ADIPOSE-DERIVED STEM CELLS FOR DENTAL TISSUE ENGINEERING

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ABSTRACT

Mesenchymal stem cells are valuable for regenerative dental research. However, the use of adipose-derived cells (ADCs) within regenerative dentistry remains relatively unexplored. This project aimed to determine an optimal method for the isolation of rat ADCs, to evaluate the influence of cell selection and cryo-storage on MSC phenotype, and compare the relative stemness and dentinogenic capacity of ADCs with bone marrow (BMDCs) and dental pulp-derived cells (DPDCs). Digestion with type-I collagenase for 30 minutes at 37ºC released the greatest number of viable and proliferative ADCs from inguinal adipose tissue. FACS and sqRT-PCR profiling indicated that ADCs shared similar levels of MSC markers (e.g. CD73, CD90, CD105) with BMDCS and DPDCs. The expression of MSC markers was also increased following cryo-storage for all cell types. Alizarin red staining, SEM and micro-CT analyses indicated that the osteogenic differentiation capacity of ADCs appeared lower than that of BMDCs and DPDCs. The FACS procedure reduced cell viability and CD29/CD90 cells had limited osteogenic differentiation capacity when compared to unsorted cell populations. Dentine matrix component (DMCs) supplementation (1 µg/mL) increased the volume of mineralised deposits in ADC, BMDC and DPDC cultures, as well as the expression of odontogenic markers (DMP1 and DSPP) in ADC and BMDC cultures. In conclusion ADCs have an odontogenic capacity, although this may be limited when compared with BMDCs and DPDCs. These findings indicate that when compared with BMDCs or DPDCs, ADCs may have a comparatively limited applicability for dental tissue engineering. However, ADCs can be isolated in comparatively large numbers with relatively little patient discomfort when compared with BMDCs and DPDCs, and these and previous studies have indicated that ADCs can be induced towards a dentinogenic phenotype.

Dedicated to my mother, Clare Davies

A very special person

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"Should I kill myself, or have a cup of coffee?"

Albert Camus

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LIST OF ABBREVIATIONS

α-MEM	Alpha-Modified Essential Medium
ACP	Amorphous Calcium Phosphate
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
ADC	Adipose-Derived Cell
ADM	Adrenomedullin
ADSC	Adipose-Derived Stem Cell
ALP	Alkaline Phosphatase
aP2	Adipocyte Protein 2
BGP	Beta-Glycerophosphate
BMDC	Bone Marrow-Derived Cell
BMP	Bone Morphogenetic Protein
BMSC	Bone Marrow Stem Cell
BSE	Backscattered Electron
BSP	Bone Sialoprotein
CBFA1	Core Binding Factor Alpha 1
CEJ	Cemento-Enamel Junction
COL1-αI	Collagen Type-I Alpha 1
DEJ	Dentine-Enamel Junction
Dex	Dexamethasone
DFSC	Dental Follicle Stem Cell
DMC	Dentine Matrix Component

DMEM	Dulbecco's Modified Eagle Medium
DMP1	Dentine Matrix Protein 1
DMP2	Dentine Matrix Protein 2
DNA	Deoxyribonucleic Acid
DPDC	Dental Pulp-Derived Cell
DPP	Dentine Phosphoprotein
DPSC	Dental Pulp Stem Cell
DSP	Dentine Sialoprotein
DSPP	Dentine Sialophosphoprotein
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ESC	Embryonic Stem Cell
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
GAG	Glycosaminoglycan
HA	Hydroxyapatite
HGF	Hepatocyte Growth Factor
IGF	Insulin-Like Growth Factor
IL	Interleukin
ISCT	International Society of Cellular Therapy
LPL	Lipoprotein Lipase
MACS	Magnetic Activated Cell Sorting

Micro-CT	Micro-Computed Tomography
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cell
MTA	Mineral Tri-Oxide Aggregate
MTT	(3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NCP	Non-Collagenous Protein
NFI-C	Nuclear Factor I/C
NLR	NOD-Like Receptor
OC	Osteocalcin
ON	Osteonectin
OP	Osteopontin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PDLSC	Periodontal Ligament Stem Cell
PG	Proteoglycan
PPARγ	Peroxisome Proliferator-Activated Protein Gamma
RER	Rough Endoplasmic Reticulum
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
SCAP	Stem Cell from the Apical Papilla
SCPP	Secretory Calcium-Binding Phosphoprotein
SE	Secondary Electron

SEM	Scanning Electron Microscopy
SHED	Stem Cells from Human Exfoliated Deciduous Teeth
SIBLING	Small Integrin-Binding Ligand, N-Linked Glycoprotein
SLRP	Small Leucine-Rich Proteoglycan
SVF	Stromal Vascular Fraction
TCP	Tissue Culture Polystyrene
TGF	Transforming Growth Factor
TLR	Toll-Like Receptor
TNFα	Tumour Necrosis Factor Alpha
VEGF	Vascular Endothelial Growth Factor

1.0 INTRODUCTION

1.1 Dentine-Pulp Complex

The dentine-pulp complex (Figure 1.1) comprises of two tissues that although compositionally and structurally distinct are embryologically similar, dentine and dental pulp (Linde and Goldberg, 1993). These two tissues function together in the maintenance of homeostasis and have interlinked roles in the pathological and reparative responses of the tooth. The dental pulp is a gelatinous innervated connective tissue containing a rich source of vasculature and is located centrally within the tooth. The pulp is surrounded by a rigid calcified tissue called dentine, which represents the major constituent of teeth. Dentine matrix is produced by post-mitotic cells called odontoblasts that line the border of the dental pulp. In reaction to tooth damage, these odontoblasts are able to up-regulate their activity and rapidly deposit a protective layer of dentine to prevent the invasion of pathogens. More severe damage is reliant on the proliferation, migration and differentiation of multipotent cells located within the pulp. The role of the dentine-pulp complex during tooth damage and the ways in which this can be exploited for tissue engineering research will be discussed in the following chapters.

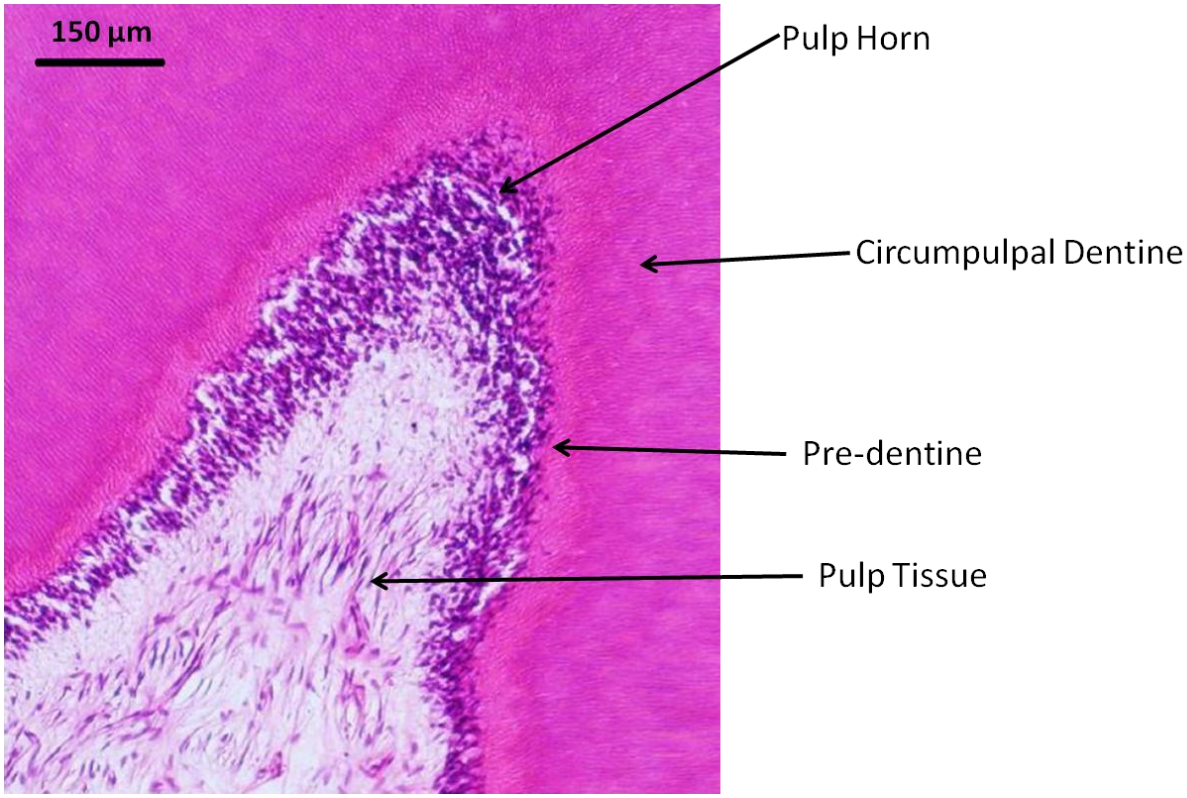

Figure 1.1 Histological section displaying the organisation of the dentine-pulp complex in a human third molar with haematoxylin and eosin staining (H&E) (Image was captured at x10 magnification). Image reproduced courtesy of Dr P. Tomson and Miss S. Finney (School of Dentistry, University of Birmingham).

1.2 Dentine and its Matrix

Dentine is a porous calcified tissue that represents the major constituent of teeth. This tissue is perforated by dentinal tubules that are 1-3 µm in diameter that traverse the dentinal layer, radiating out from the dentine-enamel junction (DEJ) or cemento-enamel junction (CEJ) to the pulp in mantle or apical dentine respectively (Maniatopoulos and Smith, 1983; Sigal *et al*, 1985). As dentinal tubules extend through the dentinal layer they

branch in areas of low tubule density producing a system of canaliculi similar to that found in bone (Mjor and Nordahl, 1996). Dentine comprises of an inorganic mineral phase (70 % by wt), an organic collagenous matrix (20 % by wt) and water (10 % wt). The organic matrix is predominantly made up of collagen (90 % wt), with the remainder comprising mainly of non-collagenous proteins (NCPs), glycosaminoglycans (GAGs) and proteoglycans (Veis, 1993; Butler and Ritchie, 1995; Butler, 1998). The mineral phase of dentine is composed of a carbonate-substituted hydroxyapatite (HA), which is deposited as crystallites that become nucleated through the action of NCPs present in the organic dentine matrix such as phosphoryn (Linde and Lundgren, 1995; Goldberg *et al*, 1995; Xu and Wang, 2012). The high mineral content of dentine (80 % by wt) makes it a harder tissue than bone and cementum (65 % by wt) and enables it to provide structural support to enamel (Hariri *et al*, 2013).

Dentine can be subdivided into several different types: primary, secondary and tertiary (Kuttler, 1959). Primary dentine represents the most abundant form within the tooth and is deposited before the tooth root is fully developed. Primary dentine can be further subdivided into mantle, circumpulpal, peritubular and intertubular dentines depending on its location and composition (see figure 1.2) (Goldberg *et al*, 2011). Mantle dentine is the first layer of dentine to be laid down and is located adjacent to the dentino-enamel junction (Goldberg *et al*, 2002). It is produced by developing odontoblasts, is approximately 5 - 30 μm wide, and is composed of loosely packed collagen fibrils and thicker Von Korff fibres (Bishop *et al*, 1991; Linde and Goldberg, 1993). Circumpulpal dentine is deposited following the formation of mantle dentine and accounts for the bulk of dentine within the tooth. This dentinal layer is located beneath the mantle dentine and is deposited at a rate of approximately 4 μm per day until crown and root development is complete (Vital *et al*,

2012). Circumpulpal dentine can be defined as inter-tubular dentine or intra-tubular/peritubular dentine. Intra-tubular dentine is secreted from odontoblast processes and can be observed as a lining around the dental tubules, potentially causing tubular occlusion as patients' age (Gotliv *et al*, 2006; Goldberg *et al*, 2011). Intra-tubular dentine is hyper-mineralised when compared with inter-tubular dentine and contains a relatively high proportion of glycosaminoglycans (GAGs), but comparatively few collagen fibrils (Linde and Goldberg, 1993). Inter-tubular dentine is secreted from the terminal web of the odontoblast and is located between the zones of intra-tubular dentine (Linde and Goldberg, 1993). It is composed of a densely packed type-I collagen matrix associated with larger mineralised crystalline plates than those found within intra-tubular dentine (Xu and Wang, 2012). Collagen fibrils are also coated with NCPs that have roles in the nucleation and expansion of mineral. The action of these proteins will be considered in section 1.2.1.

Secondary dentine is formed after root formation has been completed and the tooth has become functional. This type of dentine is structurally similar to primary dentine, but is intermittently deposited throughout life at a rate of approximately 0.4 μm per day (Wennberg *et al*, 1982). The boundary between primary and secondary dentine can be observed as a subtle calciotraumatic line, although the continuity of odontoblast processes between these two dentines is not disturbed. Secondary dentine exists as a layer surrounding the pulp chamber and contains tubules that are typically more curved in appearance when compared with primary dentine (Linde and Goldberg, 1993). This increased curvature is thought to result from odontoblast crowding as a result of continued matrix deposition. The deposition of secondary dentine does not occur uniformly but instead tends to be greater in areas subject to more stimuli, such as the crown. This asymmetric pattern of deposition subsequently leads to reduction and distortion of the

central pulp chamber. A third type of dentine may be deposited in response to chemical, physical or bacterial injury to the primary tissue. This is termed tertiary dentine and it can either be categorised as reactionary or reparative (Tziafas, 2004; Smith, 2012). The structure of tertiary dentine may differ somewhat from primary dentine since it is deposited rapidly in response to disease or trauma and may resultantly lack the regular tubular pattern of this tissue (Smith *et al*, 2005). The deposition of tertiary dentine in response to irritation shall be discussed in section 1.5.

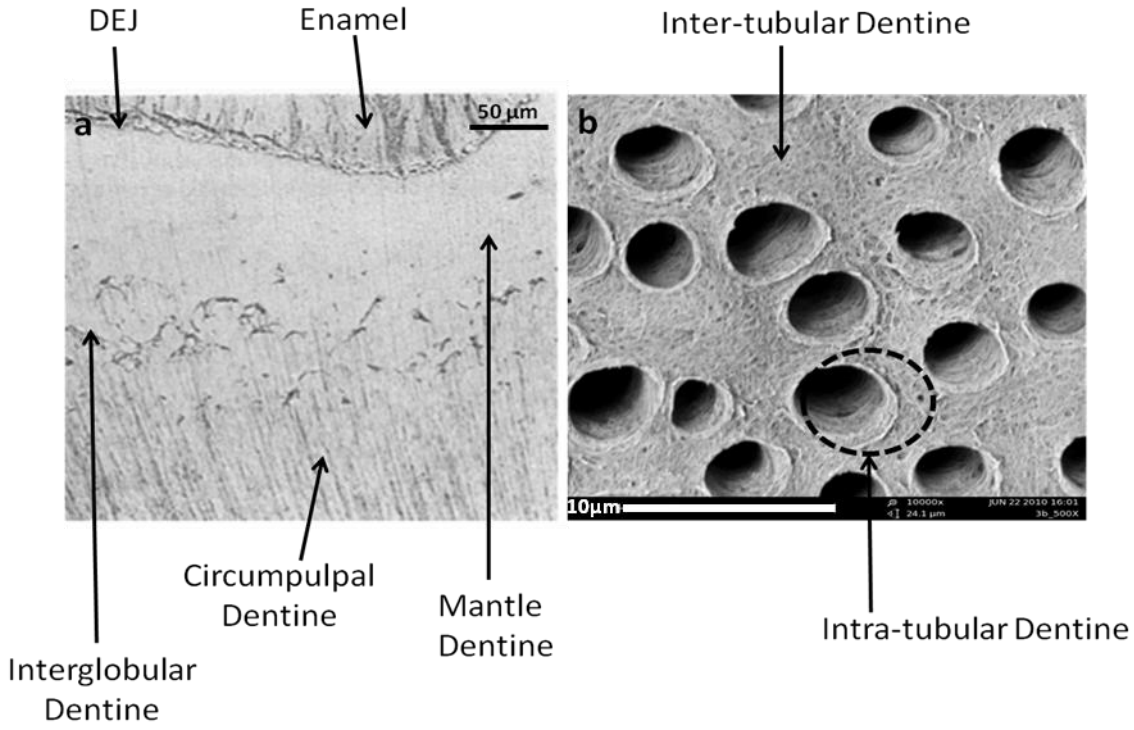

Figure 1.2 Images depicting the organisation and structure of dentine. (a) Unstained ground section depicting the location of primary mantle and circumpulpal dentines; (b) Scanning electron photomicrograph displaying the tubular arrangement of circumpulpal dentine with annotation indicating the location of inter- and intra-tubular dentines. Image **a** modified from Hayashi *et al*. (2008) and image **b** reproduced with the kind permission of E. Comar (School of Dentistry, University of Birmingham).

1.2.1 Dentine Extracellular Matrix

The dentine extracellular matrix shares many similarities with bone since both are predominantly composed of a type-I collagen network and contain proteoglycans, glycosaminoglycans and other non-collagenous proteins (NCPs) (Butler *et al*, 2003; Orsini *et al*, 2009). Collagen fibrils present in the organic dentine matrix overlap with one another creating intermittent gaps where GAGs are localised, and provide a structural template for mineralisation (Wisemann *et al*, 2005). The organic matrix is deposited by odontoblasts and concomitantly becomes mineralised by the subsequent formation and growth of carbonate-substituted apatite (this is described in more detail in section 1.4.2). Associated with the collagenous matrix are NCPs that are understood to play roles in tissue repair and homeostasis, as well as in the expansion, coordination and regulation of mineralisation (Boskey *et al*, 1991; Wu *et al*, 2008; Fujisawa and Tamura, 2012). NCPs are synthesised and secreted into the developing dentine matrix by odontoblasts where they bind to collagen fibres and initiate the nucleation of mineral. Interestingly, the types of NCP located within bone and dentine are similar and no unique marker has yet been identified to effectively differentiate the two tissues (Qin *et al*, 2001). However, quantitative differences in the expression of NCPs between the two tissues are known to exist and shall be discussed presently (Fujisawa *et al*, 1993; Goldberg and Smith, 2004).

NCPs account for approximately 10 % of all organic dentine matrix components and have been shown to have roles in adhesion and signalling as well as in mineralisation (Linde *et al*, 1989). A major family of NCPs, the **S**mall **i**ntegrin-**b**inding **li**gand **N**-linked **g**lycoproteins (SIBLING), represent a well characterised family of calcium-binding proteins that have roles in the nucleation of mineral and the coordination and regulation of

hydroxyapatite (HA) crystal formation when pre-dentine or osteoid are converted to dentine and bone, respectively (Hunter *et al*, 1996; Hao *et al*, 2009). This family currently comprises of five proteins, which contain phosphorylation, glycosylation and cell-binding (RGD) sites and include: dentine matrix protein 1 (DMP1), dentine sialophosphoprotein (DSPP), osteopontin (OP), bone sialophosphoprotein (BSP) and matrix extracellular phosphoglycoprotein (MEPE), and all are located on human chromosome 4q21 (Fisher *et al*, 2001). Both DSPP and DMP1 are present in dentinal tubules at the site of peritubular dentine and pre-dentine (Hao *et al*, 2009). DSPP is immediately cleaved into dentine phosphoprotein (DPP) and dentine sialoprotein (DSP) subunits, which account for 8 % and 50 % respectively of the total NCPs present in dentine (Zhu *et al*, 2012). Both DPP and DSP are expressed by differentiating and mature (see section 1.4) odontoblasts and participate in the binding of calcium for the conversion of pre-dentine to dentine (Butler, 1998; Begue-Kirn *et al*, 1998; Simon *et al*, 2009). These SIBLING proteins were once thought to be dentine-specific, but have since been observed in bone (Baba *et al*, 2004; Goldberg and Smith, 2004; Hao et al, 2009). However, the level of DSP in bone is 1/400 of that found in dentine and as a result a high concentration of this marker is often used to distinguish between these two hard tissues (Goldberg *et al*, 1995; Qin *et al*, 2001, 2002). DMP1 is located in the nucleus of differentiating odontoblasts where it becomes phosphorylated and is subsequently transported into the extracellular matrix (ECM) (Narayanan *et al*, 2006). This protein is proteolytically processed into N- and C-terminal fragments resulting from the cleavage of X-Asp bonds, with the full length form only being detected in dentine and not bone (Lu *et al*, 2011). The active N-terminal fragment of this protein has been implicated in the regulation of odontoblast maturation since blocking the action of this protein has demonstrated reductions in the expression of terminal

odontoblast markers such as DSP and DMP2 (Narayanan *et al*, 2001) (see section 1.4) Moreover, the presence of DMP1 is required as a downstream effector of DSPP, with the absence of this protein leading to dentine hypomineralisation (Ye *et al*, 2004; Qin et al, 2007; Chaussain *et al*, 2009; Gibson *et al*, 2013).

BSP and OP represent two well characterised NCPs that were initially identified in bone (Williams and Peacocke, 1965; Franzen and Heinegard, 1985). These two SIBLINGs have since been identified in dentine, but at significantly lower concentrations (Fujisawa *et al*, 1993). These proteins are also present in cementum (OP and BSP) and mineralising cartilage (BSP) (Fujisawa *et al*, 1993; Zhao *et al*, 2007). In fact, the comparatively reduced level of these SIBLINGs in dentine has led to them being compared with dentinal proteins such as DMP1 and DSPP for the characterisation of dentine and bone (Fujisawa *et al*, 1993). OP is a highly phosphorylated protein associated with the mediation of cell-matrix and matrix-matrix/mineral adhesion during the formation of mineralised tissues, as well as in the regulation of mineral crystal formation (McKee and Nanci, 1996; Sodek *et al*, 2000). BSP is a highly sulphated and glycosylated protein that binds to HA and cell surface integrins to promote nucleation and formation of apatite crystals in both dentine and bone (Ganss *et al*, 1999; Malaval *et al*, 2008). Both proteins are important for bone and dentine mineralisation and are up-regulated in response to mechanical wear indicating a potential role in tissue maintenance (Kubota *et al*, 1993; Carvalho *et al*, 2002).

A second family of glycoproteins found in both dentine and bone is the secretory calcium-binding phosphoprotein (SCPP) family, which contains the proteins osteocalcin (OC) and osteonectin (ON). OC is commonly used as a marker to identify the post-proliferative phase of mineralisation and has been implicated in regulating the maturation of pre-dentine

and osteoid matrix as well as in calcium ion homeostasis (Owen *et al*, 1990). The presence of this protein in dentine was demonstrated in the early 1980's, although it was detected in significantly lower concentrations than in bone (Linde *et al*, 1982). The role of this protein in the dentine ECM is not entirely understood, although several studies have demonstrated a potential function in the regulation of the mineral phase (Bleicher *et al*, 1999; Papagerakis *et al*, 2002). The SCPP ON functions in the regulation of collagen deposition within the ECM, the remodelling of mineralised tissues, as well as in the regulation of cell proliferation and the production of matrix metalloproteinases (MMPs) (Gundberg *et al*, 1984; Kawasaki *et al*, 2006; Martinek *et al*, 2007; Delany and Hankenson, 2009). The temporal expression of this protein in bone and dentine is known to vary with high levels being present within mineralised bone matrix, whereas in dentine it is typically found within un-mineralised pre-dentine layer (Mundlos *et al*, 1992; Reichert *et al*, 1992).

The NCP component of the dentine matrix also contains a diverse range of proteoglycans, enzymes such as MMPs, serum proteins and growth factors. Proteoglycans can be sub-divided into small leucine-rich proteoglycans (SLRP) and large aggregating proteoglycans. SLRPs present in dentine can be further sub-divided into two classes with class I containing decorin and biglycan and class II containing fibromodulin, lumican and osteoadherin. Members of the SLRP family of proteins function in the formation of ECM, and bind to and stabilise collagen fibrils within the organic matrix to direct and regulate both HA crystal growth and collagen fibrillogenesis (Hall *et al*, 1997). Many of the large aggregating proteoglycans are absent from the organic dentine matrix with only versican being detectable (Ruggeri *et al*, 2009. The presence of this protein in the dentine matrix

has been shown to be associated with the maintenance of ECM hydration and organisation (Sone *et al*, 2005).

