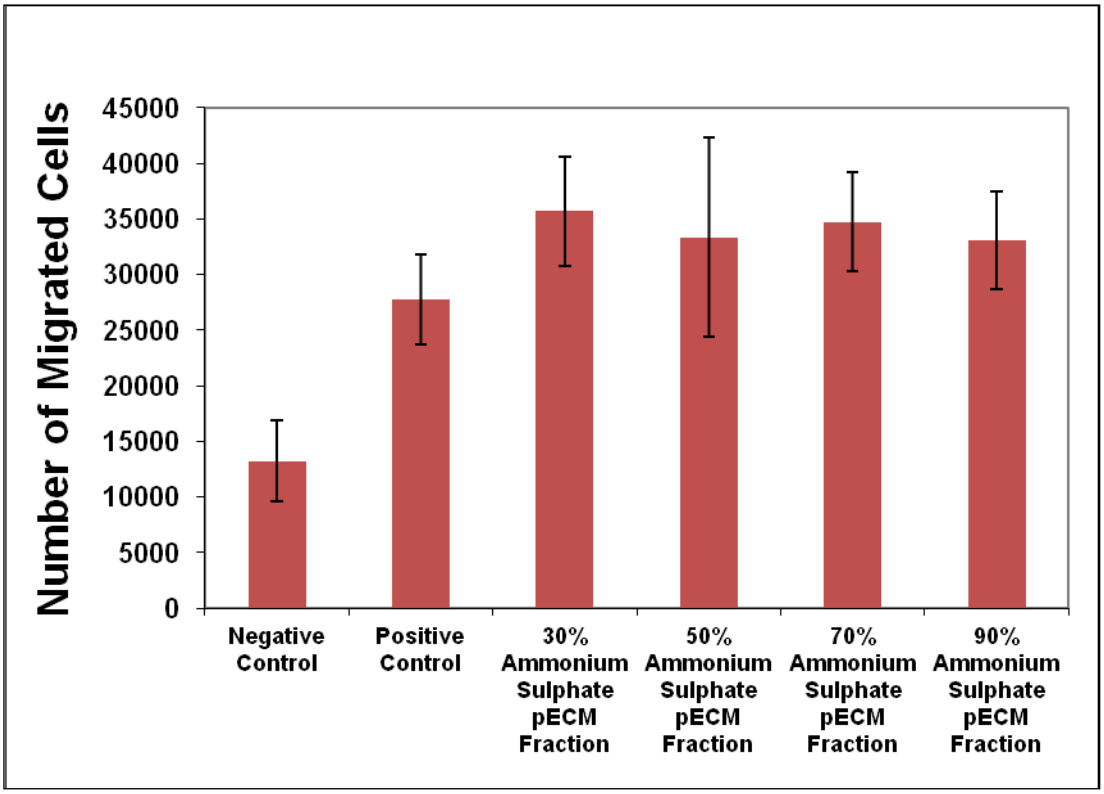
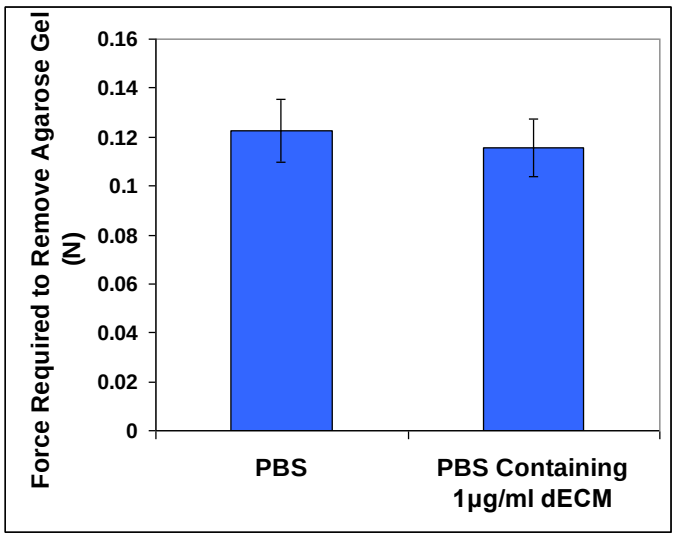


Figure 5.3 Transwell migration data showing pulp cell migration towards ammonium sulphate precipitated fractions of pECM. All fractions showed a statistically significant affect on pulp cell migration compared with the negative control. No significant differences in functional activity were identified between the different fractions of pECM molecules. N=3, standard deviation bars plotted, * = $P<0.05$.

Following the transwell migration assay analysis, an agarose spot migration assay was used with primary pulp cells to visualise the effects of dental ECM preparations on pulp cell migration. Prior to adoption of this protocol, tribometric testing (Section 2.8.2.2) was undertaken to determine whether the inclusion of ECM molecules within agarose gels affected the physical bonding of these gels to cell culture polystyrene surfaces. This was necessary to eliminate the possibility that cell migration was being influenced by physical adhesion of the gels rather than in response to biological cues.

The inclusion of dECM into agarose gels did not statistically significantly affect the strength of adhesion of the gels to the polystyrene culture surfaces (Figure 5.4). Agarose gels that had been submerged in media and incubated at 37˚C for 24 hours had a significantly reduced strength of adhesion compared with samples that had been kept dry at 4˚C for 24 hours. A reduction in adhesion strength of 70% was observed for control PBS agarose gels and of 62% for agarose gels containing 1µg/ml dECM. However, in both test conditions there was no statistically significant difference between the adhesive strength of the control PBS gels and those containing 1µg/ml dECM. This indicated that any difference in cell migration within agarose gels was due to biological interactions with the proteins and not due to the proteins affecting the adhesive properties of the gels to the underlying substrate.

A)



B)

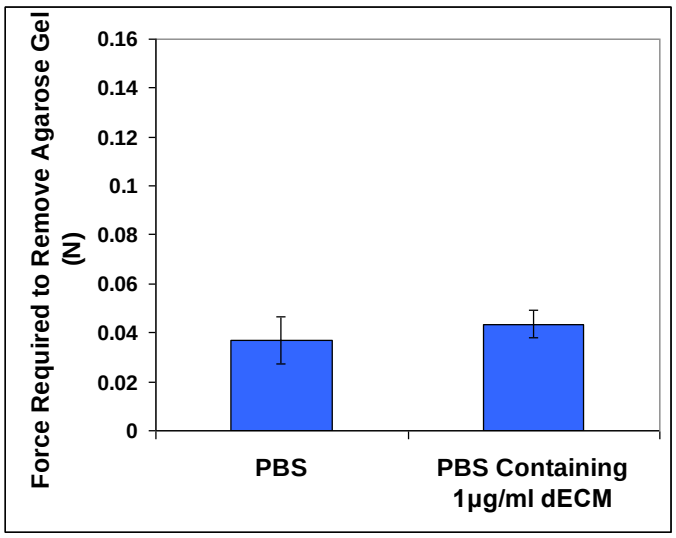
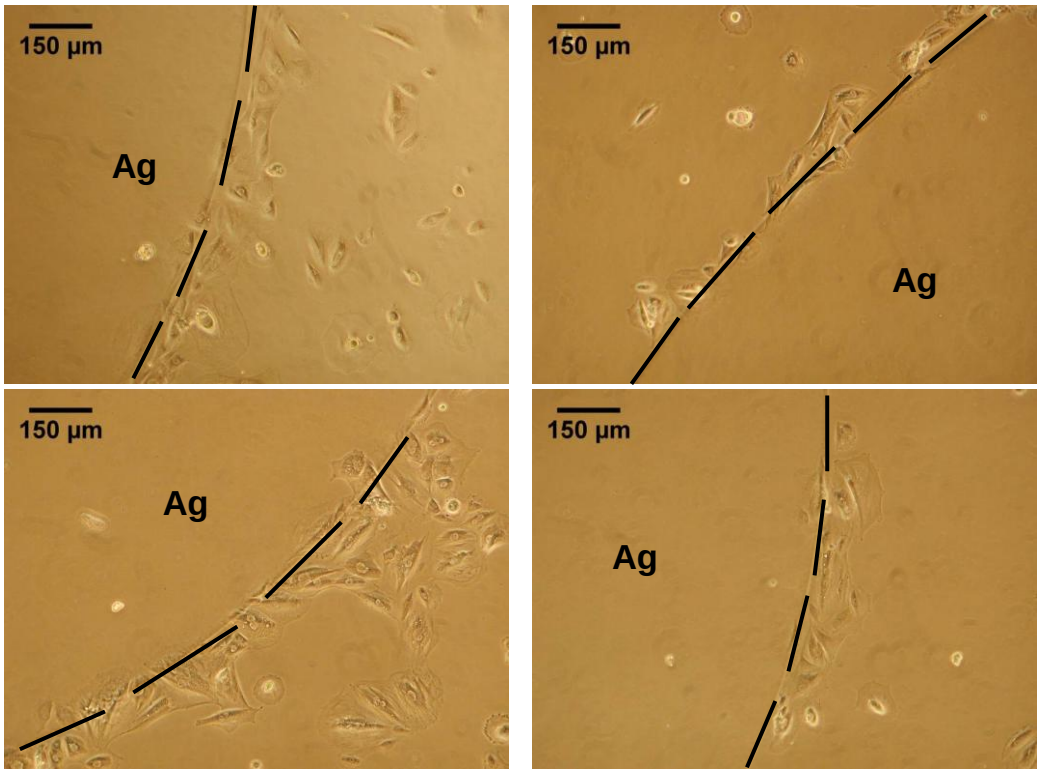


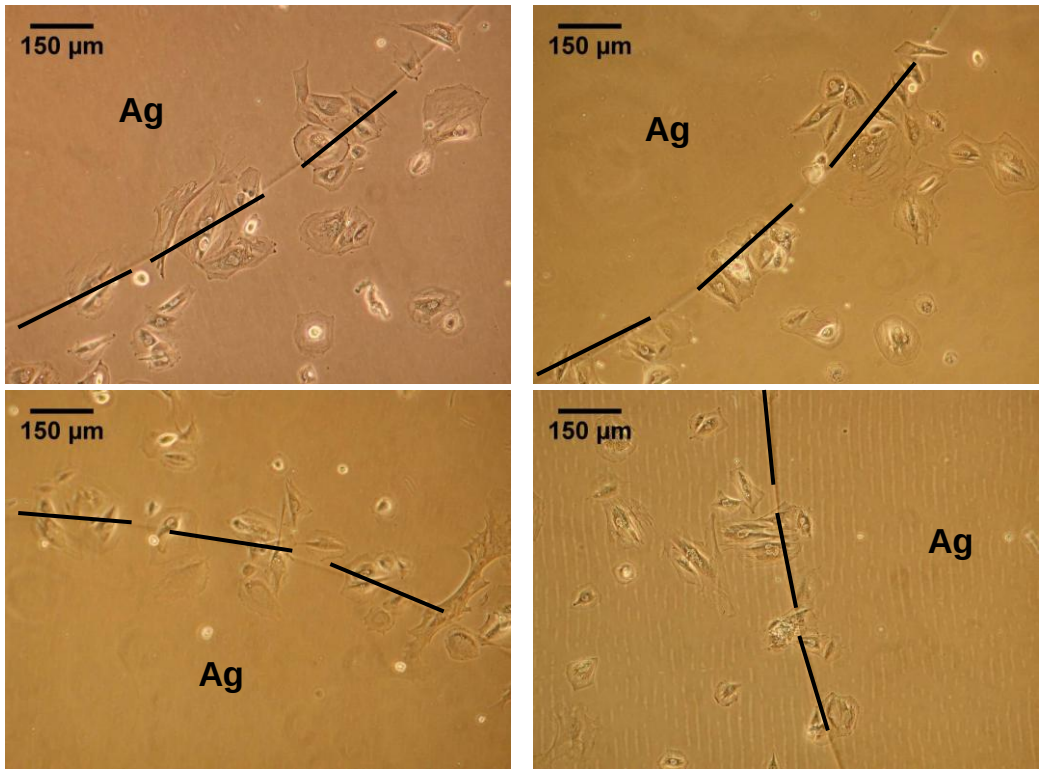
Figure 5.4 Tribometric analysis characterising the adhesive strengths of agarose gels to culture surfaces, (A) after 24 hours at 4˚C, (B) and after 24 hours submerged in media at 37˚C. A decrease in adhesion strength was detected after the gels were submerged in media at 37˚C. In both conditions, no significant difference was observed between the gels containing 1µg/ml dECM and the control PBS gels. Standard deviation bars plotted. N=5.

Using phase contrast microscopy, migration of pulp cells within the agarose gels containing pulp and dentine ECM preparations was observed after 24 hours culture (Figure 5.5). Cell count data obtained from this assay demonstrated that the inclusion of dECM enhanced the number of migrating pulp cells by 285% compared with the PBS negative control and the inclusion of pECM enhanced the number of migrating pulp cells by 100% compared with the PBS negative control (Figure 5.6). However, only the increase induced by dECM was statistically significant. These data corroborated the transwell assay data showing that pulp and dentine ECM molecules enhanced pulp cell migration.

A)


150 µm
Ag
150 µm
Ag
150 µm
Ag
150 µm
Ag

B)


150 µm
Ag
150 µm
Ag
150 µm
Ag
150 µm
Ag

C)

D)

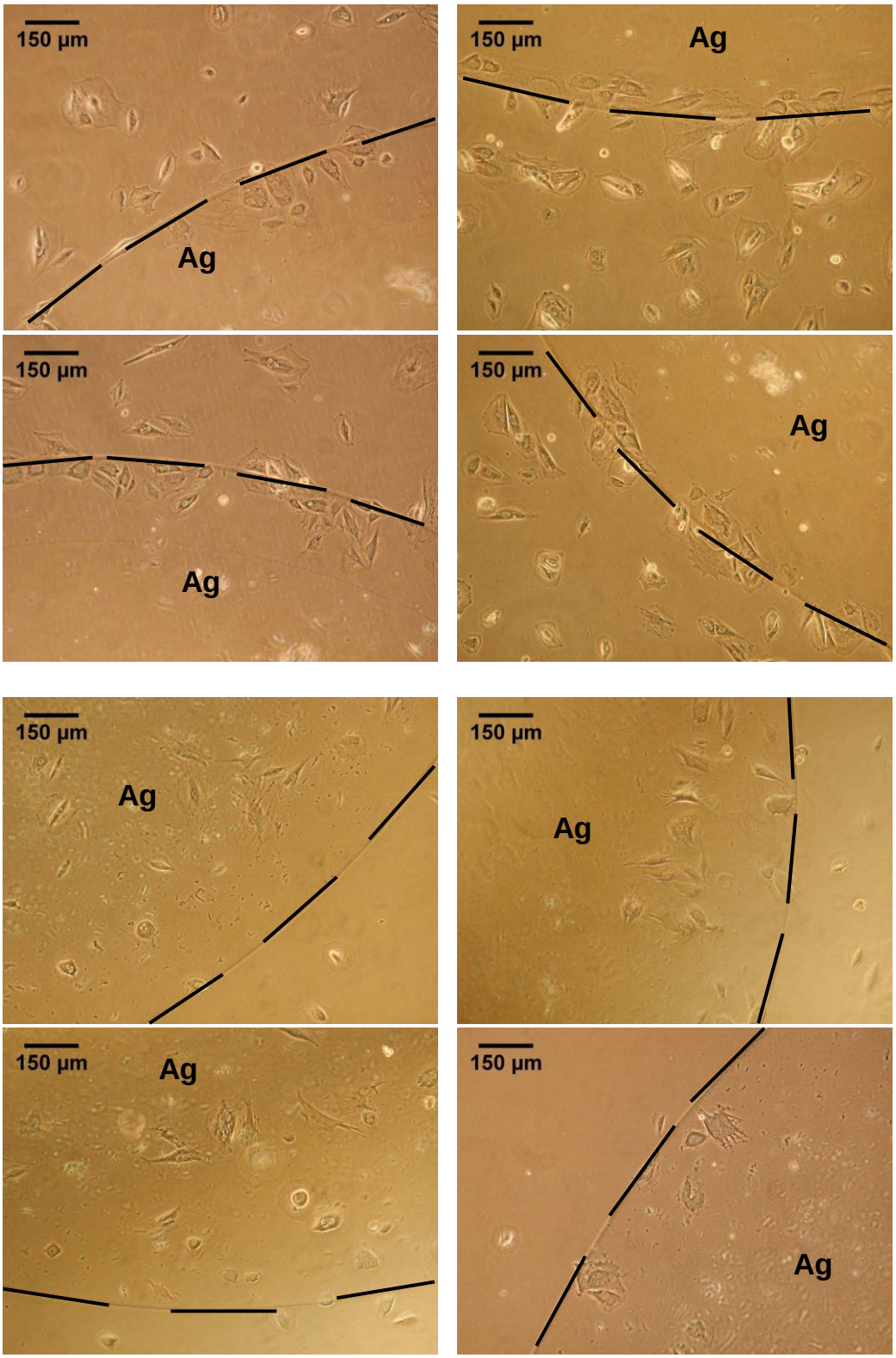


Figure 5.5 Photomicrographs of pulp cells migrating into agarose gels (Ag) containing A) PBS (negative control), B) FCS (positive control), C) dECM and D) pECM. Four replicate samples shown for each condition. pECM and dECM molecules induced pulp cell migration with increased numbers of cells observed within Ag containing ECM molecules. Dashed lines represent the edge of the agarose gels. Scale bars are shown.

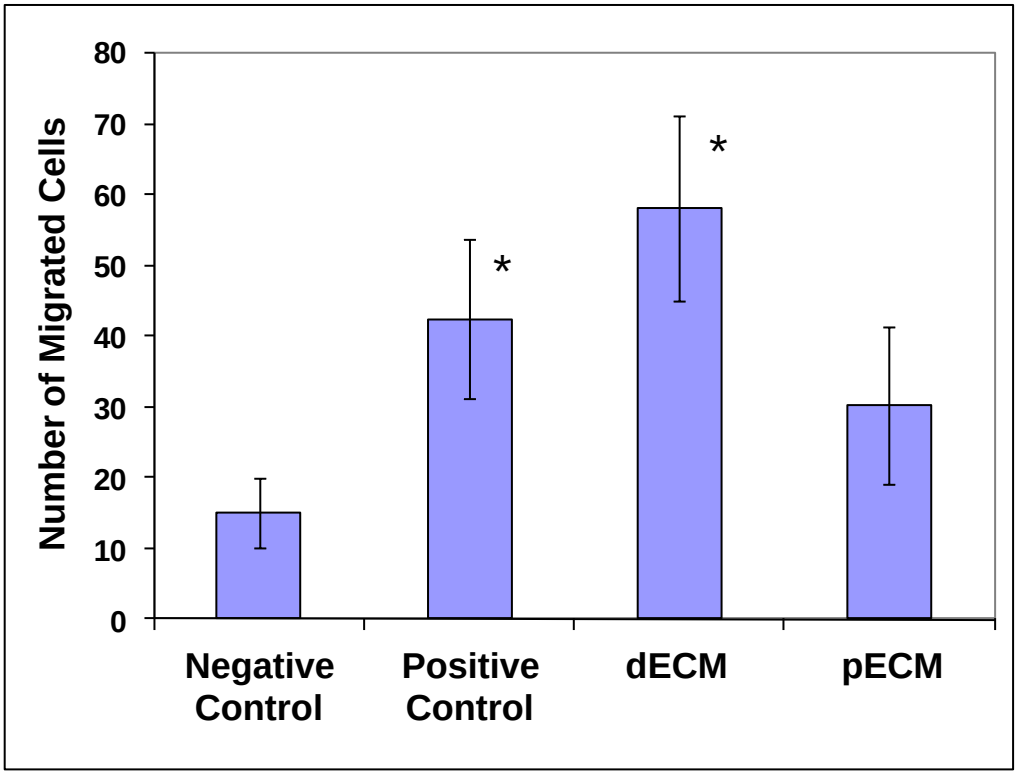


Figure 5.6 Number of pulp cells migrating into agarose gels after 24 hours culture demonstrating cell migration was enhanced by the presence of dECM and pECM molecules. The increase in pulp cell migration was statistically significant in the presence of dECM. Standard deviation bars plotted. * = $P<0.05$. N=5.

Time-lapse video microscopy of agarose spot assay data further corroborated previous results for dECM stimulated pulp cell migration (original video files seen in Disc Appendix 1). Preferential directional migration was observed into agarose gels containing dECM compared with PBS control gels (Figure 5.7). The morphology of the migrating cells appeared changed in the presence of dECM with cells appearing more uniform in control PBS gels and exhibiting more extensive processes in the presence of dECM (Figure 5.8). Analysis of video data demonstrated that after 30 hours culture, the pulp cells migrated further into dECM containing agarose gels compared with the PBS negative controls. The average distance travelled into the agarose gels in the presence of dECM was statistically significantly greater than in control agarose gels and increased from an average of 175μm to 429μm (Figure 5.8).

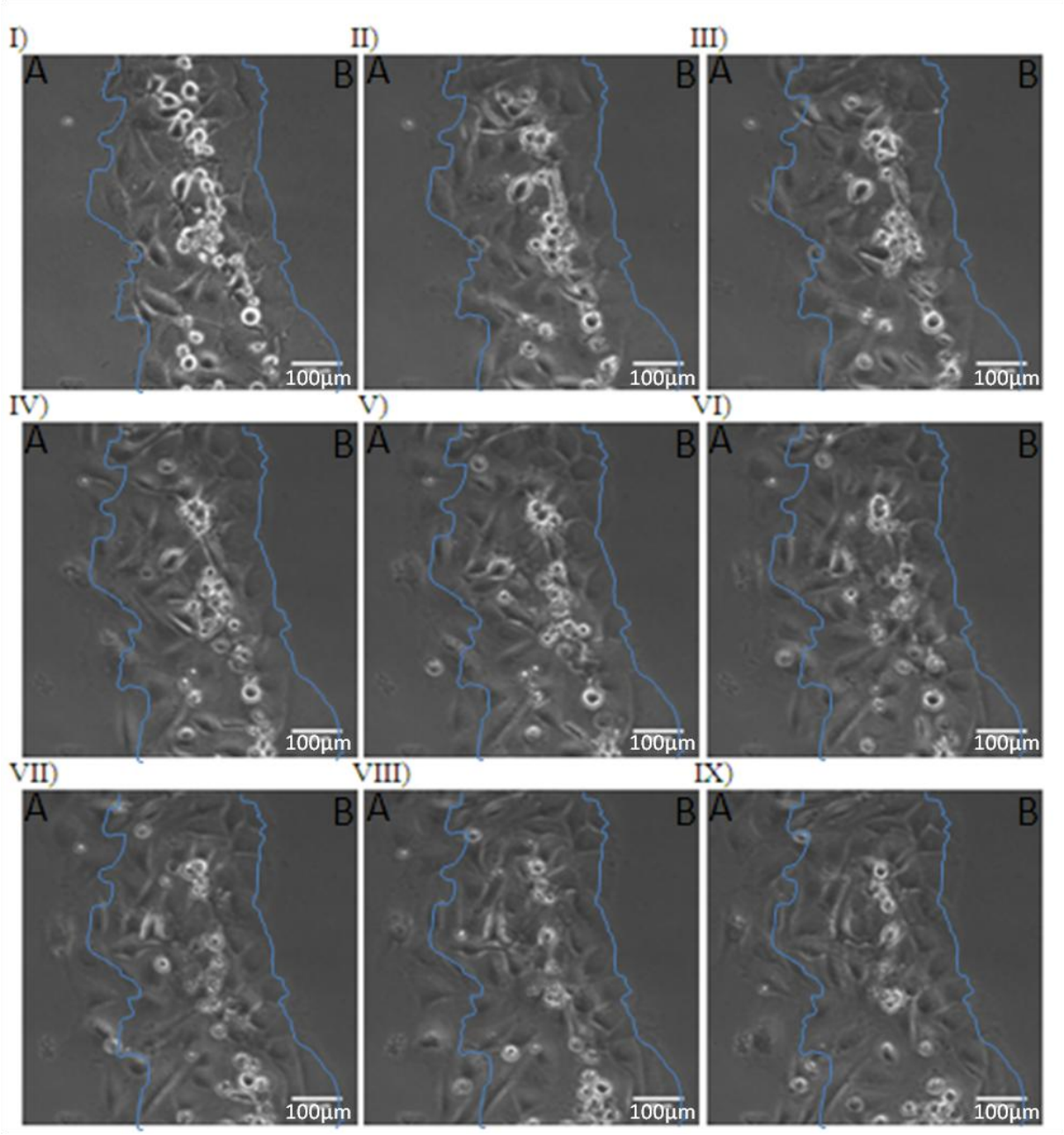


Figure 5.7 Time lapse photomicrographs taken at 2 hour intervals of cell migration into agarose containing either (A) dECM or (B) PBS control, over an 18 hour period. Blue lines represent the outer edge of pulp cells in first image. Photomicrographs illustrate preferential migration of pulp cells towards dECM (A) compared with control (B). Video file are provided in Disc Appendix 1.

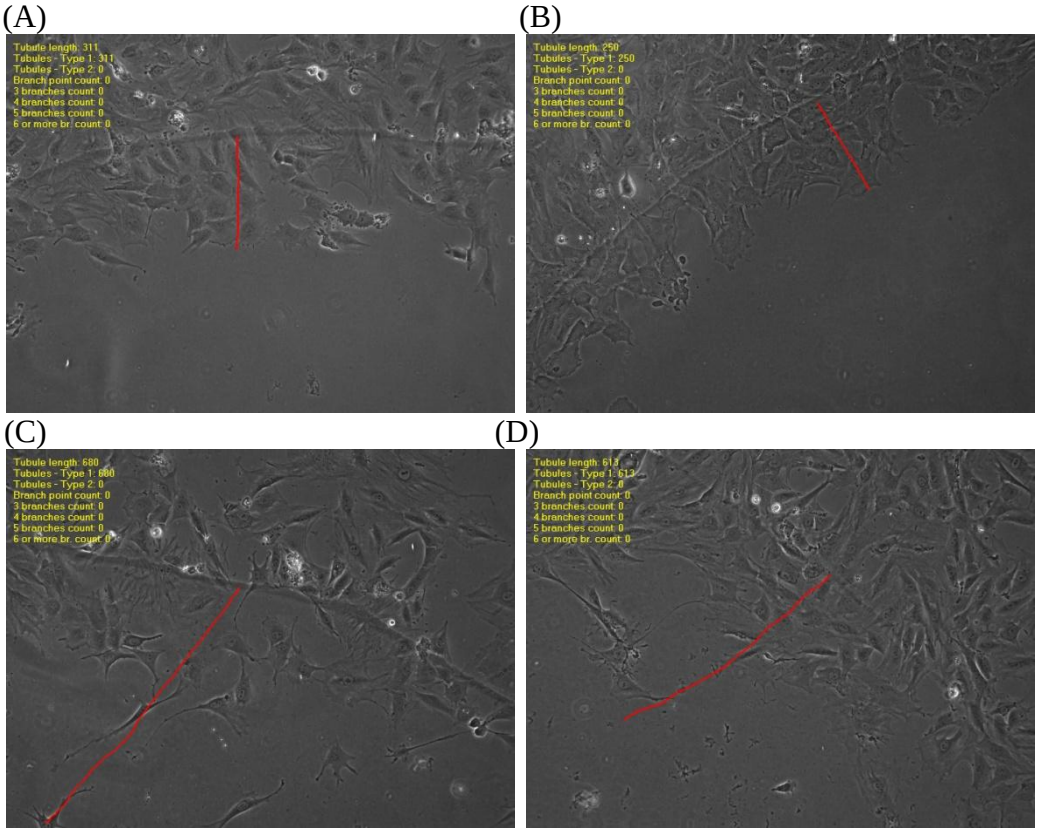


Figure 5.8 Photomicrographs of the final fields from videos obtained using Cell-IQ time lapse imaging chamber for a 30 hour period. Images show pulp cell migration into agarose gels containing PBS (A&B) and dECM (C&D), indicating dECM molecules enhanced the distance of pulp cells migration over the same time period. Analysis performed on video imagess (Chipman Tech, UK) using Cell-IQ software with red lines representing the maximum pulp cell migratory distance after 30 hours. Video files provided in Disc Appendix 1.

Fragmented dentine (>60μm dentine chips and <60μm finely powdered) were included within the agarose gels to determine whether whole dentine tissue could exert similar responses to those seen by extracted dECM molecules. Primary dental pulp cells demonstrated preferential migration towards these particles (Figure 5.9). The number of migrating pulp cells increased by 81% in the presence of dentine powder and by 37% in the presence of a dentine chip within the agarose gel compared with negative control PBS agarose gels (Figure 5.10). This increase in pulp cell migration was only statistically significant for the powdered form of dentine perhaps reflecting the large variation in results obtained using this assay. Thus, both fragments of dentine tissue and the ECM components isolated therefrom could stimulate pulp cell migration.

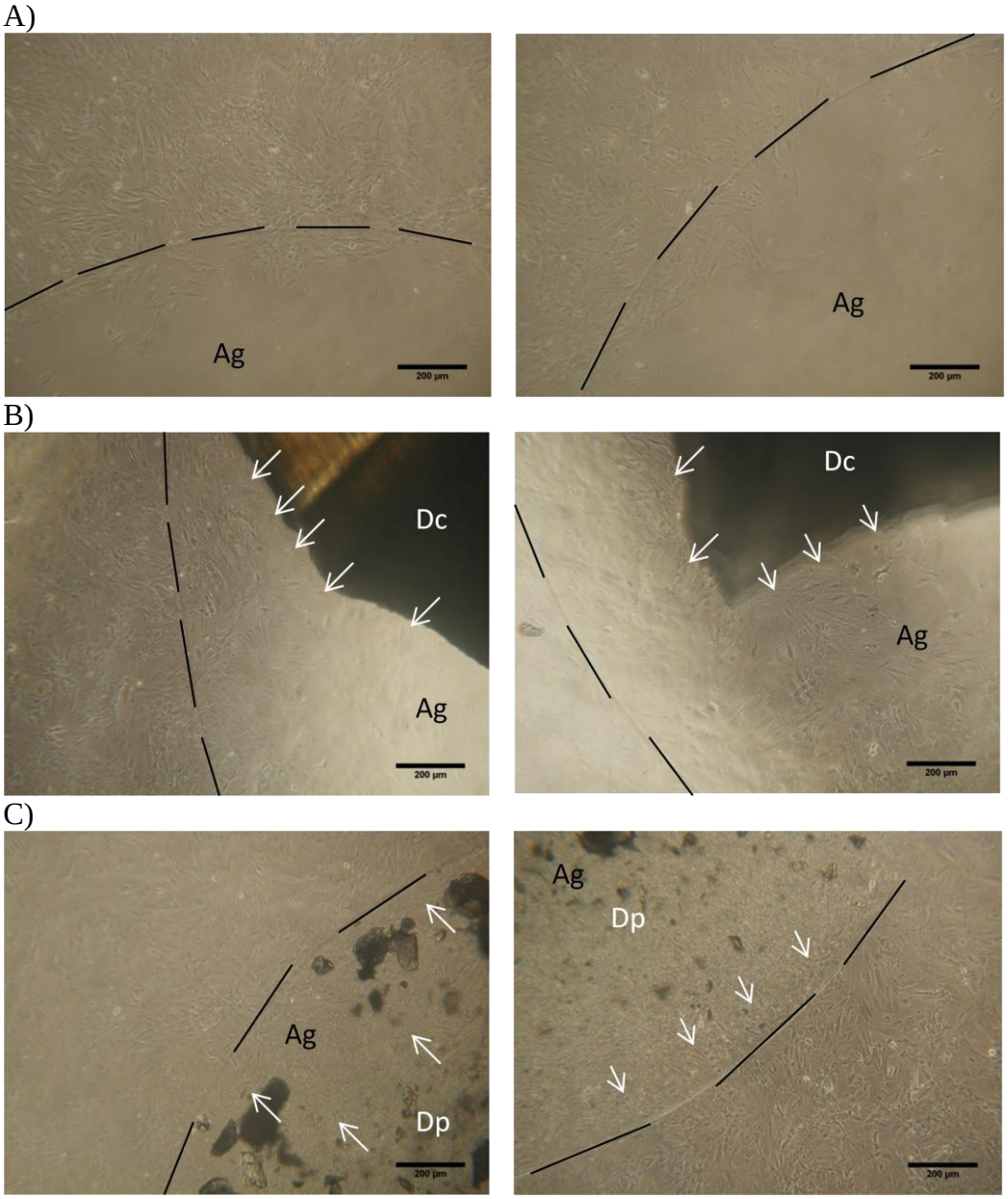


Figure 5.9 Photomicrographs of pulp cell migration into agarose gels containing A) PBS, B) dentine chips (Dc), and C) dentine powder (Dp), with arrows illustrating that pulp cells could migrate into agarose gels containing dentine. Dashed lines represent the edge of the agarose gels. Scale bars are shown.

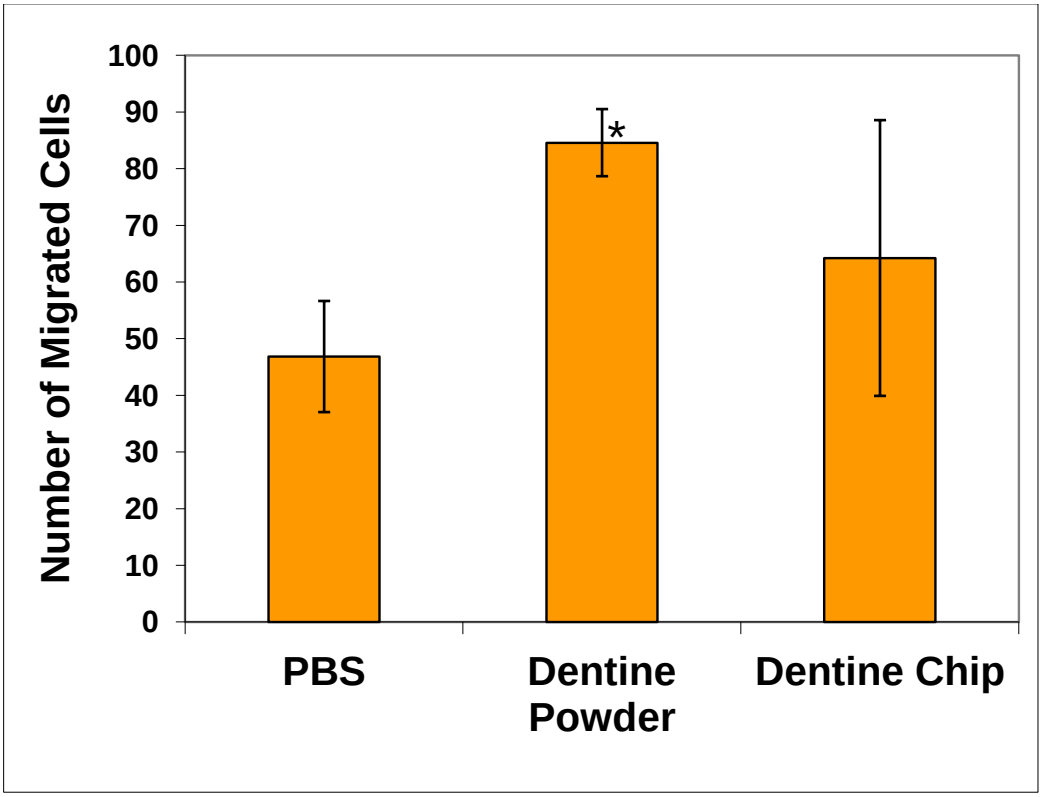


Figure 5.10 Number of cells that migrated into agarose gels after 7 days, showing pulp cell migration was enhanced by the presence of dentine. The increase in pulp cell migration was statistically significant in the presence of powdered dentine. Standard deviation bars plotted. * = $P<0.05$. N=4.

5.2 Rho Inhibition of Dental Pulp Cell Migration

To investigate the specific pathways involved in the migration of dental pulp cells, C3 transferase treatment was used to specifically inhibit the Rho-dependent pathway. Prior to C3 transferase treated pulp cells being used in migration assays, phase contrast microscopic images were obtained to determine whether the C3 transferase exposure had a visible affect on pulp cell morphology. An LDH cytotoxicity assay was also used to determine whether C3 treatment had any effect on primary pulp cell viability.

The C3 transferase treatment of dental pulp cells resulted in a visible affect on the morphology of the primary pulp cells, likely due to changes in cellular cytoskeleton (Figure 5.11). An LDH cytotoxicity assay demonstrated that with 2, 4 and 24 hours exposure to C3 transferase there was no increase in primary pulp cell release of LDH compared with untreated control pulp cell populations (Figure 5.12). Although C3 transferase treatment had a visible effect on primary pulp cell morphology these morphological changes were not due to cytotoxic effects. Primary pulp cells were treated with 1μg/ml C3 transferase prior to use in cell migration assays to determine the effects of inhibition of Rho-GTPase signalling in this assay (Bement *et al*, 2005; Mammoto *et al*, 2004).

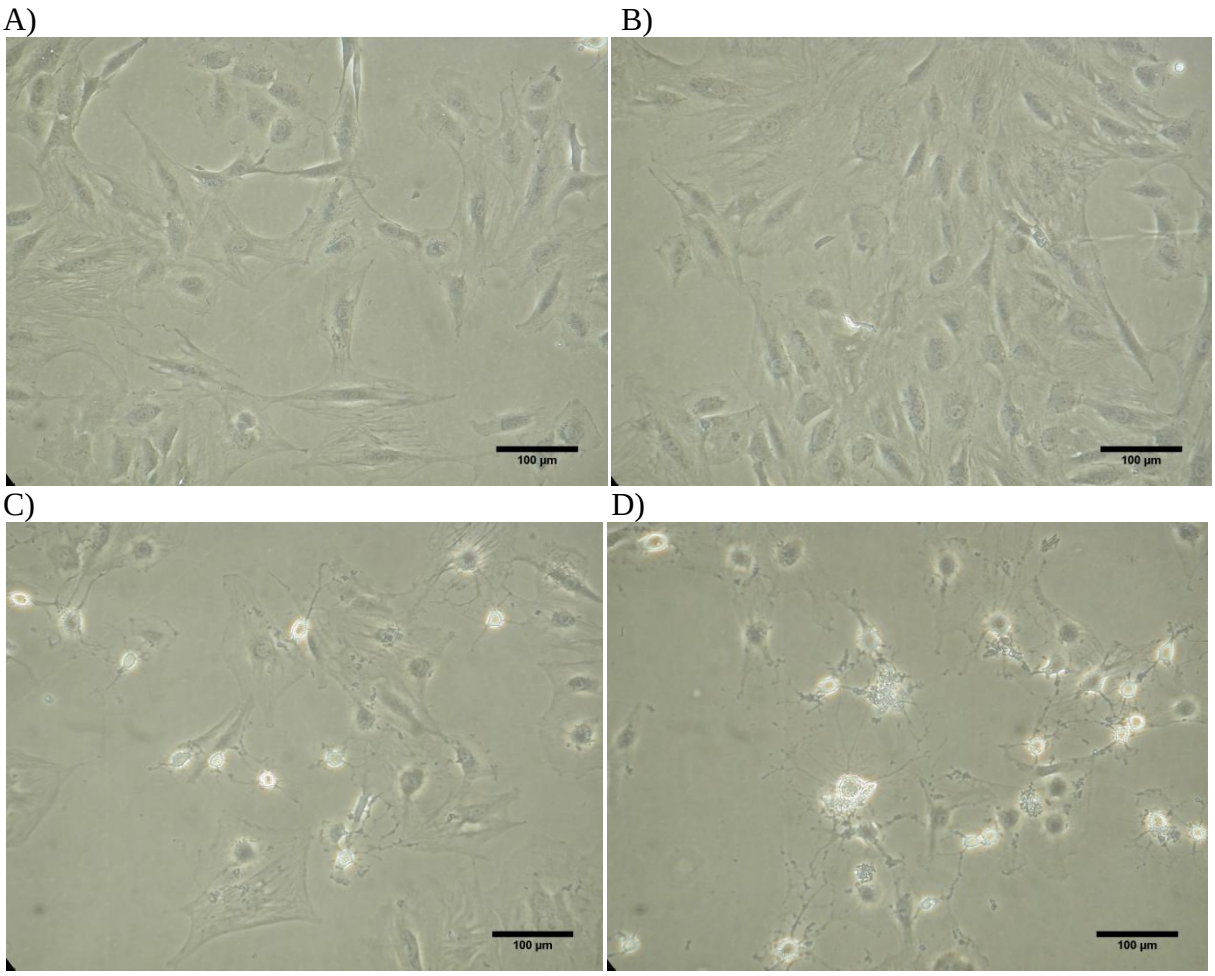


Figure 5.11 Photomicrographs showing pulp cell morphology after 4 hours exposure to control medium (A and B) or control medium containing 1µg/ml C3 transferase (C and D). Images show a change from normal cell morphology after exposure to C3 transferase.

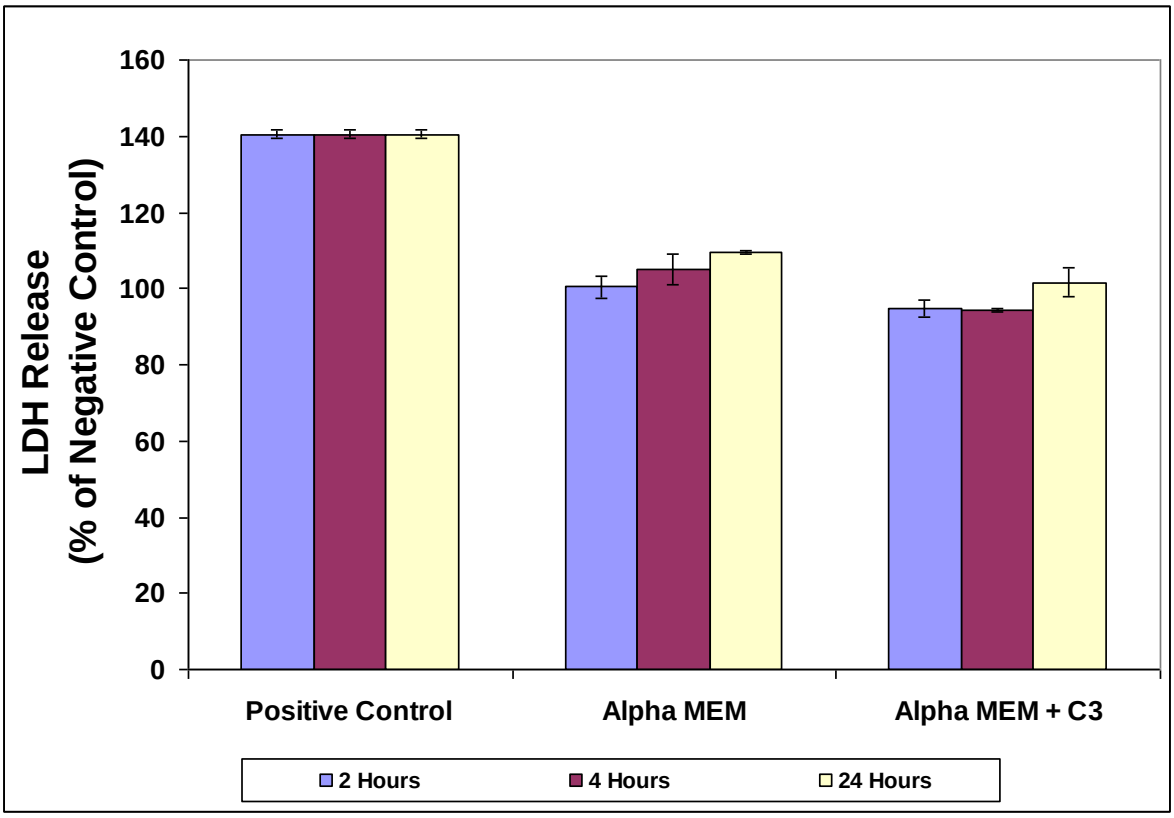


Figure 5.12 Influence of C3 transferase treatment on LDH release after 2, 4 and 24 hours for primary pulp cell populations. Alpha MEM containing 1µg/ml C3 transferase had no significant cytotoxic affect on dental pulp populations compared with alpha MEM cultures at all three time points. Standard deviation bars plotted. N=3.

A transwell migration assay demonstrated that dECM stimulated dental pulp cell migration with a statistically significant increase of 16% in the number of migrating pulp cells in the presence of dECM supplemented medium compared with the un-supplemented negative control (Figure 5.13). However, following C3 transferase treatment, the primary dental pulp cells did not respond to the dECM as demonstrated by lack of significant difference between the number of cells migrating. Combined these data demonstrated that blocking the Rho dependent pathway prevented the stimulation of dental pulp cell migration by dECM.

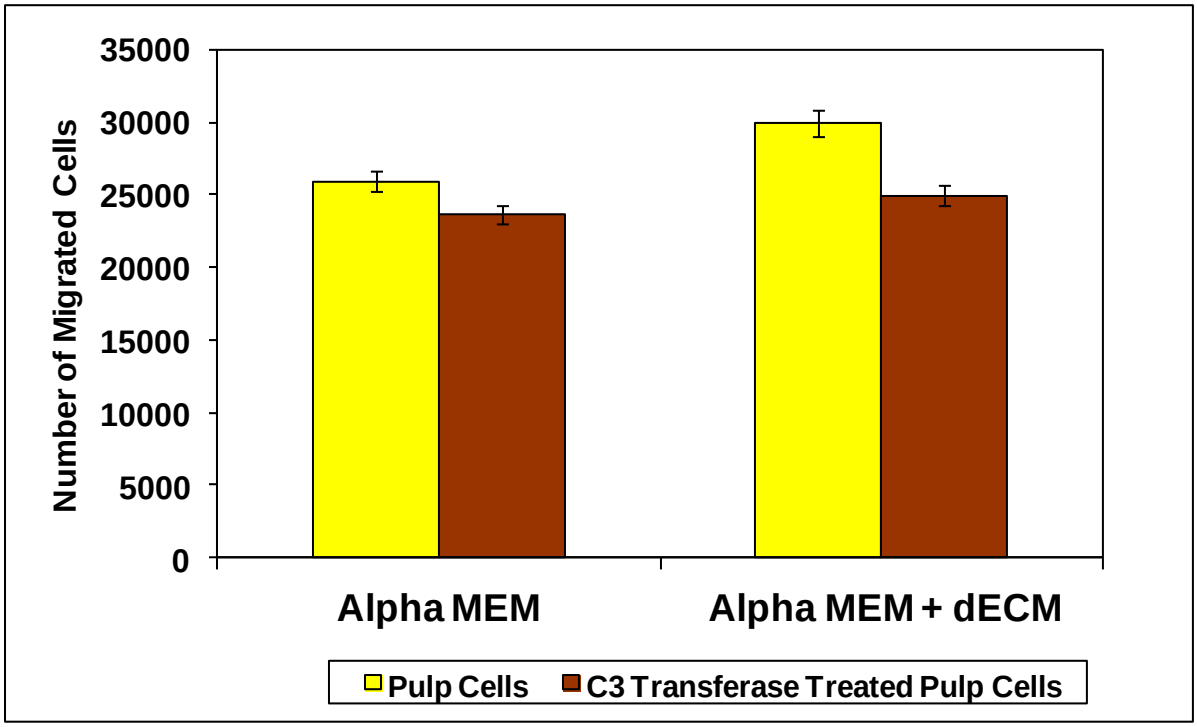


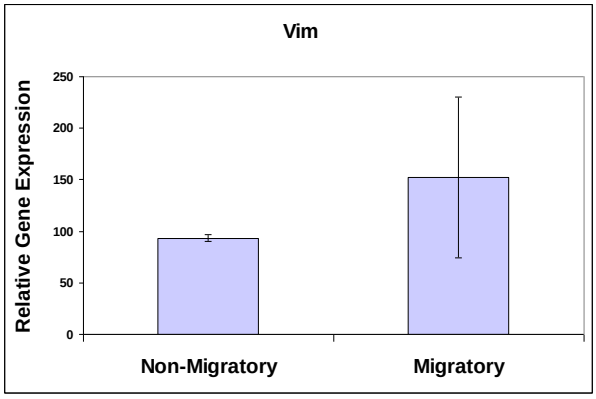
Figure 5.13 Transwell chemotaxis data quantifying pulp cell migration towards dECM. Data demonstrate that the effect of dECM on pulp cell migration was neutralised after primary pulp cells were treated with C3 transferase. N=3, standard deviation bars plotted,* = $P<0.05$.

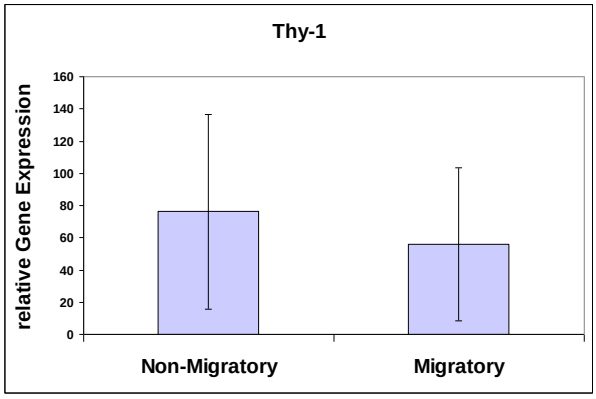
5.3 Gene Expression Analysis of Migratory and Non-Migratory Cell Populations

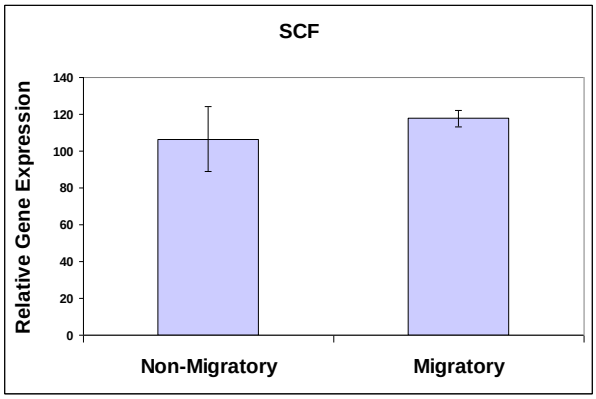
Data presented above demonstrated that dECM had a significant stimulatory effect on dental pulp cell migration. Gene expression analysis of cells which had and had not demonstrated migratory activity towards dECM was used to determine whether preferentially recruited specific pulp cell populations showed differing gene expression characteristics from the heterogeneous primary cell population studied.

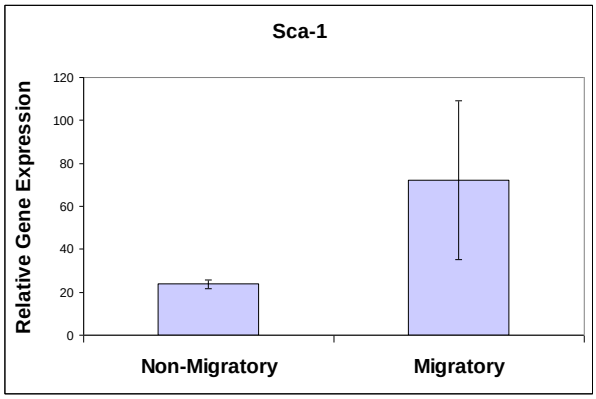
PCR analysis of primary pulp cells showed differences in gene expression between the dECM recruited cell population and the population that did not migrate in response to dECM (Figure 5.14). The migrated pulp cell population demonstrated increased expression of mesenchymal / general stem cell markers vim, Cd44, Sca-1, and Scf, but a decrease in the expression of Thy-1. These cells also demonstrated increased expression of the pluripotency markers C-myc, Sox2, Lin28, Nanog and Klf4. This analysis indicated more stem cell-like characteristics within the migrated population compared with the non-migrated pulp cells.

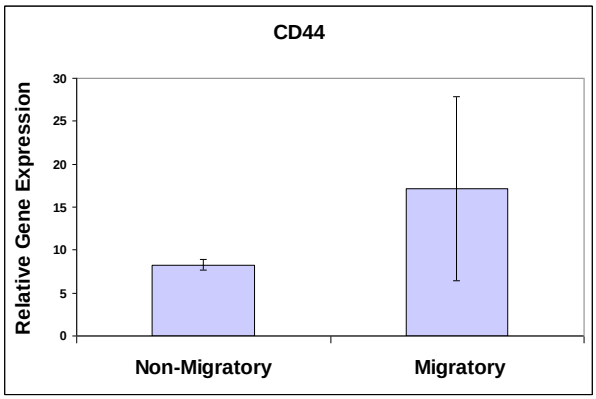
A)


Vim
Relative Gene Expression
250
200
150
100
50
0
Non-Migratory
Migratory


Thy-1
relative Gene Expression
160
140
120
100
80
60
40
20
0
Non-Migratory
Migratory


SCF
Relative Gene Expression
140
120
100
80
60
40
20
0
Non-Migratory
Migratory


Sca-1
Relative Gene Expression
120
100
80
60
40
20
0
Non-Migratory
Migratory


CD44
Relative Gene Expression
30
25
20
15
10
5
0
Non-Migratory
Migratory

B)

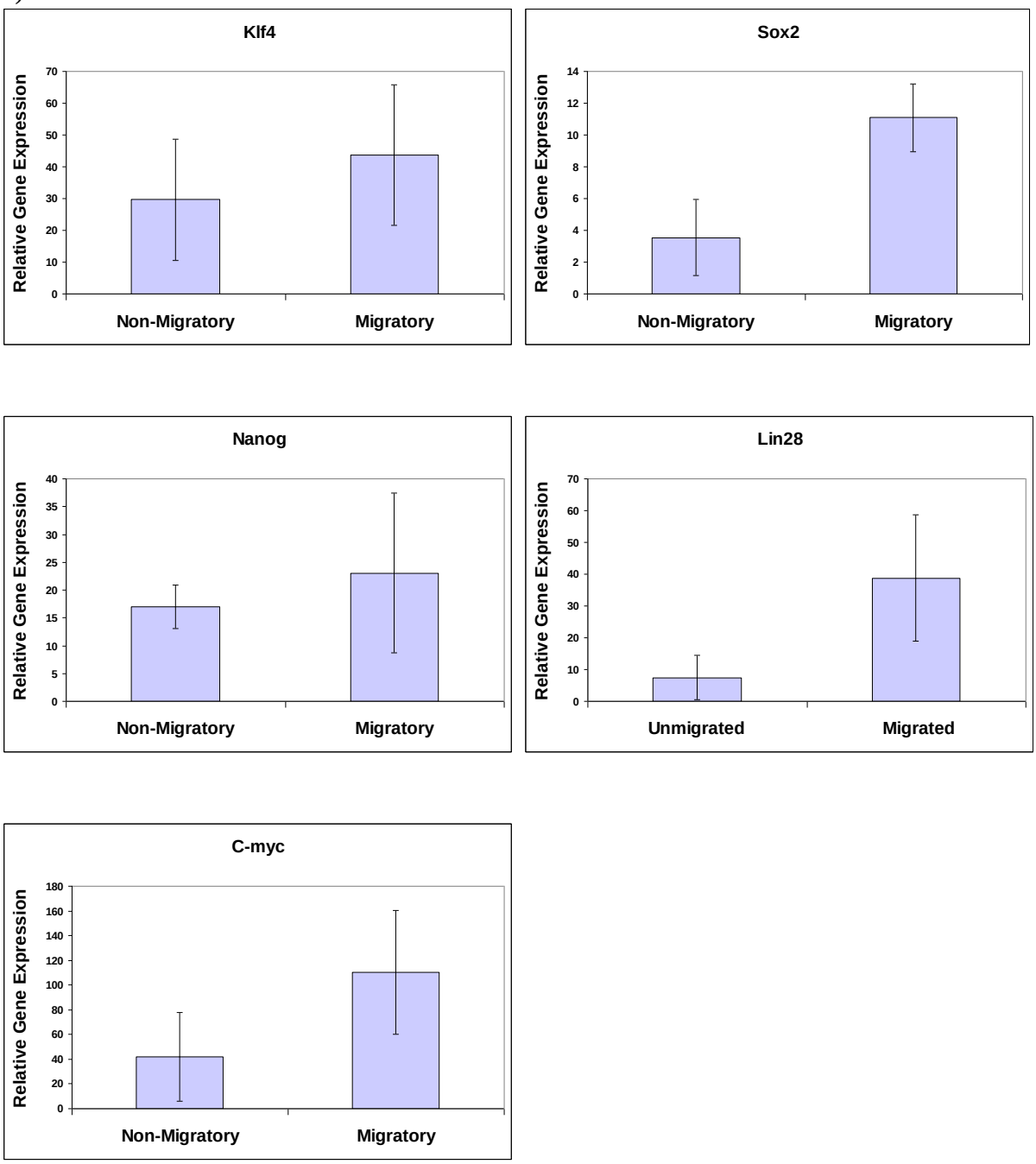


Figure 5.14 Densitometric analysis of PCR gel images (Appendix 3) demonstrating relative levels of gene expression for migratory and non-migratory pulp cells in response to dECM. Intensities were normalised against GAPDH and plotted as relative gene expression. (A) Mesenchymal / general stem cell markers, and (B) pluripotent stem cell markers. The expression of mesenchymal / general stem cell markers was increased in cells that had migrated for all markers except thy-1. The expression of pluripotent stem cell markers was also increased in the migratory population for all markers examined. Standard deviation bars plotted. N=2.