MMPs constitute a family of calcium and zinc-dependent enzymes involved in the degradation of ECM components such as collagen during organisation of the dentine matrix. These enzymes have been shown to be released from the dentine matrix following treatment with EDTA and have been implicated many dentinogenic events, such as the formation of intra-tubular dentine, the proteolytic cleavage of DSPP into its two constituents and the splitting of DMP1 into N- and C-terminal domains (Tay *et al*, 2006; Chaussain *et al*, 2009). It is also likely that MMPs play roles in the release and activation of dental growth factors such as TGF-β1 (Tatti *et al*, 2008; Boukpessi *et al*, 2008), IGFs and adrenomedullin (ADM), and may themselves be activated within the dentine matrix by various members of the SIBLING family such as BSP (MMP-2) and OP (MMP-3) (Koli *et al*, 2001). Other less well characterised NCPs located within the dentine matrix include ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), cathepsins, various serum proteins such as albumin and immunoglobulins, as well as a variety of growth factors (Orsini *et al*, 2009; Tersariol *et al*; 2010).

1.2.2 Growth Factors Present in the Dentine Matrix

The mineralised dentine matrix contains a large number of growth factors that become released through the demineralising action of bacterial acids, trauma, or the action of restorative materials such as EDTA. The release of growth factors from the dentine matrix reportedly promotes local cellular proliferation and differentiation mimicking the process of embryonic tooth development (Butler *et al*, 2003; Goldberg and Smith, 2004). Growth

factors present within the dentine matrix are synthesised and secreted by odontoblasts in the form of precursors or bound to specific binding proteins (Butler *et al*, 1995; Clemmons, 1997; Valcarce *et al*, 1999; Huang *et al*, 2008). In the ECM these factors interact with other components of the matrix such as proteoglycans - an interaction which modifies the bioactivity of these factors and prolongs their life and function (Smith *et al*, 2012). For instance, TGF-β-1 is associated with proteoglycans present in the dentine matrix such as biglycan, decorin as well as an N-terminal fragment of the TGF-β transcript called latency-associated protein (LAP) (Baker *et al*, 2009). Heparan sulphate proteoglycans located within the ECM are also able to bind these bioactive factors, such as fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), bone morphogenetic proteins (BMPs), tumour necrosis factor-α (TNF-α) and a number of interleukins (ILs) (Smith *et al*, 2001; Dreyfuss *et al*, 2009).

Active growth factors have been successfully released from the dentine matrix using dental restorative agents such as EDTA or pulp capping materials such as calcium hydroxide and mineral tri-oxide aggregates (MTA) (Smith and Smith, 1998; Ferracane *et al*, 2013). In terms of yield, EDTA-extraction appears to be far superior in its ability to solubilise larger amounts of glycosaminoglycans (GAGs), non-collagenous proteins (NCPs) and transforming growth factor-β (TGF-β) than MTA or calcium hydroxide solutions (Graham *et al*, 2006; Tomson *et al*, 2007). The addition of these dental materials *in vivo* has been shown to release growth factors present within the dentine matrix that promote the proliferation and differentiation of odontoblast-like cells in a dose-dependent manner, with the accompanying formation of tertiary dentine matrix (Smith and Leaver, 1981; Zhao *et al*, 2000; Smith *et al*, 2001; Deque *et al*, 2006). EDTA, MTA and calcium hydroxide

solubilisation can also be used as a means of isolating the active growth factors and bioactive components from mechanically pulverised dentine to produce a putative dentinogenic supplement, as shall be discussed in the following paragraphs.

Given the structural and functional similarities of dentine and bone it is not surprising that these tissues contain a similar profile of growth factors. Growth factors common to both tissues include: TGF-β superfamily members such as TGF-β1, 2 and 3; insulin-like growth factors 1 and 2 (IGF-1 and 2); fibroblast growth factor 2 (FGF2); platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (Begue-Kirn *et al*, 1994; Ruch *et al*, 1995; Cassidy *et al*, 1997; Veis *et al*, 2000; Silva *et al*, 2004). Members of the bone morphogenetic protein family have been identified during the developmental stages of dentinogenesis but the identification of these factors in mature dentine extracts is inconsistent (Thomadakis *et al*, 1999; Casagrande *et al*, 2010; Jagr *et al*, 2012). BMP family members are consistently present in demineralised bone extracts and are involved in the induction, maintenance and repair of this tissue (Hanamura *et al*, 1980; Takahashi *et al*, 1987). Other growth factors such as IGF-1 and 2 have also been detected in dentine and bone although their dentinal concentrations remain comparatively low (Finkelman *et al*, 1990). One of the most characterised growth factors present in the dentine matrix is TGF-β1. This growth factor or cytokine is an inducer of odontoblast differentiation and reparative dentine formation as well as an immunosuppressant involved in maintaining dentine-pulp homeostasis following tooth injury (Begue-Kirn *et al*, 1994; D'Souza *et al*, 1998; Dobie *et al*, 2002; Li *et al*, 2011).

The application of growth factors present in the dentine matrix to cell cultures either independently or in combination with crude dentine matrix extracts has been shown to

promote odontogenic differentiation in multipotent cell types such as dental pulp stem cells (DPSCs) (Nie *et al*, 2004; Deng *et al*, 2005; Liu *et al*, 2005). The *in vivo* application of dentine matrix components (DMCs) into dental cavities has been shown promote odontogenic differentiation, as defined by the presence of polarised, columnar cells containing widened cisternae and long filamentous processes, as well as the formation of tertiary dentine (Smith *et al*., 1990; Smith *et al*, 1994; Tziafas *et al*, 1995; Smith and Lesot, 2001). Studies applying EDTA-extracted DMCs to adult stem cells *in vitro* have indicated that these NCPs had a cell-specific effect, promoting the odontoblast-like differentiation of DPSCs, but only having a comparably limited effect on the mineralisation of stem cells isolated from adipose or bone marrow tissues (Chun *et al*, 2011). The action of TGF-β1 appears to be highly important to the odontogenic properties of DMCs with the blocking of TGF-β1 activity in EDTA-soluble DMCs diminishing the odontogenic activity of these bioactive extracts (Begue-Kirn *et al*, 1992). The purification of DMCs using heparin-affinity chromatography, which has been shown to be able to select for growth factors such as TGF-β1, has also been shown to further enhance the odontogenic effects of these extracts indicating that the cellular response to DMCs may primarily be reliant on the activity of a few key factors (Begue-Kirn *et al*, 1992).

Cells formed in the aforementioned studies are defined as odontoblast-like. Such cells are typically defined as cells that possess odontoblast characteristics that differentiate them from other similar cell types such as osteoblasts. These cells can be identified using morphological observation to assess whether the cells are elongated, polarised and secreting a tubular dentine matrix (Tziafas *et al*, 1993; Tziafas *et al*, 1995; Janebodin *et al*, 2011). Therefore, odontoblast-like cells are morphologically similar to primary odontoblasts. However, unlike primary odontoblasts, their formation does not result from

signals originating from the oral epithelium (Goldberg and Smith, 2004). Odontoblast-like cells commonly express dentine-related proteins, such as DMP1 and DSPP, and may feature comparative reductions in the expression of BSP and OP when compared with osteoblasts (Narayanan *et al*, 2001; Lei *et al*, 2013). The tertiary dentine matrix produced by odontoblast-like cells can either have a tubular and organised pattern characteristic of primary dentine or feature a more disorganised scar-like calcified fibro-dentine arrangement (Tziafas and Kodonas, 2010). Comparative microarray studies contrasting the mRNA profiles of odontoblast-like cells formed by the induced differentiation of progenitor cells with that of primary odontoblasts derived from human third molars have displayed only minor differences between the two cell types (Paakkonen *et al*, 2007). The differences observed between these cells related to the expression of genes involved with cell growth and cell cycle, and could be accounted for by the post-mitotic state of primary odontoblasts (Paakkonen *et al*, 2009). Other differences related to genes involved in the recognition of stimuli that are likely to be linked to the sensory function of primary odontoblasts during tertiary dentinogenesis (Paakkonen *et al*, 2009). Such observations indicate that odontoblast-like cells are morphologically and transcriptionally similar to primary odontoblasts.

1.3 Dental Pulp

Dental pulp is a gelatinous mesenchymal tissue that has nutritional, sensory, formative, defensive and protective roles in the maintenance of the tooth. The cells of the pulp derive from ecto-mesenchymal cells that condense and form the dental papilla, which further differentiate to form the mature pulp (Linde, 1985). The pulp has two distinct regions: the

coronal pulp, which represents the centrally located region within the tooth crown and the radicular pulp, which is situated within the apical portion of the tooth. Surrounding the pulp is an odontoblast layer that intermittently secretes secondary dentine matrix during an individual's lifespan and consequently reduces the size of the pulp chamber as the tooth ages (Paewinsky *et al*, 2005). Dendritic cells and free nerve endings are associated with the odontoblast layer and provide a means of sensing injurious stimuli for the protection of the underlying pulp (Couve *et al*, 2013). Subjacent to the odontoblasts layer is the central pulp chamber containing the cell-free (zone of Weil) and cell-rich zones as well as a pulpal core, which contains a rich vascular network, a neural supply and a large number of cells (see figure 1.3) (Provenza, 1958; Zhang et al, 1998). The cell-free zone is located immediately beneath the odontoblast layer and represents a 40 μm wide richly innervated area of the pulp containing fibroblast processes and a capillary network (Linde and Goldberg, 1993). The pulp contains a heterogeneous mixture of cells including fibroblasts, odontoblasts, white blood cells, macrophages, as well as a multipotent progenitor population (Goldberg and Smith, 2004). This collection of cells is predominantly localised to the cell-rich zone of the pulp. However, the exact location of the progenitor population is at present not fully understood (see section 1.6.1). The heterogeneous collection of cells within the pulp is surrounded by a fibrous extracellular matrix that is rich in type-I and type-III collagen fibres, providing pulp architecture and elasticity, as well as a mixture of GAGs and PGs (Goldberg and Smith, 2004). Cells located within the pulp have been shown to express members of the transforming growth factor-β (TGF-β) superfamily, as well as VEGF, FGF-2 and PDGF. These factors are associated with the maintenance of tissue homeostasis, as well as progenitor cell proliferation, migration and differentiation

following dental injury (see section 1.2.1 and 1.2.2) (Toyono *et al*, 1997; Sloan *et al,* 1999; Matsushita *et al*, 2000; Kim *et al*, 2010).

The pulp is a well innervated tissue containing both sensory and autonomic nerves that enable it to perform vasomotor and defensive functions (Okamura *et al*, 1995; Rodd and Boissonade, 2001). Sensory nerves within the pulp are principally located below the cell rich zone and form the plexus of Raschkow (Linde and Goldberg, 1993). As sensory neurons of the plexus migrate into the cell-rich zone of the pulp and beyond reaching the surrounding odontoblast layer they lose their myelin coating and function as pain receptors involved in the initiation of tertiary dentinogenesis (Magloire *et al*, 2010). The pulp is also richly vascularised with arterioles running centrally and branching off to form capillary networks at the periphery of the tissue. These capillaries are most evident during active dentinogenic phases and are closely related to the secretory activity of odontoblasts (Yoshida and Ohshima, 1996). The capillary network provides the circumpulpal odontoblasts with a source of nutrients and oxygen, as well as disposing of metabolites (Provenza, 1958). The network is also responsible for supplying a systemic source of raw materials required for the mineralisation of pre-dentine at the calcification front (Yoshida and Ohshima, 1996). Increases in blood pressure resulting from pulpal inflammation activate neurons that transduce the pain stimulus into action potentials that are received by the brain. The presence of shunt vessels that are able to open in response to increased blood flow are considered to be important for controlling increases in intrapulpal pressure resulting from inflammation due to the action of dental caries (Kim *et al*, 1992). Such direct control over blood pressure is particularly important within the pulp due to its confinement within a structurally rigid wall of dentine and enamel.

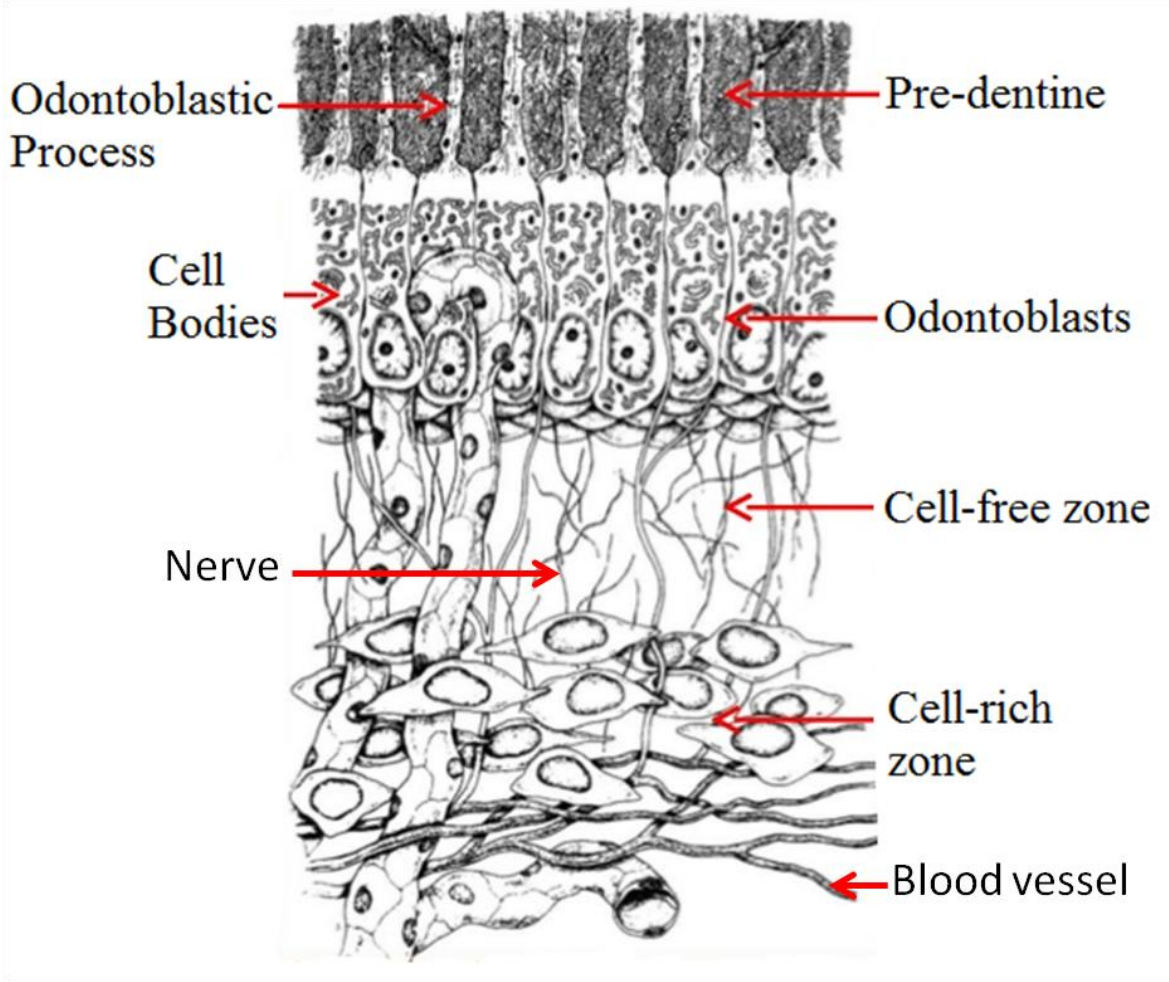

Figure 1.3 Illustration depicting the cell-rich and cell-free zones of the dental pulp along with the surrounding odontoblast layer and accompanying pre-dentine matrix. The cell-rich zone contains a rich network of blood vessels that supply the pulp with oxygen and nutrients, as well as a collection of neurons that are able to transduce stimuli resulting from injury. Modified from Ten Cate, Oral Histology. (2007)

1.4 Odontoblasts

Odontoblasts are large columnar cells that line the periphery of the dental pulp. These cells function in the intracellular accumulation of calcium and the secretion of a dentine ECM, as well as in the maintenance of tissue homeostasis (Linde and Goldberg, 1993). The morphology of these cells is known to differ depending on their level of maturity (see figure 1.4) and the location of these cells in the odontogenic layer, since odontoblasts located near the crown are larger than those located near the root (Simon *et al*, 2009). Immature odontoblasts are also relatively small cells containing limited cytoplasm and an under-developed RER and Golgi complex. As these cells begin to mature and become secretory they develop an extensive rough endoplasmic reticulum (RER), Golgi apparatus, multiple mitochondria, lysosomes and secretory vesicles (Sasaki and Garant, 1996; Simon *et al*, 2009). These intracellular organelles are required for the formation and secretion of an organic collagenous matrix that forms pre-dentine followed by the deposition of calcium phosphate required for dentine maturation. Odontoblasts lie on the formative surface of dentine and as the thickness of this matrix increases it forces the odontoblasts to move pulpally (Larmas, 2008). As the tissue mineralises, the remaining odontoblast processes become surrounded by a mineralised matrix forming the characteristic tubules associated with dentine (Nino-Barrera *et al*, 2013). As these cells become less active they become more cuboidal with less cytoplasm and decreased numbers of RER complexes (Couve, 1986; Simon *et al*, 2009). As odontoblasts mature the secretory apparatus becomes reduced through the action of autophagic activity, the remaining cellular organelles become relocated to the base of the cell and the odontoblast becomes quiescent (Couve, 1986; Couve *et al*, 2013) (see figure 1.4).

Odontoblasts are partly characterised by an elongated cytoplasmic process that extends from the cell body into dentinal tubules. The exact depth of the process is a highly debated topic with some studies claiming that the process extends to reach the dentinal surface (Maniatopoulos and Smith, 1983; Aubin, 1985; Sigal, 1985), while others claim that processes are limited to the inner pulpal third of dentine (Brannstrom and Garberoglio, 1972; Holland and Rogers, 1975). It is thought that the exact length of the odontoblast process is related to age with evidence indicating that the process only reaches the dentinal surface at early developmental stages (Byers and Sugaya, 1995). These processes contain no organelles but comprise of a large collection of structural proteins such as actin, vimentin and tubulin as well as secretory vesicles (Sigal *et al*, 1985). Lateral branches extend from the main process and are thought to provide routes for the diffusion of nutrients and matrix molecules as well as facilitating cell-cell and cell-matrix interactions and communication (Arana-Chavez and Massa, 2004). During dentinogenesis, these processes function in the transport of secretory vesicles containing pro-collagen, which is deposited in the developing ECM (Garces-Ortiz *et al*, 2013). Odontoblast processes also facilitate the movement of calcium into pre-dentine during the formation of mature dentine (Linde, 1995). Furthermore the odontoblast process also acts as sensory receptor, either directly or hydrodynamically, that is able to detect environmental alterations (Holland, 1985). Developing dentine matrix forms around these elongated cellular processes, eventually mineralising to form a dentinal tubule (see section 1.4.2).

Odontoblasts also have a defensive role and are involved in the initiation of inflammatory responses following pathogenic invasion of the dentinal tubules (Couve *et al*, 2013). Due to the fact that odontoblasts extend their long cellular processes through the dentinal layer they represent the first cells to be encountered by microorganisms that successfully

penetrate through the protective enamel and dentine layers of the tooth (Cooper *et al*, 2010). Adjacent odontoblasts are connected by inter-cellular junctions that provide an impervious barrier, which is the first line of defence against pathogenic pulpal invasion (Holland, 1975). The odontoblast layer is closely associated with dendritic cells and lymphocytes that are thought to sense and react to the infiltration of exposed dentinal tubules (Ohshima *et al*, 1995). Odontoblasts have also been shown to express bacteria-recognising Toll-like receptors (TLRs) and NOD-like receptors (NLR) on their cell surfaces that can recognise bacterial components and respond by producing cytokines that initiate an inflammatory response (Keller *et* al, 2010; Farges *et al*, 2011). This has been corroborated by studies *in vitro* demonstrating that odontoblasts respond to the presence of bacterial components such as lipoteichoic acid (LTA) that are common to gram positive bacteria primarily found in dentinal caries (Carrouel *et al*, 2013; Farges *et al*, 2011).

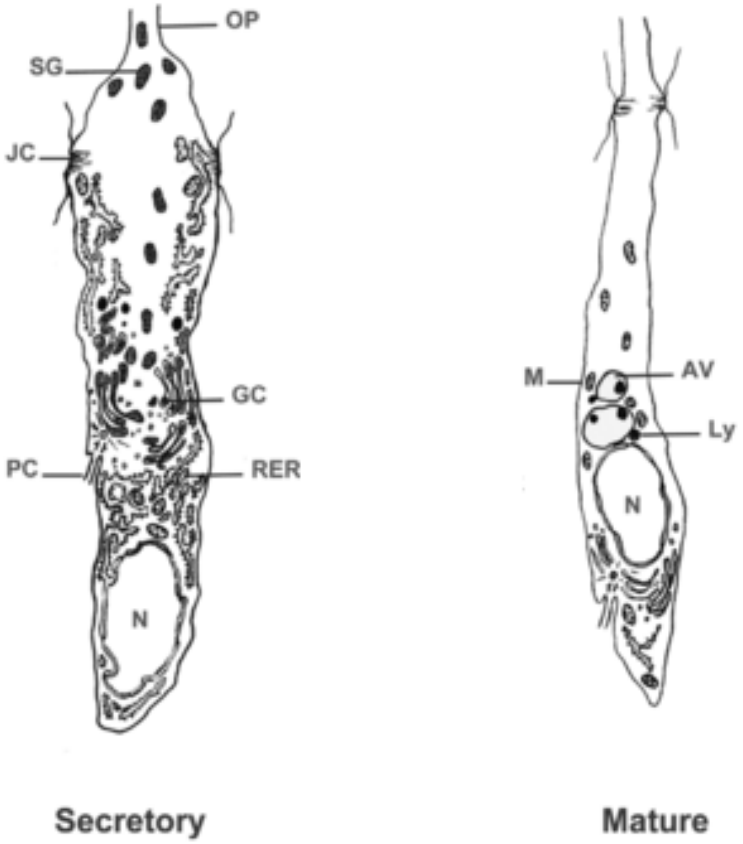

Figure 1.4 Illustration of the morphological and intra-cellular changes associated with odontoblast maturation. Secretory odontoblasts form the primary dentine matrix and contain an extensive RER and Golgi complex required for the transportation of organic and inorganic matrix constituents. As the tooth ages odontoblasts reduce their secretory activity and become mature. Mature odontoblasts are characterised by the presence of autophagic vesicles and mediate the deposition of secondary dentine. GC, Golgi complex; JC, junctional complexes; Ly, lysosome; M, mitochondria; N, nucleus; OP, odontoblastic process; PC, primary cilium; RER, rough endoplasmic reticulum; SG, secretory granules; AV, autophagic vesicles (modified from Couve and Schmachtenberg, 2011).

1.4.1 Odontoblast Development

Understanding the signalling processes involved in physiological odontoblast induction and differentiation is important if we are to develop biological mechanisms for dental repair. The generation of dentine-producing odontoblasts represents a major research challenge in regenerative dentistry and could provide a biological basis for regenerative strategies. Odontoblasts are derived from migrating cranial neural crest cells that form a layer of oral ecto-mesenchyme that condenses around the epithelial bud. The odontogenic mesenchyme forms cells of the dental papilla during the cap stage of tooth morphogenesis, during which reciprocal interactions that occur between the early oral epithelium and underlying mesenchyme results in the formation of the developing epithelial tooth bud (Ruch et al, 1995; Sharpe, 2001; Lisi *et al*, 2003). Signals derived from the dental epithelium are sequestered and immobilised within the basement membrane and presented to peripheral dental papilla cells during the induction of odontoblast differentiation, leading to the polarization of odontoblasts (Ruch *et al*, 1995). The differentiation of these cells is characterised by withdrawal from the cell cycle, cytological polarisation and the secretion of pre-dentine/dentine matrix (Lesot *et al*, 2001). Currently, knowledge of the mechanisms leading to odontoblast differentiation is far from complete. However, studies have indicated that members of the transforming growth factor-β family, such as TGF-β1, 2 and 3, as well as BMP-2, -4 and -6 are capable of inducing odontoblast differentiation *in vitro* (Smith and Lesot, 2001; Begue-Kirn *et al*, 1992; Li *et al*, 2011; Kawai *et al*, 2013). It is likely that combinations of these factors are likely to originate from the oral epithelium, basement membrane and primary enamel knot during tooth morphogenesis (Ruch *et al*, 1995; Lesot *et al*, 2001). In particular, the action of BMP-2 and TGF-β1 have been demonstrated to promote the expression of transcription factors involved in the

differentiation of odontoblasts, such as core-binding factor 1 (Cbfa1) and DSPP respectively (Ducy et al, 1997; He *et al*, 2004). Odontoblast differentiation has also been shown to be accompanied by the coordinated expression of unique sets of genes like FGF-1 and -2, and nuclear factor I-C (NFI-C), indicating that a complex network of factors may be immobilised and presented by the basement membrane to induce odontoblast differentiation *in vivo* (Ruch *et al*, 1995; Unda *et al*, 2001; Lisi *et al*, 2003; Lee *et al*, 2009). Other factors involved in the differentiation process include systemically derived molecules such as IGF-1, which has been shown to stimulate cytological differentiation, such as the re-organisation of microfilaments and fibronectin during odontoblast polarisation, but not functional differentiation of these cells (Lesot *et al*, 2001).

Odontoblast differentiation is also accompanied by cytoskeletal reorganisation. Alteration in the cytoskeletal arrangement of differentiating odontoblasts results in the polarisation of these cells and has an influence on their function, as does the compartmentalisation of intracellular organelles through the formation of a terminal web, which separates the odontoblastic process from the cell body (Linde, 1985; Lesot et al, 1985; Linde and Goldberg, 1993). Interaction between odontoblasts and molecules located in the pre-dentine and dentine matrix, such as the high molecular weight glycoprotein fibronectin, may also be instrumental in determining cytoskeletal organisation during odontoblast terminal differentiation (Thesleff *et al*, 1979; Ruch *et al*, 1995; Yoshiba *et al*, 1996). For instance, fibronectin has been observed to interact with three high molecular weight odontoblast membrane proteins and influence the organisation of microfilaments in these cells (Lesot *et al*, 1992; Yoshiba *et al*, 1996). Inter-cellular interactions in the odontoblast layer resulting from junctions present between odontoblasts are also considered important for the cytological (cytoskeletal reorganisation) and functional activity of these cells, with

alterations in odontoblast activity resulting from an increase in the number and size of junctional complexes formed during odontoblast maturation (Couve, 1986; Ushiyama, 1989; Simon *et al*, 2009).

Terminal odontoblast differentiation is characterised by the transcription of NCPs such as DMP1, DMP2 and DSPP with the accompanying formation of a mineralised dentine matrix. The pattern of NCP expression by odontoblasts varies as these cells differentiate, with each NCP expressed in odontoblasts at specific and overlapping time points (Hao *et al*, 2004). For instance DMP1 levels are decreased in secretory odontoblasts following the mineralisation of pre-dentine, while DSPP and DMP2 levels are sustained in mature odontoblasts (D'Souza *et al*, 1997; Hao *et al*, 2004; Simon *et al*, 2009). Differences in the expression patterns of these three proteins may be explained by the fact that DMP1 has been shown to be important in regulating the transcription of many odontoblast-associated genes such as DSPP and DMP2 (Narayanan *et al*, 2001). The expression of DSPP has also been linked with BMP2, a growth factor expressed during the early stages of tooth morphogenesis (see section 1.2.2). Pathologies relating to the absence of DSPP include dentinogenesis imperfecta type II and III and indicate that this NCP is also likely to have a role in the initiation of odontogenesis (Chen *et al*, 2009). DSPP is further regulated during in odontoblasts by interactions with transcription factors involved in the early stages of dentine mineralisation, such as Cbfa1 and Osterix (Chen *et al*, 2005).

Cbfa1 (also called Runx2) and Osterix represent two important transcription factors required for odontoblast differentiation and the synthesis of many SIBLINGs involved in the progression of dentine mineralisation. Many of the key factors involved in the mineralisation of pre-dentine are also common to bone and are important for the

calcification of osteoid matrix (Staines *et al*, 2012). Cbfa1 is a member of the runt/Cbfa transcription factor family and has a master regulatory role in the development of osteoblasts, and consequently bone; while Osterix is activated downstream of Cbfa1 (Ducy, 2000; Huang *et al*, 2004). These transcription factors have been proposed to play a similar role in the differentiation of odontoblasts, although their temporal expression and relative concentrations may differ from those in bone (Chen *et al*, 2009). Absence of Cbfa1 and Osterix has been linked with severe skeletal abnormalities as a result of arrested osteoblast differentiation, along with dental abnormalities such as osteogenesis imperfecta (Mundlos et al, 1997; Lapunzina *et al*, 2010). Studies analysing the effects of Cbfa1 and Osterix expression on odontogenic genes have demonstrated correlations between the expression of these genes and key odontoblast markers such as DSPP and DMP1, linking these two transcription factors with odontoblast-associated gene transcription (Kobayashi *et al*, 2005; Chen *et al*, 2009). The expression of Cbfa1 and Osterix overlap within the developing dental and osteogenic mesenchyme, although only Osterix is strongly expressed in mature odontoblasts (E12 to E16), whereas both are strongly expressed in the mature osteoblasts indicating that Cbfa1 may be primarily required for the initiation of dentine mineralisation (Ducy, 2000; (Chen *et al*, 2009). This is further confirmed by the temporal role of Cbfa1 in the determination of odontoblast lineage commitment in progenitor cell populations located in the dental papilla (Gaikwad *et al*, 2001). Indeed, the involvement of these factors during tooth development is thought to be distinct from their roles during bone morphogenesis, functioning as potential regulators during epithelium-mesenchymal signalling for the progression of tooth morphogenesis (D'Souza *et al*, 1999).

1.4.2 The Role of the Odontoblast in Dentine Maturation

Odontoblasts synthesise pro-collagen, which is packaged in transport vesicles that migrate to the Golgi complex where the collagen pre-cursor becomes glycosylated (Weinstock and Leblond, 1974). Following post-translational modification in the Golgi complex pro-collagen is transported in secretory vesicles to the secretory pole of the odontoblast where these vesicles fuse with the cell membrane and the pro-collagen is exocytosed proximally into the surrounding ECM (Sato *et al*, 2009). Once deposited in the ECM the pro-collagen becomes activated, losing its amino acid and carboxy-terminal fragments, and forms the collagen fibrils of pre-dentine (Weinstock and Leblond, 1974; Frank, 1979). NCPs of the developing dentine matrix such as proteoglycans, phosphoproteins, SCPPs such as osteocalcin, and SIBLINGs such as DMP1, BSP and ON, are secreted along a similar pathway but their exocytosis occurs at the distal end of the odontoblast near the mineralisation front (Linde and Lundgren, 1995). The rate of NCP synthesis, transport and secretion is also much faster than that of collagen requiring minutes rather than days for these NCPs to reach the ECM. Following secretion NCPs localise within the dentine matrix and play extensive roles in the nucleation, expansion and regulation of hydroxyapatite deposition within the dental matrix (Huang *et al*, 2008; Hao *et al*, 2009).

The maturation of dentine is characterised by the mineralisation of pre-dentine and requires the deposition of calcium and phosphate into the organic matrix. Once exported into the pre-dentine matrix the calcium and phosphate ions nucleate and expand forming the calcified dentine matrix (Linde and Lundgren, 1995). The process of mineralisation in mantle dentine initiates with the formation of mineral crystals from matrix-bound vesicles that have been synthesised and exported by primary odontoblasts (see figure 1.4.2)

(Eisenmann and Glick, 1972; Sisca and Provenza, 1972). It is thought that matrix vesicles provide an environment within which the solubility product for calcium phosphate is exceeded to enable crystallisation (Boskey et al, 1978; Boskey *et al*, 1997). Recent studies have indicated that matrix vesicles found within the odontoblast processes may also have an accompanying role in the mineralisation of the circumpulpal dentine matrix with these vesicles shown to bud from the odontoblast and attach to the wall of the dentinal tubules (Garces-Ortiz *et al*, 2013). However, the mineralisation of circumpulpal dentine is primarily considered to lack the involvement of matrix vesicles, with the deposited mineral instead being associated with collagen fibrils and having a more globular appearance (Stratmann *et al*, 1997; Takano *et al*, 2000). This is thought to occur by movement of calcium and phosphate ions from the vascular network of the subodontoblastic area, across the odontoblast cell layer and towards the mineralisation front. In order to achieve this high level of calcium transport odontoblasts require a means of facilitating the movement of intracellular and extracellular calcium. At present the exact mechanism of calcium transport within odontoblasts and the surrounding ECM is not fully understood but is most likely achieved though the presence of calcium channels and Na^{+}/Ca^{2+} exchangers (Lundgren and Linde, 1988, 1992). Furthermore, a high Ca^{2+}-activated ATPase activity has been recognised in the membranes of vesicles localised in the distal portion of the odontoblast cell body, as well as in the odontoblast plasma membrane, which may be responsible for coordinating the movement of ions (Granstrom et al., 1978; Lundgren and Linde, 1988). The regulation of odontoblast calcium transport has also been linked with the presence of calcium binding proteins of the cytoskeleton and cell membrane such as dentine matrix protein 4 and calbindin (Magloire et al, 1988; Linde, 1985; Hao et al, 2007). Many NCPs (e.g. DPP, BSP, OP, ON, OC) (see section 1.2.1) present within the

dentine matrix have been shown to have similar roles in collagen mineralisation as found in bone, acting as both mineral nucleators and inhibitors of crystal growth (Wallwork *et al*, 2002). A study investigating the effect of eliminating the NCPs on bone mineralisation has indicated that the remaining collagenous structure was no longer able to support the deposition of HA, which is also likely to be the case for dentine due to the structural and functional similarities of these tissues (Termine *et al*, 1981).

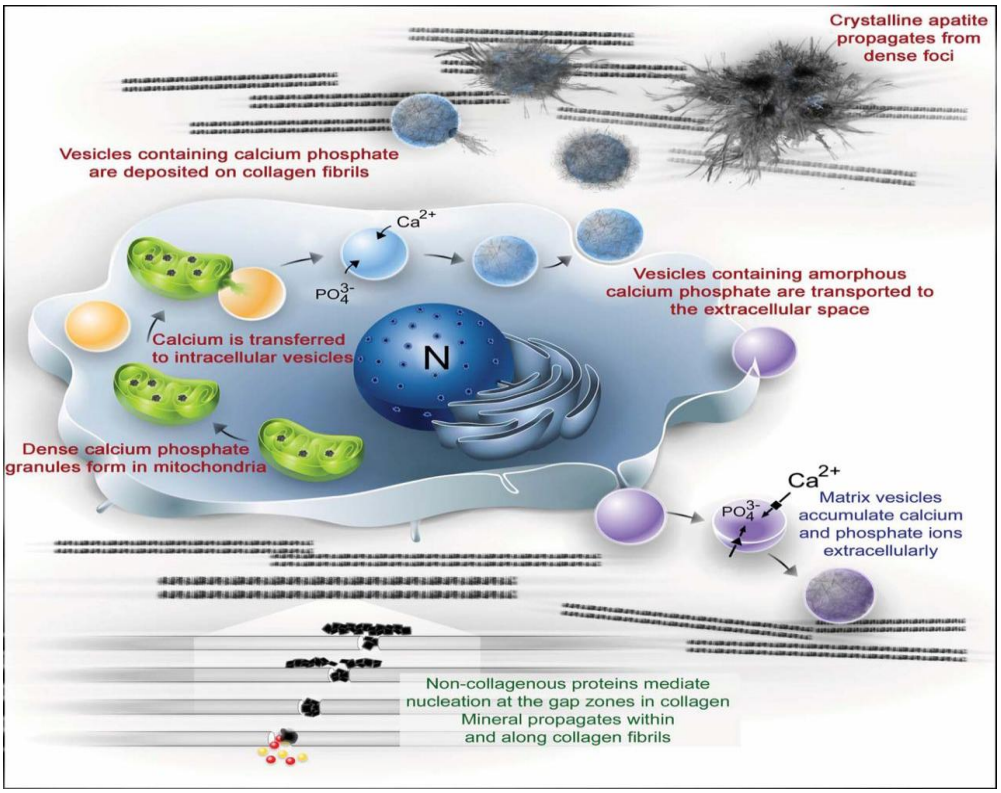

Figure 1.4.2 Diagrammatic representation of the mechanisms of intra-cellular and extra-cellular transport during dentine maturation. The diagram illustrates the presence of matrix vesicles produced by secretory odontoblasts and highlights their role during the initial stages of mantle dentine mineralisation. The diagram also shows the binding of hydroxyapatite to collagen fibrils of the organic dentine matrix, which is primary mode of mineralisation in circumpulpal dentine. Image reproduced from Boonrungsiman et al. (2012).

1.5 Tertiary Dentinogenesis

The action of irritants, dentinal caries or physical trauma on the tooth can facilitate the invasion of micro-organisms within the pulp where the harmful toxins they produce can cause damage. In response to this damage the pulp deposits a layer of tertiary dentine at the pulp-dentine interface. Tertiary dentine is a rapidly deposited matrix that can vary from an ordered tubular arrangement that is structurally similar to primary dentine to a more disordered, atubular matrix containing cellular inclusions (osteodentine) (Linde and Goldberg, 1993). Tertiary dentinogenesis can be sub-divided into two classes depending on the severity of stimulus and the cells that respond to that stimulus. The cells that increase their biological activity in response to the stimulus are either local primary odontoblasts located within the vicinity of the damage or a population of multipotent progenitor cells likely to reside in the dental pulp that proliferate, migrate to the site of damage and differentiate to form odontoblast-like cells (Linde and Goldberg, 1993; Smith et al, 2001; Sangwan et al, 2013).

1.5.1 Reactionary Dentinogenesis

Reactionary dentinogenesis is defined by the capacity of odontoblasts to up-regulate their biological and secretory activity in response to caries or trauma (Smith *et al*, 1995). The biological mechanisms underlying reactionary dentinogenesis are thought to result from the action of endogenous growth factors and bioactive molecules released from the dentine matrix following tooth damage (see figure 1.5). The bioactive molecules released possess a similar inductive potential to those produced by the enamel epithelium and basement membrane during tooth morphogenesis (Smith *et al*, 1995). Tertiary dentine deposited as a

result of reactionary dentinogenesis typically has tubular continuity with secondary dentine, although the exact structure of tertiary dentine depends on the degree of irritation, with more severe damage resulting in a less organised matrix containing a higher number of cellular inclusions (Tziafas, 2010).

1.5.2 Reparative Dentinogenesis

The exposure of dentine by caries, operative procedures or treatment with therapeutic dental materials such as EDTA results in the death of odontoblasts and requires a population of progenitor cells located within the pulp to differentiate to form odontoblast-like cells (see figure 1.5) (Magloire *et al*, 2001). These cells deposit a reparative dentine matrix at the site of injury in a process termed reparative dentinogenesis (Lesot *et al*, 1993; Harichane *et al*, 2011). Similar to reactionary dentinogenesis an injurious stimulus leads to the release of endogenous growth factors and bioactive molecules from the dentine matrix. These dissociated factors initiate cell recruitment, proliferation and differentiation within the dentine-pulp complex and the deposition of tertiary dentine, which provides a protective barrier between the pulp and the external environment (Simon *et al*, 2010).

Reparative dentinogenesis is characterised by the formation of new odontoblast-like cells from local stem/progenitor cells located within the pulp. However, the identity of the stem/progenitor population(s) associated with reparative dentinogenesis remains elusive. Multiple progenitor cell niches have been identified within the dental pulp (Sloan and Smith, 2007; Sloan and Waddington, 2009). The cell-rich layer of Hohl, which lies adjacent to the odontoblast layer, is one example of a niche that contains a population of

undifferentiated mesenchymal cells (Cotton, 1967). These cells are embryologically similar to pre-odontoblasts but lack the final signals leading to odontoblast terminal differentiation (Tziafas, 1995). In continually erupting rodent incisors, progenitor cells can also be found within the dental papilla situated below the inner enamel epithelium and the mesenchymal region of the cervical loop, with these cells demonstrating the potential for odontogenic differentiation both *in vitro* and *in vivo* (Balic and Mina, 2010). Pericytes also represent a population of multipotent cells that have been localised within the vasculature of the dental pulp (Carlile et al, 2000; Janebodin *et al*, 2013). There is however much debate relating to the derivation of pericytes with some reports claiming they represent a stage in the differentiation of mesenchymal stem cells (MSCs), while other reports claim that MSCs are in fact of vascular origin (Caplan, 2008; Crisan *et al*, 2008). Further evidence corroborating the role of pericytes during reparative dentinogenesis was derived from lineage tracing experiments demonstrating that vasculature-derived cells were capable of acting as an MSC-like population for the generation of odontoblasts-like cells following dental trauma (Feng *et al*, 2011). The mature pulp also contains a multipotent stem cell population referred to as dental pulp stem cells (DPSCs) that are derived from the oral ecto-mesenchyme during tooth morphogenesis (Gronthos *et al*, 2000; Wang *et al*, 2013). DPSCs represent a potential source of odontoblast precursors with many studies documenting the odontogenic potential of these cells both *in vivo* and *in vitro* (Huang *et al*, 2006; Huang *et al*, 2010; Gronthos *et al*, 2011).

Corroboration of the role of DPSCs during reparative dentinogenesis has been provided by the evaluation of DPSC signalling pathways following dental injury. Studies have confirmed that Notch receptors associated with MSC differentiation become activated on DPSCs following dental injury (Lovschall *et al*, 2005; Zhang *et al*, 2008). Further proof of the involvement of DPSCs in reparative dentinogenesis was obtained from comparative analysis of the gene expression profiles of pulp cells derived from healthy teeth with those from mildly carious teeth (Paakkonen *et al*, 2005). This comparison identified two notch ligands (Jagged2 and JaG2 and mitogen-activated protein kinase) implicated in the differentiation of progenitor cells towards replacement odontoblasts following tooth damage. These ligands were expressed by cells derived from carious pulp tissue, but absent from healthy tissue and prove that DPSCs are involved at some level in dentinogenic response to tooth injury (Paakkonen *et al*, 2005; Mitsiadis *et al*, 1999).

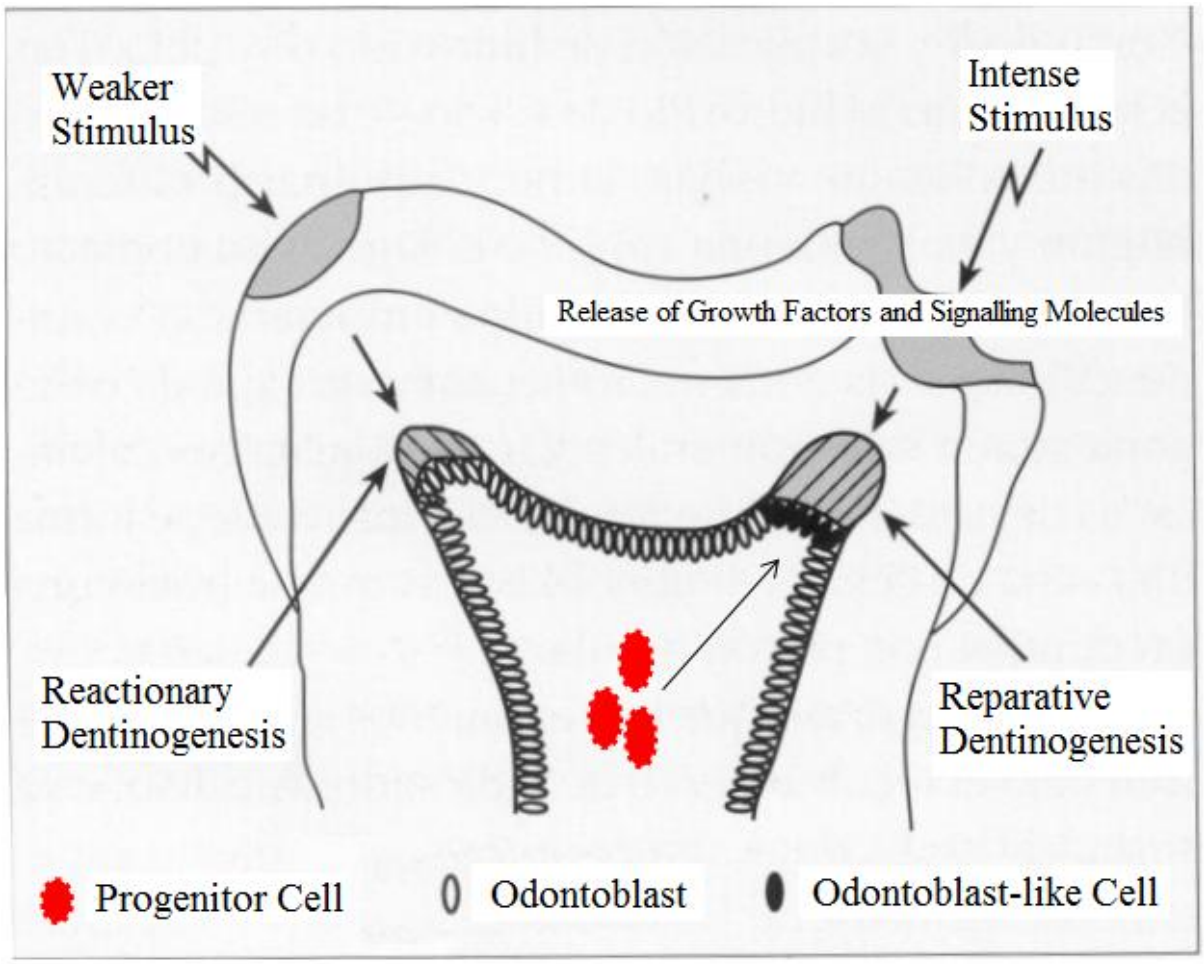

Figure 1.5 Diagrammatic representation of a cross section of a tooth illustrating the key events required for the deposition of tertiary dentine. The figure demonstrates the up-regulation of odontoblasts during reactionary dentinogenesis, as well as the proliferation and recruitment of pulpally located stem/progenitor cells required for the production of reparative dentine in response to growth factors and signalling molecules released following tooth damage. Modified from Smith *et al*. (1995).

1.6 Stem Cells

Stem cells are undifferentiated cells with an unlimited self-renewal capacity that are capable of differentiating towards specialised cell types in response to specific molecular cues. These cells are capable of symmetric divisions, creating two daughter cells both endowed with stem cell properties, or asymmetric divisions in which one stem cell and one tissue progenitor cell is produced. Stem cells can be subdivided based on their differentiation potentials into totipotent, pluripotent and multipotent types. These cells are highly valuable for the progression of regenerative clinical therapies, pharmaceutical testing, and the development of bioprosthetics (Egusa *et al*, 2012). The following chapters will define stem cell subsets and outline the role of these cells in regenerative dentistry.

1.6.1 Embryonic Stem Cells

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of the blastocyst and give rise to all the somatic cells types present in an embryo. Stable ESC lines were first developed in 1981 and are characterised by an indefinite self-renewal capacity and the potential to form cells of all three embryonic germ layers (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al*, 1998). The potential of ESCs for regenerative medicine is great with clinical trials currently underway for the treatment of degenerative eye conditions, Stargardt's macular dystrophy and age-related macular degeneration (Schwartz *et al*, 2012). The study of ESC signalling pathways that regulate self-renewal and differentiation is also providing insights into early embryonic development, cancer and the potential of these cells for regenerative medicine (Evans, 2011).

1.6.2 Post-Natal Stem Cells

1.6.2.1 Mesenchymal Stem Cells

The term mesenchymal stem cell did not originate until 1991, two decades after the initial discovery of colony-forming multipotent cells within the bone marrow (Friedenstein *et al*, 1970; Caplan, 1991). These cells have since been isolated from almost every bodily tissue and have proved useful for the advancement of many regenerative therapies, such as bone, cardiac and neuronal regeneration (Zavan *et a*l, 2010; Loughran et al, 2013; Lopez and Daigle, 2013). MSCs are currently defined by three criteria established by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT). The definition states that to be defined as an MSC a cell must: be able to adhere to tissue culture polystyrene; be CD73, CD90, and CD105 positive and CD45, CD34, CD14, CD11b, CD79, CD19 and HLA-DR negative; and have the ability to differentiate into osteoblasts, chondroblasts and adipocytes *in vitro* (Dominici *et al*, 2006). Further collation of positive markers used for the characterisation of MSCs has indicated that CD29 and CD44 also represent valuable markers that are highly conserved between species (see table 1.6.2.1) (Harting *et al*, 2008; Mafi *et al*, 2011). A more recent development has seen the identification of several markers of stem cell renewal and maintenance once thought to be exclusive to embryonic stem cells (see section 1.6.1) in adult MSCs, specifically Oct4, Nanog and SOX2 (Riekstina *et al*, 2009; Tsai and Hung, 2012). These markers function in the maintenance of embryonic stem cell self-renewal and regulate the expression of loci involved in pluripotency and differentiation. Studies characterising the roles of these factors in post-natal stem cells are as yet limited, although

it has been proposed that they are likely to function in MSC maintenance of cell plasticity (Kashyap *et al*, 2009; Varlakhanova *et al*, 2010; Shyh-Chang, 2013).