6.0 THE EFFECTS OF PULP ECM ON DENTAL PULP CELL DIFFERENTIATION

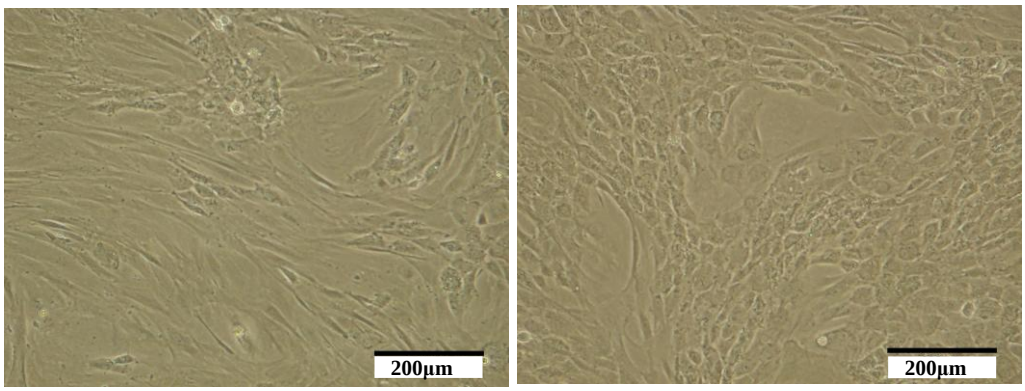
Dental pulp cells are reported to contain a population of multipotential stem cell characteristics (Gronthos *et al*, 2002; Guimarães *et al*, 2011). During dental repair processes, such as reparative dentinogenesis, the cell populations that give rise to the new generation of odontoblast-like cells are reported to reside within the dental pulp (Smith *et al*, 1990). The differentiation of these cells during reparative processes occurs within the pulp ECM environment and previous studies have shown that ECM constituents can influence the differentiation of stem cells (Martino *et al*, 2009; Rowlands *et al*, 2008). Although ECM molecules have been shown to influence cell differentiation, no study has reported the effects of pulp ECM preparations on pulp cell differentiation. The current investigations were performed to investigate the potentiality of dental pulp cells within a physiologically relevant environment.

6.1 Osteogenic Differentiation

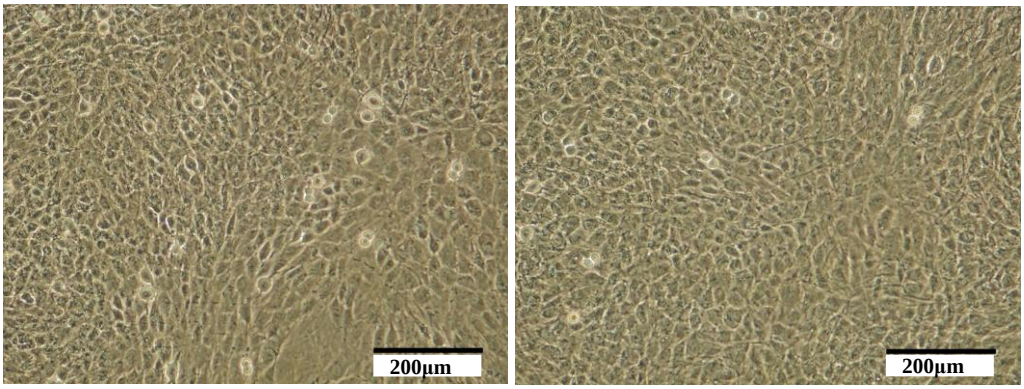
The cell morphological images from phase contrast microscopy indicated that primary pulp cells seeded on control (uncoated tissue culture grade polystyrene) culture surfaces and pECM coated surfaces could respond to stimulation with osteogenic medium (Method 2.7.1.1) with changes in morphology (Figure 6.1). Primary pulp cells became smaller and more rounded following exposure to osteogenic medium and alizarin red staining demonstrated an increase in positive staining compared with controls (Figures 6.2 and 6.3). After 2 weeks exposure to osteogenic medium, the pulp cells on control and pECM surfaces showed a statistically significant increase in alizarin red staining compared with cells exposed to control culture medium. This increase in staining in response to osteogenic medium was greater for the pulp cells cultured on pECM compared with cells cultured on the control polystyrene culture surface. Normalising total mineral stained for the number of cells present showed that the increased staining on the pECM compared with the control surface was still apparent and therefore was not simply a reflection of increased cell numbers (Figures 6.2 and 6.3). After 3 weeks exposure to osteogenic medium, there was a significant increase in alizarin red staining compared with that observed after 2 weeks exposure. Again, the increased staining observed on control culture surfaces and pECM surfaces was statistically significant compared with cultures in the absence of osteogenic medium and the effects were greater with the pECM than the control culture surface. These data demonstrated that primary pulp cells responded to stimulation with osteogenic medium showing morphological changes associated with differentiation as reflected in the increased mineralisation shown by alizarin red staining. The increase in alizarin red staining on pECM relative to control cultures showed enhanced mineralisation in response to the pECM environment. Cells cultured on pECM, then re-seeded on control surfaces did not show this enhanced alizarin red staining compared with control

surfaces (Figure 6.4) demonstrating the importance of continued contact with the pECM for the enhanced mineralisation effect.

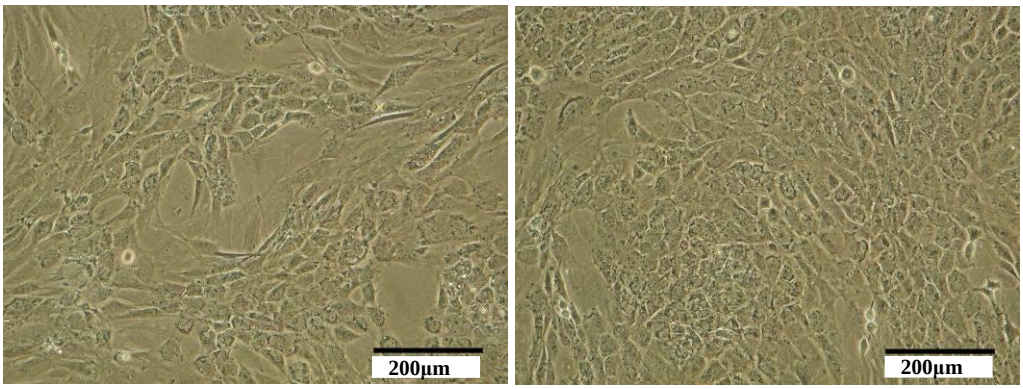
A)



B)



C)



D)

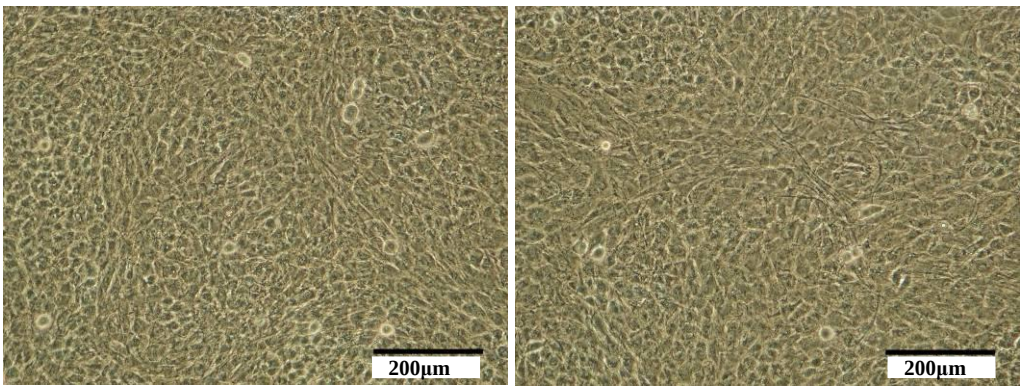
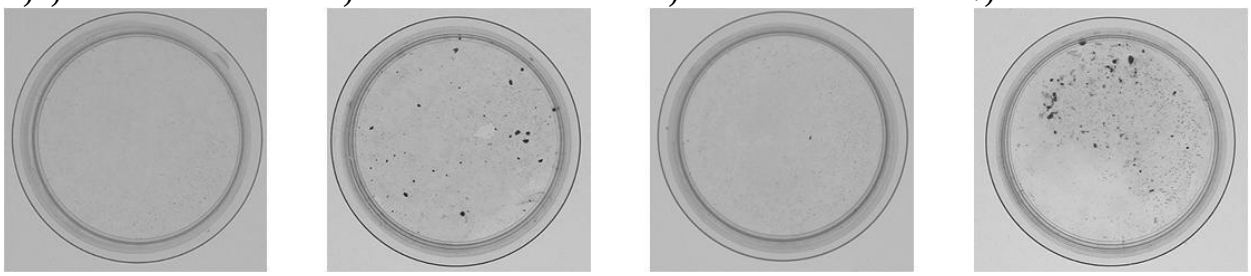
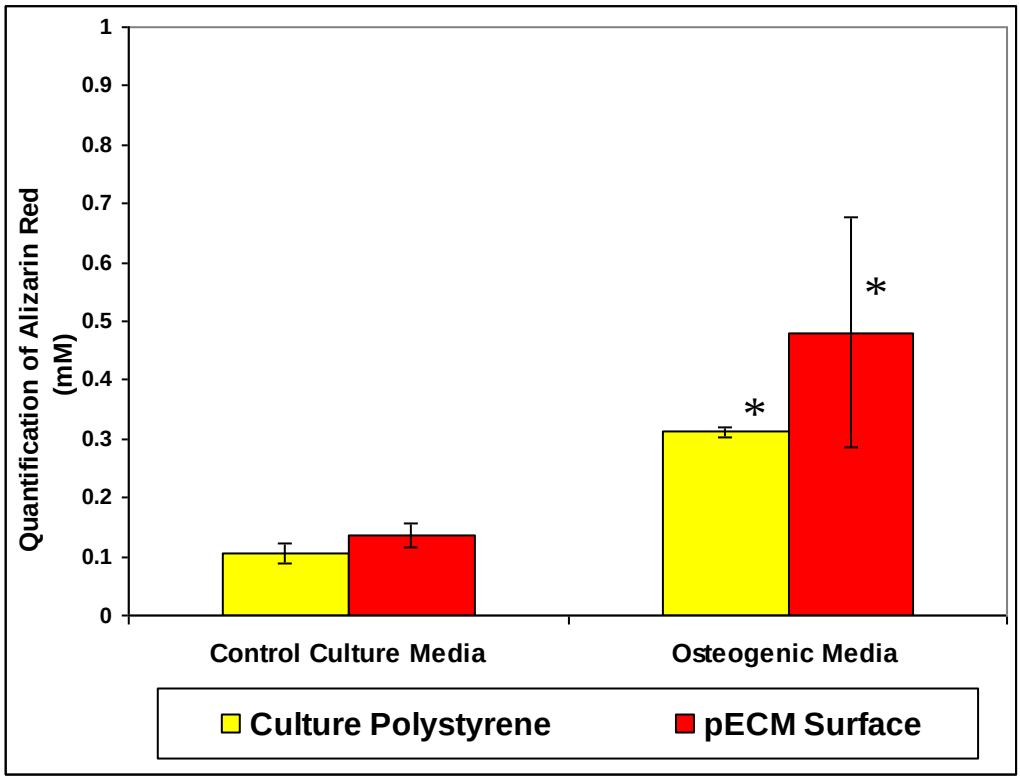


Figure 6.1 Phase contrast photomicrographs of primary pulp cells after 14 days culture in different conditions; A= Culture polystyrene with αMEM, B= Culture polystyrene with osteogenic medium, C= pECM with αMEM, D= pECM with osteogenic medium. Images illustrate a change towards a smaller and more rounded cell morphology on both surfaces in response to osteogenic medium. Scale bars are shown.

A) i) ii) iii) iv)



B)



(C)

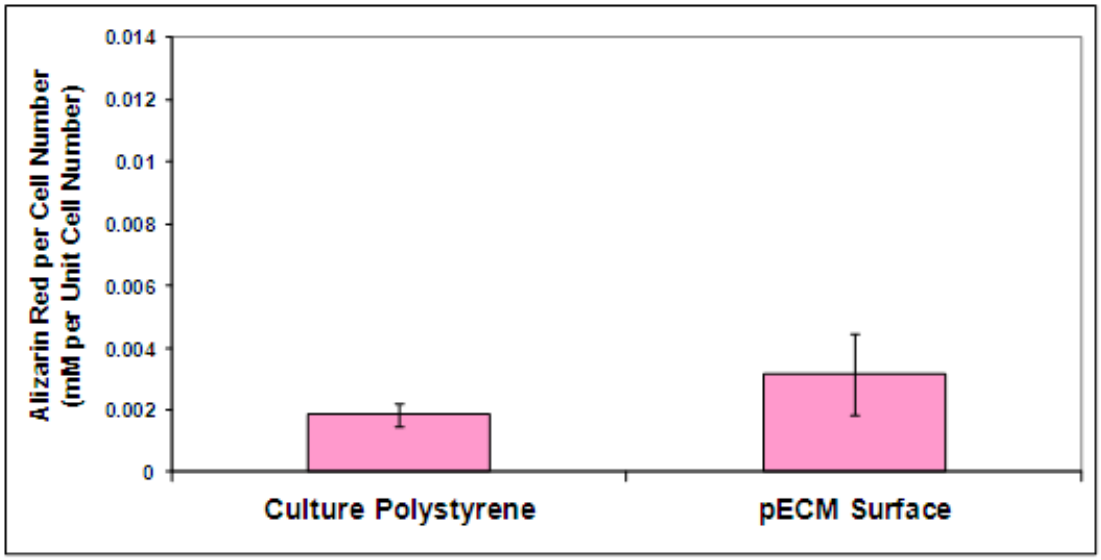
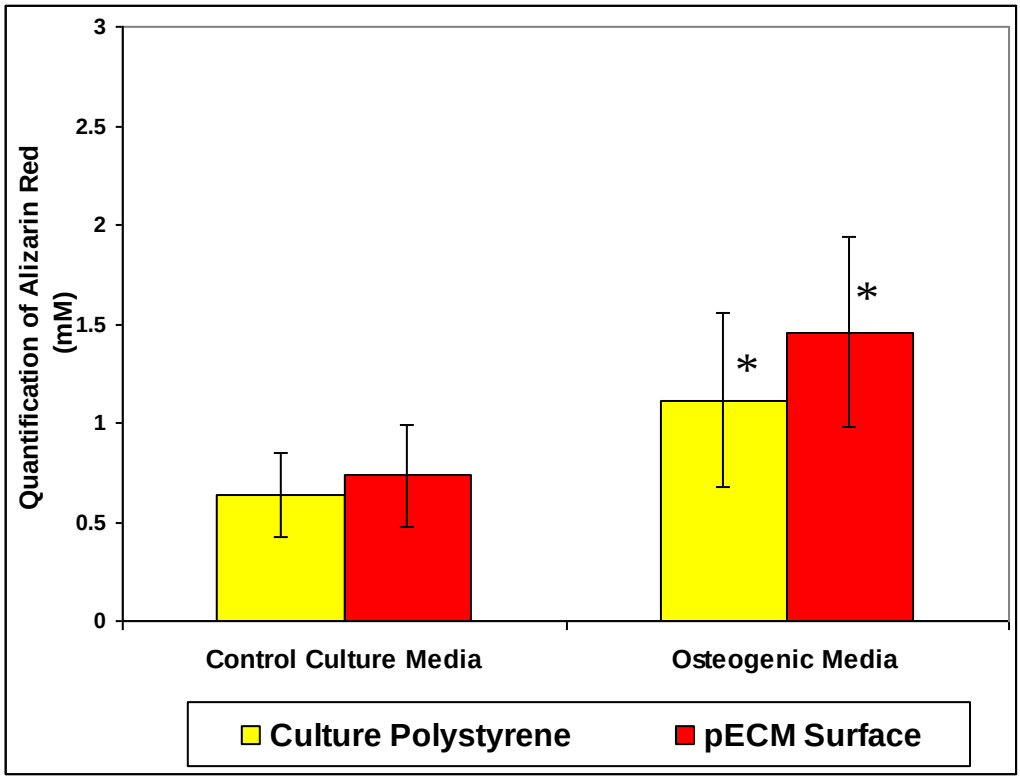


Figure 6.2 Analysis of alizarin red staining of cells after two weeks culture in osteogenic medium. A) photographs of stained cultures on i=culture polystyrene with αMEM, ii= culture polystyrene with osteogenic medium, iii= pECM with αMEM and iv= pECM with osteogenic medium B) Quantification of alizarin red staining of pulp cell cultures illustrated that there was a statistically significant increase in staining after exposure to osteogenic medium on both surfaces. This increase was greater on pECM coated surfaces compared with control surfaces, although the difference was not statistically significant. C) Quantification of alizarin red staining of osteogenic cultures per cell demonstrated that the increase in staining on pECM was not due to different numbers of cells. Standard deviation bars plotted. * = $P<0.05$. N=3.

A)



B)

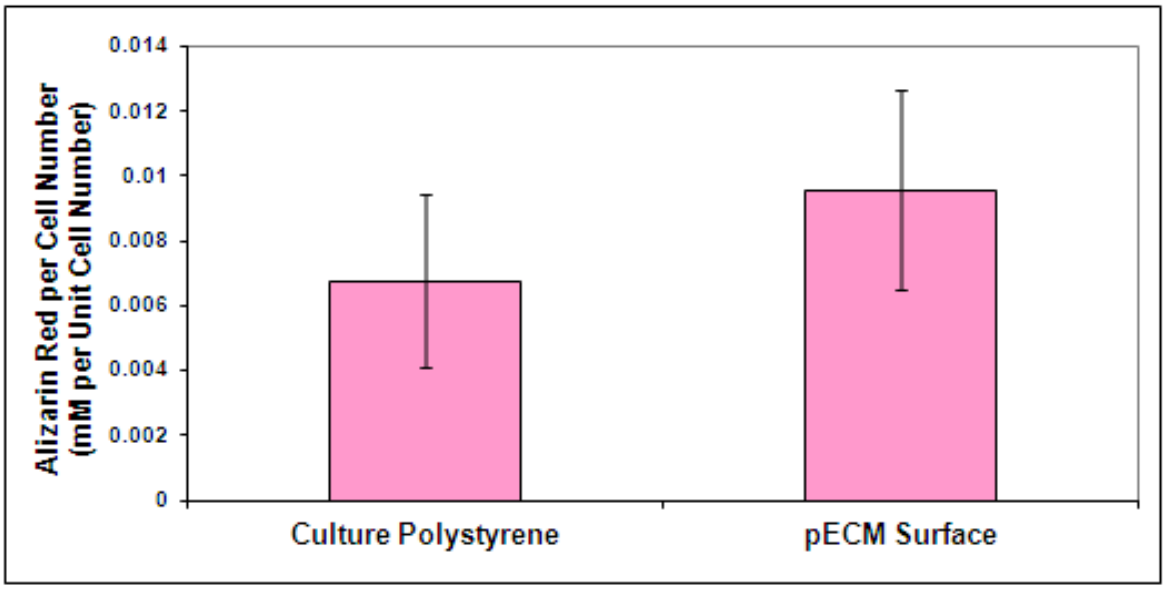


Figure 6.3 Alizarin red staining analysis of cells after three weeks culture in osteogenic medium. A) Quantification of alizarin red staining of pulp cell cultures. B) Quantification of alizarin red staining of osteogenic cultures per cell illustrated that there was a statistically significant increase in alizarin red uptake after exposure to osteogenic medium on both surfaces. This increase appeared greater on pECM coated surfaces compared with control surfaces, although the increase was not statistically significant. Standard deviation bars plotted. * = $P<0.05$. N=3.

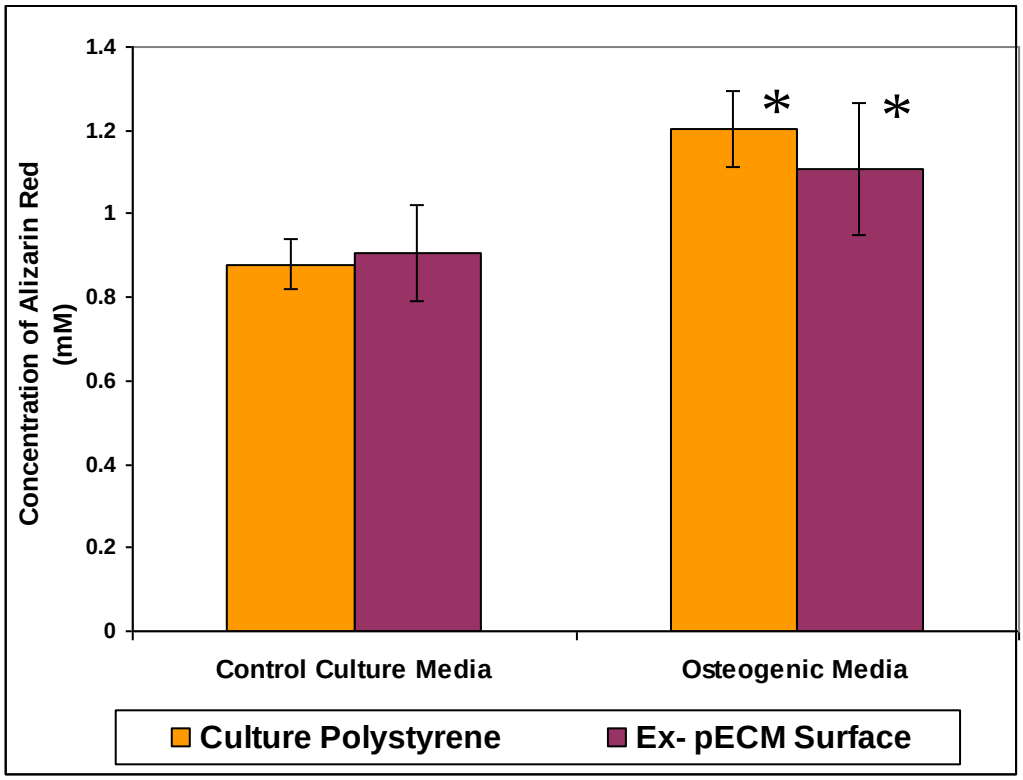
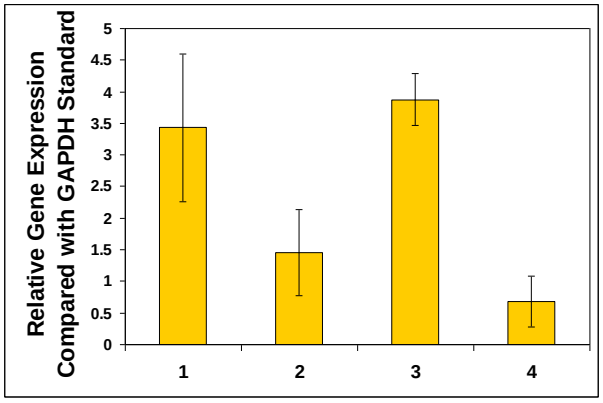


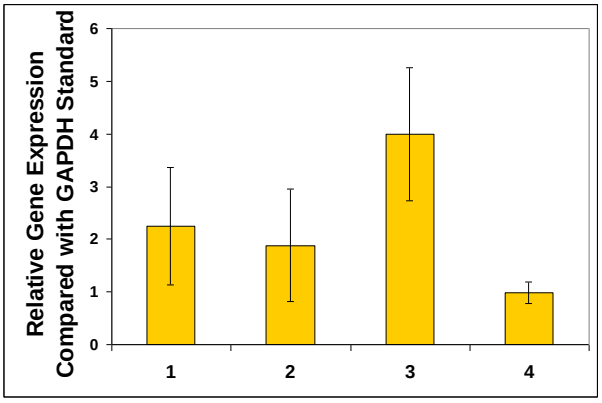
Figure 6.4 Alizarin red staining of cells after three weeks culture in osteogenic medium illustrated that there was a statistically significant increase in alizarin red uptake after exposure to osteogenic medium in both conditions. There was no statistical difference between the cells always cultured on polystyrene and those that had been removed from pECM and seeded on culture polystyrene. Standard deviation bars plotted. * = $P<0.05$. N=3.

Gene expression analysis confirmed that osteogenic stimulation influenced primary pulp cells at a molecular level. Pulp cells exposed to osteogenic medium on both control and pECM coated surfaces showed increased expression of the differentiation markers, nestin and DMP-1, as well as the expression of markers of mineralisation, osteocalcin and osteoadherin. Interestingly, other markers associated with mineralisation (osteopontin, alkaline phosphatase and collagen type I alpha) were down-regulated following two weeks exposure to osteogenic medium.

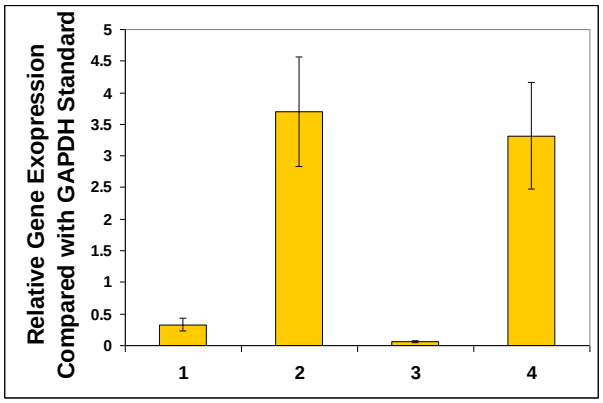
A) Coll-Iα



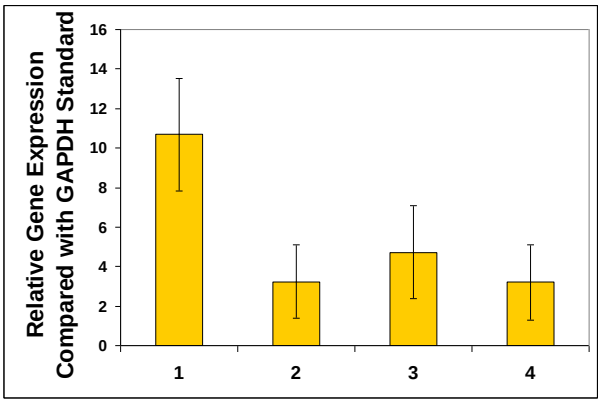
B) ALP



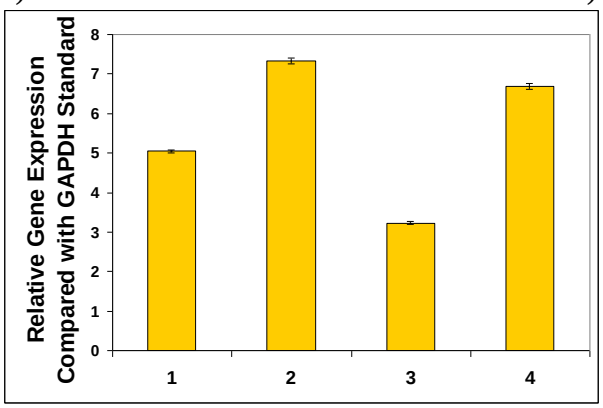
C) OC



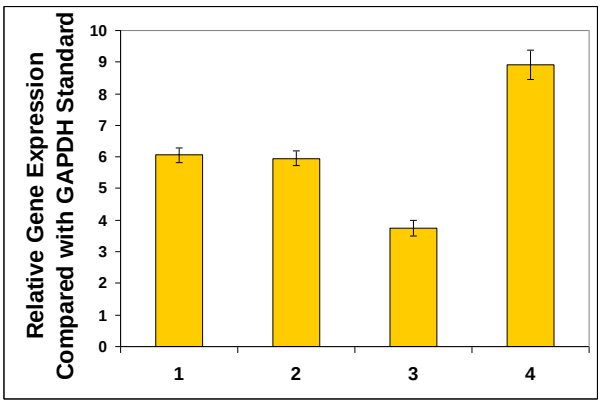
D) OP



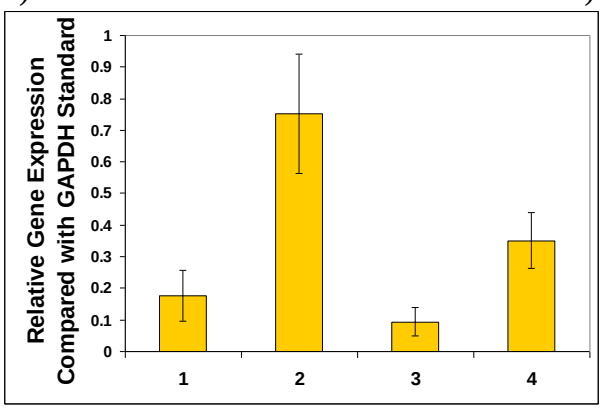
E) OA



F) ON



G) Nestin



H) DMP-1

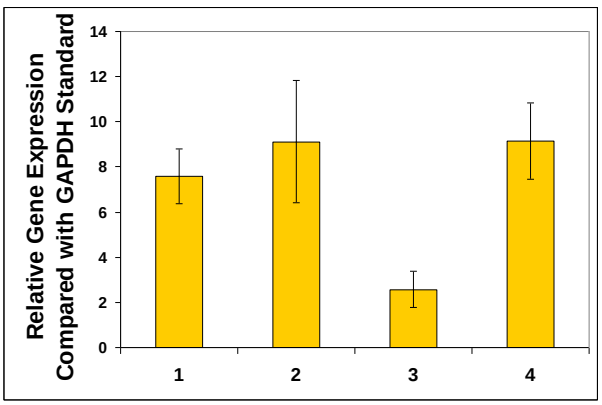
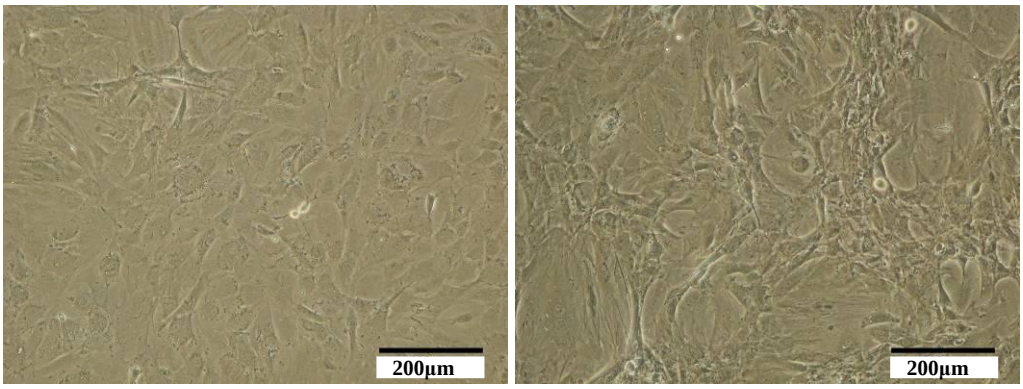


Figure 6.5 Gene expression profiles for transcripts that demonstrated a difference in expression between pulp cells cultured in osteogenic medium compared with controls, with cells exposed to osteogenic medium expressing higher levels of DMP-1, Nestin, OA and OC on both surfaces. Samples were normalised to the GAPDH housekeeping gene and normalised densitometric values of amplified products represented graphically from PCR gel images shown in Appendix 4. Cell cultures analysed were **1**= pulp cells on polystyrene with control medium, **2**= pulp cells on polystyrene with osteogenic medium, **3**= pulp cells on pECM with control medium, **4**= pulp cells on pECM with osteogenic medium. N=2.
A. Collagen type I alpha (Coll-Iα); **B.** Alkaline Phosphatase (ALP); **C.** Osteocalcin (OC);
D. Osteopontin (OP); **E.** Osteoadherin (OA); **F.** Osteonectin (ON); **G.** Nestin; **H.** Dentine Matrix Protein 1 (DMP-1).