Bone marrow stem cells are perhaps the best characterised of all the post-natal stem cells. Since the bone marrow contains minimal ECM, these cells can be isolated without the use of digestive enzymes typically employed during the isolation of MSCs from other tissues, such as adipose-derived stem cells (ADSCs) and dental pulp-derived stem cells (DPSCs) (Bianco *et al*, 2001). The estimated proportion of stem cells within the bone marrow is approximately 0.001-0.1 %, which is lower than the proportion of MSCs found in adipose tissue (approximately 10 %) (Wexler *et al*, 2003; Zuk, 2010). MSCs isolated from bone marrow were first identified as a population of stromal osteogenic progenitor cells that could be distinguished from the haematopoietic population based on their ability to adhere to tissue culture polystyrene and their fibroblast-like morphology (Friedenstein *et al*, 1970). Additionally, transplantation of these cells *in vivo* demonstrated they had a multi-differentiation capacity (Friedenstein *et al*, 1987). Subsequently BMSCs have become the most widely used MSCs for regenerative research with studies demonstrating the ability of these cells to differentiate to neurogenic and myogenic lineages (Woodbury *et al*, 2000; Gojo *et al*, 2003). Furthermore, the number of clinical trials involving BMSCs far exceeds those of other MSC (Chen *et al*, 2006; Segers and Lee, 2008; Nery *et al*, 2013).

Marker	Functional Details
CD29	An integrin transmembrane receptor involved in the assembly of ECM components (collagen, laminin and fibronectin) (Cybulsky et al, 1992)
CD44	Cell membrane glycoprotein that tethers to the ECM and monitors changes that influence cell survival, proliferation and differentiation (Aruffo et al, 1990; Ponta et al, 2003)
CD73	Plasma membrane located nucleotidase involved in cell adhesion as well as immunity, inflammation and the regulation of apoptosis (Ohta et al, 2013; Mikhailov et al, 2008; Antonioli et al, 2013)
CD90	GPI-anchored glycoprotein involved in cell-cell and cell-matrix interactions and the regulation of inflammation (Rege and Hagood, 2006; Wandel et al, 2012)
CD105	Membrane glycoprotein component of the TGF-β receptor complex that has associations with the vascular system (Li et al, 2003; Rius et al, 1998)

Table 1.6.2.1 Table illustrating the five most common markers used for the characterisation and isolation of human, mouse and rat-derived MSCs and their related biological functions. Markers were selected based on the frequency of their use in MSC isolation studies throughout the literature.

1.6.2.1.1 Adipose-Derived Stem Cells

Adipose tissue can be macroscopically divided into at least five types with each serving its own specific function: white, brown, mammary, bone marrow-specific and mechanical (Gimble *et al*, 2007). White adipose tissue is the most prevalent throughout the body and represents the most abundant source of ADSCs (Algire *et al*, 2013). Adipose tissue is a loose connective tissue largely composed of mature adipocytes but also containing stromal vascular cells such as fibroblasts, smooth muscle cells, pericytes, endothelial cells and adipogenic progenitor cells (Niemela *et al*, 2007). Adipose tissue primarily functions as a store of energy in the form of lipids but also acts as a major endocrine organ and provides bodily insulation (Kershaw and Flier, 2004).

Knowledge of progenitor cell types within adipose tissue dates back to the late 1980's when cells capable of adipogenic differentiation were first isolated (Deslex, 1987; Hauner, 1989). These cells were termed pre-adipocytes and it took over ten years for the multipotent capacity of these cells to be recognised (Zuk *et al*, 2001, Gimble and Guilak, 2003). The anatomical location from which ADSCs are harvested has no observable influence on morphology, proliferation or immunophenotype (Jurgens *et al*, 2008; Kaewkhaw *et al*, 2011). However, recent evidence has indicated that the anatomical site of derivation in human donors may influence the differentiation capacity of these cells (Requicha *et al*, 2012). Subcutaneous and perinephric adipose tissue have also been shown to contain multipotent cells that possess an increased neurogenic capacity (Kaewkhaw *et al*, 2011). Furthermore, the differentiation potential of ADSCs may be inversely related to the depth of adipose tissue from which they are obtained, with ADSCs

isolated from superficial fat possessing an improved differentiation potential when compared with those isolated from deeper adipose layers (Aksu *et al*, 2008).

A variety of terms have been employed to define the tissue culture polystyrene adherent population of multipotent cells isolated from adipose tissue (Gimble *et al*, 2007). To eliminate any discrepancies in nomenclature, the International Fat Applied Technology Society (IFATS) reached a consensus to adopt the term "adipose-derived stromal/stem cells" (Mitchell *et al*, 2006; Gimble *et al*, 2007). ADSCs are most often described as cells obtainable from the cultured stromal vascular fraction (SVF) of an adipose digest that adhere to tissue culture polystyrene and give rise to the colony forming unit fibroblast (CFU-F) (Zuk *et al*, 2001; Gimble *et al*, 2007). These cells like other MSCs should express markers defined by the ISCT (see section 1.6.2.1) and demonstrate the ability to differentiate toward several specific lineages including adipogenic, osteogenic or chondrogenic, if cultured under suitable conditions (Gimble *et al*, 2007). A recent definition by IFATS characterised ADSCs as a CD45-CD235a-CD31-CD34+ cell type that can be distinguished from BMSCs by the presence of CD36 and absence of CD106 (Bourin *et al*, 2013).

Over the last two decades various studies have shown that ADSCs can be differentiated into a variety of cell types such as oligodendrocytes and Schwann cells, epidermal cells, hepatocytes, and pancreatic islets (Seo *et al*, 2005; Timper *et al*, 2006; Kingham *et al*, 2007; Banas *et al*, 2007; Chandra *et al*, 2009; Mitzuno and Nambu, 2011). Freshly isolated lipoaspirates contain relatively low levels of contaminating pericytes, endothelial cells, or smooth muscle cells (Zuk *et al*, 2001), while stem and progenitor cells within freshly isolated adipose tissue represent up to 10 % of the total cell population with

approximately 0.05 - 2 × 10^5 stem cells isolated per gram of adipose tissue, a much larger proportion than present in bone marrow (see section 1.6.2.1) (Oedayrajsingh-Varma *et al*, 2006; Zuk, 2007; Fraser *et al*, 2008). At present the precise localisation of ADSCs remain unclear with proposals suggesting they are a subpopulation of stem cells located within the vasculature that exit the circulation and become lodged within adipose tissue (see section 1.6.3) (Crisan *et al*, 2008; Lin *et al*, 2010; Zimmerlin *et al*, 2010).

If MSCs are to be potentially useful in regenerative medicine, they should be abundant, easily isolated and harvested using minimally invasive procedures, differentiate towards multiple lineages in a reproducible manner and be safely and effectively transplanted into an autogenic or allogenic host (Gimble *et al*, 2007). ADSCs therefore potentially represent a model source of MSCs, since these cells can be isolated in large quantities under local anaesthesia with little patient discomfort in contrast to bone marrow isolations, which often result in pain as well as high donor-site morbidity (Zuk *et al*, 2001; Schaffler and Buchler, 2007). ADSCs have also been shown to be less prone to reductions in differentiation capacity and the expression of MSC markers associated with increased donor age or prolonged *in vitro* expansion than other MSCs. In fact, ADSCs have been shown to retain a stable differentiation capacity and MSC marker profile for ≥70 population doublings, almost double that of BMSC populations (Bruder *et al*, 1997; Bianco *et al*, 2001; De Ugarte et al, 2003; Wu *et al*, 2013).

1.6.2.1.2 Dental Stem Cells

The cells responsible for forming tertiary dentine following exposure of the pulp with subsequent differentiation into odontoblast-like cells were initially identified as fibroblasts

and endothelial cells based on local proliferation of these cells after tooth damage (Fitzgerald, 1979). It took another two decades for these odontoblast-forming cells to be recognised as a resident stem cell population existing within the pulp tissue (Gronthos *et al*, 2000). These cells were of neural crest origin and ontological analysis demonstrated that they continued to express genes related to blood vessel and neuron formation when cultured *in vitro* (Kim *et al*, 2011). DPSCs have since been shown to be a highly proliferative when compared with MSCs derived from bone marrow, potentially due to a comparatively increased expression of cell cycling mediators such as IGF-1 and cyclin-dependant kinase 6 (Shi *et al*, 2005). DPSCs have also been shown to possess a multi-differentiation potential and have demonstrated the capacity to form dentine-like tissue both *in vitro* and *in vivo* (Gronthos *et al*, 2002; Yu *et al*, 2006; Huang *et al*, 2010; Casagrande *et al*, 2011). These cells have express MSC markers defined by the ISCT and are commonly identified by the presence of the cell surface protein, Stro1 (Yang *et al*, 2007; Yang *et al*, 2009).

Characterisation of DPSCs led to the discovery of further multipotent populations of cells within the oral environment. Stem cells from human exfoliated deciduous teeth (SHED) were documented three years after DPSCs and have since been shown to possess a higher rate of proliferation and the capacity to differentiate towards odontogenic and osteogenic lineages (Miura *et al*, 2003). Soon after the discovery of SHED cells, a population of stem cells were isolated from the periodontal ligament of human third molar roots (Seo *et al*, 2004). Periodontal ligament stem cells (PDLSC) demonstrated the ability to form cementum-like tissue *in vivo* and have since been isolated from the alveolar bone surface (Seo *et al*, 2004; Wang *et al*, 2011). Stem cells from the apical papilla (SCAP) were the next population of multipotent dental stem cells to be reported (Sonoyama *et al*, 2006).

These cells demonstrated a similar proliferative capacity and multi-differentiation potential to SHEDs (Sonoyama *et al*, 2008). The most recently identified population of MSCs are the gingiva-derived stem cells (Tomar *et al*, 2010). Studies have indicated that stem cells derived from dental tissues typically have a greater proliferative potential when compared with BMSCs (Gronthos *et al*, 2000; Alge *et al*, 2010; Tamaki *et al*, 2013). However, due to the comparatively low volume of tissue obtainable from dental sources and the loss of MSC phenotype with increased passage (typically reported at around passage 10), the future clinical applicability of these cells remains debatable (Jing *et al*, 2007; Patel *et al*, 2009).

1.6.2.1.3 The Accuracy of MSC Phenotyping

No individual marker outlined by the ISCT (see section 1.6.2.1) is unique to MSCs. Conflicting results have also emerged throughout the literature regarding the expression of ISCT negative markers, as well as other commonly used MSC markers such as CD10, CD34, CD36, CD44, CD49d, CD49f, CD106 and CD146 (Mafi *et al*, 2011; Pachon *et al*, 2011). For instance, in many studies MSCs isolated from adipose tissue are described as having a CD34+ CD31- immuno-phenotype, while remaining positive for other MSC markers such as CD105, CD90, and CD73 (Dominici *et al*, 2006; Lin *et al*, 2010). ADCs are also unique in the sense that their expression of Stro1, one of the most common adult stems cell markers, is routinely disputed (Gronthos *et al*, 2001; Lin *et al*, 2008, 2010, 2011). Indeed, ambiguities relating to differences in the expression of cell surface markers are widespread throughout the literature and may be due to the semi-quantitative nature of certain profiling methods (e.g. sqRT-PCR). Differences may also be related to the fact that

immunophenotypic studies are often performed using different species even though information regarding the transferability of MSC marker characteristics among these species is limited (see section 1.6.3). Furthermore, the use of non-standardised isolation protocols for the dissociation of MSCs (particularly ADSCs and DPSCs) may also contribute to variations in the immunophenotype of these cells (Horn *et al*, 2011; Baer and Geiger, 2012; Hilkens *et al*, 2013).

Differences have also been documented in the ability of MSCs sourced from different tissues to differentiate towards osteogenic, adipogenic and chondrogenic lineages equally (Al-Nbaheen *et al*, 2013). Indeed, many studies have indicated that ADSCs may have a comparatively reduced osteogenic potential in comparison with BMSCs or DPSCs (De Ugarte *et al*, 2003; Zaminy *et al*, 2008; Hayashi *et al*, 2008). However, in contrast the adipogenic, myogenic and cardiomyogenic potentials of ADSCs appear relatvely increased increased when compared with BMSCs or DPSCs (Im *et al*, 2005; Huang *et al*, 2005; Pachon-Pena *et al*, 2011). Gronthos *et al.* (2000) have further indicated differences in the rate of proliferation and colony-forming unit capacity of BMSCs and DPSCs, as well as differences in the appearance of mineral deposited by these MSCs following osteogenic culture (Gronthos *et al*, 2000). Moreover, variations have also been documented relating to the secretion of various paracrine factors by MSCs isolated from different tissues with ADSCs demonstrating a comparatively high expression of IGF-1, VEGF-D and IL-8 when compared with BMSCs (Hsiao *et al*, 2012).

1.6.2.1.4 Inter- and Intra-Species Differences between MSCs

As mentioned in Section 1.6.2.1, a profile of markers has been specified by the ISCT for the selection of MSCs from heterogeneous populations. However the majority of studies defining stem cell surface markers are often conducted using human or murine MSCs with less data available for rat-derived MSCs. Such a gap in knowledge makes the identification of MSCs within rats difficult and relies on the cross-transferability of information obtained using human or mouse-derived MSCs. The immunophenotyping of rat-derived MSCs has demonstrated positivity for ISCT defined markers, such as CD73, CD90 and CD105 (Karaoz *et al*, 2009; Harting *et al*, 2008; Barzilay *et al*, 2009). However, such profiles have been primarily constructed using MSCs derived from rat bone marrow and no reports have been published comparing rat BMSCs with MSCs sourced from other tissues such as adipose or dental pulp. A lack of commercially available antibodies is likely to be a contributing factor in the limited immunophenotyping of rat-derived MSCs. Ambiguities relating to the expression of defined MSC markers may result from studies using antibodies targeted against humans or mice in other related species, even though there may only be limited cross-reactivity (Boxall and Jones, 2012). A comprehensive evaluation of markers used for the selection of MSCs among different species has indicated that CD29 and CD44 represent the most widely expressed surface antigens, while certain ISCT recommended markers are frequently absent from MSCs isolated from pig, goat, sheep and horse (Boxall and Jones, 2012). Evidently the need for in-depth and comparative profiling of MSCs sourced from different anatomical locations in a wide number of species is important for the progression of tissue engineering research.

1.6.3 Stem Cell Niche

The concept of a stem cell niche was originally proposed in the late seventies when scientists noted that haematopoietic stem cells were regulated by their association with discrete cellular microenvironments (Dexter *et al*, 1977; Schofield, 1978; Allen, 1978). The stem cell niche has since been shown to play a pivotal role in controlling the participation of its cells in tissue maintenance, repair and regeneration through the action of endocrine and paracrine factors involved in Notch, Wnt and hedgehog (hh) signalling pathways (Mitsiadis *et al*, 2007, 2011). The niche may comprise of a wide network of various cell types that function in the maintenance of MSCs, or simply be refined to signals derived from the basement membrane of the ECM and other non-cellular components such as NCPs, inorganic ions and metabolic products (Scadden *et al*, 2006; Hynes, 2009). It has been proposed that the regulation of asymmetric divisions by the basement membrane may be crucial for stem cell polarity and the prevention of uncontrolled cell divisions that may lead to the development of cancers (Singh, 2012). Recently a model of a non-specialised stem cell niche has been proposed (Ema and Suda, 2012). The non-specialised niche is thought to comprise of a wide range of cells such as MSCs, fibroblasts, adipocytes, vascular endothelial cells, neurons and blood cells. The signals involved in MSC maintenance in this instance are far more complex since they originate from multiple cell types and may affect MSCs both directly and indirectly. Hence, the concept of the stem cell niche is a complex topic that needs to be further studied in order to provide a basis for manipulation of MSCs for regenerative medicine.

Many MSCs routinely used in tissue engineering research are understood to reside in a perivascular niche (Zannettino *et al*, 2008). Both DPSCs and BMSCs have been identified

within perivascular regions using Stro1 and CD146 localisation (Shi and Gronthos, 2003; Kolf *et al*, 2007). CD146 represents a cell surface glycoprotein that has been identified as a pericyte marker and its co-localisation with Stro1 raised many questions as to the potential perivasular microenvironment of MSCs, or whether pericytes are in fact MSCs (Caplan, 2008). Moreover, the expression of α-smooth muscle actin, a protein primarily associated with blood vessels, and CD106, a vascular cell adhesion molecule associated with endothelial cells of the blood vessel wall, by DPSCs and ADSCs has further strengthened this hypothesis (da Silva Meirelles, 2006; Kolf *et al*, 2007). Furthermore, the localisation pattern of MSC markers within the capillaries, arteries, smooth muscle and endothelium of adipose tissue has indicated that this stem cell population may in fact reflect a group of vascular precursor stem cells at multiple stages of differentiation (Lin *et al*, 2008). This hypothesis has been developed by studies demonstrating the capacity of cells within the adipose SVF to form endothelial cells and aid the formation of blood vessels (Miranville *et al*, 2004; Park *et al*, 2013; Sahar *et al*, 2012). The origin of these vascular cells and their association with MSCs at present remains uncertain and further studies are required to elucidate whether MSCs originate from the local mesenchyme and migrate to these perivascular locations, or if these stem cells are actually derived from the vasculature and acquire their tissue specificity through the action of local signals (Orbay *et al*, 2012). However, it has been implied that the perivascular location of stem cells would facilitate their dispersal throughout the post-natal organism (Feng *et al*, 2010).

1.6.4 ADSCs for Dental Tissue Engineering

Current dental treatments are restorative and provide no biological basis for tooth regeneration. Cellular therapies may eliminate the need for dental implants that can lead to tooth crowding and potential periodontal bone defects caused by direct integration with the maxilla or mandible. Post-natal stem cells represent the ideal cell choice for dental regeneration since these cells have the capacity to differentiate towards odontoblasts, ameloblasts and cementoblasts under the appropriate culture conditions (Wen *et al*, 2011; Jiang *et al*, 2014). Of these post-natal stem cells DPSCs are the most thoroughly characterised for dental regeneration. However, ADSCs represent a more abundant and easily harvested population of stem cells that have shown dentinogenic potential in several studies (Wu *et al*, 2008; Jiang *et al*, 2008; Zuk, 2010).

Mineralisation of adipose tissue to produce ectopic calcified bone is a feature of several diseases, such as Paget's disease, Lupus profundus or subcutaneous fat necrosis (Clarke and Williams, 1975; Shakelford *et al*, 1975). This is indicative of an inherent capacity of cells within the adipose tissue to differentiate towards a mineral-producing cell type. However, as yet, the potential for ADSCs in dental tissue engineering remains relatively unexplored. A small number of investigations into the odontogenic capacity of these cells have yielded some encouraging results (Jing *et al*, 2008; Wu *et al*, 2008). For instance, the over-expression DSPP using a transfected epigenetic adenovirus (Ad-DSPP) vector in ADSCs has been shown to induce odontoblast-like cell differentiation (Wu *et al*, 2008). Over-expression of DSPP was found to enhance the expression of genes associated with mineralisation and odontogenesis (Cbfa1, BSP, OCN and DMP1) as well as increasing the expression of alkaline phosphatase and the formation of mineralised nodules, thus

suggesting an odontogenic potential (Wu *et al*, 2008). Additionally, three-dimensional culture of ADSCs in a growth factor-rich medium has been shown to promote the formation of dental bud structures expressing markers associated with dental morphogenesis (Ferro *et al*, 2011). Moreover, a recent study demonstrated that ADSCs could differentiate to produce innervated and vascularised tooth-like structures when implanted into extraction sockets of the rabbit dental alveolus (Hung *et al*, 2011). Together, these studies indicate that ADSCs represent a promising, albeit relatively unexplored, stem cell source for dental regenerative therapies.

1.7 Project Aims

It is hypothesised that ADCs represent an abundant and easily accessible source of stem cells that can be utilised for dental tissue engineering. This project aims to investigate the odontogenic capacity of adipose-derived cells (ADCs) and examine how this compares with multipotent cells obtained from bone marrow (BMDCs) and dental pulp (DPDCs) tissue. To meet this aim, the following research objectives were addressed:

1. **Developing an optimal method for the dissociation and isolation of ADCs from adipose tissue**

The effect of several digestive enzymes and incubation periods were compared to analyse the release of ADCs from rat inguinal adipose tissue. The growth kinetics of ADCs was determined together with the ability of these cells to form viable colonies.

2. **Comparison of the stem cell profiles of multipotent cells isolated from three distinct tissue sources**

The present study directly aimed to compare the "stemness" of ADCs, BMDCs and DPDCs isolated from donor-matched animals using standardised protocols to provide an accurate profile of MSCs isolated from these different tissues. Growth kinetics and differentiation potentials were compared, while FACS and sqRT-PCR were used to generate comparative MSC marker profiles.

3. **Determination of the influence of FACS on primary cell populations**

The study aimed to evaluate the effectiveness of FACS as a method of MSC selection. CD29/CD90 positive ADCs, BMDCs and DPDCs were FACS selected and their viabilities, MSC marker profiles and differentiation potentials compared with relevant unsorted primary cells derived from the same tissues.

4. **Determination of the effect of cryo-storage on multipotent cells**

This study aimed to determine whether the process of cryo-storage affected the viability and stem cell properties of MSC-like cells. ADCs, BMDCs and DPDCs were subjected to cryo-storage, revived and their viability and MSC marker profiles compared with primary cells (which had not undergone cryo-storage) from the same tissues.

5. **The ability of dentine matrix components to promote odontogenic differentiation**

This study investigated the ability of dentine matrix components (DMCs) to act as a putative dentinogenic supplement to promote the odontogenic differentiation of ADCs, BMDCs and DPDCs. Odontogenic differentiation was evaluated by comparing the effects

of DMCs on the expression of odontoblast associated transcripts and the formation of mineral *in vitro*.

Investigating the *in vitro* odontogenic capacity of ADSCs and comparing these results with those of BMSCs and DPSCs should provide a better understanding to enable the application of adult stem cells for regenerative dentistry.

2.0 MATERIALS AND METHODS

2.1 Cell Culture

For each experiment ADCs, BMDCs and DPDCs were isolated from three independent rats (three biological repeats). Cells derived from each rat were assayed three times (technical repeats) and combined to calculate a mean.

2.1.1 Cell Culture Media and Reagents

2.1.1.1 Primary cell culture medium

Growth medium consisted of alpha-modified minimum essential medium (α-MEM) (Biosera, UK), containing 2 mM L-glutamine (Sigma-Aldrich, UK) supplemented with 1 % penicillin/streptomycin/amphotericin (100 units/mL penicillin with 100 µg/mL streptomycin and 2.5 µg/mL amphotericin) (Sigma-Aldrich, UK), and 10 % (v/v) foetal bovine serum (FBS) (Biosera, UK).

2.1.1.2 Phosphate buffered saline (PBS)

PBS comprised of 7.8 g NaCl (Sigma-Aldrich, UK), 1.5 g K_2HPO_4 (Sigma-Aldrich, UK) and 0.2 g KH_2PO_4 (Sigma-Aldrich, UK) in 1 L of distilled water and adjusted to a pH of 7.5. The solution was autoclaved at 121 °C to sterilise prior to use.

2.1.2 Tissue and Cell Isolation

Adipose tissue, bone marrow and dental pulp tissue were isolated from 100-120 g, 6 week old male Wistar-Hann rats that had been killed by cervical dislocation (Aston University, Pharmaceutical Sciences Animal House, Birmingham, UK).

2.1.2.1 Adipose Tissue

2.1.2.2 Isolating the Rodent Inguinal Fat Pad

Inguinal fat pads were removed by making incisions at the right and left hand sides of the abdomen using a scalpel and the adipose tissue separated from the dermis using toothed forceps. Adipose tissue was placed in a universal tube containing α-MEM (Biosera, UK) supplemented with 10% (v/v) FBS (Biosera, UK) and 1 % penicillin/streptomycin/amphotericin (100 units/mL penicillin with 100 µg/mL streptomycin and 2.5 µg/mL amphotericin) (Sigma-Aldrich, UK). The tissue was washed three times in sterile PBS (Section 2.1.1.2), placed onto a glass slide (Fisher Scientific, UK) within a 90 x 16 mm petri dish (Sarstedt, UK) and minced to give pieces of approximately $1mm^3$ in size. α-MEM + 10 % (v/v) FBS was added to the extracted adipose tissue to prevent desiccation.

2.1.2.3 Establishing a Method for Adipose Tissue Digestion

Several digestive enzymes were compared for the dissociation of cells from adipose tissue. Minced adipose tissue was placed into 15 mL Falcon® tubes (VWR, UK), incubated at 37 ºC and subjected to one of the digestive treatments outlined in Table 2.1.2.3 The method yielding the highest number of cells was adopted within the protocol for adipose cell isolation (see Section 2.1.2.4).

Digestive Enzyme(s)	Concentration/Volume	Digestion Period (mins)
Type I Collagenase (C0130, Sigma-Aldrich, UK)	1 g/L	15
		30
		45
Trypsin/EDTA (25200-072, Gibco, UK)	2.5 g/L trypsin in 0.38 g/L EDTA	30
		45
Type I Collagenase + Trypsin/EDTA	1 g/L Collagenase + 2.5 g/L trypsin in 0.38 g/L EDTA	15
		30
		45
Accutase (A1110501, Gibco, UK)	10 mL per 75 cm^2 culture area	15
		30
		45

Table 2.1.2.3 Table comparing several digestive methods for the dissociation of a heterogeneous population of cells from freshly isolated adipose tissue. All concentrations were based on supplier's guidelines.

2.1.2.4 Adipose Cell Isolation

Minced adipose tissue (15 g) was placed into 15 mL Falcon® tubes (VWR, UK) containing Collagenase type I isolated from *Clostridium histolyticum* (1 g/L) (C0130, Sigma-Aldrich, UK), 1 % (w/v) BSA (Sigma-Aldrich, UK) in sterile PBS (see Table 2.1.1.2). Tissue was digested with collagenase for 30 minutes at 37 °C in a rotary hybridisation oven (SI20H, Stuart Scientific, UK). After incubation, collagenase activity was neutralised with an equal volume of α-MEM (Biosera, UK) supplemented with 10 % (v/v) FBS (primary

culture medium) (Gibco, UK) and the cell suspension was passed through a 70 μm sieve (Millipore, UK) to enable single cell isolation. The resulting cell suspension was centrifuged at 1600 g (Eppendorf 5804R, Eppendorf, UK) for 5 minutes, after which the supernatant was aspirated and the remaining pellet re-suspended in 1 mL primary culture medium (see section 2.1.1.1). Adipose derived cells were seeded in 75 cm^2 culture flasks (Nunc, UK) containing primary culture medium, and incubated at 37 ºC in a 5 % CO_2 atmosphere. Culture medium was replaced every three days until the cells became 80 – 90 % confluent.

2.1.3 Bone Marrow

2.1.3.1 Dissection of Rodent Femora

Femora were isolated using scalpel, scissors and tweezers to tease away surrounding muscle and ligaments. This was achieved by making a longitudinal incision down the thigh and peeling back the skin to expose the femur and surrounding soft tissue. Soft tissue was stripped away from the femoral bones using a scalpel and tweezers. Femora were then dislocated from the pelvic girdle and placed in universal tubes containing 10 mL sterile α-MEM (Gibco, UK).

2.1.3.2 Bone Marrow Cell Isolation

Each femur was lightly sprayed with 70 % (v/v) ethanol and the epiphyses removed using scissors. A 20 mL syringe (BD Bioscience, UK) containing primary culture medium (see section 2.1.1.1) was attached to a 22 gauge needle (Appleton Woods, UK), which was inserted into the femoral cavity. Medium was then passed through the cavity to dislodge

the bone marrow and enable its collection in universal tubes. Flushed bone marrow was then passed through a 70 μm cell strainer (Millipore, UK) and collected in a 50 mL Falcon® tube (VWR, UK). Cells were pelletted by centrifugation using an Eppendorf 5804R centrifuge (Eppendorf, UK) at 900 g for a period of 5 minutes and re-suspended in primary culture medium (see section 2.1.1.1). Cell suspensions were seeded into 75 cm^2 culture flasks (Nunc, UK) containing α-MEM (Biosera, UK) supplemented with 10 % (v/v) FBS. Medium was changed every three days until cultures became approximately 80 % confluent.

2.1.4 Dental Pulp

2.1.4.1 Dissection of Rodent Incisors

Maxillary and mandibular incisors were dissected by removing the surrounding hard and soft tissues with scissors and scalpel to carefully remove the surrounding jaw bone. Incisors were placed in universal tubes containing sterile primary culture medium (see section 2.1.1.1) and stored for no longer than 1 hour prior to use.

2.1.4.2 Dental Pulp Isolation

Incisors were lightly sprayed with 70 % (v/v) ethanol and the inner pulp exposed by cutting along the longitudinal axis. Each incisor was placed onto a glass slide (Fisher Scientific, UK) within a petri dish (Sarstedt, UK), teased open and the dental pulp removed from the pulp chamber using a scalpel and forceps. Medium was added to the extracted pulp to prevent desiccation, the tissue minced into pieces of approximately 1 mm^3 and digested for 30 minutes at 37 °C in a rotary hybridisation oven (SI20H, Stuart Scientific,

UK) using 0.25 % (w/v) trypsin, 1 mM EDTA·4Na [2.5 g/L trypsin in 0.38 g/L EDTA (25200-072, Gibco, UK)]. After incubation, an equal volume of primary culture medium (see section 2.1.1.1) was added to neutralise trypsin activity. The resulting cell suspension was passed through a 70 μm cell strainer (Millipore, UK) and centrifuged (Eppendorf 5804R, Eppendorf, UK) for 5 minutes at 900 g. The supernatant was aspirated and the pellet re-suspended in 1 mL α-MEM (Gibco, UK). Cells were seeded in 25 cm^2 culture flasks (Nunc, UK) overnight at 37 °C, 5 % CO_2 to enable cell attachment to the tissue culture polystyrene. The following day, a further 4 mL α-MEM supplemented with 20 % FBS (v/v) was added to each culture (Gale *et al*, 2011). Culture medium was changed every three days until cultures became approximately 80 – 90 % confluent.

2.1.5 Cell Culture Assays

2.1.5.1 Cell Counting

10 μL of a cell suspension was added to an equal volume of 0.4 % (w/v) Trypan blue cell stain (Sigma-Aldrich, UK). The suspension was mixed thoroughly using a Gilson pipette, transferred to a Neubauer haemocytometer (Hawksley, UK) and viable cells counted manually under a variable relief contrast (VAREL) microscope (Zeiss Axiovert 25) (Zeiss, UK). Five counts per sample were recorded and an average calculated. Suspensions were then diluted according to the required seeding density and cultured at 37 ° in 5 % CO_2 in an RS Galaxy S+ incubator (RS Biotech).

2.1.5.2 Growth Kinetics and Population Doubling Time Analysis

Freshly isolated adipose tissue, bone marrow and dental pulp were incubated with 160 mM NH_4Cl (BDH Laboratory Supplies, UK) at room temperature for 5 minutes to lyse any erythrocytes present (Stroo *et al*, 2009). Each extraction was then centrifuged at 1200 g using an Eppendorf 5804R centrifuge (Eppendorf, UK) for 5 minutes and the resulting pellet re-suspended in 1 mL primary culture medium (see section 2.1.1.1). Cells were seeded at a density of 3.5 x 10^4 cells per 25 cm^2 flask (Nunc, UK) and cultured for 2, 4, 6, 8, 10, 12 and 14 days in osteogenic (see section 2.6.1.1), odontogenic (see section 2.6.1.2), or primary culture medium (see section 2.1.1.1) – with and without the addition of 1 µg/mL human dentine matrix components (see section 2.3). Culture flasks were removed from the RS Galaxy S+ incubator (RS Biotech) on days 2, 4, 6, 8, 10, 12 and 14 and the adherent cells treated with 0.25 % (w/v) trypsin, 1 mM EDTA (25200-072, Gibco, UK) to promote cellular detachment. Subsequently, cells were counted using an improved Neubauer haemocytometer (Hawksley, UK) (see section 2.1.5.1). The time taken for a population of cells to double in number was calculated for freshly isolated, and passage 1 and 2 cells, using the following formula (Lawson and Pederson, 1987):

$$PDT = t \log 2 / \log Nt - \text{Log } N0$$

PDT = generation time
t = time period
Nt = number of cells at time point t
N0 = number of cells at time point zero

2.1.6 Storage of Tissue Cell Isolates

Adipose-derived (ADCs), bone marrow-derived (BMDCs) and dental pulp-derived (DPDCs) cells were stored in liquid nitrogen (BOC Gases) prior to future use. To prepare the cells for storage, detached monolayers were centrifuged (Eppendorf 5804R, Eppendorf, UK) at 900 g for a period of 5 minutes, the supernatant aspirated, and the pellet re-suspended in 10 % (v/v) FBS (Gibco, UK) containing 10 % (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK). The cell suspension was transferred to cryogenic vials (Corning, UK) that were prepared for liquid nitrogen storage by incubation at 4 ºC for 1 hour, then at -20 ºC for 2 hours and finally -80 ºC overnight. Frozen cell suspensions were then transferred to vats of liquid nitrogen where they were stored until required.

When required, vials of cells were recovered from storage by thawing in a 37 ºC RS Galaxy S+ incubator (RS Biotech) for approximately 5 minutes. To remove residual DMSO prior to culture, the contents of each vial were transferred to 15 mL Falcon® tubes containing 5 mL α-MEM + 10 % (v/v) FBS (Biosera, UK) and centrifuged (Eppendorf 5804R, Eppendorf, UK) at 900 g for 5 minutes. The supernatant was aspirated and the pellet re-suspended and seeded in a 25 cm^2 culture flask (Nunc, UK).

To determine cell viability following storage, cells were cultured until approximately 80 % confluent. The cell monolayer was then detached using 0.25 % (w/v) trypsin, 1 mM EDTA·4Na [2.5 g/L trypsin in 0.38 g/L EDTA (25200-072, Gibco, UK)], centrifuged (Eppendorf 5804R, Eppendorf, UK) at 900 g for 5 minutes, neutralised with α-MEM + 10 % (v/v) FBS and the resulting suspensions transferred to 15 mL Falcon® tubes (VWR, UK). Cell suspensions were incubated at 4 ºC for a total period of 5 hours using a Denley orbital mixer (Appiely Quality Services, Ltd, UK) to provide constant agitation. The

number of viable cells was counted every 30 minutes during this incubation period by adding 0.4 % (v/v) Trypan blue solution (Sigma-Aldrich, UK) to an equal volume of cell suspension and manually counting the cells using an improved Neubauer haemocytometer (Hawksley, UK) (see section 2.1.5.1).

2.2 Fluorescence Activated Cell Sorting (FACS)

2.2.1 Cell Preparation and Analysis

FACS was employed to assess the relative expression of the surface proteins CD29 and CD90, which are reportedly common to mesenchymal stem cells (Dominici *et al*, 2006; Mafi *et al*, 2011). Confluent cultures had their media aspirated and were washed with sterile PBS (see section 2.1.1.2) prior to addition of 5 mL of 0.2 % (w/v) EDTA (Gibco, UK). Each culture was then incubated at 37 ° to promote cell detachment. An equal volume of primary culture medium (see section 2.1.1.1) was added to neutralise the reaction and 10 μL of the cell suspension removed for use in cell counting (see section 2.1.5.1). The cell suspension was centrifuged (Eppendorf 5804R, Eppendorf, UK) at 400 g for 5 minutes and the pellet re-suspended in sufficient sterile PBS to provide a final cell concentration of 10 x 10^6 cells/mL. Non-specific binding sites were blocked by incubating the cell suspension with 1 μg/mL of anti-rat Fc block (BD Pharmingen, UK) in sterile PBS for 10 minutes at room temperature. Cells were then washed using sterile PBS, centrifuged (Eppendorf 5804R, Eppendorf, UK) at 400 g, and re-suspended in FACS buffer (ice cold sterile PBS supplemented with 1% (v/v) FBS). 1 mL of FACS buffer containing approximately 10^6 cells was transferred to sterile 1.5 mL Eppendorf tubes (Eppendorf, UK) and primary conjugated antibodies (Table 2.2.1) were added at a

concentration of 0.5 μg/mL in sterile PBS supplemented with 3 % (w/v) BSA. The cell suspensions were then incubated in the dark at room temperature for 30 minutes to promote antibody binding. Cell suspensions were then washed using sterile PBS and centrifuged (Eppendorf 5804R, Eppendorf, UK) at 400 g for 5 minutes - this process was repeated three times, after which the pellet was re-suspended in 1 mL FACS buffer. The cells were stored on ice and transported to the FACS Facility at the Functional Genomics, Proteomics and Metabolomics Facility, University of Birmingham. Cells were sorted using a FACSAria™ II cell sorter (BD Pharmingen, UK) and results analysed using CellQuest Pro software (BD Pharmingen, UK). Sorted cells were placed on ice and transported to the School of Dentistry, University of Birmingham, where cell viability was immediately assessed using the method described in Section 2.1.5.1.

After determining cell viability, FACS cell suspensions were centrifuged (Eppendorf 5804R, Eppendorf, UK) at 900 g to pellet cells, and the supernatant aspirated. Sorted populations were then either used directly for differentiation assays or expanded to increase cell numbers (indirect use). For expansion, cells were cultured in 25 cm^3 culture flasks containing 20 % (v/v) FBS supplemented growth medium and grown until approximately 80 % confluent. Cells were detached using 0.25 % (v/v) trypsin, 1 mM EDTA·4Na [2.5 g/L trypsin in 0.38 g/L EDTA (25200-072, Gibco, UK)], centrifuged (Eppendorf 5804R, Eppendorf, UK) at 900 g for 5 minutes and the resulting pellets re-suspended in growth medium or re-profiled using FACS as previously described (Section 2.2.1). To assess multilineage differentiation, FACS cell suspensions or re-suspended pellets obtained following expansion were seeded at 1 x 10^5 cells per well in 35 mm^2 culture dishes (Sarstedt, UK) containing α-MEM supplemented with 20 % (v/v) FBS

(Biosera, UK) for 24 hours. Following expansion, growth medium was aspirated and replaced with osteogenic (Section 2.6.1.1) or adipogenic media (Section 2.6.2.1).

Target Antigen	Conjugated Fluorophore	Excitation/Emission Wavelength (nm)	Manufacturer/Catalogue Number
CD29 (Integrin β1)	APC	650; 755/767	eBiosciences/17-0291
CD90 (Thy-1)	FITC	495/519	eBiosciences/11-0900

Table 2.2.1 Details of antibodies used for the FACS profiling of MSC-associated markers for cells isolated from adipose, bone marrow and dental pulp tissue.

2.2.2 Live/Dead Assay

Live/dead assay using ethidium bromide/acridine fluorescent markers was used to discriminate between living and dead cells (Mironova *et al*, 2007). Acridine orange (AO) (Sigma-Aldrich, UK) is able to cross membranes of viable cells and intercalate with the DNA resulting in the emission of a green fluorescence, while ethidium bromide (EtBr) (Helena Bioscience, UK) is only able to intercalate with the DNA of cells with damaged cell membranes, emitting an orange fluorescence. Therefore, live cells appear green while the up-take of EthBr by damaged/dead cells effectively overrides the fluorescent signal

emitted by AO, thereby causing dead cells to appear orange. The live/dead reagent was prepared by dissolving 50 mg EtBr in 1 mL 95 % (v/v) ethanol, adding 15 mg of AO and diluted with 49 mL dH_2O (Gale *et al*, 2011). The stock solution was thoroughly mixed using a vortex mixer, 1 mL aliquots distributed in opaque-walled Eppendorf tubes (Eppendorf, UK) and stored at -20 ºC. 500 µL of a 1 % (v/v) working solution was diluted in $d.H_2O$ and placed in 15 mL Falcon® tubes (VWR, UK) containing FACS analysed cells and incubated at room temperature in the dark for 5 minutes with constant agitation. 10 µL of each cell suspension was added to an improved Neubauer haemocytometer, where live/dead cells were visualised and counted (*N*=5) using a TE300 Nikon Eclipse fluorescent microscope (Nikon, UK) with a 480-520 nm filter.

2.3 Preparation of Dentine Matrix Components

2.3.1 Dentine Extraction

Ethical approval was obtained for the collection of non-carious human teeth from patients attending the Birmingham Dental Hospital (ethical approval reference: BCHDent286.1471.TB). Freshly extracted teeth were placed in PBS containing 15 mM of the bacteriostatic agent sodium azide (BDH Laboratory Supplies, UK), washed under running water for several hours to remove debris and stored at -20 °C. Teeth were further cleaned using a prophylaxis paste to remove contaminants (BDH Laboratory Supplies, UK), then cut longitudinally into approximately 1 mm thick sections using a low speed diamond saw (Isomet Buehler, USA) with an MK303 102 mm diamond blade to allow for easy extraction of the pulp and enamel removal. A scalpel was used to remove central pulp tissue from the tooth slices, while remaining enamel was removed using bone

clippers. Isolated dentine slices were weighed using an Ohaus high precision analytical balance (Ohaus, UK) and pulverised using a Glen Creston percussion mill (Spex 6700 Freezer/Mill) under constant liquid nitrogen cooling. The resulting dentine powder was passed through a 60 mesh sieve (Millipore, UK) and the weight recorded.

2.3.2 Solubilisation of Dentine Matrix Components

A 10 % (w/v) EDTA extraction solution (pH 7.2) was prepared containing 10 mM N-ethylmaleimide (NEM) (Sigma-Aldrich, UK) and 5 mM phenylmethylsulphonyl fluoride (Sigma-Aldrich, UK) to inhibit the action of proteases.

To dissolve the inorganic mineralised component of the dentine and solubilise the non-collagenous organic matrix, 5 g of powdered dentine was placed in 20 mL EDTA-extraction solution (described above) at 4 ºC with constant agitation. On a daily basis, the mixture was centrifuged at 3600 g using an Eppendorf 5804R centrifuge (Eppendorf, UK) for 20 minutes, the EDTA-soluble fraction was pooled in 1 litre collection vessels (BDH Laboratory Supplies, UK) and stored at -20 ºC prior to further use. Each EDTA-soluble fraction had its absorbance measured at 280 nm using a Philips UV/VIS spectrophotometer (Philips, UK) to provide a daily indication of organic content present within the extract. The dentine pellet was then re-suspended in 20 mL of fresh EDTA-extraction solution and incubated at 4 ºC for a further 24 hours. This cycle of incubation, centrifugation, removal and pooling of the EDTA-soluble fraction, and re-suspension of the dentine pellet was repeated daily for a total period of 14 days.

Dialysis was performed to remove and replace the EDTA-extraction solution with dH_2O. Pooled EDTA-soluble extracts were transferred to 19 mm dialysis tubing (14000 MWCO) (Fisher Scientific, UK) and exhaustively dialysed against dH_2O for a period of 14 days at

4 ºC, with daily water changes. Following dialysis, extracts were transferred to round-bottomed flasks and shell frozen under liquid nitrogen. Extracts were lyophilised for 24 hours using an Edwards 4K Modulyo freeze dryer (Edwards High Vacuum Int, UK). The resultant lyophilised extracts were stored at -20 ºC until required.

2.4 Characterisation of Dentine Matrix Components

2.4.1 Dye-Binding Assay for the Quantification of Non-Collagenous Proteins

A method developed by Bradford (1976) was employed to analyse the total non-collagenous protein content of EDTA-extracted dentine matrix components (DMCs). Bradford reagent was prepared by dissolving 20 mg Coomassie Brilliant Blue G-250 (Sigma-Aldrich, UK) in 10 mL of 95% (v/v) methanol, added to 20 mL of 85 % (v/v) phosphoric acid and made up to 200 mL with dH_2O. The final reagent was filtered using number 1 Whatman filter paper (Whatman Int, UK) to remove any insoluble material prior to use.

100 µL of Bradford reagent was added to a micro-cuvette, diluted with 900 µL of dH_2O and the absorbance measured at 595 nm using a Philips UV/VIS spectrometer (Philips, UK) to provide a reference blank. A standard curve was produced by adding 100 µL of bovine serum albumin (BSA) (Sigma-Aldrich, UK) and using this to produce serial dilutions ranging from 0-10 µg/mL (see figure 2.4.1). Test samples were generated by dissolving 1 mg of EDTA-extracted dentine matrix components in 1 mL dH_2O and the total NCP content evaluated by adding 100 µL of Bradford reagent. Each sample was mixed thoroughly and allowed to stand for 5 minutes to ensure thorough binding of the

Bradford reagent. Absorbance values were then measured at 595 nm using a Philips UV/VIS spectrometer (Philips, UK). Values obtained for test samples were plotted and extrapolated to provide a measurement of total NCP concentration.

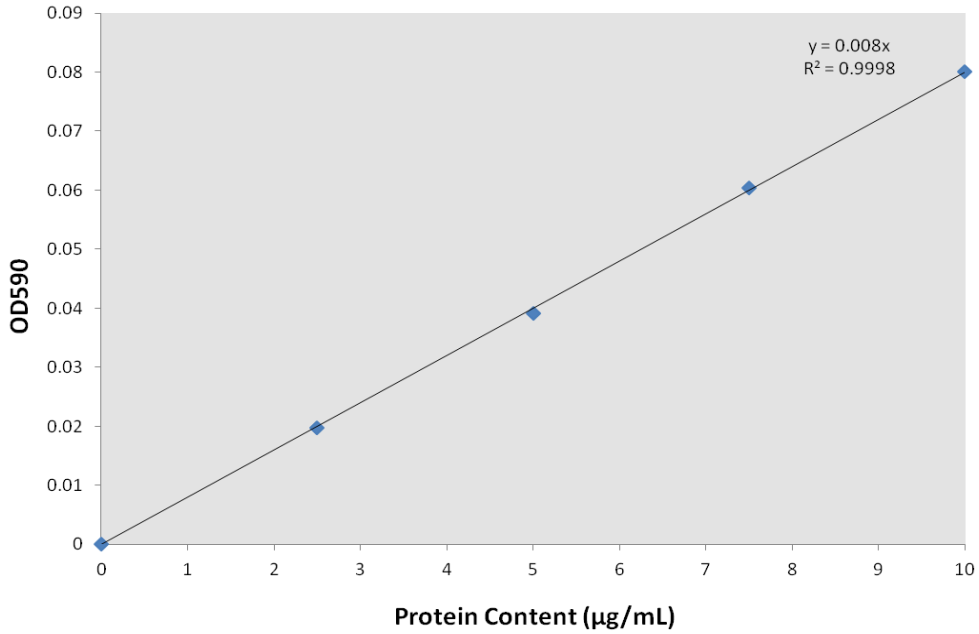

Figure 2.4.1 Standard curve displaying a series of absorbance values obtained at 595 nm for known non-collagenous protein concentrations ranging from 0-10 µg/mL. Both the equation for the line and R^2 value are displayed. The R^2 value obtained indicates a low lineage variance with a value > 0.99.

2.4.2 Dye-Binding Assay for Glycosaminoglycan Quantification

A method developed by Farndale et al. (1986) was used to quantify total glycosaminoglycan (GAG) content based on staining with dimethyl methylene blue dye. Farndale reagent was prepared by dissolving 4 mg dimethyl methylene blue (Hopkin & Williams Ltd, UK), 0.75 g glycine (BDH Laboratory Supplies, UK) and 0.584 g sodium chloride (Sigma-Aldrich, UK) in 250 mL dH_2O. The working solution was then passed

through number 1 Whatman filter paper (Whatman International Ltd, UK) to remove any insoluble material prior to use.

1 mL of the Farndale reagent was added to 100 µL dH_2O in a micro-cuvette and the absorbance measured at 525 nm using a Philips UV/VIS spectrometer (Philips, UK) to provide a reference blank. 100 µL of GAG-chain standard, chondroitin sulphate (Sigma-Aldrich, UK) was added to 0.5 M acetic acid and used to produce serial dilutions ranging from 0 to 5 µg/mL (see figure 2.4.2). Test samples were prepared by dissolving 1 mg of EDTA-extracted dentine matrix components in 1 mL dH_2O. Total GAG content was evaluated by adding 100 µL of sample to 1 mL Farndale reagent in a micro-cuvette. Each sample was mixed thoroughly and the absorbance immediately measured at 525 nm using a UV/VIS spectrometer (Philips, UK). Values obtained for test samples were plotted and extrapolated to provide a measurement of total GAG concentration.