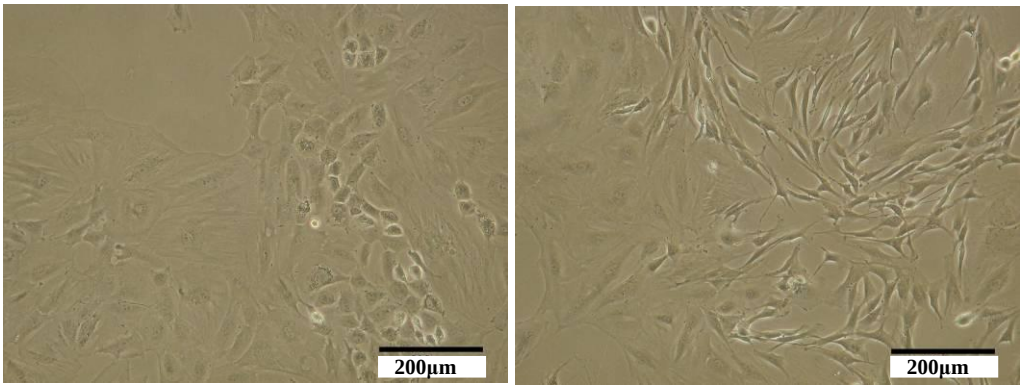
6.2 Adipogenic Differentiation

Phase contrast microscopy indicated that the primary pulp cells cultured on control polystyrene and pECM surfaces responded to the adipogenic medium (Method 2.7.2.1) with changes in morphology (Figure 6.6). Two distinct morphologies developed within the primary pulp cells exposed to adipogenic stimulants and these were populations of cells with small cell bodies and no visible processes being extended and populations of cells with cell bodies and long extended processes. Oil red O staining of cells demonstrated that within the primary pulp cell population on both control and pECM surfaces, some cells were positively stained following exposure to adipogenic stimulation (Figure 6.7). Positively stained cells demonstrated a characteristic ring of stained lipid inclusions. These data indicated that primary pulp cells could respond to adipogenic stimulation to differentiate into cells with adipocyte-like characteristics. Although oil red O staining confirms an increase in adipogenic character in cells exposed to differentiation signals, quantification of staining was not sensitive enough to show any differences between cells responding on control surfaces and cells responding on pECM coated surfaces (Figure 6.8).

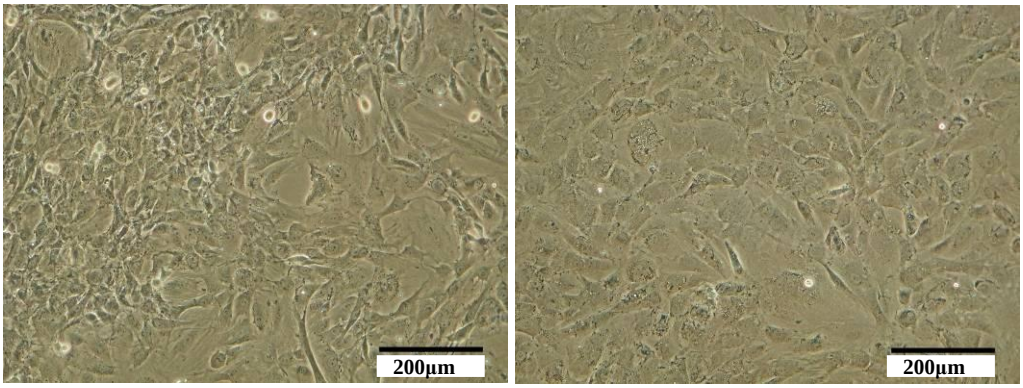
A)



B)



C)



D)

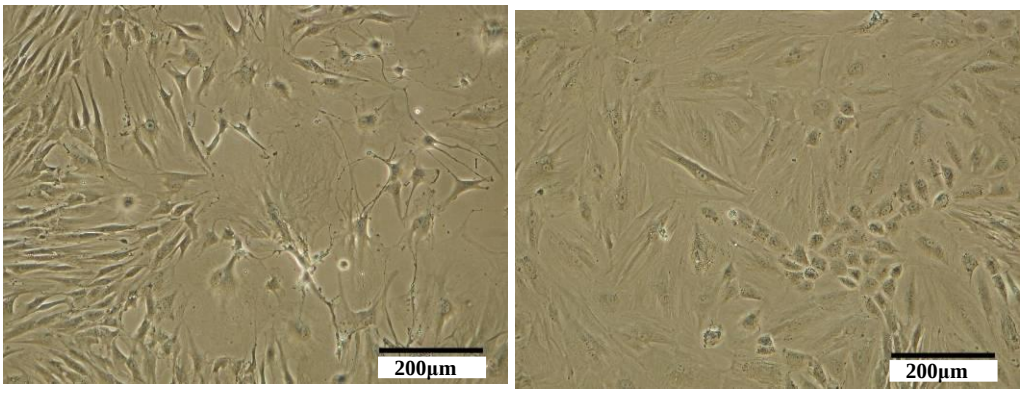
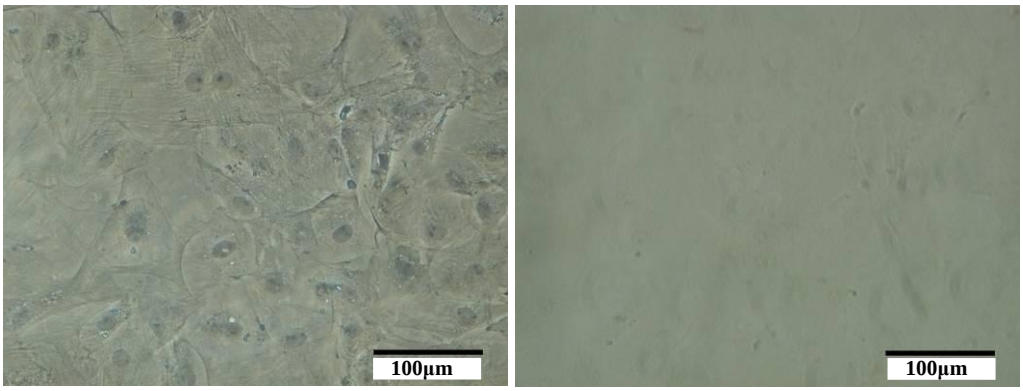
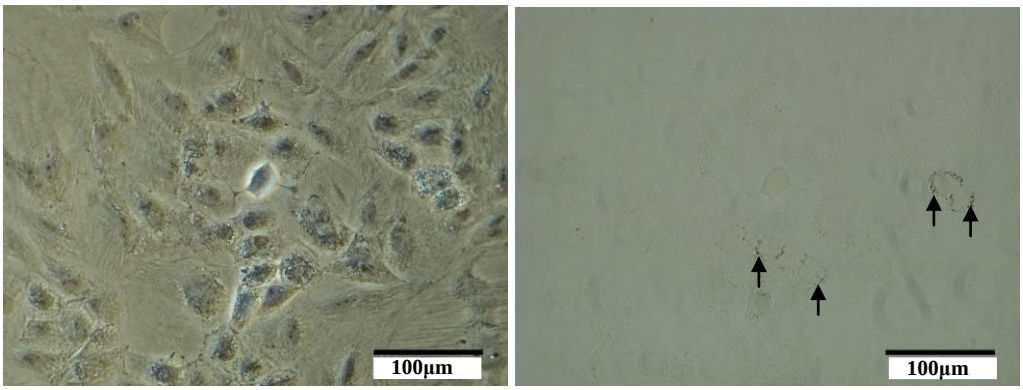


Figure 6.6 Photomicrographs of primary pulp cells after 14 days culture in different media; A= culture polystyrene with αMEM, B= culture polystyrene with adipogenic medium, C= pECM with αMEM, D= pECM with adipogenic medium. Images illustrate a change in cell morphology on both surfaces in response to the adipogenic medium. Populations of cells with small cell bodies and no visible extended processes and populations of cells with thin cell bodies and long extended processes developed in response to adipogenic stimulation. Scale bars are shown.

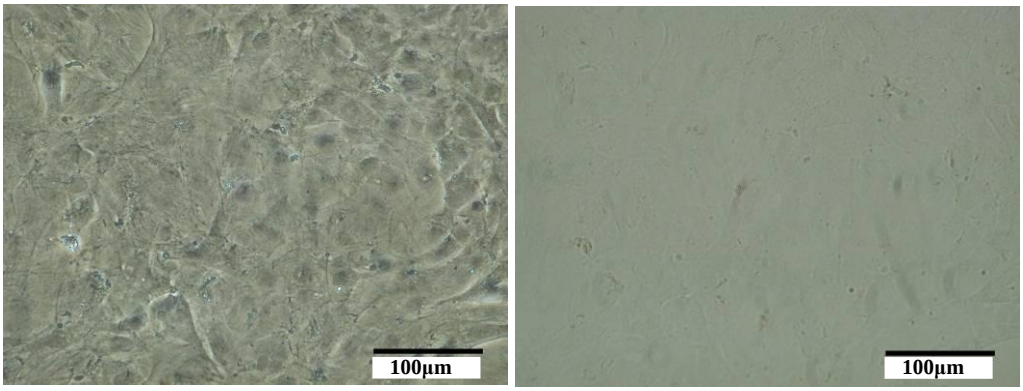
A) I II



B) I II



C) I II



D) I II

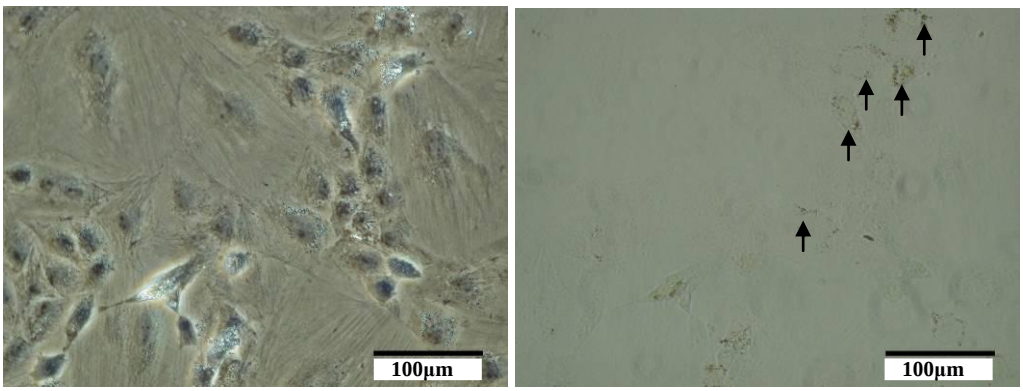
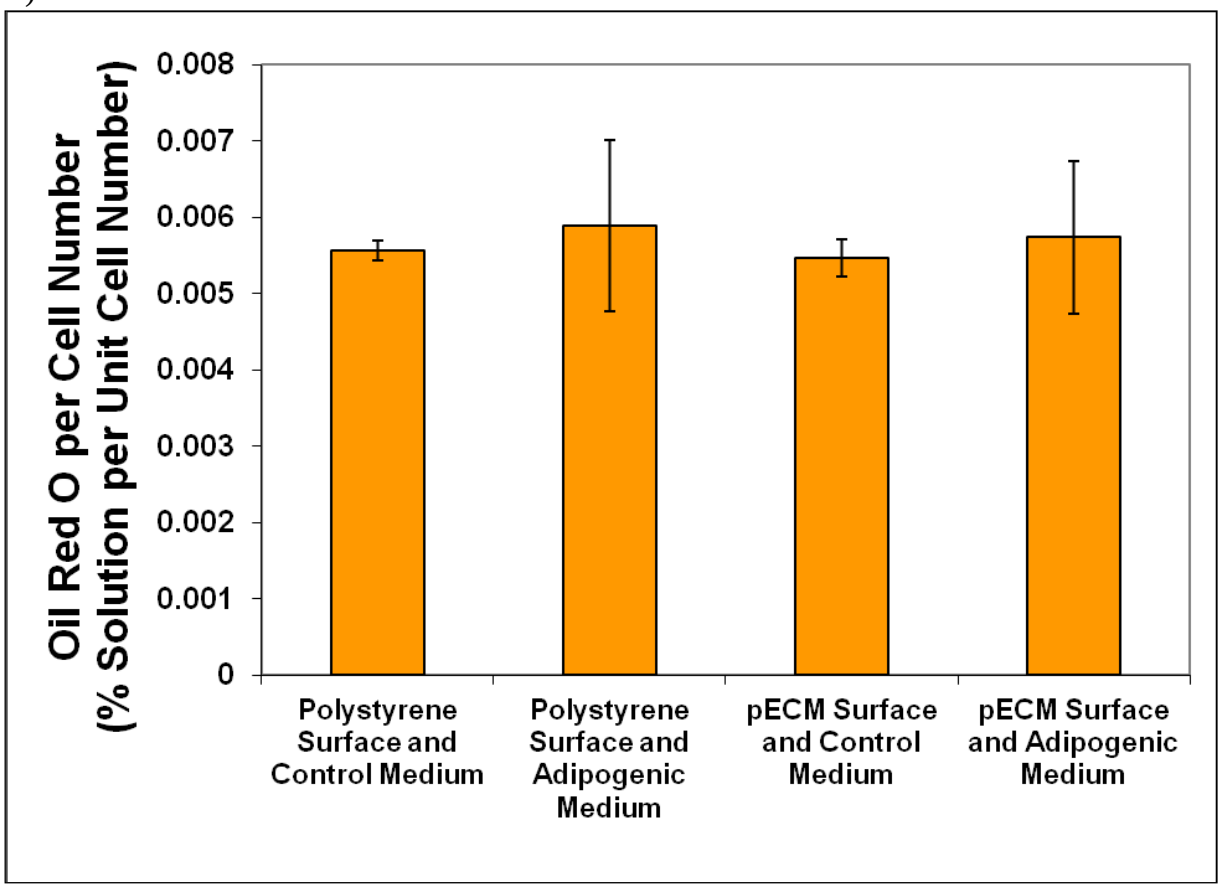


Figure 6.7 Photomicrographs of primary pulp cells stained with oil red O. A= Culture polystyrene with αMEM, B= Culture polystyrene with adipogenic medium, C= pECM with αMEM, D= pECM with adipogenic medium. I= phase contrast image; II= light microscopy image. Arrows illustrate positive oil red O staining of lipid inclusions in pulp cell cultures treated with adipogenic media. Scale bars are shown.

A)



B) i) ii) iii) iv)

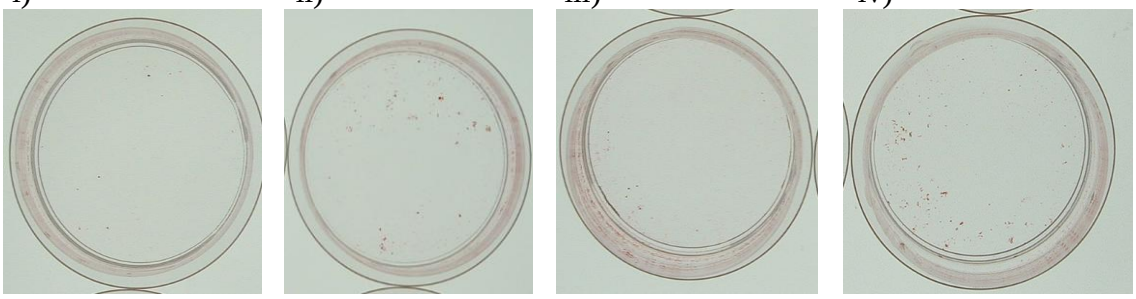
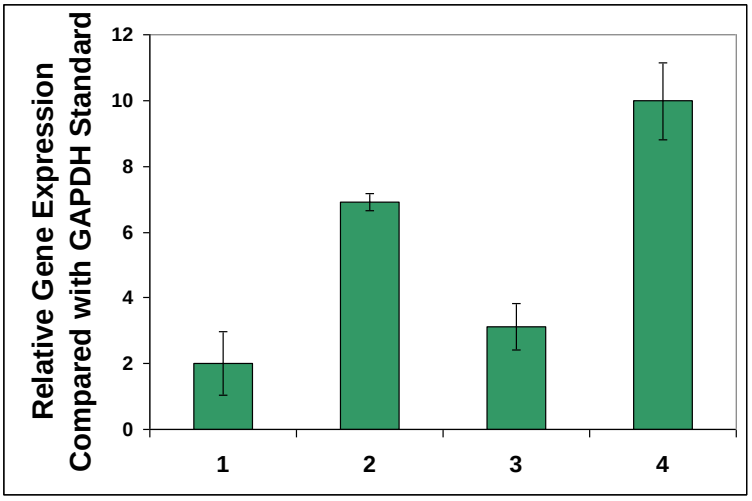


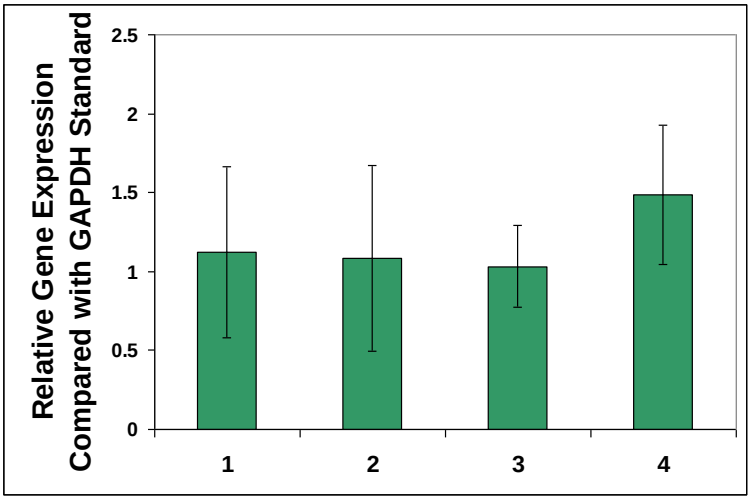
Figure 6.8 Oil red O staining of cells after three weeks culture in adipogenic medium. A) Quantification of oil red O staining of pulp cell cultures, B) photographs of stained cultures; i=culture polystyrene with αMEM, ii= culture polystyrene with adipogenic medium, iii= pECM with αMEM and iv= pECM with adipogenic medium. Data illustrated that there was a small increase in oil red O uptake after exposure to adipogenic medium on both surfaces, although these differences were not statistically significant. Standard deviation bars plotted. N=3.

Gene expression analysis confirmed that adipogenic stimulation influenced pulp cells at a molecular level. Pulp cells exposed to adipogenic medium on both control and pECM coated surfaces increased expression of the adipocyte marker, adipocyte protein 2. There was a decrease in the expression of the early adipogenic differentiation marker, peroxisome proliferator-activated receptor -2, in pulp cells following two weeks exposure to adipogenic medium. Minimal difference was observed in the expression of lipoprotein lipase, a gene involved in lipid metabolism, with an increase only detected in the pulp cells exposed to adipogenic medium cultured on pECM coated surfaces.

A) aP2



B) LPL



C) PPAR2

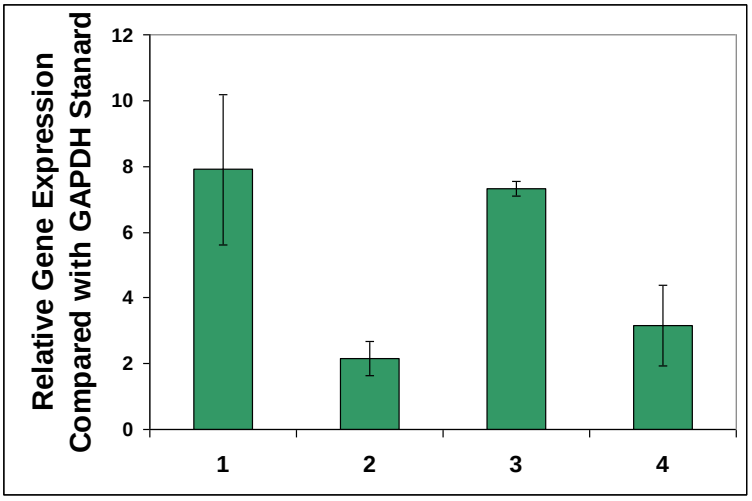


Figure 6.9 Gene expression patterns for transcripts that demonstrated a difference in expression between pulp cells cultured in adipogenic medium compared with controls. Following adipogenic stimulation, primary pulp cells expressed significantly higher levels of aP2 and significantly lower levels of PPAR2. Samples were normalised to the GAPDH housekeeping gene and normalised densitometric values of amplified products were represented graphically from PCR gel images shown in Appendix 5. Cell cultures analysed were **1**= pulp cells on polystyrene with control medium, **2**= pulp cells on polystyrene with adipogenic medium, **3**= pulp cells on pECM with control medium, **4**= pulp cells on pECM with adipogenic medium. N=2.
A. Adipocyte protein 2 (aP2); **B.** Lipoprotein lipase (LPL); **C.** Peroxisome proliferator-activated receptor -2 (PPAR2).

7.0 ANTIBACTERIAL ACTIVITY OF PULP AND DENTINE MATRIX MOLECULES

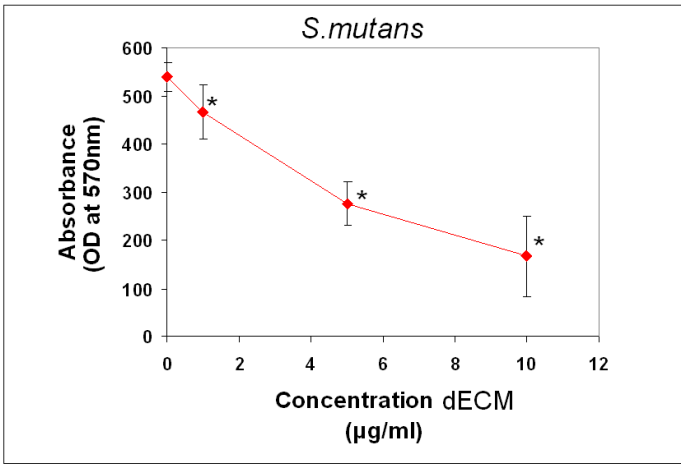
Recently, ECM derived from tissues such as small intestine and bladder have been shown to possess antimicrobial activity (Sarikaya *et al*, 2002) and biological scaffolds containing ECM molecules have shown resistance to bacterial infection (Badylak *et al*, 1994; Badylak *et al*, 2003; Ruiz *et al*, 2005; Mantovani *et al*, 2003). Ammonium sulphate-derived fractions of differently charged ECM components, as well as degradation products, from liver and bladder also have demonstrable antibacterial activity against *Staphyloccus aureus* (skin) and *Escherichia coli* (gut) indicating the presence of either a potent individual antibacterial constituent or a series of molecules acting synergistically (Brennan *et al*, 2006).

Dentine and pulp are known to contain a range of naturally occurring antimicrobial peptides (AMPs), including neuropeptides substance P (SP), neurokinin A (NKA), calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP) and adrenomedullin (ADM) (Awawdeh et al, 2002; El-Karim et al, 2003; El-Karim et al, 2006; Tomson et al, 2007). These molecules may play an important role in host tissue defence following infection and should be considered in the management of infected dental tissues. This chapter therefore examines the hypothesis that total ECM preparations from dentine and pulp possess antimicrobial activity.

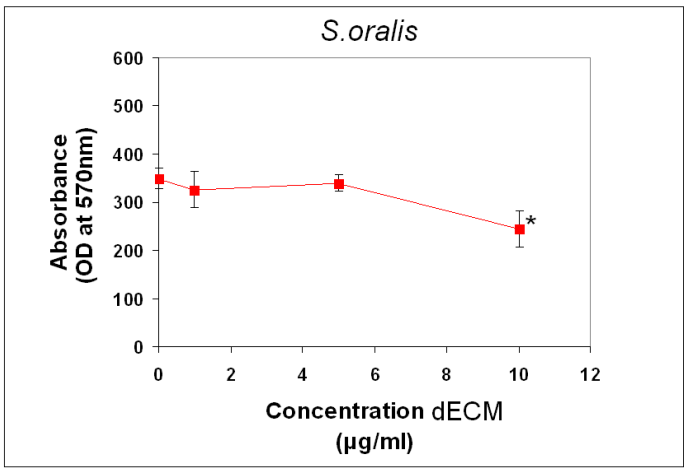
7.1 The Effect of Dentine ECM (dECM) on Bacterial Growth

S.mutans, S.oralis and *E.faecalis* are three species of anaerobic bacteria associated with infected dental tissues (Marsh 1994; Portenier *et al*, 2003). dECM demonstrated differential antibacterial effects against *S.mutans, S.oralis* and *E.faecalis* (Figure 7.1). The greatest effect was observed against *S.mutans* with each concentration (1, 5 and 10µg/ml) showing a statistically significant decrease ($P<0.05$) in bacterial growth compared with the PBS negative control. Only at a concentration of 10µg/ml, did dECM show a statistically significant decrease in *S.oralis* and *E.faecalis* growth. At a concentration of 10µg/ml dECM, the reduction in *S.mutans* growth could be observed within the wells as decreased turbidity was indicative of less growth (Figure 7.3). The reduction in bacterial growth was no longer apparent when the dECM was removed from the growth environment (Method 2.6.2), indicating that the effect was bacteriostatic and not bacteriocidal (Figure 7.2).

(A)



(B)



(C)

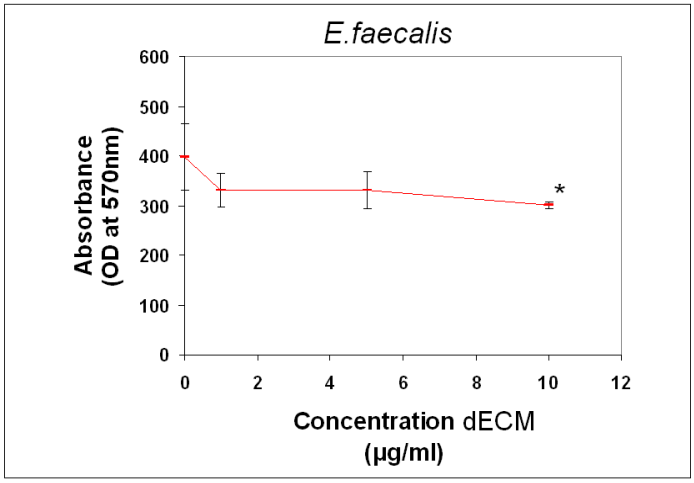


Figure 7.1 Increasing dECM concentration reduced bacterial growth, A) *S.mutans* B) *S.oralis* and C) *E.faecalis*, at 24 hours culture. Decrease in absorbance values (read at a wavelength of 570nm) were all statistically significant for *S.mutans* compared with the PBS negative control. Only at a concentration of 10µg/ml did dECM show a statistically significant decrease against *S.oralis* and *E.faecalis* compared with the negative control. Standard deviation bars plotted. * = $P<0.05$. N=5.