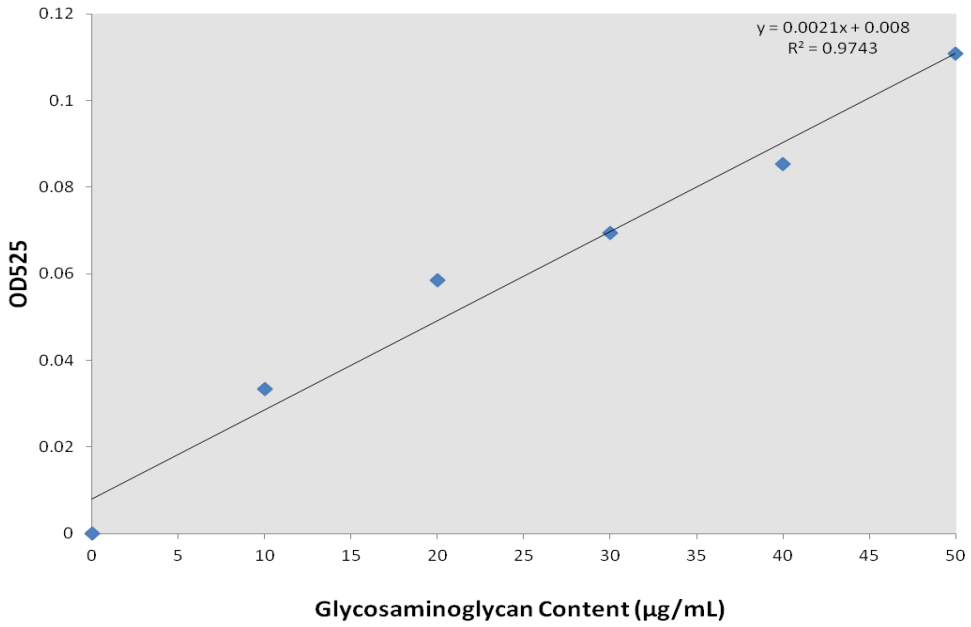

Figure 2.4.2 Standard curve displaying a series of absorbance values obtained at 525 nm for known GAG concentrations ranging from 0-5 µg/mL. Both the equation for the line and R^2 value are included. The R^2 value obtained indicates a low lineage variance with a value > 0.97.

2.5 Using Dentine Matrix Components (DMCs) for Odontogenic Induction

2.5.1 Colony forming unit fibroblast (CFU-F)

Passage 2 ADCs, BMDCs and DPDCs were seeded in 35 mm^2 culture dishes (Sarstedt, UK) at a density of 500 cells per dish and cultured in α-MEM supplemented with 10 % (v/v) FBS (Gibco, UK), with or without the addition of 1 µg/mL DMCs. 1 µg/mL DMCs was selected from preliminary experiments that indicated that this concentration promoted the most significant increases in the mineral content and odontogenic gene expression of cell cultures. After 6 days, cultures were immediately fixed using 10 % (v/v) formalin and

stained with 0.1 % (w/v) toluidine blue (Sigma-Aldrich, UK). Cultures were then washed three times with PBS to remove any unbound dye, and CFU-F visualised and counted manually under a Nikon TE-DH100w camera attached to a Nikon Eclipse TE300 microscope (Nikon, UK).

2.5.2 Metabolic Activity Analysis

The colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay provides a measure of cellular metabolic activity through the action of mitochondrial NADPH-dependent oxido-reductase enzymes. The reduction of this dye leads to the formation of an insoluble purple formazan product that can be quantified spectrophotometrically to provide a measure of metabolic activity. 10 µL of 5 mg/mL MTT solution was added to ADC, BMDC and DPDC cultures grown in 6.86 mm^3 wells containing 2 mL α-MEM + 10 % (v/v) FBS (Corning, UK) and left to incubate for 4 hours at 37 ºC. After incubation, culture plates were removed, 50 µL DMSO (Sigma-Aldrich, UK) added and the absorbance determined at 570 nm using an ELx800 plate reader (Biotek, UK). To provide a measure of metabolic activity per cell, counts were performed as previously described (see section 2.1.5.1).

2.6 Multi-Lineage Differentiation

To assess the differentiation potential of passage two ADCs, BMDCs and DPDCs, these cells were seeded on 35 mm^2 polystyrene culture surfaces (Sarstedt, UK) containing primary growth medium (see section 2.1.1.1) at a density of $1x10^5$ cells/well. Cells were cultured for a period of 24 hours after which the medium was aspirated and replaced with

osteogenic (see section 2.6.1.1), adipogenic (see section 2.6.2.1) or odontogenic media (see section 2.6.1.2).

2.6.1 Osteogenic/Odontogenic Induction

2.6.1.1 Osteogenic Medium

Basic growth medium was supplemented with factors necessary for osteogenic induction: 50 μg/mL ascorbic acid (Sigma-Aldrich, UK) and 10 mM β-glycerophosphate (Sigma-Aldrich, UK) (Castano-Izquierdo et al, 2007; Kyllonen et al, 2013).

The influence of dexamethasone on mineralisation was assessed by culturing passage 2 ADCs in osteogenic medium containing varying concentrations of dexamethasone (Sigma-Aldrich, UK). Dexamethasone was added to osteogenic medium at concentrations of 10^{-7}, 10^{-8}, and 10^{-9} M, and medium was replaced every three days for a period of three weeks. Images of cellular morphologies were obtained using a Nikon TE-DH100w camera attached to a Nikon Eclipse TE300 microscope (Nikon, UK) every seven days and each culture stained with alizarin red after three weeks incubation to evaluate the amount of mineral present (Section 2.6.1.3).

2.6.1.2 Odontogenic Medium

Odontogenic culture medium consisted of osteogenic medium (see section 2.6.1.1) supplemented with dentine matrix components (DMCs) (see section 2.3) (Deng *et al*, 2005; Liu *et al*, 2005; Smith *et al*, 2001). DMCs were added and cellular responses assessed at concentrations of 0.1, 1, and 10 μg/mL.

2.6.1.3 Alizarin Red Staining and Quantification

Alizarin red stain (ARS) (Sigma-Aldrich, UK) was used to visualise calcium deposition in mineralising cultures. The stain used comprised of 2 mg/mL 40 mM ARS in 0.5 M acetic acid (BDH Laboratory Supplies, UK) and its pH adjusted to 4.2 using 1 % (v/v) ammonium hydroxide (BDH Laboratory Supplies, UK) (Gregory *et al*, 2004). Prior to staining, the number of cells present in each well was manually counted in different fields (N = 5) of view using a graticule mounted on a Nikon Eclipse TE300 microscope (Nikon, UK). Each well was washed using PBS and fixed with 10 % (v/v) formaldehyde (VWR, UK) at 4 °C for 20 minutes. ARS was added to each well and incubated at room temperature for 20 minutes to allow ARS binding of calcium deposits. Cells were washed five times with PBS to remove any unbound stain, and visualised and photographed using a Nikon TE-DH100w camera attached to a Nikon Eclipse TE300 microscope (Nikon, UK).

ARS bound within osteogenic cultures was solubilised from the cultures by incubation with 300 µL 10 % (v/v) acetic acid (VWR, UK) at room temperature for 30 minutes. A cell scraper (Corning, UK) was used to remove the loosely attached monolayer and cells transferred to a 1.5 mL Eppendorf tube (Eppendorf, UK). The detached monolayer and alizarin red mixture was vortexed for 30 seconds to disaggregate the monolayer and placed on a block heater at 85 °C for 10 minutes. The resultant cell slurry was placed on ice for 5 minutes and then centrifuged (Eppendorf 5804R, Eppendorf, UK) at 20,000 g for 15 minutes. Supernatant containing the extracted ARS was removed and 200 µL 10 % (v/v) ammonium hydroxide (BDH Laboratory Supplies, UK) was added. The supernatant was transferred to 6.35 mm^2 wells of a flat bottomed opaque-walled assay plate (Corning, UK) and the absorbance measured at 405 nm using an ELx800 plate reader (Biotek, UK).

2.6.2 Adipogenic Induction

2.6.2.1 Adipogenic Medium

Basic growth medium was supplemented with factors necessary for adipogenic induction: 0.5 mM 1-methyl-3-isobutylxantine (Sigma-Aldrich, UK), 60 μM indomethacin (Sigma-Aldrich, UK), 0.5 μM hydrocortisone (Sigma-Aldrich, UK) (Gronthos *et al*, 2002; Zhang *et al*, 2008).

2.6.2.2 Oil Red O Staining and Quantification

Oil red O (ORO) stain is a lysochromic dye used to visualise lipid droplets in adipogenic cultures. An ORO stock solution was prepared by dissolving 0.5 g of ORO stain (VWR, UK) in 200 mL 60 % (v/v) propan-2-ol (VWR, UK), which was heated at 56 °C for 1 hour and then left to cool at room temperature (Zhang *et al*, 2008). A working solution was prepared by adding three parts ORO stock solution to two parts distilled water, which was filtered immediately to remove any un-dissolved stain. Cultures were initially washed twice with PBS, and representative fields (N = 5) counted manually using a graticule mounted on a Zeiss Axiovert 25 microscope (Zeiss, UK). ORO working solution was added to the cultures, which were incubated for 40 minutes at room temperature with constant agitation. Cultures were then washed once with 60 % (v/v) propan-2-ol (VWR, UK), followed by two consecutive washes in PBS to remove any unbound ORO. Stained monolayers were visualised and photographed using a Nikon TE-DH100w camera attached to a Nikon Eclipse TE300 microscope (Nikon, UK).

Bound ORO dye was eluted from cell monolayers by the addition of 200 μL of 60 % (v/v) propan-2-ol (VWR, UK) and incubated at room temperature for 20 minutes with constant

agitation. 60 µL volumes were transferred to an opaque-walled 96 well assay plate (Corning, UK) and the absorbance measured at 500 nm using an ELx800 plate reader (Biotek, UK).

2.7 Gene Expression Analysis

2.7.1 RNA Isolation

RNA was isolated from cell cultures using the Qiagen RNeasy minikit according to the manufacturer's instructions (Qiagen, UK). Medium was aspirated from cultures and the cells washed with PBS. Cells were lysed by the addition of 600 µL RLT lysis buffer supplemented with 1 % (v/v) β-mercaptoethanol. An equivalent volume of 70 % (v/v) ethanol was added to the cell lysate, which was briefly vortexed. 700 µL of the mixture was transferred to an RNeasy mini-column, which drained into a 2 mL collection tube. RNeasy mini-columns containing cell lysate were centrifuged (Eppendorf 5804R, Eppendorf, UK) for 15 seconds at 8,000 g and the elute discarded. Successive washing steps were performed at 8,000 g (Eppendorf 5804R, Eppendorf, UK) using 700 µL of kit wash buffer for 15 seconds, 500 µL of ethanol kit buffer for 15 seconds, and 500 µL of ethanol kit buffer for 2 minutes. Each RNeasy spin column was then transferred to a 1.5 mL flat-bottomed collection tube (Appleton Woods, UK) and 30 µL molecular grade RNase-free water added to the column membrane. The assembly was centrifuged (Eppendorf 5804R, Eppendorf, UK) at 8000 g for 1 minute to remove any RNA bound to the membrane.

2.7.2 RNA Analysis

To determine the concentration of RNA isolated (see section 2.7.1) the absorbance of each sample at 260 nm was determined using a Biophotometer (Eppendorf, UK). RNA content was further analysed by electrophoresis by running each lysate on a 1 % (w/v) agarose gel (WebScientific, UK) to determine RNA presence and integrity (see figure 2.7.2). The agarose gel was produced by adding 1 % (w/v) agarose to 60 mL of 1x tris(hydroxymethyl)-aminomethane (TAE) buffer (Qiagen, UK) and boiling using a microwave until dissolved. The gel was allowed to cool at room temperature to approximately 60 ºC and 3 μL of 10,000X SYBR gold nucleic acid stain staining solution (Invitrogen, UK) added. The mixture was manually transferred to a gel tray, a well-forming comb inserted, and left to cool at room temperature. 1 μL of the extracted RNA was added to 3 μL RNase-free water and 1 μL 10X loading buffer (Promega, UK). This mixture was loaded into wells in the 1 % (w/v) agarose gel and electrophoresed at 120 V for ~40 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 were visualised by UV illumination.

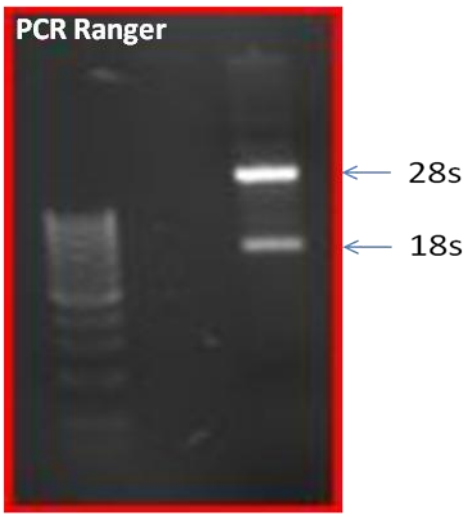

Figure 2.7.2 Representative image of electrophoresed RNA on a 1 % (w/v) agarose gel with 18S and 28S bands visible. 4 µL of PCR ranger 100 bp RNA ladder was used to provide a measure of RNA band size (Norgen, UK).

2.7.3 Reverse Transcription of RNA

RNA was reverse transcribed into cDNA using the QIAGEN Omniscript RT kit according to the manufacturer's instructions (Qiagen, UK). Approximately 2 µg of RNA was diluted in a total volume of 12 µL of sterile molecular grade water (BDH Laboratory Supplies, UK). 2 µL of 10 µM oligo-dT primer (Ambion, UK) was added to the RNA solution and the mixture incubated at 80 ºC for 10 minutes, and then quenched on ice for 5 minutes to stop the reaction. A PCR master-mix was generated by combining 2 µL reverse transcriptase buffer, 2 µL deoxynucleoside triphosphates (dNTP), 1 µL RNase inhibitor (Promega, UK) and 1 µL omniscript reverse transcriptase; which was then added to the quenched RNA mixture. The solution was mixed using a vortex mixer and incubated at 37 ºC for 1 hour to allow the synthesis of double stranded cDNA. Finally, the mixture was heated to 95 ºC for 5 minutes to halt the reaction.

2.7.4 Concentration and Purification of Synthesised cDNA

20 μL of synthesised cDNA was added to Microcon YM-30 spin column (Millipore, UK) containing 460 μL molecular grade water (BDH Laboratory Supplies, UK). The mixture was centrifuged (Eppendorf 5804R, Eppendorf, UK) at 10,000 g for 2 minutes and the elute discarded. The volume of liquid remaining in the spin column was measured; if over 50 μL was still present, the mixture was further centrifuged (Eppendorf 5804R, Eppendorf, UK) at 10,000 g for successive 1 minute periods. When the volume of cDNA mixture remaining in the spin column was below 50 μL, the spin column was inverted, transferred to a sterile 1.5 mL collection tube and centrifuged (Eppendorf 5804R, Eppendorf, UK) at 8000 g for 1 minute to collect the concentrated cDNA. To gain an estimate of the concentration of cDNA, the absorbance was measured at 260 nm using a Biophotometer (Eppendorf, UK).

2.7.5 Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction (sqRT-PCR) Amplification

A REDTaq mastermix was produced, comprising of 12.5 μL REDTaq ready mix (Sigma-Aldrich, UK), 12.5 μL of molecular grade water (BDH Laboratory Supplies, UK), 1 μL of 1 μM forward primer (Invitrogen, UK) and 1 μL of 1 μM reverse primer (Invitrogen, UK). 6 μL of PCR mastermix was added to individual 0.2 mL PCR tubes (Appleton Woods, UK) to which 50-100 ng of cDNA was added. All components were mixed thoroughly using a vortex mixer and transferred to a GeneAmp 2700 PCR Thermocycler (Applied Biosystems, UK). The amplification parameters were optimised for each gene, details of which are provided in Table 2.7.5. Parameters common to all genes were: an initial denaturation temperature of 94 ºC for 5 minutes, followed by a 94 ºC treatment for

20 seconds, during which dsDNA was separated to give ssDNA. This step would allow DNA to be bound by primers and accessed by the Taq polymerase enzyme. The creation of ssDNA was followed by a 20 second cycle at 60/60.5 ºC for primer annealing to ssDNA and a period of primer extension at 68 ºC for 20 seconds. After the final 68 °C cycle, the primers entered an extension phase at 72 ºC for 10 minutes.

Primer	Primer Sequence (5'→ 3')	Annealing Temperature (°C)	Cycle Number
Normalisation			
GAPDH	F-CCCATCACCATCTTCCAGGAGC; R-CCAGTGAGCTTCCCGTTCAGC	60.5	21-27
Pluripotent Markers			
Klf4	F-ATCATGGTCAAGTTCCCAGC; R-ACCAAGGACCATCGTTTAGG	60.5	35
C-myc	F-CTTACTGAGGAAACGGCGAG; R-GCCCTATGTACACCGGAAGA	60.5	35
Nanog	F-TATCGTTTTGAGGGGTGAGG; R-CAGCTGGCACTGGTTTATCA	60.5	35
Lin28	F-TTTCTTGTTTCCCCCAAATG; R-AGAGGGGCTGGTTGTAAGGT	60.5	35
Sox-2	F-ATACAAGGGAATTGGGAGGG; R-AAACCCAGCAAGAACCCTTT	60.5	35
Multipotent Markers			
CD44	F-TGGGTTTACCCAGCTGAATC; R-CTTGCGAAAGCATCAACAAA	60.5	33-37
CD105 (Endoglin)	F-TTCAGCTTTCTCCTCCGTGT; R-TGTGGTTGGTACTGCTGCTC	60.5	41-45
CD73 (5'-nucleotidase)	F-GGACTGATTGATCCCCTCCT; R-TTGTCCCTGGATTTGAGAGG	60.5	25

CD29 (Integrin β1)	F-AATGGAGTGAATGGGACAGG; R-TCTGTGAAGCCCAGAGGTTT	60.5	25
CD90 (Thy1)	F-AGCTCTTTGATCTGCCGTGT; R-CTGCAGGCAATCCAATTTTT	60.5	26
Osteogenic/Odontogenic Markers			
Core-Binding Factor Subunit 1 (Cfbα1)	F-GCCGGGAATGATGAGAACTA; R-GGACCGTCCACTGTCACTTT	60.5	35
Osterix	F- CAGCCTGCAGCAAGTTTGG; R- TTT TCCCAGGGCTGTTGAGT	60.5	35
Collagen Type 1α1 (Col1α1)	F-CACCCTCAAGAGCCTGAGTC; R-GCTTCTTTTCCTTGGGGTTC	60.5	35
Osteopontin (OP)	F- AAGCCTGACCCATCTCAGAA; R- GCAACTGGGATGACCTTGAT	60.5	25
Osteonectin (ON)	F-AAACATGGCAAGGTGTGTGA; R-AGGTGACCAGGACGTTTTTG	60.5	22
Bone Sialoprotein (BSP)	F-ATGGAGATGGCGATAGTTCG; R-TCCACTTCTGCTTCTTCGTTC	60.5	35
Alkaline Phosphatase (ALP)	F- CTCCGGATCCTGACAAAGAA; R- ACGTGGGGGATGTAGTTCTG	60.5	30
Dentine Sialophosphoprotein (DSPP)	F-TGCATTTTGAAGTGTCTCGC; R-CCTCCTGTCTTGGTGTGGTT	60.5	25
Dentine Matrix Protein 1 (DMP1)	F-CGGCTGGTGGTCTCTCTAAG; R-CATCACTGTGG GGTCCTTG	60.5	30
Nestin	F-CATTTAGATGCTCCCCAGGA; R-AATCCCCATCTACCCCACTC	60.5	31
Adipogenic Markers			
Adipocyte Protein 2 (aP2)	F-TGGAAACTCGTCTCCAGTGA; R-GCTCATGCCCTTTGCTAAAC	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

2.7.6 Gel Electrophoresis of RT-PCR products

A 1.5 % (w/v) agarose gel was produced as previously described (Section 2.7.2) and 3 µL of 0.5 µg/mL ethidium bromide (Helena Bioscience, UK) added to aid visualisation. The solidified gel was transferred to an electrophoresis tank filled with 1 x tris-acetate-EDTA (TAE) running buffer (Qiagen, UK) and 5.5 µL of amplified cDNA was added to each well. Samples were electrophoresed at 120 V for a period of 40 minutes. The gel was then removed from the eletrophoresis tank and visualised by UV illumination using a G:Box gel documentation and analysis unit (Syngene, UK) and images captured using accompanying Genesnap software (Syngene, UK). Semi-quantitative values of band intensities were extrapolated from these images using the GeneTools image analysis software (Syngene, UK).

2.8 Mineral Analysis

2.8.1 Micro-Computed Tomography

A polychromatic SkyScan-1172 µCT scanner (SkyScan, Kontich, Belgium) was used to analyse mineral deposited in passage 2 ADC, BMDC and DPDC cultures after three weeks differentiation in 35 mm^2 petri dishes (Sarstedt, UK) containing osteogenic medium (Section 2.6.1.1). Osteogenic medium was removed and the cultures fixed with 10 % (v/v) formalin (VWR, UK) and whole dishes analysed in air using an isotropic voxel size of 10 µm, which was able to provide whole dish scans while maintaining high resolution images. An electrical potential of 40 kV was applied, and a 0.5 mm aluminium filter installed to limit the number of low energy (softer) photons reaching the sample, thereby

reducing beam-hardening and attenuation artefacts that may cause dark bands or streaks and thereby lower image quality. All samples were scanned under identical conditions, with each dish independently mounted on a revolving turntable with a programmed rotation step of 0.4 degrees, and scanned using a current of 120 mA and an exposure time of 1100 ms. To produce a phantom for system calibration, di-potassium phosphate (K_2HPO_4) was solubilised at concentrations of 0, 250, 500, 750, 1000, 1250, and 1500 mg/cm^3. Following scanning, each cross-section was reconstructed with a 32-bit dynamic range and converted to an 8-bit bitmap (BMP) to allow visualisation and analysis using format conversion software (Skyscan, Kontich, Belgium), with each pixel having a representative grey value between 0 and 255 (black and white). Grey values were extrapolated to provide corresponding mineral density measurements (mg/cm^3), which were colour coded using Fiji software (National Institute of Health, USA) to represent increasing densities.

2.8.2 Scanning Electron Microscopy (SEM)

SEM was used to analyse mineral present following 21 days culture in osteogenic (see section 2.6.1.1) and control (α-MEM + 10 % (v/v) FBS) medium. Samples were fixed with 2.5 % (v/v) glutaraldehyde (Agar Scientific, UK) in a 0.1 M sodium cacodylate buffer (Fisher Scientific, UK) for 30 minutes at room temperature. Samples were dried using a progressive series of alcohol dehydration steps with increasing concentrations of (v/v) ethanol (Sigma-Aldrich, UK) (20 - 100 %), followed by overnight incubation with hexamethyldisilazane (HMDS) (Sigma-Aldrich, UK) in a fume hood for complete dehydration of each sample. The tissue culture polystyrene surfaces on which cells were grown, fixed and dehydrated were cut into sections of approximately 3 cm^2 using a pair of

wire cutters. Cut sections were fixed to aluminium SEM stubs (Agar Scientific, UK) using conductive Acheson electrodag tape (Agar Scientific, UK). Samples were sputter coated with gold under vacuum using an Emitech K550X coater for 2 minutes at 25 mA. Secondary (SE) and backscattered (BSE) electron micrographs were generated for each sample using an accelerating voltage of 10 kV using a Zeiss EVO MA10 scanning electron microscope (Zeiss, UK).

Energy dispersive X-ray spectroscopy (EDS) was used to provide an elemental analysis of the mineral present in osteogenic cultures. Ashed inorganic material (see section 2.8.3) was affixed to aluminium SEM stubs (Agar Scientific, UK) as described above. Samples were sputter coated with gold under vacuum using an Emitech K550X coater for 2 minutes at 25 mA. Secondary and backscattered electron micrographs were generated for each sample using an accelerating voltage of 15 kV in a Zeiss EVO MA10 scanning electron microscope (Zeiss, UK). Elemental analysis of inorganic material was achieved via the analysis of X-rays emitted by atoms present within the material using an energy dispersive X-ray spectrometer (EDS-INCA, Oxford Instruments, UK).

2.8.3 Quantification of Total Inorganic Content

ADCs, BMDCs and DPDCs were cultured in osteogenic (see section 2.6.1.1) medium for 21 days. Medium was aspirated from culture dishes, the cells washed once with PBS, and fixed in 10 % (v/v) formalin (VWR, UK) for 20 minutes. Following fixation, the cell layer containing synthesised mineral was manually dissociated from the culture dish using a cell scraper (Corning, UK), placed on a glass slide (Fisher Scientific, UK) and finely diced using a scalpel. Glass slides were weighed using an Ohaus high precision analytical balance (Ohaus, UK) before and after the addition of dissociated culture contents and the

total weight of combined organic and inorganic matter quantified. Glass slides were then transferred to an ashing furnace (Carbolite AAF 11/3, UK) and heated at 600 ºC for 15 minutes to remove any organic material and reduce any inorganic mineral to ash, without altering the mineral phase (Holager, 1970; Lim and Liboff, 1972). The total weight of the ashed inorganic content was then weighed and subtracted from the value obtained for total organic/inorganic content to provide a measure of the total mineralised portion of osteogenically cultured cells.