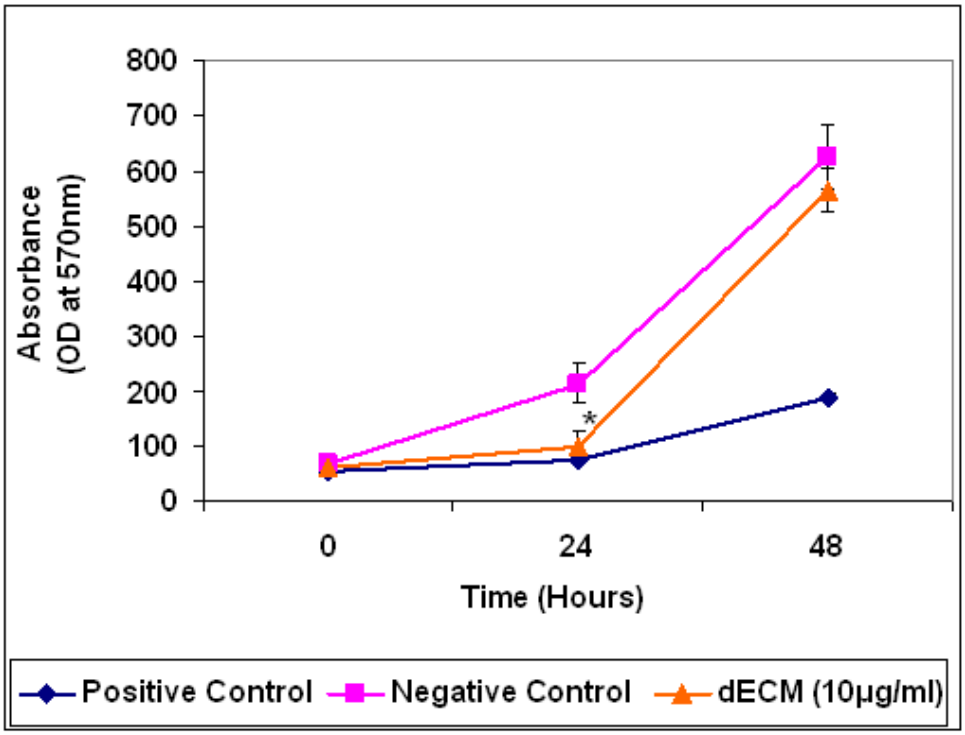


Figure 7.2 Removal of dECM from the *S.mutan* bacteria growth environment at 24 hours removed the inhibitory effect on growth. At 24 hours, the presence of dECM demonstrated a statistically significant decrease (* = $P<0.05$) in bacterial growth relative to the negative control and no statistically significant difference from positive control. At 48 hours following removal of the cells from dECM, no statistically significant decrease in bacterial growth compared with the PBS negative control was observed, although there was a statistically significant increase in growth compared with the positive control (0.5µg/ml Penicillin/streptomycin). Turbidity read at a wavelength of 570nm. Standard deviation bars are plotted. N = 3.

A) B)

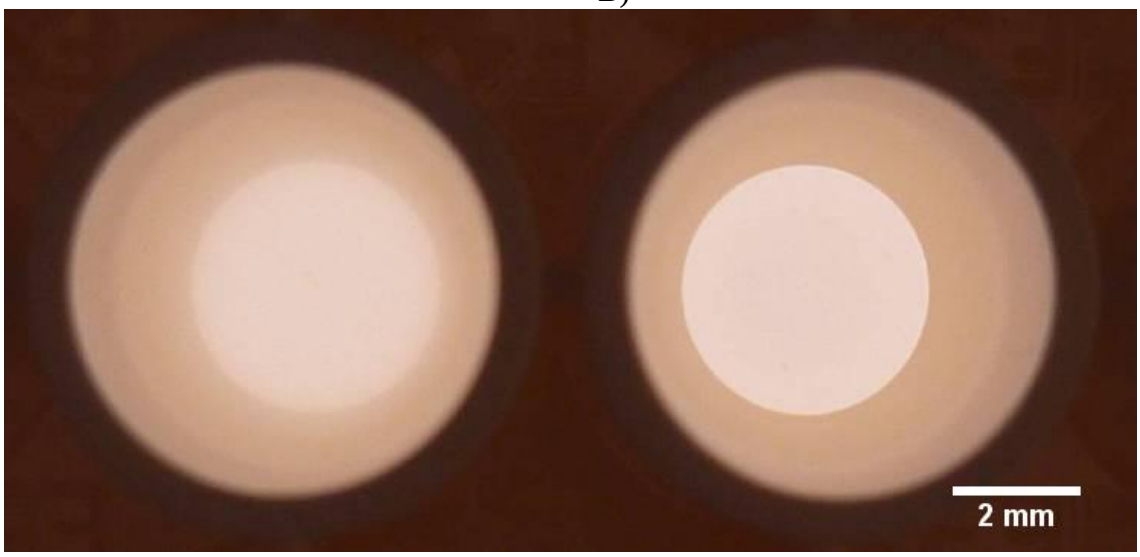


Figure 7.3 Photograph of bacteria wells after 24 hours growth showing the effect of dECM on *S.mutans* bacterial growth. A) Control PBS supplemented bacteria broth, B) PBS containing 10μg/ml dECM supplemented bacteria broth. The Well containing dECM is visibly less turbid due to lower levels of bacteria growth.

7.2 The Effect of Pulp ECM on Bacterial Growth

Ammonium sulphate fractions of pECM demonstrated antibacterial activity against *S.mutans* to differing extents (Figure 7.4). The antimicrobial activities detected with each fraction were all statistically significantly different to the negative control. There was however no statistically significant difference in activity between the pECM fractions precipitated at 50%, 70% and 90% ammonium sulphate saturation. The fractions precipitated at 50%, 70% and 90% showed a statistically significant greater decrease in bacterial growth compared with the pellet precipitated at 30% ammonium sulphate saturation. These data indicated that there were a number of molecules within the pECM fractions that showed antibacterial effects. Again, the reduction in bacterial growth was no longer apparent when the pECM was removed from the growth environment indicating that the effect was bacteriostatic and not bacteriocidal (Figure 7.5). The bacteriostatic trend was similar to that observed with the matrix molecules from dentine.

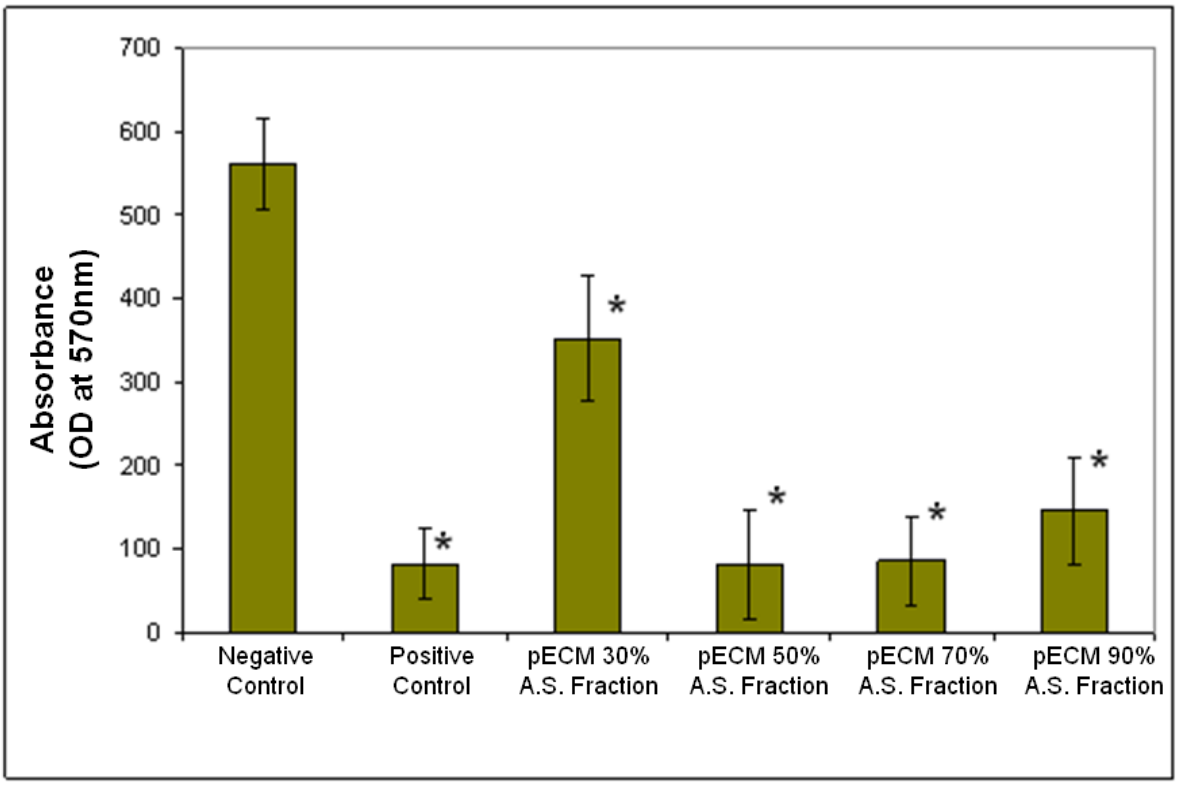


Figure 7.4 Ammonium sulphate (A.S.) pECM fractions (10µg/ml) reduced *S.mutans* growth after 24 hours. All fractions showed a statistically significant decrease in bacterial growth compared with the PBS negative control. Standard deviation bars are plotted. Turbidity was determined at a wavelength of 570nm. * = $P<0.05$. N=5.

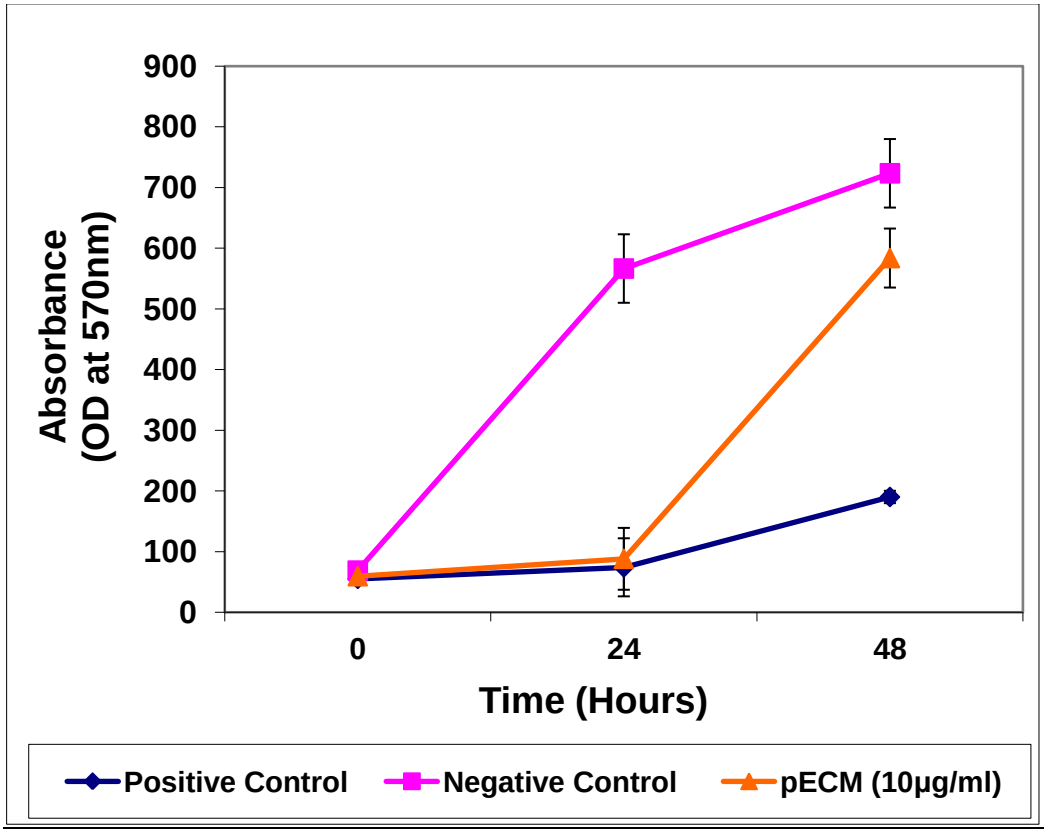
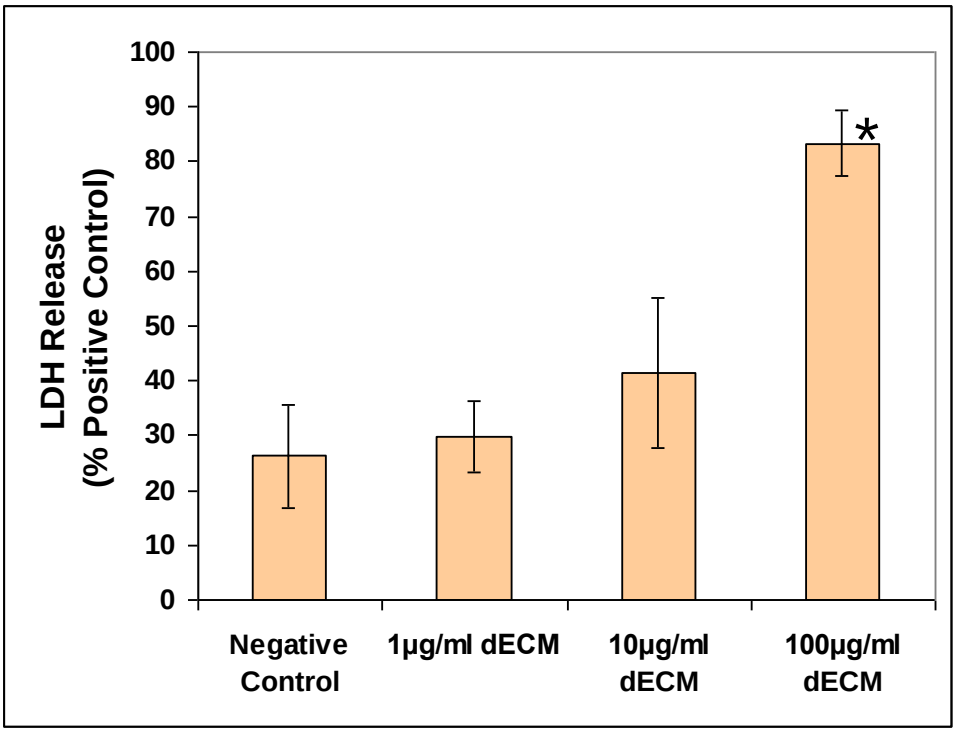


Figure 7.5 Removal of pECM from the bacterial growth environment at 24 hours reversed the inhibitory effect on bacterial growth. At 24 hours, the presence of pECM showed a statistically significant decrease (* = $P<0.05$) in bacterial growth relative to the negative control and no statistically significant difference from the positive control. At 48 hours, removal of pECM after 24 hours resulted in no statistically significant decrease in bacterial growth compared with the PBS negative control, although a statistically significant increase in growth compared with the positive control (0.5µg/ml Penicillin/streptomycin) was observed. Standard deviation bars are plotted. Turbidity read at a wavelength of 570nm. N=3.

7.3 Assessment of Cytotoxicity of Dental ECM Preparations on Pulp Cells

To determine whether the reduced levels of bacterial growth were due to specific antibacterial action or general cytotoxic effects of dental ECM molecules, a LDH cytotoxicity assay was performed using primary pulp populations and a range of concentrations of dECM and pECM. LDH release following cellular exposure to test conditions can be used as a measure of the cytotoxicity and dECM increased LDH release from primary pulp cells at the highest concentration examined (100μg/ml) (Figure 7.6). However, only small increases in LDH release were detected at the lower concentrations (1 and 10μg/ml), levels which were previously demonstrated to possess antimicrobial activity. Increases in LDH release at 1 and 10μg/ml dECM were not statistically significant compared to PBS negative control. At all three concentrations, pECM had no significant affect on dental pulp cell LDH release. These data demonstrated that at ECM concentrations of 1 and 10μg/ml for both dECM and pECM, there was no significant cytotoxic effect on primary pulp cells. These findings corroborate the conclusion that the reduction in bacterial growth seen at 1 and 10μg/ml was due to a bacteriostatic effect and not a general cytotoxic effect.

A)



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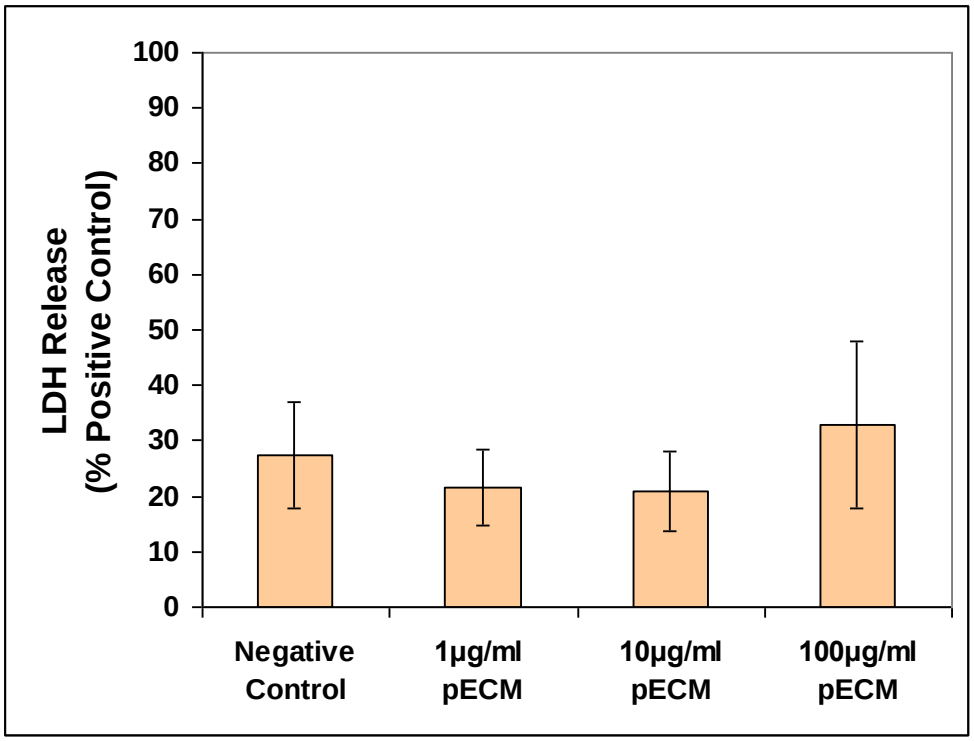


Figure 7.6 Graphs showing LDH release from pulp cells following exposure to A) dECM and B) pECM. Increasing concentration of dECM resulted in raised levels of LDH release from dental pulp cells, although this was only statistically significant at 100µg/ml compared with the PBS negative control. pECM molecules had no significant cytotoxic affect on dental pulp cells compared with the PBS negative control. Standard deviation bars plotted. * = $P<0.05$. N=4.

8.0 ENGINEERING OF ALGINATE AND DENTAL PULP CELL 3D CONSTRUCTS

Tooth tissue engineering is an area of increasing interest with several different methodologies being investigated ultimately aiming to develop a 3D bioengineered tooth for future tooth replacement therapies. Tooth bioengineering methods can be classified as scaffold-based or scaffold-free (Zheng *et al*, 2011). Tooth-like structures have reportedly been generated by scaffold-free methods such as tissue recombination (Hu *et al*, 2006), cell pellet engineering (Yu *et al*, 2006), and chimeric tooth engineering (Nakao *et al*, 2007). Tooth and tooth germ-derived cells have been used to generate tooth-like structures in scaffold-based approaches using several different materials as scaffolds including poly(lactide-co-glycolide) and polyglycolide (Young *et al*, 2002; Duailibi *et al*, 2008), silk fibroin (Xu *et al*, 2008), hydroxyapatite / tricalcium phosphate, collagen matrix and titanium mesh (Zhang *et al*, 2006) and a variety of calcium phosphate composites (Zheng *et al*, 2011). However, all of these methods have relied on *in vivo* transplantation to develop the tooth construct and to date are unable to generate tooth constructs that mimic the exact shape and size of a natural tooth (Zheng *et al*, 2011).

This chapter investigates the suitability of alginate as a scaffold material for dental pulp cell culture. Pulp ECM proteins were included within the scaffold to provide an environment more physiologically relevant and representative of the dental pulp. Dentine matrix components were included in a dentinogenic medium, as well as ascorbic acid and β-glycerophosphate, to provide morphogenic signals relevant to an *in vivo* dental repair environment. These conditions were used to investigate formation of tooth-like constructs that more accurately resembled the shape and size of a natural tooth without the need for *in vivo* transplantation.

8.1 Encapsulation of Dental Pulp Cells

Assays were performed on alginate encapsulated pulp cells seeded at a density of $5x10^5$cells/ml, a similar density to that used for the encapsulation of other cell types in previous alginate studies (Hunt *et al*, 2009; Hunt *et al*, 2010). Alginate scaffolds of 1%, 3% and 5% were used to determine if the density of the scaffold material could influence cell behaviour, these densities were also at a level similar to that of alginate scaffolds used in previous studies (Hunt *et al*, 2009; Hunt *et al*, 2010). Prior to long term lineage induction studies, the viability of the cells after alginate encapsulation was assessed to determine whether alginate was appropriate for use as a scaffold material for primary pulp cell culture (Method 2.11.1). Tri-sodium citrate was used to release pulp cells after 3, 7 and 14 days encapsulation periods, trypan blue cell counts were performed and cells were seeded on culture polystyrene surfaces. No significant change in viable cell number (Figure 8.1) was seen within any of the different percentage alginate scaffolds examined. The inclusion of pECM within the alginate scaffolds also showed no significant change in viable cell number (Figure 8.1). These data indicating that cells had not proliferated over the two week alginate encapsulation period. Trypan blue cell counts (Figure 8.1) and photomicrographs (Figure 8.2) of released pulp cells showed membrane integrity and the ability to adhere to polystyrene surfaces with retention of a normal morphology, indicating that although cells were not proliferating during encapsulation, there was no apparent loss of viability even during long term encapsulation culture. As a consequence, alginate was then used as a 3D scaffold material to investigate the effect of lineage inductive medium on encapsulated pulp cells.

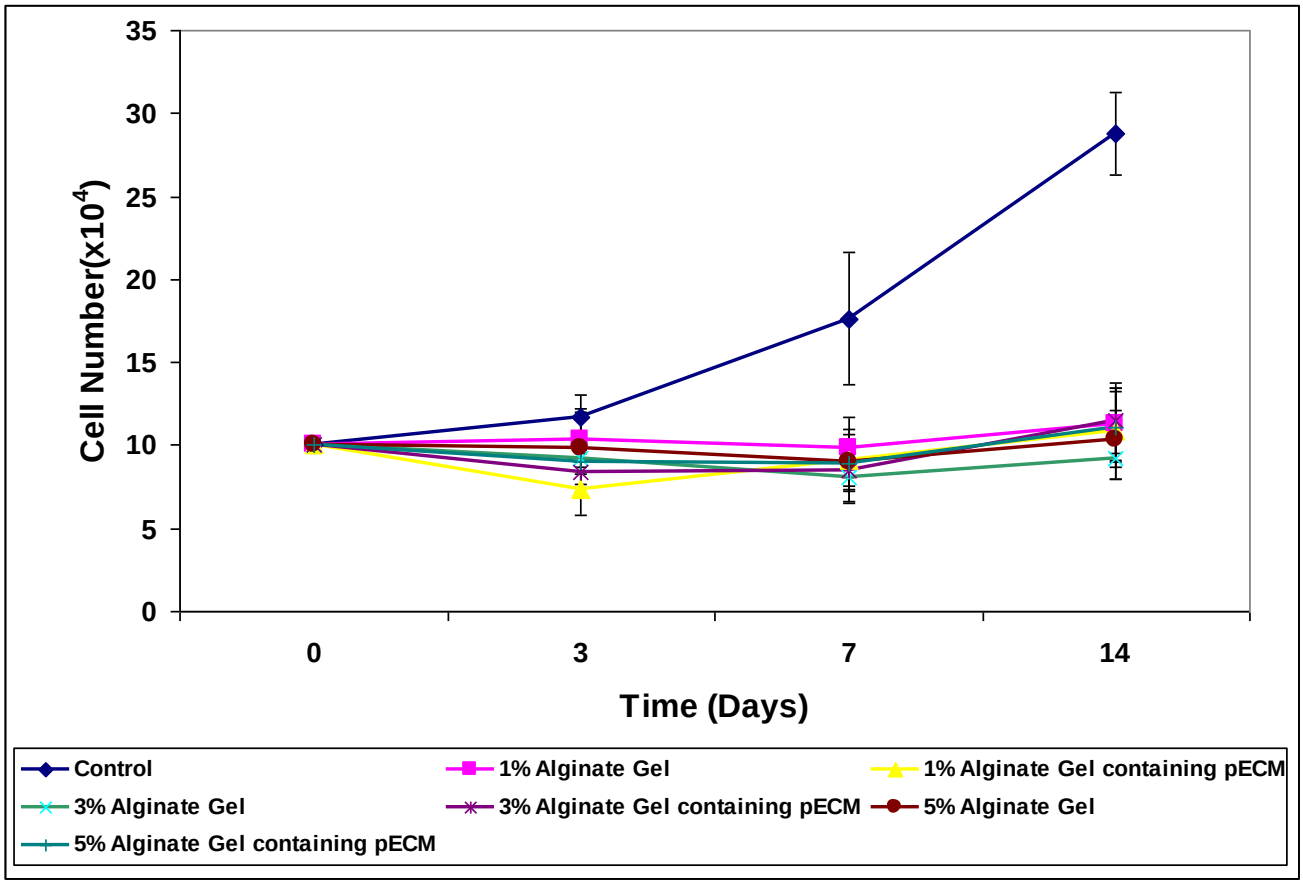


Figure 8.1 Graph showing the number of tri-sodium citrate released viable cells from different concentrations of alginate gels in the presence or absence of pulp ECM molecules over a two week period. The number of viable cells released from the gels was determined by Trypan blue staining and standard cell counts remained relatively constant over the two week period for all six gel conditions. There was no statistical difference in cell number between the six alginate gel conditions. Cell numbers released from gel encapsulation were significantly lower than the control of un-encapsulated cells grown on tissue culture polystyrene at all three time points ($P<0.05$). Standard deviation bars are shown. N=4.

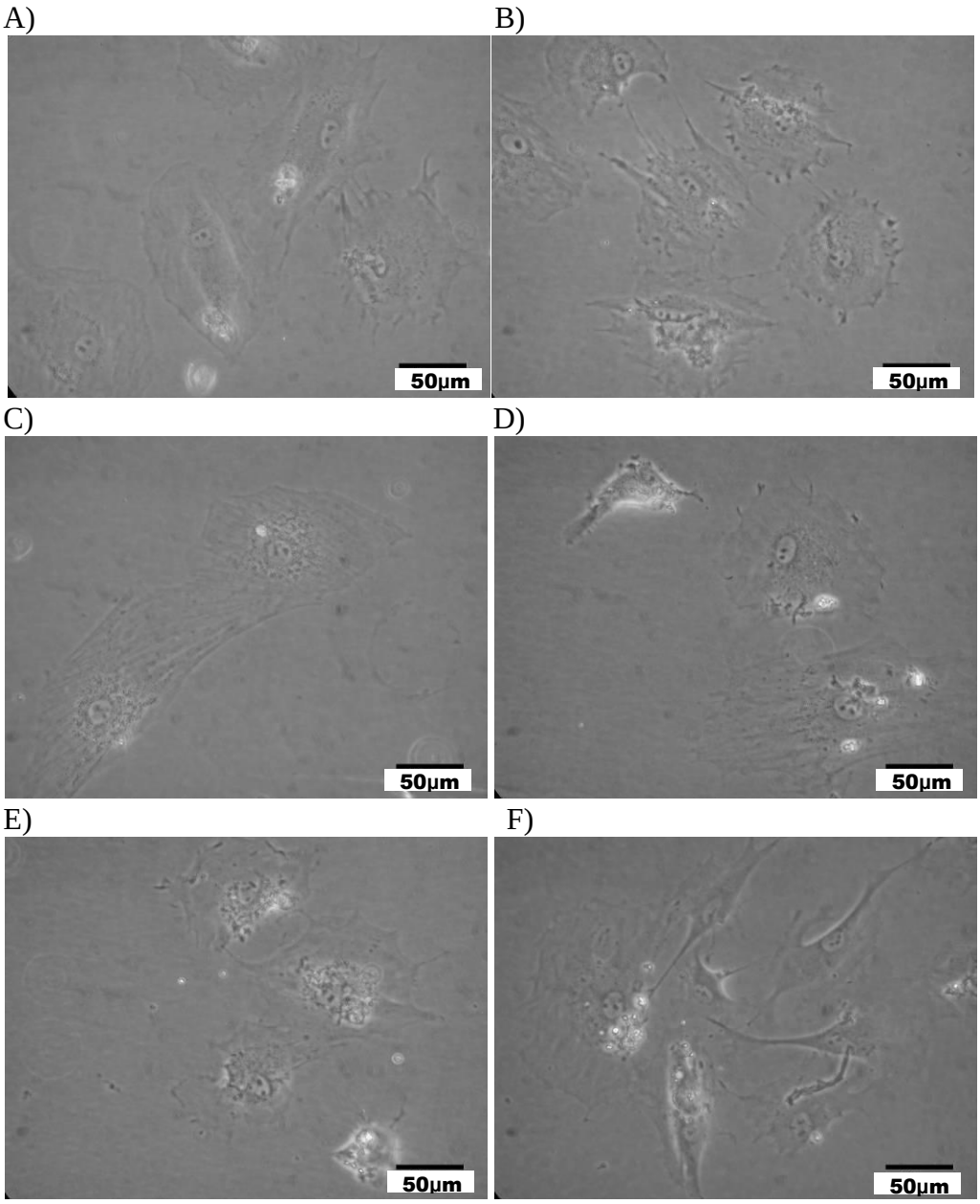


Figure 8.2 Photomicrographs of released primary pulp cells after culture for one week in different alginate gel conditions. (A) 1% alginate gel (B) 1% alginate gel with pECM molecules (C) 3% alginate gel (D) 3% alginate gel with pECM molecules (E) 5% alginate gel (F) 5% alginate gel with pECM molecules. Phase contrast images are representative of cells released from all six gel conditions showing that released cells could adhere to polystyrene surfaces and retained a morphological appearance similar to controls.

8.2 Lineage Induction of Encapsulated Dental Pulp Cells

There was a gradual change towards a more opaque white gel surface appearance in gels exposed to osteogenic medium (Figure 8.3AB). MicroCT data obtained from scanned gels were viewed as a 2D slice (Figure 8.3CD) or a 3D reconstruction (Figure 8.3EF). Both views showed no change in pixel density inside gels, however a large increase in pixel density around the outer surface of the gels exposed to osteogenic medium was evident. The outer surface pixel density was found to be at a level similar to that of a hydroxyapatite mineral phantom composed of tetracalcium phosphate (TTCP)/ dicalcium phosphate anhydrous (DCPA) powder and α-tricalcium phosphate (α -TCP) powder (Hofmann *et al*, 2007). These preliminary data indicated that primary pulp cells could still respond to osteogenic signals by differentiating and secreting mineral as previously shown in 2D cultures (Figure 6.1). As a result, 3D alginate scaffolds were investigated further with both osteogenic and dentinogenic media. MicroCT scans were performed each week over a 5 week time course, allowing the pulp cells sufficient time to respond to inductive signals, to visualise early mineralisation and how this changes over time.

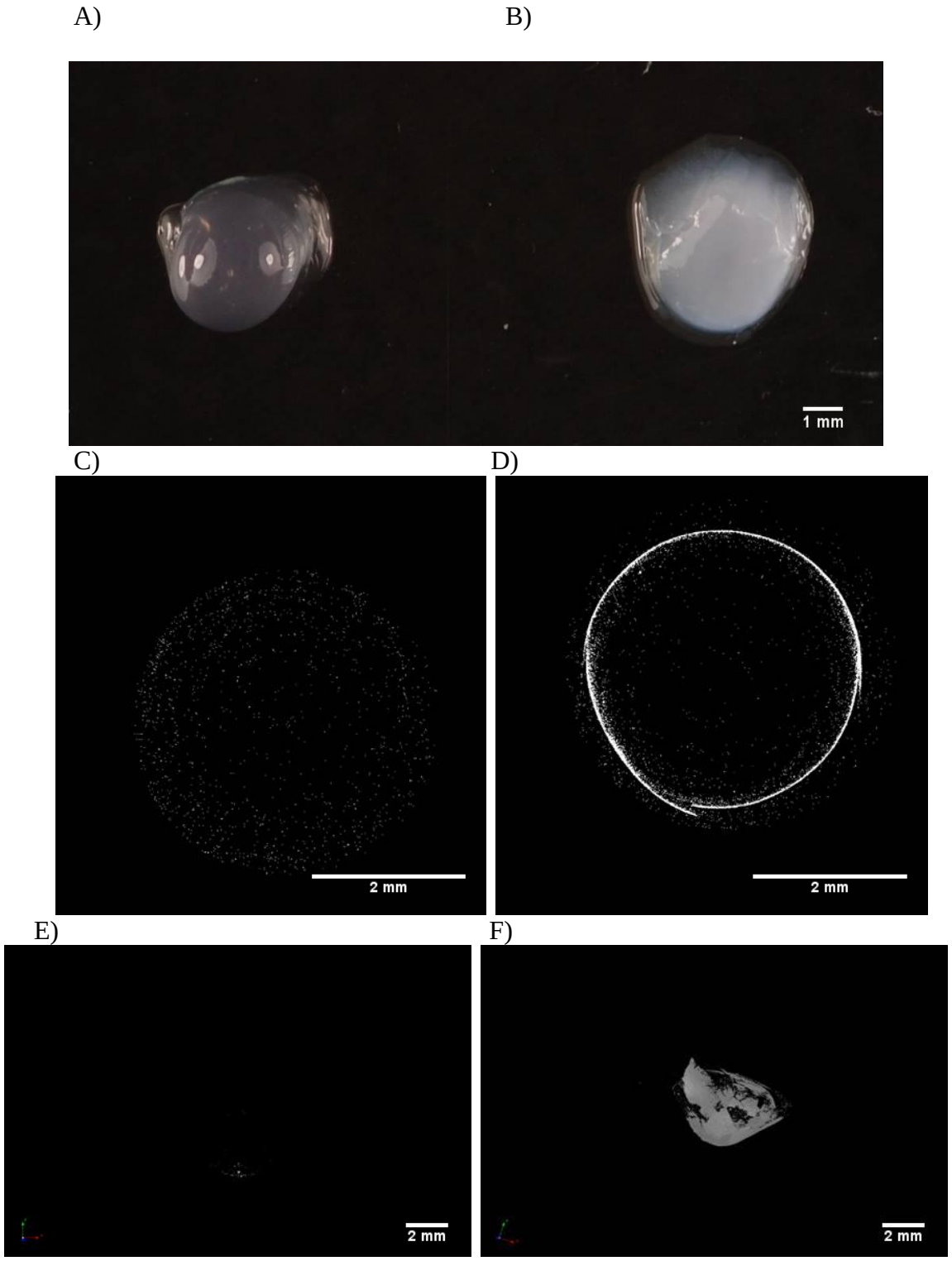


Figure 8.3 Images from the pilot study showing deposition of mineral of outer surface of alginate gels containing pulp cells after exposure to osteogenic medium. Photographs of alginate gels after 5 weeks culture in A) control medium or B) osteogenic medium. MicroCT 2D images of the sagittal plane of the alginate gels after 5 weeks culture in C) control medium and D) osteogenic medium. MicroCT images of 3D reconstructions of alginate gels after 5 weeks culture in E) control medium and F) osteogenic medium.

Cross sections of microCT scanned alginate gels showed increased pixel density over the surface of gels exposed to osteogenic and dentinogenic media from week 3 of culture, and the number of pixels above the hydroxyapatite threshold increased at 4 and 5 weeks (Figure 8.4). Quantification of pixels in scanned gels confirmed an increase in the number of pixels on the outer surface of the gels with a density above the level of the hydroxyapatite mineral phantom in osteogenic and dentinogenic conditions (Figure 8.5).

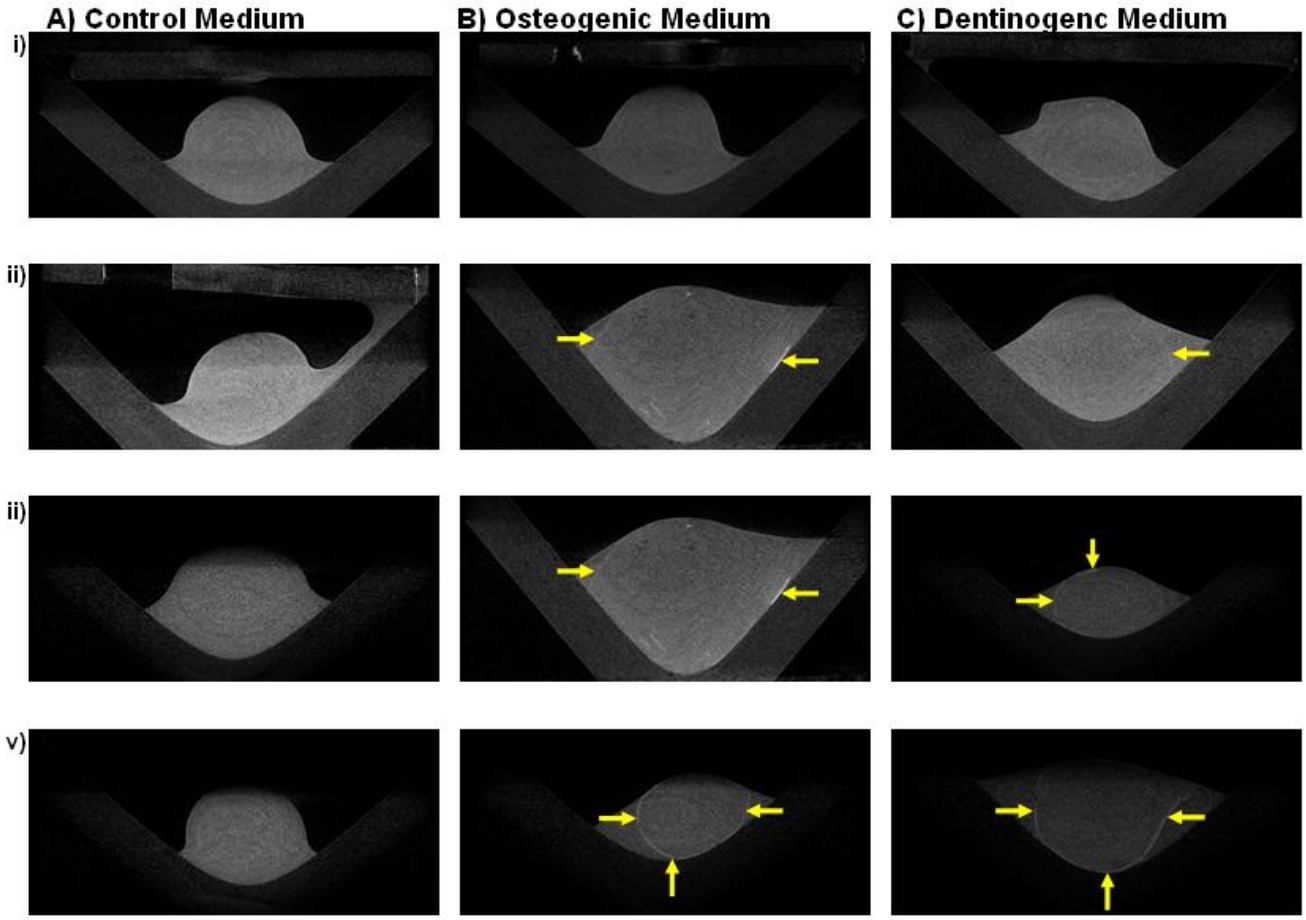


Figure 8.4 Cross sectional MicroCT images of 3% alginate gel containing pECM molecules and primary pulp cells after culture in A) control medium, B) osteogenic medium, and C) dentinogenc medium, for i) 2 weeks, ii) 3 weeks, iii) 4 weeks, and iv) 5 weeks. Arrows indicate areas on the outer surface of gels where pixel density is above the mineral threshold when exposed to osteogenic and dentinogenic media.

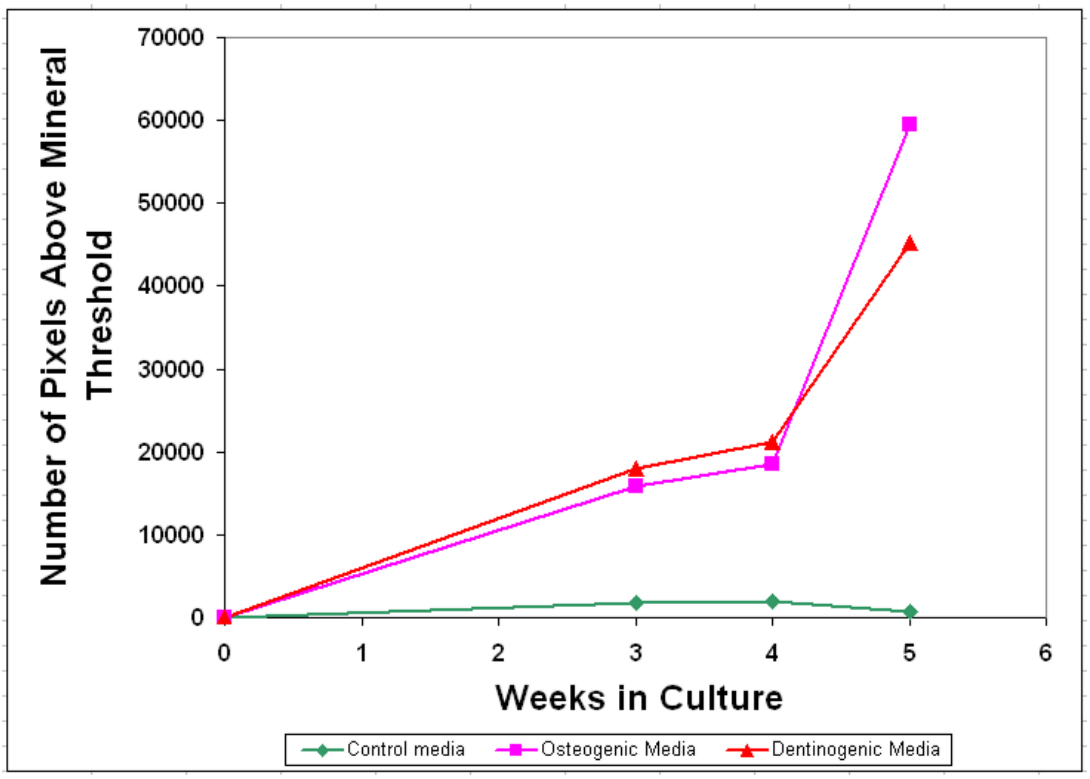


Figure 8.5 Graph showing number of pixels above the intensity of the hydroxyapatite mineral threshold in microCT images of the scanned alginate gels. Data showed that both osteogenic and dentinogenic media caused an increase in the number of pixels above the hydroxyapatite mineral threshold (n=1).

Histological analysis of haematoxylin and eosin stained alginate gel sections showed increased eosinophillic protein staining over the outer surface of the gels exposed to osteogenic and dentinogenic media (Figure 8.6). This may have indicated that only the outer surface cells were capable of differentiating and secreting protein that was mineralised. Combined, microCT data and histological staining both indicated that mineral formation only occurred on the outer surfaces of the gels exposed to inductive signals.

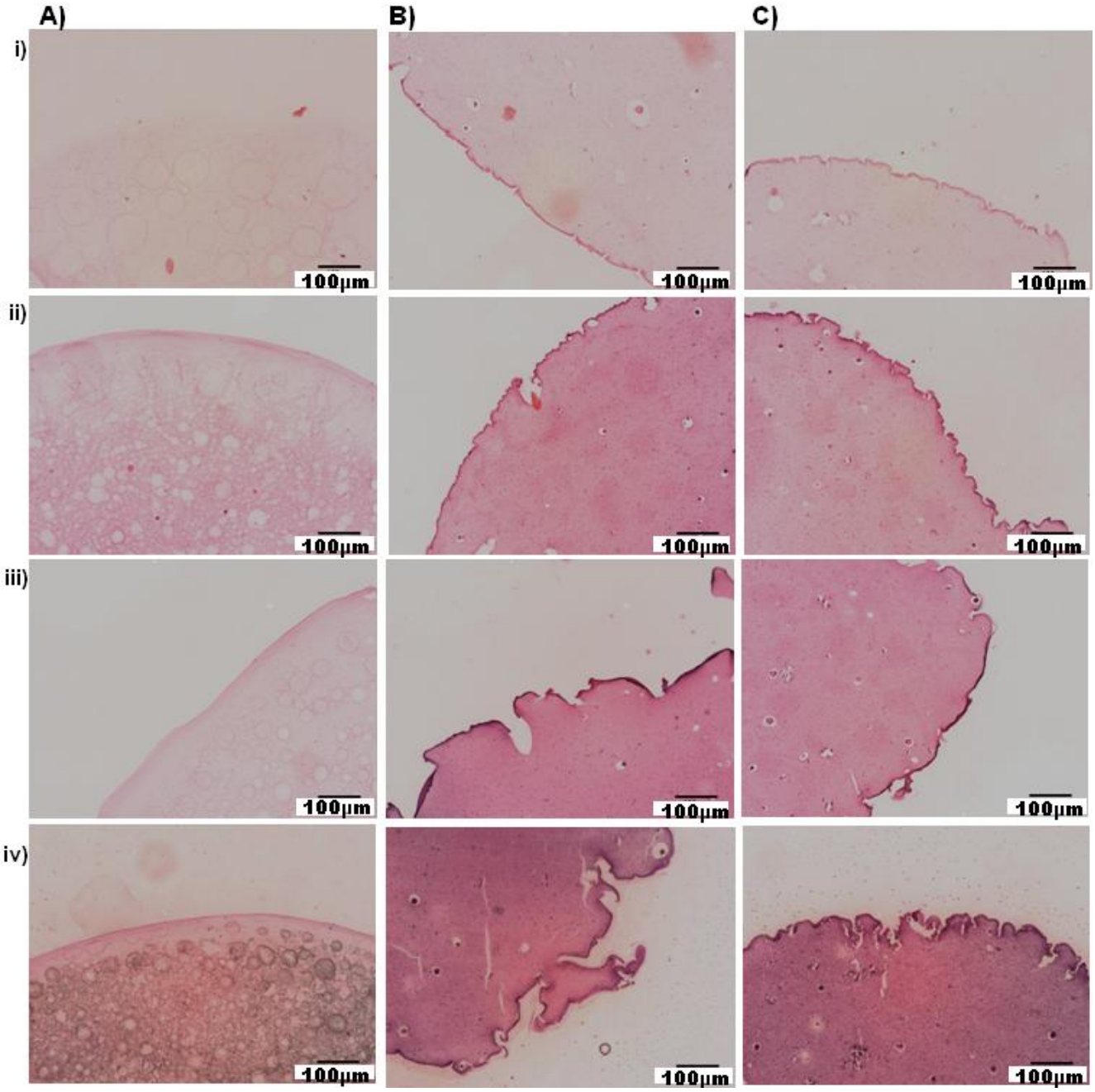


Figure 8.6 Photomicrographs of histological sections of the 3% alginate gels containing pECM molecules and primary pulp cells stained with haemotoxylin and eosin after culture in A) control medium, B) osteogenic medium, and C) dentinogenc medium, for i) 2 weeks, ii) 3 weeks, iii) 4 weeks, and iv) 5 weeks. Images show increased staining on the outer surface of gels exposed to osteogenic and dentinogenic media.

8.3 Engineering a Tooth Shaped Dental Pulp Cell-Alginate Construct

Photographs of tooth shaped alginate constructs (Method 2.11) containing pECM proteins and encapsulated dental pulp cells (Figure 8.7) showed that the constructs changed towards a more opaque white appearance when exposed to dentinogenic medium for 4 and 5 week time periods. MicroCT scans of alginate gels containing encapsulated pulp cells exposed to dentinogenic medium showed increases in outer surface pixel density after 4 weeks (Figure 8.8.I) and 5 weeks (Figure 8.8.II). Histological analysis (Figure 8.9) showed the outer surface of alginate gels containing encapsulated pulp cell that were exposed to dentinogenic medium were stained with alizarin red. SEM images (Figure 8.10.A) showed a change in surface topography from a smooth uniform appearance toward a more roughened and irregular appearance after exposure to dentinogenic medium. EDS analysis (Figure 8.10.B) demonstrated an increase in key elements of mineralised tissue with the atomic percentage of calcium increasing from 2.8% to 6.2% and phosphate from 0.8% to 3.2% after exposure to dentinogenic medium.

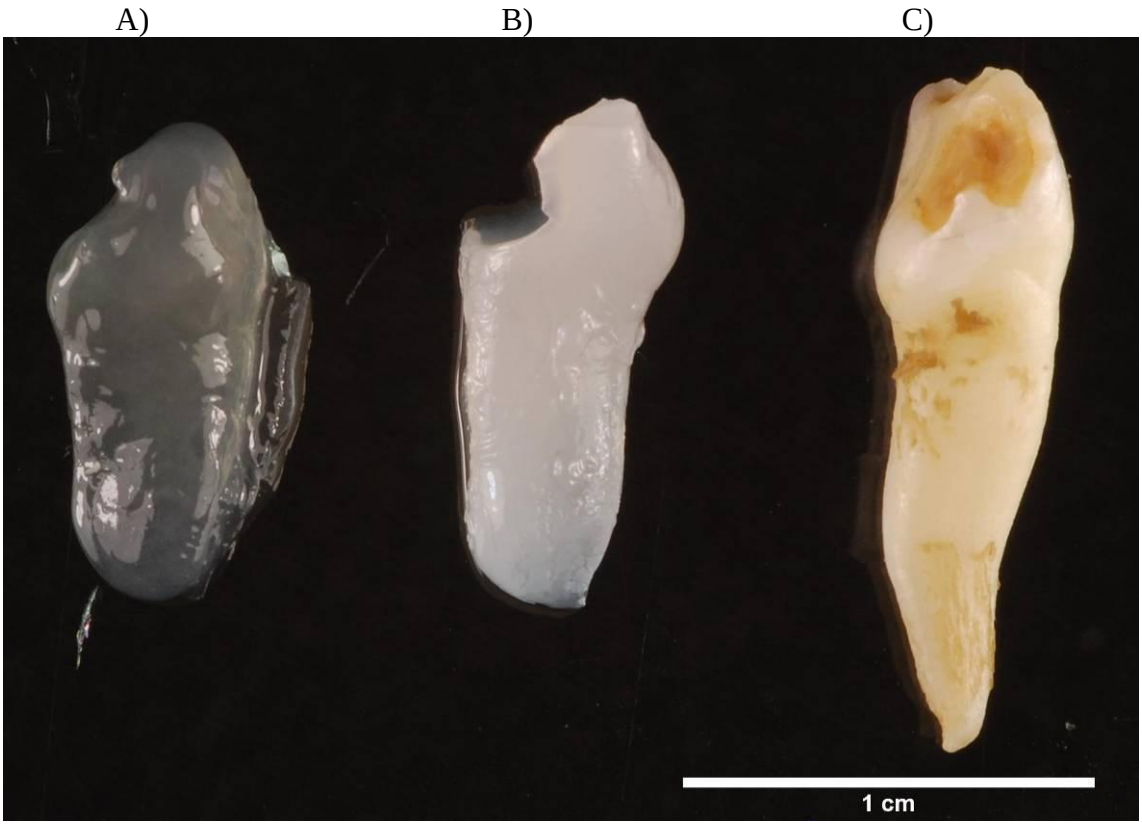
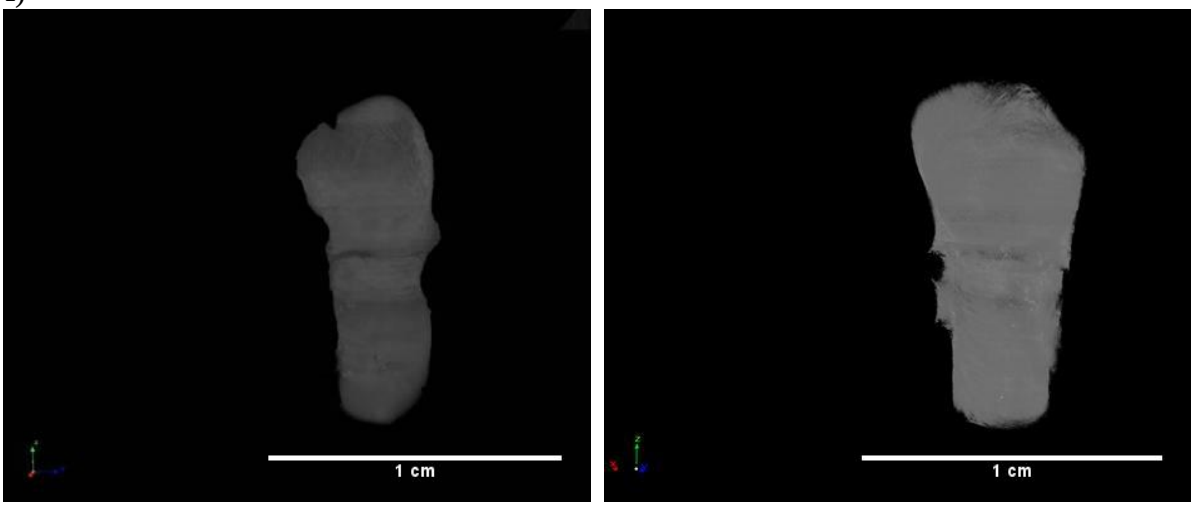


Figure 8.7 Photograph of A) encapsulated pulp cells in tooth shaped alginate construct after 5 weeks in control medium, B) encapsulated pulp cells in tooth shaped alginate construct after 5 weeks in dentinogenic medium, and C) human deciduous tooth used for production of alginate gel mould (Method section 2.11).

I) **II)**

A)



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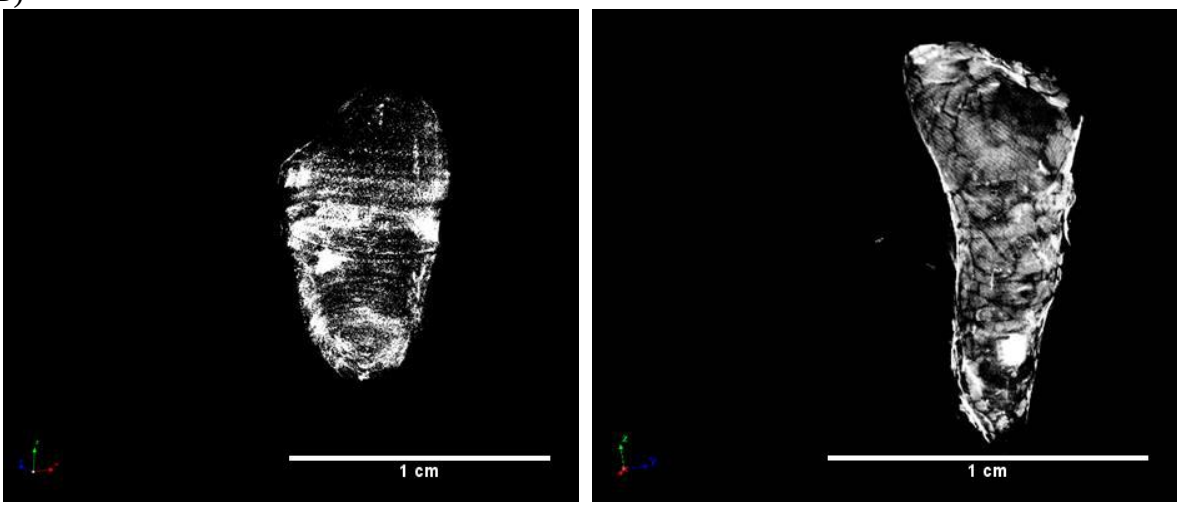


Figure 8.8 Image analysis of microCT scanned tooth shaped alginate gels after I) 4 weeks and (II) 5 weeks in A) control medium, B) dentinogenic medium (image thresholded to pixel intensity above average level of control).