To quantify levels of calcium and phosphate, the ashed inorganic material needed to be solubilised. Inorganic material, as well as a hydroxyapatite standard (Sigma-Aldrich, UK), was solubilised in 10 % (v/v) hydrochloric acid (BDH Laboratory Supplies, UK). Following solubilisation, the acidic solutions were gradually neutralised using 0.1 % (w/v) ammonium hydrochloride (BDH Laboratory Supplies, UK) (Miles *et al*., 2001).

2.8.4 Determination of Calcium and Phosphorus Content

The concentration of calcium and phosphorus present in ashed osteogenic cultures (see section 2.8.3) was quantified using established methods (Chen *et al*, 1956; Robinson & Weatherall, 1968). To determine calcium content, a calcein reagent was prepared by dissolving 5 mg calcein (Biotium Inc, UK) in 25 mL 3 M KOH (Sigma-Aldrich, UK) and diluted with 100 mL with dH_2O. Calcium carbonate was dried at 105 ºC for one hour using a muffle furnace (Carbolite AAF 11/3, UK), followed by further dehydration at 60 ºC for 1 hour. To determine phosphorus content, a phosphorus reagent was prepared by making two solutions: solution A, containing 10 % (v/v) ascorbic acid (Sigma-Aldrich, UK) dissolved in 25 mL double distilled water ($d.d.H_2O$), and solution B, containing

0.625 % (v/v) ammonium molybdate (Sigma-Aldrich, UK) dissolved in 1.5 N sulphuric acid (BDH Laboratory Supplies, UK) and made up to 100 mL with d.d.H_2O. A working phosphorus reagent was then prepared by adding one part solution A with four parts solution B.

Calcium standards were prepared by drying calcium carbonate (Sigma-Aldrich, UK) at 105 °C for 1 hour, followed by further dehydration at 60 °C for 1 hour. 12.5 mg of dried calcium carbonate was dissolved in 13.5 mL 2 M perchloric acid (VWR, UK), and made up to 1 litre with d.d.H_2O. This 5 μg/mL calcium stock solution was used to make serial dilutions with calcium concentrations ranging from 2-4 μg/mL. Phosphorus standards ranging from 0.5-100 mg/L were produced by dissolving 0.22 g of potassium di-hydrogen orthophosphate (Sigma-Aldrich, UK) in 500 mL d.d.H_2O and using this stock solution for serial dilutions. Test samples were generated by culturing passage 2 ADCs, BMDCs and DPDCs in osteogenic medium (see section 2.6.1.1) for 21 days, ashing the resulting mineral phase, and solubilising the inorganic material (see section 2.8.3). Total calcium content was evaluated by adding 1 mL of solubilised inorganic material to micro-cuvettes containing 1 mL of calcein reagent. All samples were thoroughly mixed using a vortex mixer and the absorbance of each immediately read at 506 nm using a UV/VIS spectrometer (Philips, UK), with 2 mL of calcein reagent used to provide a reference blank. The absorbance values for calcium carbonate standards were used to produce a standard curve (see figure 2.8.4a). Values obtained for test samples were plotted and extrapolated to provide a measurement of total calcium content.

Total phosphorus content was evaluated by adding 2 mL of solubilised inorganic material to tubes containing 2 mL of freshly made phosphorus reagent, mixing thoroughly and

incubating for 2 hours at 37 ºC in a water bath. Samples were removed from the water bath and left to cool for 5 minutes before being transferred to micro-cuvettes and absorbance measured at 820 nm using a UV/VIS spectrometer (Philips, UK), with 2 mL of phosphorus reagent used to provide a reference blank. The absorbance values for potassium di-hydrogen orthophosphate standards were used to produce a standard curve (see figure 2.8.4b). Values obtained for test samples were plotted and extrapolated to provide a measurement of total phosphorus content.

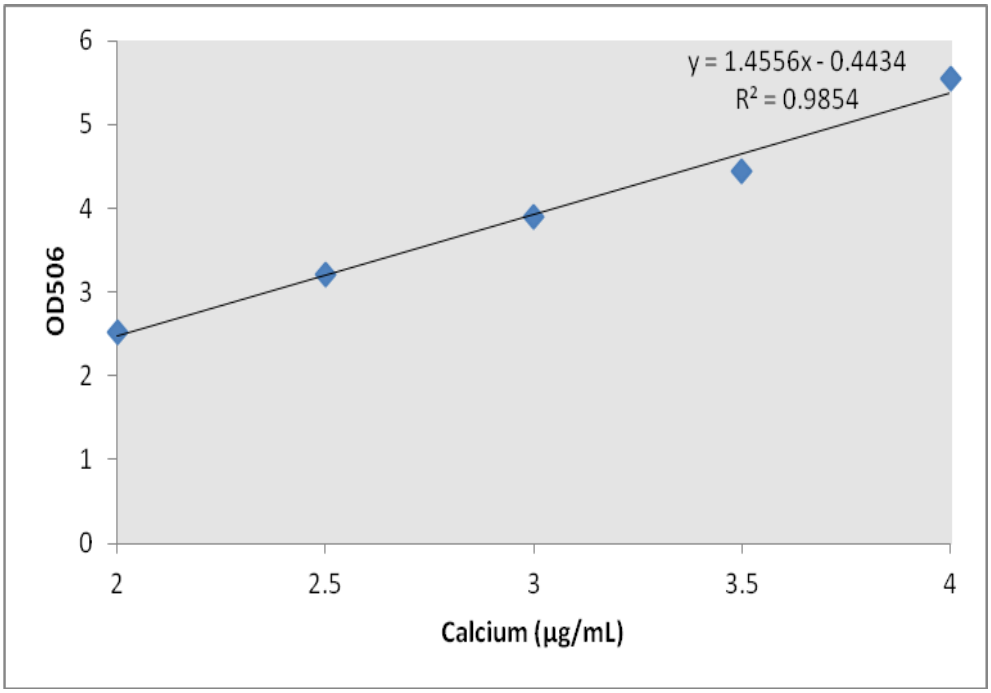

Figure 2.8.4a Standard curve displaying a series of absorbance values obtained at 506 nm for known calcium concentrations ranging from 2-4 µg/mL. Both the equation for the line and R^2 value are displayed. The R^2 value obtained indicates a low lineage variance with a value >0.98.

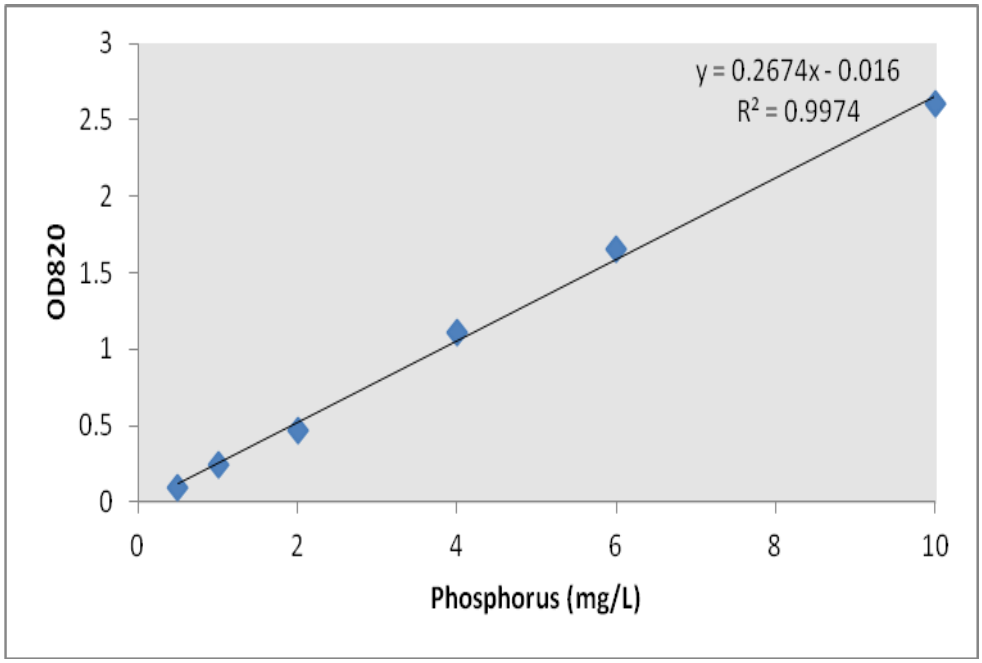

Figure 2.8.4b Standard curve displaying a series of absorbance values obtained at 820 nm for known phosphorus concentrations ranging from 0.5-10 mg/L. Both the equation for the line and R^2 value are displayed. The R^2 value obtained indicates a low lineage variance with a value >0.99.

2.8.5 Fourier Transform Infra-red Spectroscopy (FTIR)

FTIR was used to qualitatively compare phosphate and carbonate peaks of the inorganic mineral phase of odontogenic cultures with those obtained for powdered human dentine and reagent grade hydroxyapatite (289396-25G, Sigma-Aldrich, UK). Dentine powder was obtained from human non-carious teeth (ethical approval reference: BCHDent 286.1471.TB), which were cleaned, cut and pulverised as previously described (see section 2.3.1). 100 mg of dentine and hydroxyapatite powders were compressed with 1800 N force applied by an Instron 5544 electromechanical testing system (Instron, UK) to create discs of 1.2 mm diameter. Passage 2 ADCs, BMDCs and DPDCs were cultured in

osteogenic medium for 21 days (Section 2.6.1.1), the media aspirated and the remaining cultures heated at 600 °C to eliminate any organic content (Section 2.8.3). Ashed samples were deposited in an FTIR sample cup (13 mm, 3 mm thickness), the surface levelled and analysed using an FTIR spectrometer (Nicolet 520, Nicolet Instrument Corp., Madison, WI) operating with 32 scans at a resolution of 4 cm^{-1} within a wavelength range of 585-2000 cm^{-1}.

2.9 Statistical Analysis

All data were expressed as mean ± standard deviation and analysed using Microsoft Excel (Microsoft Corporation, USA) and SPSS software (SPSS Inc, USA). Basic statistical analyses were performed using the paired Students T-test, while the one way analysis of variance (ANOVA) with Bonferroni *post hoc* was used to determine significance between cell types and treatments. P values < 0.05 were considered significantly significant.

3.0 RESULTS

3.0 ISOLATION OF ADIPOSE-DERIVED CELLS

Adipose tissue potentially represents an abundant and accessible source of mesenchymal stem cells that can be utilised for regenerative dentistry (Gimble *et al*, 2007). However, methods of isolating adipose-derived cells (ADCs) have not yet been standardised. Variations in the methodology used for ADC isolation are thought to contribute to discrepancies in the immunophenotype and differentiation capacity of these cells (see section 1.6.2.1.3). Therefore, to utilise MSCs from adipose tissue for regenerative purposes the identification and development of an efficient method of ADC dissociation is required. This study compared the relative capacity of several enzymes for the dissociation of cells from inguinal adipose tissue, and evaluated the effect of each approach on the growth kinetics and colony forming capacity of the cells obtained.

3.1 Enzymatic Dissociation of Cells

Incubation with type I collagenase for 30 minutes released the largest number of cells (Table 3.1), with this method releasing approximately 24 % more cells than the next best method (30 minute treatment with type I collagenase/trypsin). 15 minute incubations with each of the enzymes released significantly ($P<0.05$) fewer cells when compared with 30 or 60 minute incubations, with a 15 minute type I collagenase treatment releasing 73 % fewer cells than 30 minute treatment with the same enzyme. Both Trypsin and accutase digestions were relatively ineffective at dissociating cells from adipose tissue, with 30

minute treatments with either of these enzymes releasing significantly ($P<0.05$) fewer cells than a 30 minute incubation using type I collagenase.

Treatment	Digestion Period (mins)	Cells Released per Gram Adipose Tissue (± Percentage SD)
Type I Collagenase (1 g/L)	15	481,798 ± 25 %
	30	1,766,553 ± 40 % *
	60	585,516 ± 35 %
Trypsin (2.5 g/L trypsin in 0.38 g/L EDTA)	30	286,180 ± 31 %
	60	258,302 ± 46 %
Type I Collagenase + Trypsin (1 g/L Collagenase + 2.5 g/L trypsin in 0.38 g/L EDTA)	15	692,041 ± 31 %
	30	1,335,669 ± 41% *
Accutase (10 mL per 75 cm^2 culture area)	15	270,780 ± 27 %
	30	452,851 ± 34 %
	60	382,021 ± 29 %

Table 3.1 Numbers of cells released per gram of inguinal adipose tissue following incubation at 37 °C with different digestive enzymes for periods of 15, 30 and 60 minutes (Mean ± SD, n=5, * = $P<0.05$).

3.1.1 The Influence of Digestion Time on Cell Growth

Digestion periods of 30 and 60 minutes were most effective for the release of cells from adipose tissue. Therefore, the effect of 30 and 60 minute digestion periods on the growth of dissociated cells was next compared.

3.1.1.1 Type I Collagenase Digestion

Following 3 days in culture, the number of cells declined by 44 % and 69 % for enzyme digestion periods of 30 and 60 minutes respectively (see figure 3.1.1a) indicating that the 60 minute digestion caused a comparatively significant ($P<0.05$) reduction in cell viability when compared with the 30 minute treatment. Cell numbers increased after the third day of culture for both 30 and 60 minute treatments with increases by day 6 of 97 % and 96 % respectively. After 6 days in culture, cell numbers stabilised for ADCs dissociated using 30 and 60 minute type I collagenase treatments. After 12 days culture, the 30 minute digestion resulted in a higher yield of cells with a significantly higher cell number than the 60 minute digestion (1.8 fold higher).

3.1.1.2 Trypsin/EDTA Digestion

Following 3 days culture the number of cells declined by 87 % and 96 % for incubations of 30 and 60 minutes, respectively (see figure 3.1.1b). Cell numbers plateaued at the third day of culture with no further cell loss. Cells derived using a 60 minute incubation period with trypsin demonstrated no substantial increase in numbers when compared with the 30 minute treatment but maintained a constant number of cells until day 12. Cells derived using 30 minute trypsin incubation also plateaued between days 3 and 6, but began to rise steadily between days 6 and 9 with a significant ($P<0.05$) increase of 92 %. After 12 days

culture, a 30 minute digestion generated the highest yield of cells with a significantly ($P<0.05$) higher final cell number than a 60 minute digestion (60 fold higher).

3.1.1.3 Accutase Digestion

Following 3 days in culture the number of cells declined by 98 % and 99 % from the initial seeding density for both the 30 and 60 minute digestion periods respectively (see figure 3.1.1c). Cell numbers obtained using both 30 and 60 minute digestions plateaued at day 3. Following the plateau only a minor increase of 6 % was observed between days 3 and 12 for both treatments.

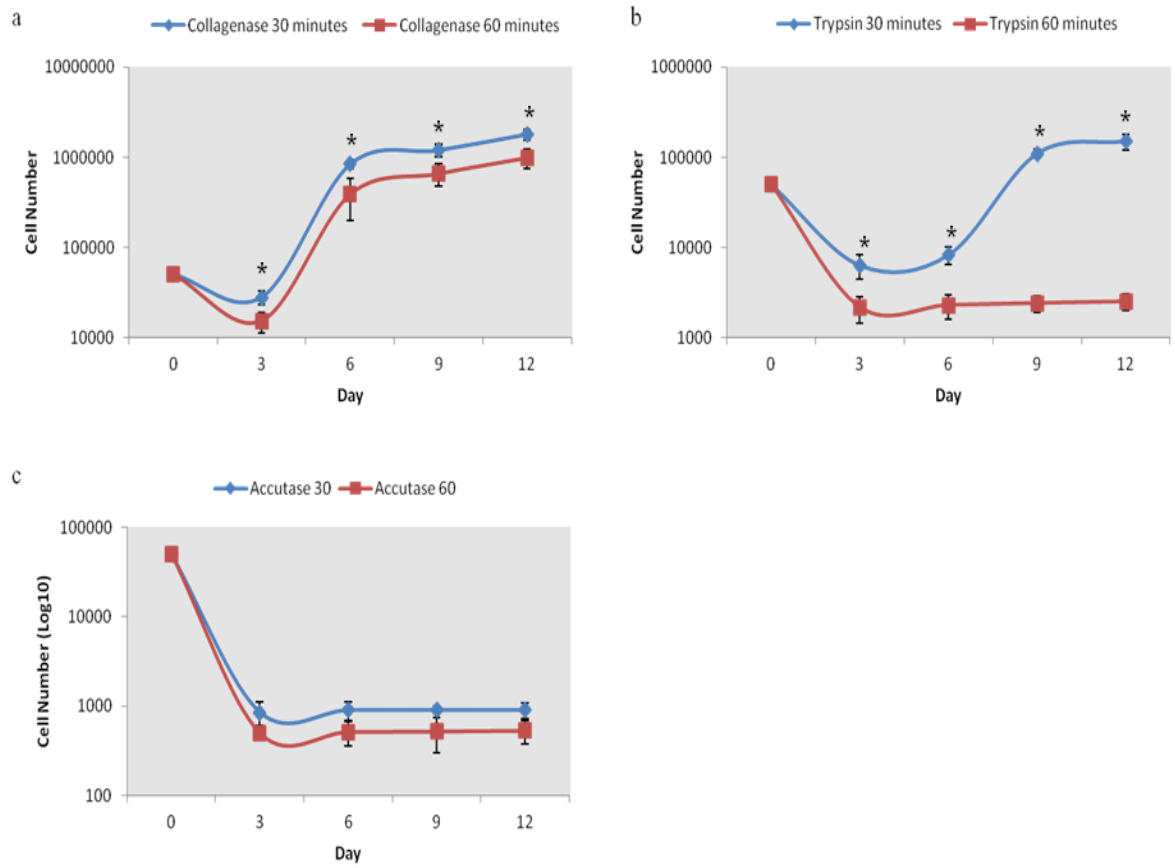

Figure 3.1.1 Growth curves displaying the effect of incubation time on the dissociation of ADCs. Adipose tissue was treated with (a) type I collagenase, (b) Trypsin or (c) Accutase and incubated for periods of 30 or 60 minutes. Resulting cell isolates were cultured over a period of 12 days with cell counts performed at 3 day intervals (Mean ± SD, *n*=5, * = P<0.05).

3.1.2 Influence of Digestive Treatment on Colony-Forming Frequency

ADC cultures were stained with a 0.1 % Toluidine blue solution to visualise the number of colonies present in 25 cm^2 culture dishes after 8 days culture (see figure 3.1.2). Digestion for 30 minutes with type I collagenase or a combination of collagenase plus Trypsin

yielded significantly (P<0.05) higher numbers of cells (Figure 3.1.1) and significantly (P<0.05) more colonies per dish (44 and 40 colonies, respectively) than any other treatment. Cells released using a 30 minute period of digestion formed significantly (P<0.05) more colonies per dish than following a 60 minute digestion. Treatment with either Accutase or Trypsin generated significantly (P<0.05) fewer colonies per dish than any other treatment, with ≤3 colonies obtained for each.

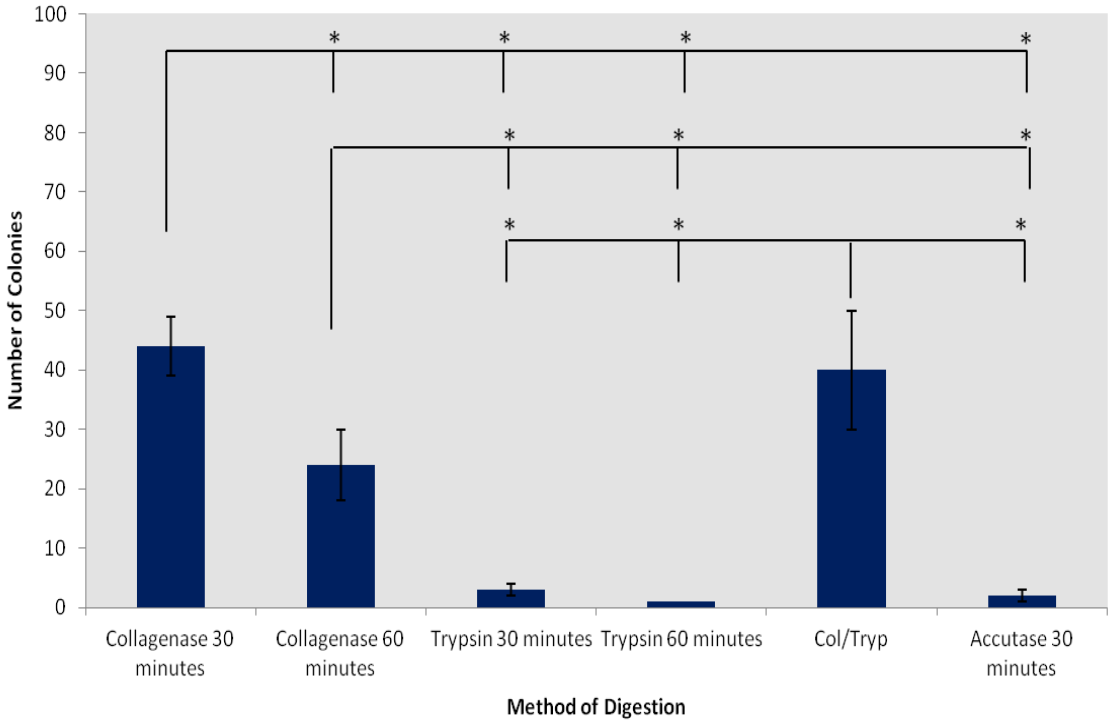

Figure 3.1.2 The number of colonies formed within ADC cultures derived using independent enzyme treatments and incubation periods. Cells were cultured in 25 cm^2 flasks for a period of 8 days, stained with 0.1 % Toluidine blue and the number of colonies counted manually under a phase contrast microscope. Colonies were defined as ≥50 cells and were counted per dish (Mean ± SD, *n*=5, * = P<0.05).

3.1.3 Comparison of 30 Minute Digestive Treatments on Cell Growth

For all enzyme regimes tested a 30 minute digestion period dissociated higher numbers of cells than a 15 or 60 minute digestion (Section 3.1). ADCs obtained using a 30 minute digestion period demonstrated increased growth rates (Section 3.1.1) and colony frequencies (Section 3.1.2) when compared with other treatments. Therefore, comparative growth profiles of cells derived from adipose tissue following enzymatic digestion for 30 minutes were compared. Following 3 days culture the numbers of viable cells present were reduced for all digestive methods. Cells obtained using type I collagenase or a combination of type I collagenase plus trypsin were least affected, with significantly ($P<0.05$) more viable cells present in these cultures after 3 days than in ADC cultures obtained using Accutase or Trypsin dissociation (Figure 3.1.3). Cells derived using type I collagenase or a combination of collagenase and trypsin treatment demonstrated significant ($P<0.05$) increases in cell numbers following 3 days in culture. In contrast, Trypsin digested adipose cultures showed no increase in cell numbers until day 6, after which the number of cells increased by > 90 % until day 12. Cell obtained following accutase treatment plateaued at day 3 and only increased by 6 % by day 12. Final cell numbers were significantly ($P<0.05$) higher for cultures derived using type I collagenase or combined collagenase plus trypsin treatment, with final cell counts of 18×10^6 and 15×10^6, respectively.