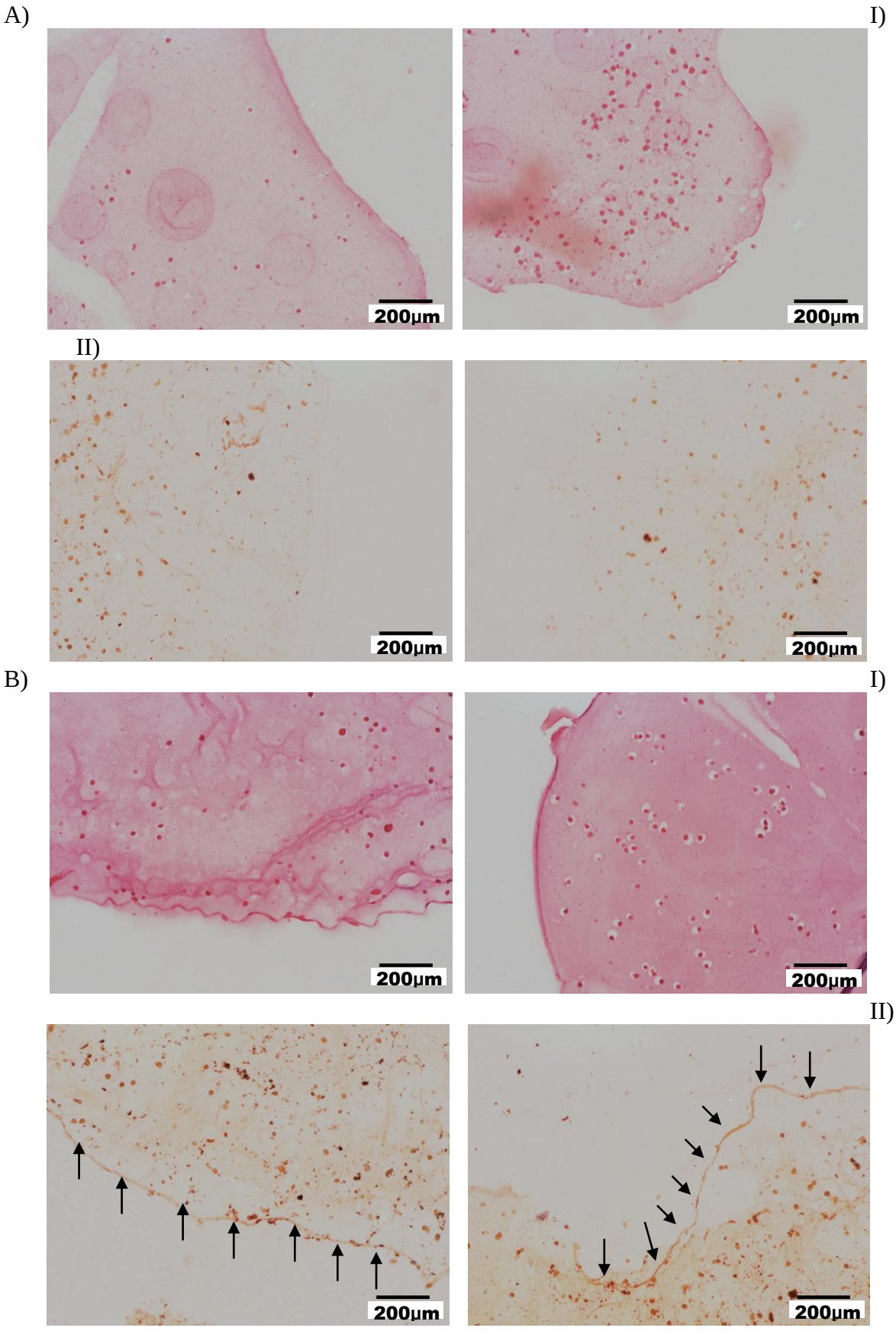
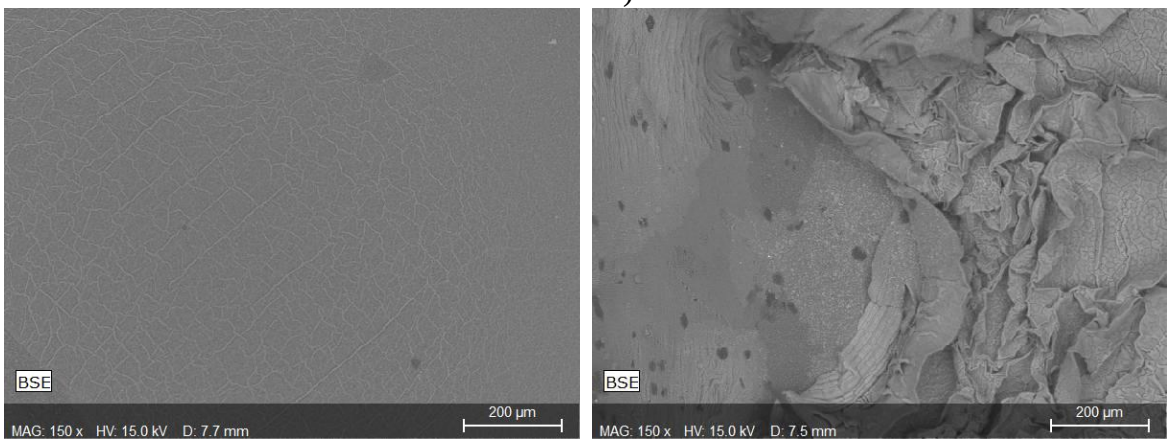

A)
I)
200µm
200µm
II)
200µm
200µm
B)
I)
200µm
200µm
II)
200µm
200µm

Figure 8.9 Photomicrographs of histological sections of tooth shaped alginate gels containing pECM molecules and primary pulp cells stained with I) haematoxylin and eosin II) alizarin red after 5 weeks culture in A) control medium, B) dentinogenic medium. Images show increased alizarin red staining on the outer surface of gels exposed to dentinogenic medium.

A)

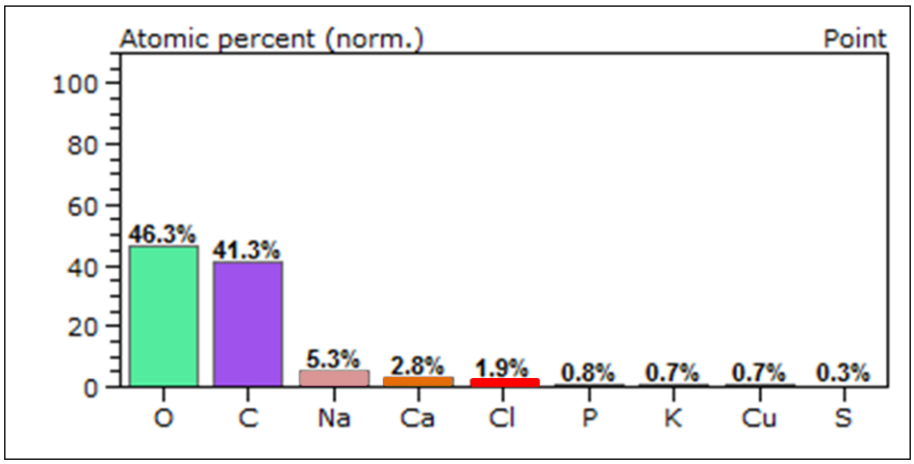
I)

II)



B)

I



II

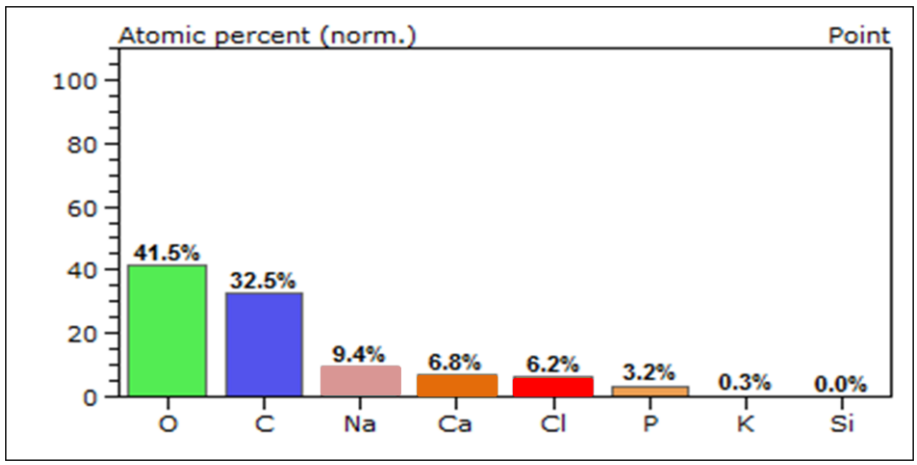


Figure 8.10 SEM analysis of the alginate tooth construct surfaces. A) SEM images and B) EDS analysis after I) 5 weeks culture in control medium, II) 5 weeks culture in dentinogenic medium. Analysis shows a change in surface topography from a smooth uniform appearance toward a more roughened and irregular appearance and an increase in key elements of mineralised tissue with the atomic percentage of calcium increasing from 2.8% to 6.2% and phosphate from 0.8% to 3.2% after exposure to dentinogenic medium.

9.0 DISCUSSION

9.1 Pulp ECM

Previously, studies have shown that individual ECM molecules can influence the behaviour of dental pulp cells including fibronectin and laminin enhancing adhesion (Zhu *et al*, 1998), dentonin enhancing proliferation (Liu *et al*, 2004; Six *et al*, 2007), DMP1 inducing differentiation (Almushayt *et al*, 2006), and TGF-β1 inducing migration (Howard *et al*, 2010). As well as the individual functional influences of the molecules, there may also be synergistic activities between the molecules. Therefore, in the present study, ECM molecules were isolated from pulpal tissue using a method previously shown to extract ECM in a functionally active state from other connective tissue (Bellon *et al*, 1988). The novel pECM preparation was shown to contain numerous peptides with sizes ranging from 3kDa to over 200kDa and the presence of the three main components of the ECM (collagens, NCPs and GAGs) was also confirmed. Proteomic analysis of pulp ECM extracts also identified the presence of a large number of proteins. These included known pulp ECM containing proteins such as decorin, tenascin, versican core protein, hyaluronan and proteoglycan link protein 1 and fibronectin (Martinez *et al*, 2004; Pearson *et al*, 1986). The collagens present in the pulp ECM preparation were identified as type I and type III and this supports previous studies that these collagen types comprise over 97% of pulp derived collagen (Shuttleworth *et al*, 1978; Tsuzaki *et al*, 1990). These proteomic results therefore indicate for the first time that the extraction method used in this study can successfully obtain preparations of ECM from pulp. The physiologically relevant whole pECM extracts were used in a variety of mechanistic and functional assays which demonstrated that they were indeed able to significantly affect dental pulp cell behaviour in terms of proliferation (Section 9.3), gene expression (Section 9.4),

differentiation (Section 9.5), and migration (Section 9.6), as well as possessing antimicrobial activity (Section 9.10).

9.2 Cell Adhesion to Pulp ECM

Pulpal ECM coated surfaces were generated to mimic the *in vivo* environment where primary pulpal cells naturally reside. The coating approach was shown to successfully generate surfaces with adsorbed proteins as demonstrated by positive Coomassie blue staining. While the resultant surfaces comprised of an adsorbed layer of soluble pECM proteins, these surfaces differed from native pulp by the absence of substantial amounts of insoluble structural proteins, such as collagen types 1 and 3. Importantly, although these surfaces were coated with pECM molecules, it was not possible to control the spatial oritentation of these molecules during coating and thus, replication of the native pulp ECM architecture must be questioned.

These pECM coated surfaces were shown to have no significant influence on the number of cells which initially adhered at 4 hours. Cell membrane bound adhesion molecules, such as integrins, have been shown to specifically bind to known ECM components, including fibronectin (Buck, 1987) and studies of pre-adsorption of fibronectin to culture substrates have revealed increased fibroblast adhesion in comparison with controls (Horbett, 1994). Fibronectin coatings have also been used to preferentially isolate distinct progenitor stem cell populations from dental pulp (Waddington *et al*, 2009). This enhanced binding may reflect transient expression of a 165kDA protein by mesenchymal cells from dental pulp that can bind to extracellular fibronectin (Lesot *et al*, 1989; Lesot *et al*, 2001). However, the pECM

preparation used to coat the culture surfaces in the present study was heterogeneous in composition, possibly leading to a range of complex cell interactions occurring between cell surface receptors and matrix molecules which did not fully mimic all of the interactions occurring physiologically. Although fibronectin was shown to be present in the pECM and subsequent experiments demonstrated that pECM molecules could modify other functional activities, there was no significant alteration in overall cell adhesion. Nevertheless, although the overall number of adherent cells did not change there may have been alterations to the adhesion of specific cell populations, which may have represented a relatively minor proportion of the total cells present. Whilst this may not have had a significant contribution to the overall cell count, such a phenomenon may still have been important functionally. Indeed, the primary pulp cells used in this investigation have been reported to be heterogeneous in nature (Gronthos *et al*, 2000) and included various cells types with different morphologies that may have differing affinities for specific ECM components present on the coated surface. A recent study using a microarray of 14 different ECM proteins demonstrated that cell-type specific adhesion profiles exist (Kuschel *et al*, 2006). It may therefore be possible that although the number of cells that adhered to control and ECM surfaces was not changed, the relative proportions of cell present may have been altered as a result of specific interactions with ECM proteins. Further studies should consider characterisation of the adherent cell populations and any changes in their nature during adherence. The present study allowed 4 hours for cell adhesion to occur; earlier time points may illustrate differences in the number of cells that initially adhered.

9.3 Cell Proliferation on Pulp ECM

Molecules in the ECM can alter the growth and proliferation of cells by binding to cell surface receptors and activating intracellular signalling pathways. These are complex signalling pathways and the same ECM molecules can have differing effects when acting on different cell types (Kiefer *et al*, 2001; Henriet *et al*, 2000). The growth and proliferation of bone marrow MSCs were not promoted when these cells were cultured on collagen I and fibronectin coated-surfaces (Song *et al*, 2008). In the present study, adsorbed ECM molecules derived from pulpal tissue were shown to decrease cell proliferation in the three cell types analysed (MDPC-23 odontoblast-like cells, primary bone marrow cells and primary pulpal cells), which included two that reportedly contain populations of mesenchymal stem cells.

The reduced proliferation rates seen for cells that were adherent to adsorbed ECM extracts may have been more representative of the *in vivo* situation where the cells may be in a more quiescent state until activated by host signalling. Indeed, previous studies have shown various stem cell populations to be slow cycling within their niche environment (Cotsarelis *et al*, 1990; Morris and Potten 1994; Booth and Potten, 2000). One study also identified that the slow cycling stem cell population was found in close proximity to ECM rich areas within their niche environment (Bi *et al*, 2007) and it has been reported that these ECM components play a key role in maintaining the balance of quiescence, self-renewal and cell-fate commitment (Bi *et al*, 2007). Potentially, the mechanism for ECM exerting this action on cell proliferation may occur by three routes; direct signalling, indirect signalling and physical forces (Young, 2011). Direct cellular binding to ECM proteins, such as tenascin C, osteopontin, biglycan and decorin has been found to regulate the proliferation of neural,

haematopoietic and bone marrow stem cells (Bi *et al*, 2005; Garcion *et al*, 2004; Nilsson *et al*, 2005; Ohta *et al*, 1998; Stier *et al*, 2005). Indirect action can occur as ECM components have been shown to modulate the bioactivity of growth factors and cytokines, such as TGF-β (Gliezes *et al*, 1997; Smith *et al*, 1998; Baker *et al*, 2009), TNF-α (Tufvesson and Westergren-Thorsson, 2002) and PDGF (Nili *et al*, 2003). The bioactivity of these molecules can be modulated by the ECM sequestering growth factors and thereby hindering receptor binding of growth factors to cells (Hildebrand *et al*, 1994; Santra *et al*, 2002; Baker *et al*, 2009), or by activating latent growth factors by proteolytic processing (Gliezes *et al*, 1997; Tufvesson and Westergren-Thorsson, 2002; Nili *et al*, 2003). By modulating the bioactivity of these factors, the ECM will indirectly control the activity of the cells present within the environment. The third mechanism of ECM action is by physical force generated by integrin binding to ECM components, giving rise to tensional forces that affect cell shape (Ingber, 1993) and can subsequently influence functional activities, such as growth and proliferation (Ghosh and Ingber, 2007), differentiation and other stem cell characteristics (Wan *et al*, 2010; Fu *et al*, 2010). The physical tethering of cells to ECM components may modulate functional activity of the molecules and therefore supplying the ECM molecules as either soluble factors within media or bound surface molecules may exert different influences.

This study demonstrated that regardless of the mechanism, the integrity of the ECM is important as to its ability to exert its influence on cell proliferation. When the pulp ECM was subjected to enzymatic autodegradation, the decrease in cell proliferation rate was abolished. This suggested that the mechanism of action relied on specific regions of ECM components being preserved for cell binding to occur. When the pECM was subjected to enzymatic degradation, it is likely that a change in protein structure occurred, thereby preventing

functional binding. This may relate to the role of the ECM within disease whereby during the healing processes, such as reparative dentinogenesis, the pECM is subjected to enzymatic degradation by the action of bacterial proteases and host MMPs (Armstrong, 1958; Larmas, 1972 ; Tjaderhane *et al*, 1998). This degraded ECM may subsequently modulate the behaviour of stem cells within the tissue changing their activity from a more quiescent state to a greater proliferation beneficial to the wound healing response. Previous studies have shown that enzymatic breakdown of ECM during disease in other tissues results in increased proliferation of resident cells (Long *et al*, 2001; Schedin *et al*, 2000; Cowan *et al*, 2000). The present study suggests that this cellular response to enzymatic degradation of tissue specific ECM may also occur within dental pulp.

The reduced proliferation rate of pulp cells seen on ECM may not only be more representative of the *in vivo* environment, but may also provide a suitable model system for the *in vitro* culture of pulp cells. There is a balance between stem cell proliferation and differentiation (Williams *et al*, 2011) during culture and primary MSC cell cultures are often not appropriate for use after a limited number of passages as a stem cell phenotype may be lost (Bonab *et al*, 2006; Shibata *et al*, 2007; Wagner *et al*, 2008; Yu *et al*, 2010; Patel *et al*, 2009). The reduced proliferation rates on ECM seen in this study were accompanied by an increase in stem cell characteristics, relative to control polystyrene surfaces. Therefore, culture in an ECM environment may provide a more long term approach for appropriate study of a primary population containing MSCs.

9.4 Expression of Stem Cell Markers by Cells Grown on Pulp ECM

This study demonstrated that culturing primary pulp cells in an environment that contained pECM molecules affected gene and protein expression. Pulp cell populations showed decreases in the expression of the odontogenic markers DSPP and DMP-1 and increases in the expression of several markers of MSCs and pluripotency when cultured in the presence of pECM molecules. While comparatively low level expression of pluripotent markers oct4, sox2, c-myc and klf4 have been recently reported in dental pulp derived cell cultures (Liu *et al*, 2011), the present study demonstrated that the expression of these markers could be enhanced, or possibly maintained, by culture in a pECM containing environment. The increase in expression of stem cell markers and decrease in expression of odontogenic markers may suggest that pulpal cells remained in a less differentiated state when cultured in an pECM environment. These data also support the premise that pulp ECM components may contribute to the maintenance of a stem cell niche within the *in vivo* environment, which can be recapitulated *in vitro* for stem cell culture. The role of ECM components in the maintenance of stem cell niches is currently being investigated for utilisation in improved stem cell culture techniques (Kobel and Lutolf, 2010). A recent study has utilised laminin as a component of the *in vivo* stem cell niche to enhance the long term culture of embryonic stem cells with maintenance of pluripotent characteristics (Rodin *et al*, 2010). Another study used peptides derived from the matrix components vitronectin and bone sialoprotein to create surfaces beneficial for pluripotent stem cell culture (Melkourmian *et al*, 2010). This has been taken further by the generation of synthetic substrates that mimic the binding role of ECM components to cell surface glycosaminoglycans to facilitate the long term culture of pluripotent stem cells (Klim *et al*, 2010; Villa-Diaz *et al*, 2010). It has also been shown that

the loss of stem cell characteristics on culture polystyrene could be reduced by culture on ECM, and this was accompanied by higher levels of telomerase activity (Lai *et al*, 2009). The activation of telomerase has been shown to prevent telomere erosion and inhibit stem cell replicative senescence *in vitro* (Cong and Shay, 2008). From these data, it has been hypothesised that the mechanism for ECM action on stem cells in culture occurs by stabilising high levels of telomerase activity, resulting in an extended undifferentiated life-span of these cells in culture (Chen, 2010). The extended undifferentiated life-span of these cells may also be linked to the reduced proliferation rates. These studies support the present data, suggesting that the binding of cells to ECM components can enhance stem cell marker expression indicating another possible role for these molecules within the dental pulp.

An alternative explanation for these data is that instead of the pECM environment retaining the cells more readily in the state that they were harvested, it actively modifies cellular gene expression and phenotype towards a more undifferentiated state. After the discovery that pluripotency could be induced by the use of viral-mediated transduction of specific transcription factors (Oct-3/4, Sox2, C-myc, Klf4, Nanog and Lin28), there has been interest in a chemical method of activating these reprogramming factors (Takahashi *et al*, 2006; Okita *et al*, 2007; Wernig *et al*, 2007). Currently, there is significant interest in chemical and biological molecules, such as self-renewal modulators, pluripotency gene activators and reprogramming boosters for inducing or enhancing pluripotency by the action of molecules and non-viral vectors (Feng *et al*, 2009). It is therefore possible that there are growth factors or signalling molecules present within the pECM extract that may modulate cellular pluripotency.

9.5 Differentiation Capacity of Cells Grown on Pulp ECM

Previous studies have demonstrated the multilineage potential of dental pulp cell populations (Gronthos *et al*, 2002; Zhang *et al*, 2008; Guimarães *et al*, 2011). Results from the present study demonstrate that the dental pulp cell populations used possessed the ability to differentiate along at least two separate mesenchymal lineages.

After two weeks exposure to osteogenic signals, there was a visible change in dental pulp cell appearance towards a smaller and more rounded cell morphology. These stimulated populations were shown to exhibit a changed gene expression profile following exposure to mineralising signals, with increased expression of the differentiation markers, nestin and DMP-1, as well as increased expression of important markers of mineralisation, osteocalcin and osteoadherin. Interestingly, other markers associated with mineralisation, including osteopontin and alkaline phosphatase, were down-regulated following three weeks exposure to osteogenic medium. The changes in gene expression demonstrate that the dental pulp cells were able to respond to the osteogenic signals. The decrease in several markers may be explained by the fact that the cells were analysed for gene expression profile after three weeks exposure to the osteogenic signal and after this period the cultures may have been fully differentiated and undergoing extensive mineralisation. These markers may therefore be expressed at a relatively lower level at this time-point compared with cells that are in a more undifferentiated state, this would be similar to the expression pattern seen for these markers in osteoblast differentiation (Aubin, 2001). The increased relative expression of markers of full differentiation and several markers associated with mineralisation indicate the cells had differentiated and secreted mineralised ECM in response to the mineralising signals. The

positive alizarin red staining within pulp cultures exposed to osteogenic signals also confirmed that these cells responded to the signals by differentiating and forming mineralised structures.

After two weeks exposure to adipogenic signals, the dental pulp cells displayed altered cell morphologies within the cultures. Gene expression analysis demonstrated that the adipogenic signals could influence pulp cells at a molecular level. Pulp cells exposed to adipogenic medium increased expression of the adipocyte marker, adipocyte protein 2. However, there was a decrease in the expression of the early adipogenic differentiation marker peroxisome proliferator-activated receptor -2 in pulp cells following three weeks exposure to adipogenic medium. The increase in expression of adipocyte protein 2 suggests a more adipogenic phenotype of the pulp cells indicating the pulp cells could respond to these differentiation signals. The decreased expression of peroxisome proliferator-activated receptor-2 was not expected. However, as this marker expected, as this marker is expressed early in the adipogenesis process (Smith, 2002) and it may therefore be that by week three, this gene had become down-regulated. Pulp cells exposed to adipogenic signals stained positive with oil red O for lipid inclusions confirming their differentiation to a more adipogenic phenotype.

Previous studies have also shown that ECM molecules can influence the differentiation potential of stem cells (Martino *et al*, 2009; Rowlands *et al*, 2008). Indeed, the culture of embryonic stem cells on ECM has been shown to enhance their differentiation capabilities (Baharvand *et al*, 2005), while fibronectin coating of culture plasticware has subsequently been shown to enhance bone marrow MSC differentiation in contrast to collagen coatings which did not exert such effects (Sogo *et al*, 2007). The differentiation of dental pulp cells has

also been shown to be enhanced by culture on surfaces coated with ECM components such as tenascin (Matsuoka *et al*, 2010) and fibronectin (Waddington *et al*, 2009). The present study demonstrated that mineralisation may be enhanced when the process occurs in a physiologically relevant pECM environment. Quantification of alizarin red staining showed increased mineralised tissue secretion by dental pulp cells cultured in an ECM environment after two and three weeks exposure to osteogenic signals. The enhanced differentiation may be due to the pECM environment maintaining the cells in a more stem like state where they are more readily able to respond to future differentiation signals. This possibility may be supported by the fact the dental pulp cells demonstrated an initial decreased proliferation rate and an increase expression of stem markers when cultured on pECM as previously discussed. Alternatively, stimulation of differentiation may be due to pECM providing signals that promote the process, possibly from chemical groups present on the surface of pECM molecules. The surface chemistry of the pECM will likely involve reactive surface groups, such as methyl and carboxyl groups, the ratio of which can influence the switch between self-renewal and differentiation of the cells seeded in this environment (Curran *et al*, 2006). It is therefore possible that these chemical groups on the pECM surface may influence the change in cellular activity from stem cell renewal towards differentiation Although there is a lack of clarity as to the mechanism of ECM influence on the cells, whether it be maintaining cells in a more stem cell like state, reprogramming cells to a state more able to respond to osteogenic signals or providing environmental signals preferential for differentiation, this influence was lost after cells had been trypsined and re-seeded in a control environment lacking the ECM.

Quantification of adipogenesis was investigated to determine whether the enhanced differentiation capacity of pulp cells cultured on ECM occurred down multiple lineages.

However, attempts to quantify oil red O staining of lipid inclusions provided only limited data, with only very small increases in the intensity of the oil red O solutions obtained from the adipogenic cultures. These data showed no differences in apidogenic characteristic between the cells in the control and ECM environment. However, more sensitive techniques for the quantification of adipogenic differentiation, such as image analysis software, may highlight any subtle differences.

9.6 Recruitment of Pulp Cells by ECM

ECM components have been shown to play an important role in regulating the migration of cells within the wound repair environment (Clark, 2003; Pospisil *et al*, 2003; Sahni *et al*, 1998; Greiling and Clark, 1997). Specific molecules within ECMs from dental tissues (cementum, dentine and enamel), including various growth factors and proteins, have been shown to play important roles in the migration of cells during dental disease (Ogata *et al*, 1997). Indeed, dentine phosphoprotein (DPP) and dentine sialoprotein (DSP) have been shown to induce the migration of neutrophils (Silva *et al*, 2005), while TGF-β1 induces the migration of mouse dental papilla derived MDPC-23 cells (Kwon *et al*, 2010). Basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) alone, or in combination with nerve growth factor (NGF) and bone morphogenetic protein-7 (BMP7), induced pulp cell homing, angiogenesis, and mineralised tissue formation in dental repair processes (Kim *et al*, 2010). A recent study showed that the ECM proteins laminin, fibronectin and collagen type-1, as well as growth factors contained within ECM, such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and TGF-β1, and sphingosine-1-phosphate (S1P) also enhanced the migration of

a dental pulp stem cell line (Howard *et al*, 2010).

Ammonium sulphate fractionation has previously been used to separate mixtures of ECM proteins by charge, creating fractions significantly less complex in comparison with the un-fractionated form, to facilitate the identification of low-abundance proteins within the ECM (Hughes *et al*, 2010). This method was applied here to partially fractionate the pECM preparation to investigate whether the recruitment of dental pulp cells could be assigned to specific components within one fraction. Data from transwell assays demonstrated that the recruitment of dental pulp cells was induced by all ammonium sulphate fractions of the ECM with no significant differences detected. This indicated that there was a range of molecules within the pulp ECM that can induce pulp cell migration. Separation by charge did not produce fractions with differing functional activity.

The present study demonstrated that intact dentine tissue (dentine chips/fragments) and ECM extracts from dentine and pulp could enhance primary pulp cell migration, indicating dentine can release migratory signals. The physiological relevance of the ECM and cells used in this study demonstrated how these molecules may induce *in vivo* migration and suggested a potential role in stem and progenitor cell recruitment during dental repair processes such as reparative dentinogenesis.

9.7 Recruitment of Pulp Cells by ECM Breakdown Products

This study demonstrated that acidic and enzymatic breakdown products from dental ECMs could also induce primary pulp cell migration. Interestingly, the level of pulp cell migration was enhanced following acidic breakdown of dentine ECM and enzymatic breakdown of pulp ECM, compared with the untreated ECMs. As dental pulp cell migration occurs *in vivo* following severe pulpal tissue damage where ECM breakdown products will be present in the repair environment, these data suggest that breakdown products may also influence the complex signalling involved in dental wound repair. Matrix metalloproteinases (MMPs) present in the dentine have been suggested to play an important role in the remodelling of the dentine matrix following demineralisation by bacterial acids (Chaussain-Miller *et al*, 2006). Indeed, dentine matrix preparations have previously been shown to autodegrade due to proteolytic activity (Smith *et al*, 1984) and the enzymatic breakdown products of pulp and dentine matrices used in this study may mimic some of the biologically relevant breakdown products present *in vivo*. Acids secreted by plaque bacteria will also regulate matrix breakdown (Armstrong, 1958; Larmas, 1972) generating products that may influence cell migration. Acetic acid has previously been used to mimic the effects of plaque acid demineralising the dentine matrix (Kleter *et al*, 1994; Klont *et al*, 1991). Therefore, the acid breakdown of pulp and dentine ECM preparations used in this study was predicted to contain biologically relevant products. Other studies have also shown enzymatic breakdown products from other connective tissue ECM, such as skin and bladder, to induce cell migration (Brennan *et al*, 2008; Reing *et al*, 2009; Beattie *et al*, 2009). The potential mechanism of the functional action of ECM breakdown products is an area of increasing interest and a recent paper has postulated an explanation for the increased functional activity of ECM following

breakdown, seen here and in other studies, which is due to "unmasking of cryptic matrikines" (Barker, 2011). Fragments of ECM proteins released following breakdown have been observed to possess mitogenic activity and are now considered to comprise a new class of bioactive molecule termed matrikines (Davis *et al*, 2000). These molecules may possess cryptic receptor binding sites that remain masked until breakdown exposes the sites for functional activity to occur at a time when it is required (Schenk *et al*, 2003). The unmasking of cryptic matrikines is a tightly controlled process and has been implicated in regulating many of the cell-ECM interactions that occur during wound repair to control cell migration, invasion, adhesion and differentiation (Mott *et al*, 2004; Tran *et al*, 2005; Davis *et al*, 2000; Schenk *et al*, 2003). For example, during epidermal wound repair, MMPs unmask matrikines from collagen, elastin, laminin and fibronectin to enhance cellular migration and adhesion (Mott *et al*, 2004; Tran *et al*, 2005). It is therefore possible that the enhanced pulp cell migration seen in response to pulp and dentine ECM breakdown may be due to the unmasking of dental cryptic matrikines similar to those within other ECMs. This would have physiological benefits as exposure of these sites during dental disease would enhance pulp progenitor and stem cell migration to the damage environment therefore increasing dental repair.

9.8 Recruitment of Pulp Cells through the Rho Pathway

Cell migration can be divided into four stages; extension of cell processes, formation of focal adhesions, contraction of the body of the cell and tail detachment (Ridley, 2001). Three members of the rho GTPase family have been identified to participate in regulating cell migration in general and include rho, rac and cdc42 (Hall, 1998; Raftopoulou and Hall, 2004).

To date, the underlying mechanism of signalling of dental pulp cell migration is not fully defined, however, a recent study reported that members of the rho-ROCK pathway play a major role in the migration of dental pulp cells by controlling lamellipodium formation, cytoskeletal assembly and alpha smooth muscle actin expression (Cheng *et al,* 2010). This study also showed that lysophosphatidic acid (LPA) could increase dental pulp migration through the Gα12/13 protein activation of rho and that the inhibition of ROCK, a downstream target of rho, could enhance dental pulp cell migration and suggested rac activation as a possible mechanism. Currently, this finding is controversial as several studies have shown ROCK inhibition to result in a loss of cell motility in several cell types (Sumi *et al*, 2001; Kawano *et al*, 1999, Mitchison and Cramer, 1996). Active rac has been shown to inhibit rho function (Sander *et al,* 1999), and these data therefore suggest that more than one mechanism for dental pulp cell migration may be operative through either a rho or rac dependent pathway. The present study used an inhibitor to selectively inactivate rhoA, rhoB and rhoC, thereby blocking the rho dependent pathway while other related GTPases such as rac remained in an active state. These data showed dental pulp cell migration in response to dental ECM proteins was blocked following the use of this inhibitor. This demonstrated that rho to be active in dental pulp cell migration in this system and indicated that if any alternative pathways exist for dental pulp migration, such as the rac pathway, rho still likely needs to be in an active state.

9.9 Recruitment of Specific Populations within Pulp Cells

Wound repair involves the selective recruitment of circulating or tissue specific stem cell populations (Blau *et al*, 2001). The selectiveness of this recruitment depends on cell types differentially expressing cell surface receptors that respond to molecules present within the wound environment. The present study showed differences in gene expression between the dECM recruited pulp cell population and the population of pulp cells that did not migrate. The recruited pulp cells showed relatively higher levels of the expression of mesenchymal / general stem cell markers vim, cd44, sca-1, and scf, as well as relatively higher levels of the expression of pluripotency markers s-myc, sox2, lin28, nanog and klf4. This analysis indicated a more stem cell-like characteristic within the recruited population compared with the unrecruited pulp cells. The differences in cell character between the populations may be due to selective recruitment of stem cell-like populations from the heterogeneous source population. This possibility merits further investigation and clonal analysis of the recruited cells could provide a deeper insight.

This finding may have physiological significance as during dental disease, released dECM components may play a role in the selective recruitment of cells involved in wound repair. The mechanism for this selective recruitment is likely complex with numerous molecules and receptors involved. However, there is a significant lack of understanding of how MSCs target specific tissues due to a lack of understanding of the appropriate cell adhesion and migration receptors (Karp and Teo, 2009). Further characterisation of the differential cell surface receptor expression between migrated and unmigrated pulp populations, as well as further characterisation of dECM composition, may provide useful information on cells surface

receptors and ECM molecules required for selective recruitment of MSCs.

9.10 Antibacterial Activity within Dental ECMs

The present study demonstrated that dentine and pulp ECM preparations exhibited antibacterial activity against three types of anaerobic bacteria associated with infected dental tissues. These data are in agreement with previous studies, which have demonstrated that ECM extracts derived from other tissues possess antimicrobial activity (Sarikaya *et al*, 2002; Brennan *et al*, 2006). These antimicrobial properties are likely ascribed to a complex range of molecules, including AMPs that play a key role in innate immunity. Indeed, previous studies have demonstrated that neuropeptides substance P (SP), neurokinin A (NKA), calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP) are present in dental pulp (Awawdeh *et al*, 2002; El-Karim *et al*, 2003; El Karim *et al*, 2006) and possess antimicrobial activity against several types of bacteria including *S. mutans* and *E. faecalis* (El Karim *et al*, 2008). ADM (Adrenomedullin) is a multifunctional peptide also with antibacterial function directed against both gram positive and negative bacteria resident in the oral cavity (Allaker and Kapas, 2003) and is present in dentine matrix (Tomson *et al*, 2007; Musson *et al*, 2010). It is likely that the complex range of AMPs present within dental ECM is responsible for the antimicrobial activity present within this tissue.

Previously, ECM from liver and bladder have been demonstrated to possess antimicrobial activity (Brennan *et al*, 2006) and ammonium sulphate fractionation of these ECMs demonstrated that this activity was associated with a variety of low molecular weight peptides,

with a range of activity amongst the fractions (Brennan *et al*, 2006). The present study, however, demonstrated antibacterial activity in all of the pulp ECM ammonium sulphate fractions indicating that this activity was likely derived from a range of AMPs as with other ECMs. The action of pulp and dentine ECMs appeared to be bacteriostatic rather than bacteriocidal as bacterial growth was no longer apparent when the ECM was removed from the growth environment. This was in agreement with other studies that have reported the antimicrobial action of submucosal ECM from small intestine and urinary bladder (Sarikaya *et al*, 2002). The absence of any statistically significant cytotoxic effects on pulp cells at antibacterial concentrations precluded a general cytotoxic effect on all cell types. This was also in agreement with other studies that have demonstrated that AMPs present within pulp have only limited cytotoxic effects on human cells, including some pulp AMPs such as ADM that indeed have a stimulatory effect on proliferation (Musson *et al*, 2010), supporting a potential physiologically relevant bacteria specific role (El Karim *et al*, 2008).

Both *streptococci* species studied here are found in the plaque microflora (Marsh, 1994). *E. faecalis* is most commonly associated with root canal infections (Portenier and Haapasalo, 2003). Interestingly, the present study demonstrated that the ECM preparations possessed differing degrees of antibacterial activity against these three bacterial types. Due to the complex range of AMPs in these preparations, it is difficult to provide good mechanistic insight accounting for the differing degrees of antibacterial activity, however, this may relate to degree and localisation of infection. Indeed, the fact that charge dependent separation produced fractions with differing activity may suggest that molecular charge may influence activity. It is also interesting to note that the activity was greatest against *S. mutans*, and this may reflect a defence response of the dentine-pulp complex to relatively early or slowly

progressing disease.

The results presented in this study may have clinical implications for dental treatment. EDTA is commonly used for extraction of non-collagenous matrix molecules from dentine (Smith *et al*, 1979) and is also used clinically as a root canal irrigant (Haapasalo *et al*, 2010). It is now conceivable to suggest that such clinical use of EDTA may have added benefit by releasing AMPs, which aid root canal disinfection. Further characterisation of the extracted ECM components may also identify novel AMPs with improved targeted clinical antimicrobial applications.

9.11 Encapsulation of Dental Pulp Cells

Investigation into different scaffold materials for the 3D encapsulation of cells is an area of great promise for future tissue engineering applications. The present study investigated the potential for alginate to be used in combination with dental pulp cells for tooth tissue engineering. Alginate gels have previously been used to successfully encapsulate other cell types including fibroblasts (Hunt *et al*, 2009), dorsal root ganglia (Bellamkonda *et al*, 1995), chondrocyctes (Hong *et al*, 2007), MSCs (Smith *et al*, 2007), osteoblasts (Kong *et al*, 2003), hepatocytes (Khattak *et al*, 2007), and neural progenitor cells (Zielinski and Aebischer, 1994). Other studies have suggested that alginate is not an optimal scaffold material for the encapsulation of all cell types, with some cell populations decreasing in viability during encapsulation while others migrated out of the gels (Tsai *et al*, 2006). The present study demonstrated that alginate can be used to successfully encapsulate dental pulp cells without the loss of cell viability. Dental pulp cells did not proliferate during alginate encapsulation as

was previously also reported for fibroblast populations encapsulated in alginate (Hunt *et al*, 2009). Notably, however, other cell types, including myoblasts, have been reported to proliferate during alginate encapsulation (Thakur *et al*, 2010).

This study showed dental pulp cells remained viable during encapsulation within a range of alginate gel concentrations. A recent study demonstrated that altering the modulus of the supporting matrix, from values ranging between that of natural bone (93kPa) to that of soft connective tissues (11kPa), could have an appreciable influence on the differentiation capacity of dental derived cells (Viale-Bouroncle *et al*, 2011). As the mechanical properties of the alginate constructs can be easily manipulated by altering the concentration of the alginate in the gels, it would be possible to create a construct with similar mechanical properties to that of natural dental pulp tissue. In addition, alginate hydrogels have previously been used to facilitate pulpal wound healing by the inclusion of relevant growth factors, such as TGF-β (Dobie *et al*, 2002). With the inclusion of extracted pECM and manipulating concentration of alginate in the gel, a construct can therefore be created with biomechanical and biochemical signals similar to those that would be present *in vivo*.

The present study also demonstrated how encapsulated dental pulp cells could respond to differentiation and mineralisation signals to form mineralised tissue around the outer surface of the alginate gels. Previous studies have shown other cell types such as embryonic stem cells are able to produce mineralised tissue within alginate gels (Hwang *et al*, 2009). The present study used dental pulp cells and an odontogenic medium containing physiologically relevant differentiation signals to induce the formation of mineralised tissue. The pulp cells encapsulated deep within the gel showed no obvious response to the signals, but those closest

to the outer surface clearly responded by forming mineralised tissue. This may be due to differences in oxygen tension, reduced diffusion of mineralisation signals, or physical restriction of more deep encapsulated cells. This method not only demonstrated the suitability of this model for dental tissue engineering but may also, provide a useful model to study dental repair processes. The alginate model used here included pulp ECM molecules within the gel construct with the dental pulp cells and dentine ECM molecule within the mineralisation medium. Therefore, comparisons can be drawn between the mineralisation process occurring here and the processes that occur within the *in vivo* repair environment, with the gels serving as a model for the exposed pulp.

Current research into scaffold-based and scaffold-free methods for tooth tissue engineering are unable to generate tooth constructs that mimic the exact shape and size of a natural tooth (Zheng *et al*, 2011). This has led to several studies beginning the obligatory task of scaling up towards human tooth size (Xu *et al*, 2008; Abukawa *et al*, 2009). One recent study has attempted to overcome the translational hurdles of these methods by developing cell-free anatomically shaped tooth scaffolds to be used in combination with growth factors (Kim *et al*, 2010). These scaffolds were shown to produce anatomically shaped tooth-like structures *in vivo* by cell homing and without cell delivery. The present study demonstrated how alginate / pulp cell constructs could be produced to closely resemble the shape and size of a natural tooth. Whereas the cell-free scaffolds were generated using a highly accurate 3D printing method, the alginate scaffolds used in this study were generated by the simple use of dental impression materials. This study therefore demonstrates the potential for impression materials to be used in a relatively inexpensive, rapid and simple approach for producing anatomically shaped constructs as an alternative to more complex and expensive methods such as 3D

printing. However, it should be noted that this approach is currently far from the level required for tissue engineering applications. Only one cell type has been used within this model and there has been no investigation into how to vascularise the model or how the alginate will perform *in vivo* for successful long term survival.

10. CONCLUSIONS

- This study has demonstrated that a range of molecular weight ECM proteins can be extracted from pulpal tissue in a functionally active state. These molecules were used to create an *in vitro* growth environment that significantly affected cell behaviour.

- The presence of pulp ECM *in vitro* resulted in reduced pulp cell proliferation rates and increased stem cell marker expression compared with controls generated in the absence of ECM. The reduced proliferation rates observed in this study were likely more representative of the *in vivo* situation where cells are in a more quiescent state until activated by signals.

- The increased stem cell marker expression indicated that dental pulp ECM can contribute to the maintenance of a stem cell phenotype *in vitro* which may point to its role in the maintenance of a stem cell niche *in vivo*.

- The dental pulp cells used in this study were shown to have the capacity to be induced down multiple lineages. Induction on a surface containing ECM molecules enhanced the differentiation capacity of the population. These data indicate that *in vitro* culture of dental pulp cells in a pulp ECM environment provides a population with stem cell-like characteristics and that ECM molecules may influence stem cell behaviour *in vivo*.

- Another significant finding of this study was that ECM molecules released from the tooth during tissue injury, promoted pulp cell migration. The chemotactic effects of these molecules were enhanced when exposed to degradative conditions relevant to disease. ECM

molecules and their breakdown products will likely influence pulp cell migration *in vivo* during repair processes, such as reparative dentinogenesis.

- The migration of pulp cells in response to dental ECMs was shown to be dependent on an active rho pathway and the recruited cells exhibited increased stem cell marker expression. Molecules present within dental ECM may therefore selectively recruit stem cell like populations required for *in vivo* repair processes.

- Dentine and pulp ECM preparations exhibited antibacterial activity against three types of facultative anaerobic bacteria associated with infected dental tissues. The presence of antibacterial activity in all of the pulp ECM ammonium sulphate fractions indicated that the antibacterial activity was likely derived from a range of AMPs, the actions of which were shown to be bacteriostatic rather than bacteriocidal.

- The absence of any statistically significant cytotoxic effects of the dentine and pulp ECM preparations on pulp cells at concentrations which were shown to be antibacterial precluded a general deleterious effect on all cell types. These molecules likely play a role in slowing the progression of bacterial growth and invasion *in vivo* thereby contributing to tissue defence.

- Dental pulp cells encapsulated in 3D alginate structures demonstrated long term culture viability. This model was used in combination with encapsulated and culture media supplemented ECM molecules resulting in the generation of physiologically relevant culture conditions.

- Dental pulp cells were shown to exhibit mineralisation capabilities on the outer surface of the alginate gel constructs. These findings have the potential to be used for investigation into 3D dental pulp cell mineralisation and future dental tissue engineering applications.

- Ultimately, the data from this study illustrated a range of functional activities associated with dental ECM that may play important roles in cellular regulation *in vivo*. These molecules can be utilised for influencing dental pulp cell behaviour *in vitro*, for more physiologically relevant cell culture and show potential for tissue engineering applications.

11. FUTURE WORK

The data presented in this study illustrates the importance of ECM in modulating cellular behaviour. There is considerable potential to build upon the work presented here to provide a greater insight into how ECM exerts its biological influence. Although this study used a variety of techniques to identify components within the ECM, characterisation of the extracts was not exhaustive. Mass spectrometry identified 1814 different peptides that could not at present be assigned to specific proteins. There are a significant number of proteins present within the extracted pECM sample that have not been identified by this approach and therefore more thorough and detailed protein identification techniques, including protein arrays, may provide a more comprehensive list of the molecular components of the ECM.

With a more thorough characterisation of the ECM components, additional molecules of interest could be identified for further functional investigation. Blocking experiments against these target molecules, using antibodies and/or siRNAs, could be used to investigate their functional effects on proliferation, stem cell responses, antibacterial activity and cell recruitment. Such studies could provide insights into the mechanisms of ECM functional activity and identify components with clinical application for use in cell homing or as novel antibacterial agents. However, this study has demonstrated using fractionation techniques that the activities detected may be associated with a variety of molecules that may interact with each other in synergistic manner, and therefore the inhibition of one molecules activity may be compensated for by others presented within the ECM.

The significant effects of the pECM molecules on stem cell behaviours demonstrate the

potential of these molecules for future use in stem cell culture techniques. The reduced proliferation rates and increased stem cell characteristics seen in primary rodent pulp cells, when cultured on pECM compared with control culture surfaces, demonstrate that pECM environments may provide a more long term approach for appropriate study of a primary population containing MSCs. Further investigation into how the pECM environment exerts its influence on MSCs may provide useful insight into the maintenance of the stem cell niche *in vivo* that could be utilised in future clinical tissue engineering applications.

This study also highlighted the potential of ECM molecules to be used in the recruitment of MSCs within *in vitro* assays. Further characterisation of the molecules and mechanisms involved in MSC recruitment by ECM molecules may provide data useful for future stem cell homing techniques.

Further investigation into the influence of ECM in a 3D environment would provide another future research area. The present study has demonstrated how ECM molecules can be encapsulated in viable 3D constructs containing pulpal cells. This model has the potential for further development to investigate whether the functional actions of ECM observed in the 2D tissue culture model are recapitulated within the 3D environment. Investigation in this 3D model may provide data more relevant to the *in vivo* situation. The 3D model developed here also has potential for development into clinical applications. such approahes may include its use as a cell / drug delivery device to aid *in vivo* repair, or as an *in vitro* tissue engineering approach that could be used for subsequent transplant into patients for tissue replacement therapies.

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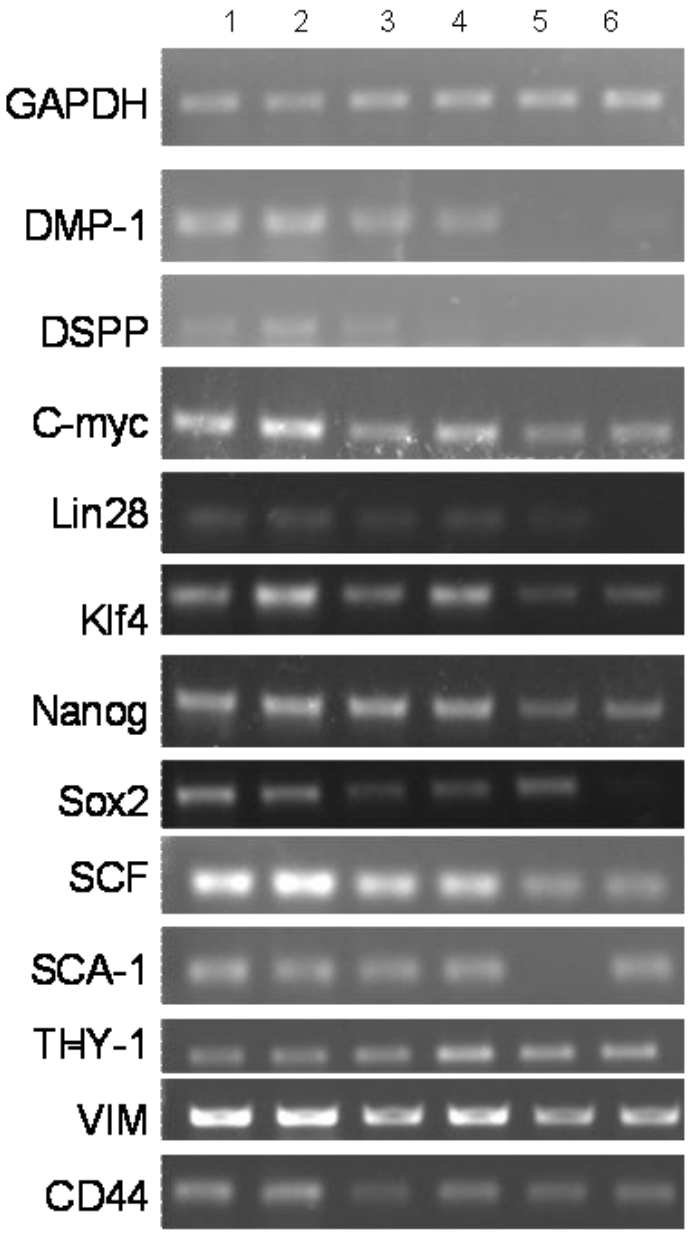
APPENDIX 1

Gel images corresponding to figure 4.9



1= MDPC-23 on culture polystyrene

2= MDPC on pECM

3= Primary pulp cells on culture polystyrene

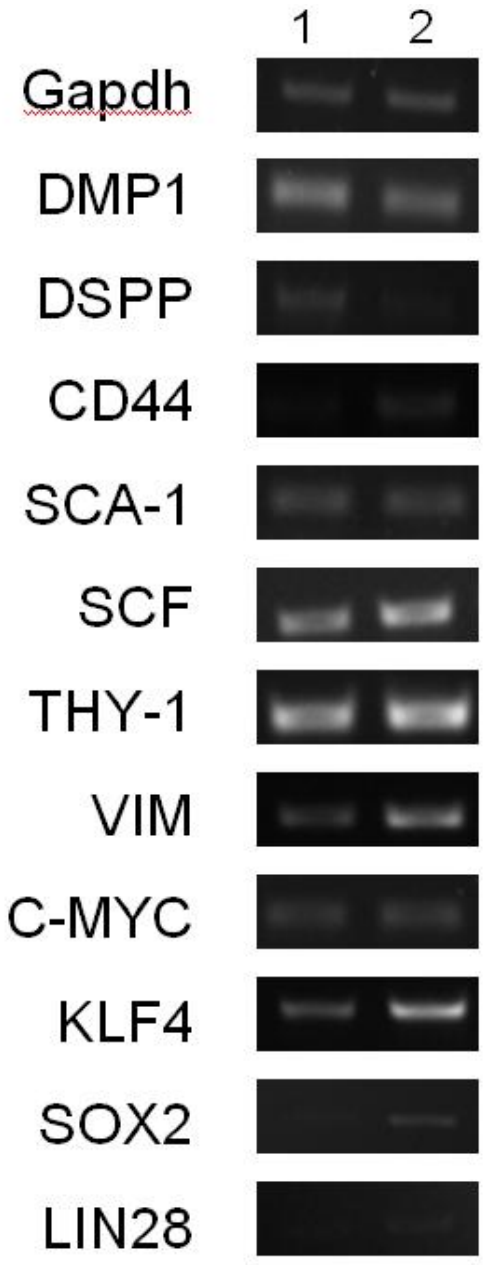
4= Primary pulp cells on pECM

5= Primary bone marrow cells on culture polystyrene

6= Primary bone marrow cells on pECM

APPENDIX 2

Gel images corresponding to figure 4.10

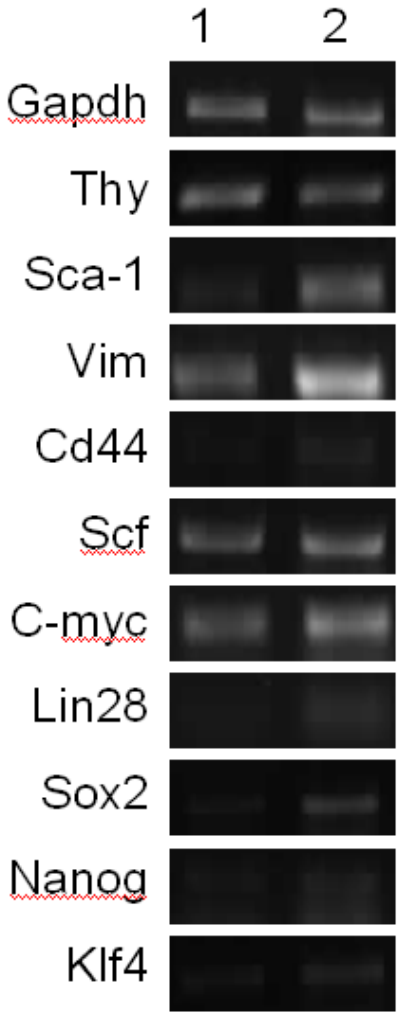


1= Primary pulp cells cultured on control polystyrene surfaces

2= Primary pulp cells cultured on ECM coated surfaces

APPENDIX 3

Gel images corresponding to figure 5.14

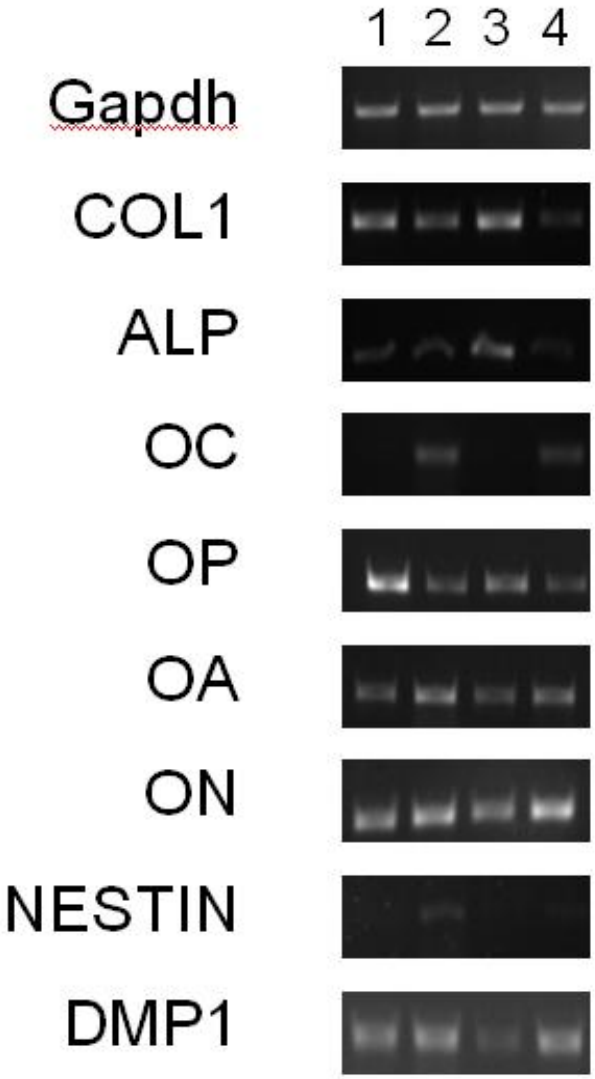


1= Unrecruited primary pulp cells

2= dECM recruited primary pulp cells

APPENDIX 4

Gel images corresponding to figure 6.5



1= Pulp cells on polystyrene with control media

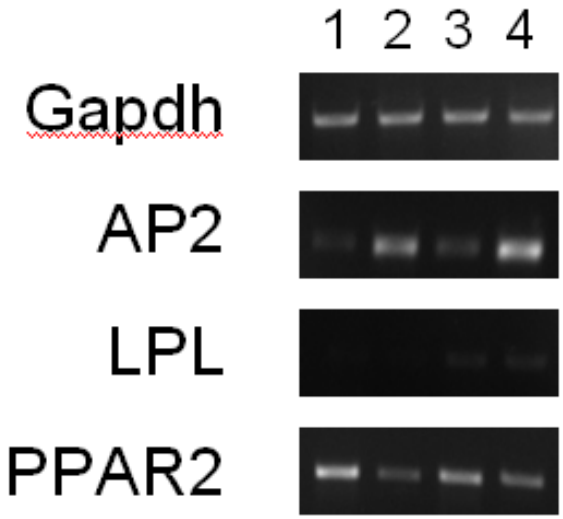
2= Pulp cells on polystyrene with osteogenic media

3= Pulp cells on ECM with control media

4= Pulp cells on ECM with osteogenic media

APPENDIX 5

Gel images corresponding to figure 6.9



1= Pulp cells on polystyrene with control media

2= Pulp cells on polystyrene with adipogenic media

3= Pulp cells on ECM with control media

4= Pulp cells on ECM with adipogenic media