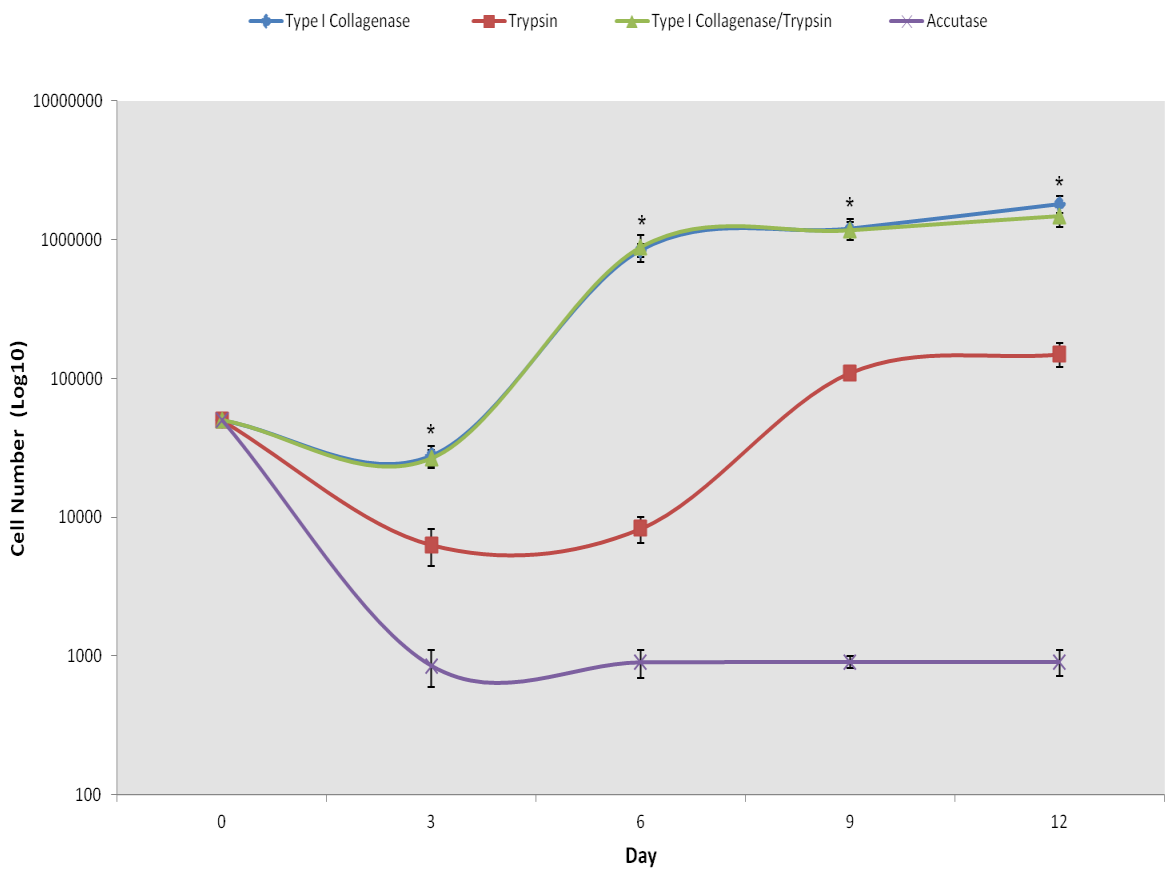

Figure 3.1.3 Growth curves for primary cells derived from adipose tissue using independent 30 minute enzyme treatments. Cells were dissociated from adipose tissue using type I collagenase, Trypsin: EDTA, Accutase, or a mixture of type I collagenase/trypsin: EDTA and their growth recorded over a period of 12 days (Mean ± SD, *n*=5, * = P<0.05).

3.1.4 Influence of Digestive Treatment on Population Doubling Time

The influence of the most effective dissociative treatments on population doubling times (PDTs) over a period of 12 days were next compared. PDTs obtained using Accutase and

60 minute Trypsin digestions were discarded due to a reduction in cell number following digestion (see figures 3.1, 3.1.1b and 3.1.1c). Cells derived using a 30 minute type I collagenase treatment had the lowest PDT and doubled on average every 44 hours. Cells derived using a combination of type I collagenase and Trypsin digestion also displayed a PDT of just 48 hours. A 60 minute incubation with type I collagenase increased the PDT by approximately 10 hours, while Trypsin digestion significantly ($P<0.05$) increased the PDT to 147 hours. This information, along with that obtained in sections 3.1, 3.1.2 and 3.1.3 indicated that 30 minutes digestion with type I collagenase was the most efficient and effective method for dissociating the highest number of viable cells from adipose tissue.

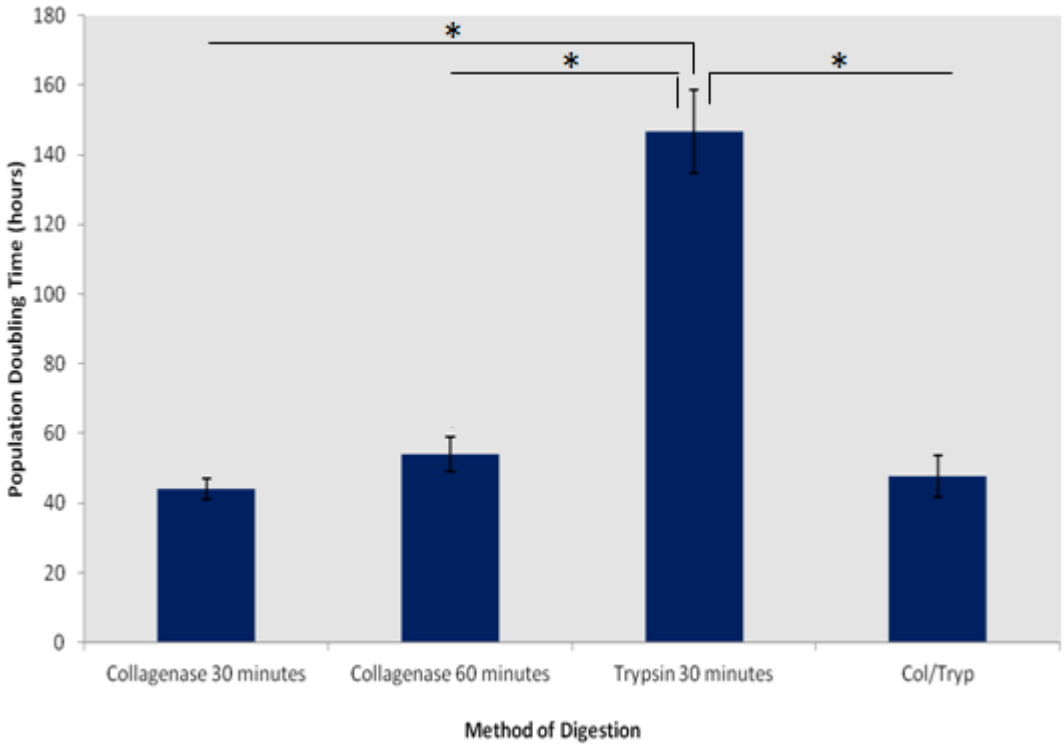

Figure 3.1.4 Graph displaying the average time taken for cells obtained using different digestive treatments to double in number. PDT was calculated over a period of 12 days using the formula displayed in section 2.1.5.2 (Mean ± SD, *n*=5, * = $P<0.05$).

4.0 COMPARATIVE ANALYSIS OF MESENCHYMAL STEM CELL SOURCES

4.1 Comparison of MSC-Like Cells Derived from Different Anatomical Sources

Mesenchymal stem cells (MSCs) can be isolated from a variety of different anatomical sites throughout the body, such as adipose tissue, bone marrow and dental pulp tissues. These cells are characterised by their ability to adhere to tissue culture polystyrene, express a profile of common surface markers and undergo multipotent differentiation (Dominici *et al*, 2006). However, a number of differences between MSCs isolated from different tissues have been demonstrated with discrepancies in marker profiles and potentially distinctive differentiation capacities reported (Mafi *et al*, 2011; Al-Nbaheen *et al*, 2013). Therefore, cells derived from adipose (ADCs), bone marrow (BMDCs) and dental pulp (DPDCs) tissues were examined using a number of stem cell characterisation assays to provide a direct comparison of their stem cell profiles.

4.1.1 Morphological Comparison

Photomicrographs taken of tissue culture polystyrene adherent cells at passage 1 and 2 using phase contrast microscopy demonstrated that BMDCs and DPDCs appeared morphologically similar demonstrating fibroblast-like appearances with several thin processes extending from the cell bodies (see figure 4.1.1). Cells appeared to become more homogenous at passage 2 with a loss of some larger cell types and a more uniform fibroblastic appearance. ADCs typically had smaller cell bodies than BMDCs or DPDCs. ADCs also presented thinner, more elongated processes with a more spindle-like appearance.

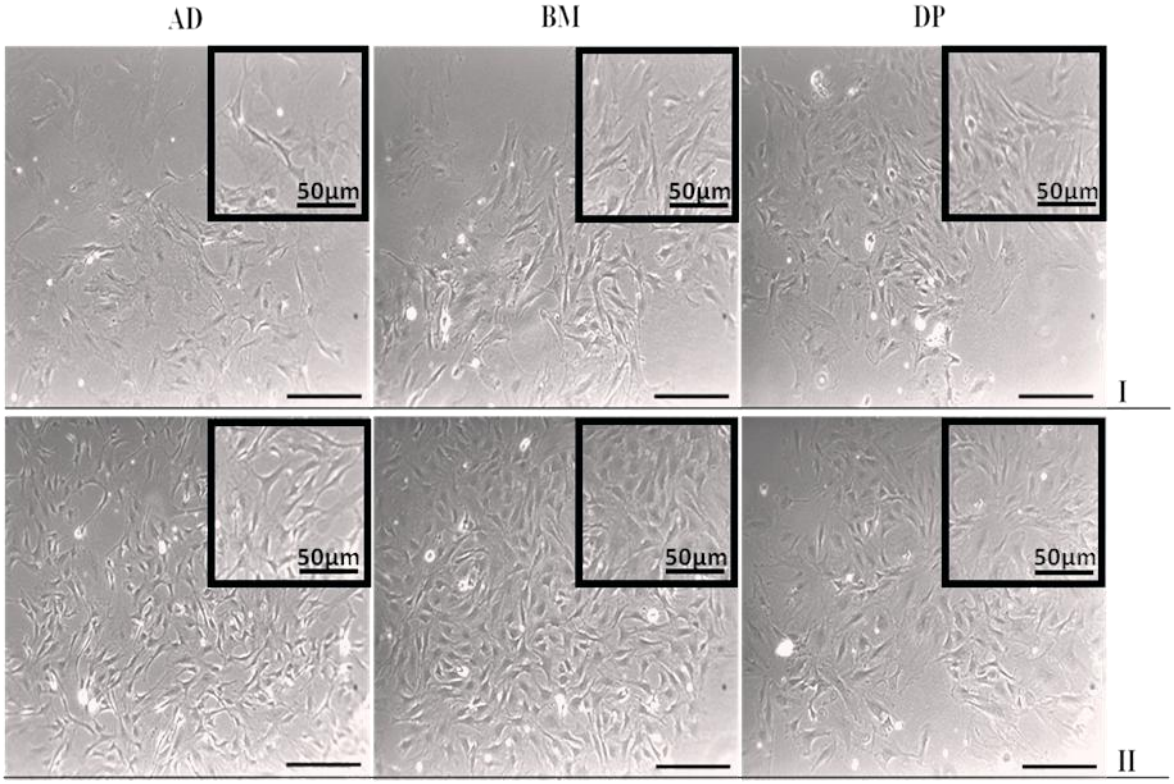

Figure 4.1.1 Representative phase contrast photomicrographs displaying passage 1 (I) and 2 (II) ADC, BMDC and DPDC cultures. Cells demonstrated fibroblast morphologies with ADCs appearing slightly smaller with a more spindle-like appearance. Scale bar represents 200 µm.

4.1.2 Growth Rates

4.1.2.1 Growth Rates for Passage 1 Cells

Cell counts obtained using Trypan blue "exclusion" staining every 3 days over a total period of 18 days showed that ADCs and BMDCs had significantly ($P < 0.05$) increased cell numbers at all time points when compared with DPDCs (see figure 4.1.2.1). ADCs and BMDCs showed 2.5 fold increases in cell numbers between days 0-3, whereas the

number of DPDCs did not increase until day 3. ADCs, BMDCs and DPDCs displayed 2.55, 2.88 and 2.5 fold increases in cell numbers between days 3-6. ADC and BMDC cultures displayed significantly ($P<0.05$) short population doubling times (PDTs) when compared with DPDC cultures during the first 9 days of culture. However, this pattern was reversed between days 9-18 with DPDCs demonstrating significantly ($P<0.05$) shorter PDTs than both ADCs and BMDCs. Cell counts at day 18 indicated that BMDC populations displayed the highest cell number with 5 % more cells than ADC cultures and significantly ($P<0.05$) more (35 %) cells than DPDC cultures.

4.1.2.2 Growth Rates for Passage 2 Cells

Passage 2 ADC, BMDC and DPDC cultures demonstrated similar growth patterns as passage 1 (Figure 4.1.2a) with ADCs and BMDCs demonstrating 4-fold increases in cell number at day 3, while DPDCs displayed a significantly ($P<0.05$) lower rate of cell growth until day 6 (Figure 4.1.2b). DPDCs demonstrated 2.8- and 2.4-fold increases in cell numbers between days 3-6 and 6-9 respectively, while the rate of cell growth in ADC and BMDC cultures began to reach a relatively constant level. Evaluation of population doubling times (PDTs) indicated that passage 2 ADC and BMDC populations displayed significantly ($P<0.05$) lower PDTs between days 0-9 than DPDCs, with this pattern being reversed after 9 days culture. Cell counts at day 18 indicated that ADCs and BMDCs achieved significantly ($P<0.05$) higher numbers of cells than DPDCs following 18 days culture, with 25 % and 23 % more cells, respectively.

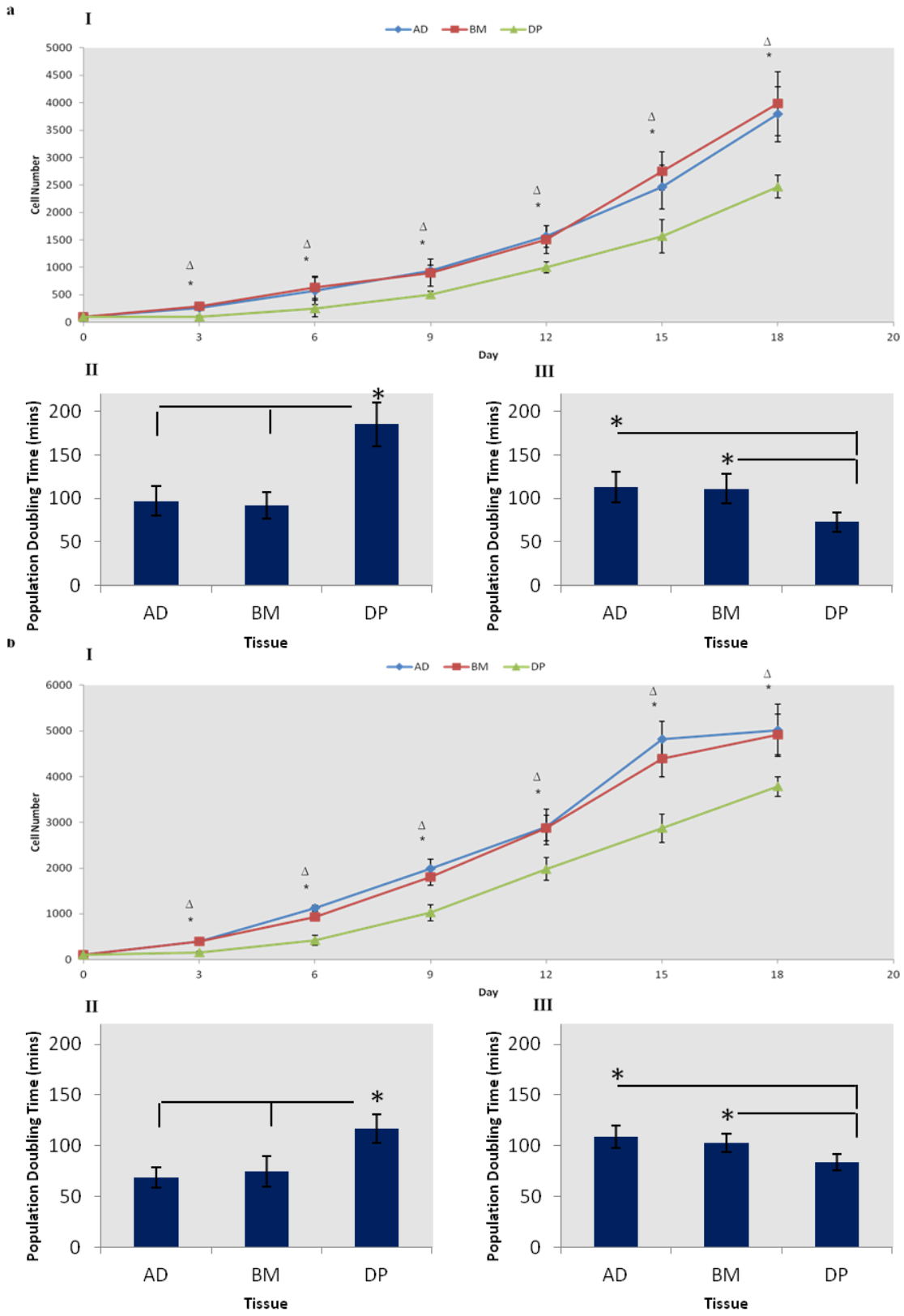

Figure 4.1.2 Growth curves (I) and population doubling times (II and III, 0-9 and 9-18 days, respectively) obtained for ADCs, BMDCs and DPDCs at passage 1 (a) and 2 (b) (Mean ±SD, n=5, * (ADC) and Δ (BMDC) = $P<0.05$).

4.1.3 Colony Formation Frequency

Quantitative analysis of the number of colonies present in each 35 mm^2 culture dish indicated that BMDC cultures contained significantly ($P<0.05$) more colonies than ADC and DPDC cultures, 2-fold and 1.5-fold respectively (see figure 4.1.3). ADC cultures contained comparatively few colonies with 1.3-fold fewer than present in DPDC cultures. Observation of all three cultures using phase contrast microscopy indicated that colonies present within ADC cultures were generally much larger than those present within BMDC and DPDC cultures with each colony having an approximately 3-fold higher cell number (see figure 4.1.3c). These data demonstrated a correlation between the relatively low colony frequency within adipose cultures and the comparatively high numbers of cells obtained after 6 days culture (see section 4.1.2).

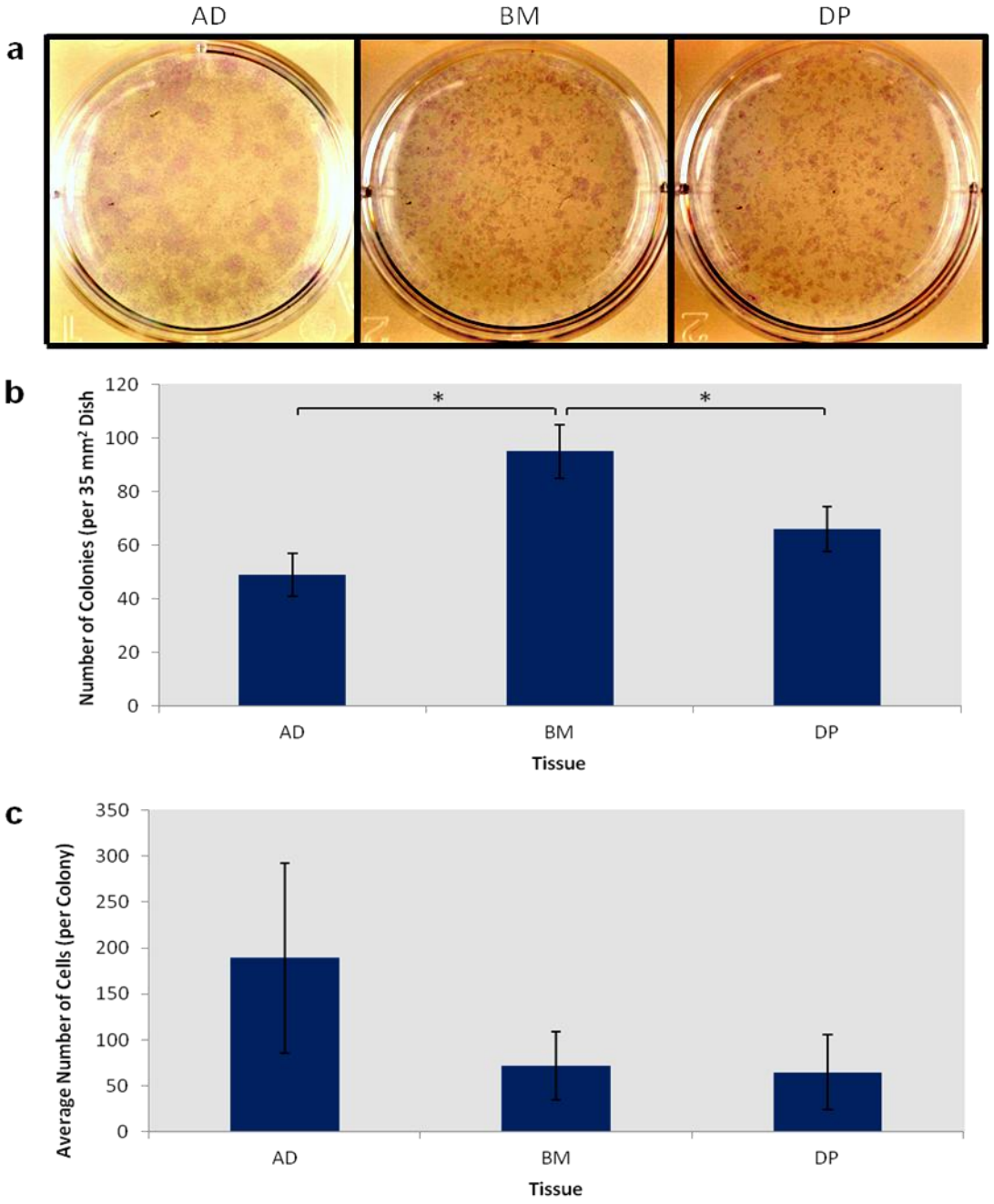

Figure 4.1.3 (a) Photomicrographs displaying Toluidine blue-stained colonies formed within passage 2 ADC, BMDC and DPDC cultures with accompanying quantification of colony number per 35 mm^2 (b) and number of cells per colony (c). colonies were defined as clusters of ≥ 50 cells and counted manually under a phase contrast microscope (see section 2.5.1) (Mean ± SD, *n*=3, * = P<0.05).

4.2 Comparative Analysis of the Expression of MSC Markers

4.2.1 FACS Profiling for CD29/CD90 Positive Populations

FACS was used to identify the proportion of cells positive for mesenchymal stem cell markers within freshly isolated, passage 1 and passage 2 ADC, BMDC and DPDC populations. CD29, CD90 and CD29/CD90 positivity was analysed within a defined region of cells that was established to avoid the selection of false positives resulting as a consequence of cell clumping or cell death (see appendix 8). CD29 and CD90 surface antigens were chosen based on guidelines presented by the ISCT, and the prevalence of these markers within MSC populations has been consistently demonstrated (Dominici *et al*, 2006; Mafi *et al*, 2011). Freshly isolated ADC populations were 91 % positive for CD29, 57 % positive for CD90 and 51 % positive for CD29/CD90 (see figure 4.2.1a). When cultured in basal growth medium at passage 1 the population of CD29, CD90 and CD29/CD90 positive cells within the total ADC population increased by 6 %, 12 % and 13 %, respectively (see figure 4.2.1b). Further expansion at passage 2 resulted in no increase in presence of CD29 positive cells but did increase the numbers of CD90 and CD29/CD90 positive cells detected by a further 13 % and 11 %, respectively (see figure 4.2.1c). Freshly isolated ADCs displayed significantly ($P<0.05$) increased numbers of CD29, CD90, and CD29/CD90 positive cells when compared with freshly isolated BMDCs (see figure 4.2.1a). However, the proportion of CD29 positive cells within BMDC populations increased by 33 % following initial subculture to levels that were 8 % higher than ADC populations (see figure 4.2.1b). No data were obtained for newly isolated DPDCs as these cells were not isolated in sufficient quantities required for FACS analysis. Expression of CD29 at passages 1 and 2 showed no variation between ADC, BMDC and DPDC

populations with each displaying between 95-99 % positivity. Expression of CD90 and CD29/CD90 in DPDC populations at passage 1 was 10 % and 8 % higher than the expression of these markers in BMDC populations and significantly ($P<0.05$) higher than their expression in ADC populations. The profile of these markers remained stable for BMDC cultures between passage 1 and 2, with only minor fluctuations in expression, while CD90 and CD29/CD90 expression increased for passage 2 DPDC cultures by 4 % and 4.5 %, respectively. These data demonstrated that tissue culture polystyrene selection was able to increase the proportion of cells expressing MSC-associated markers in ADC, BMDC and DPDC populations and provide similar numbers of CD29, CD90 and CD29/CD90 positive cells in all three populations.

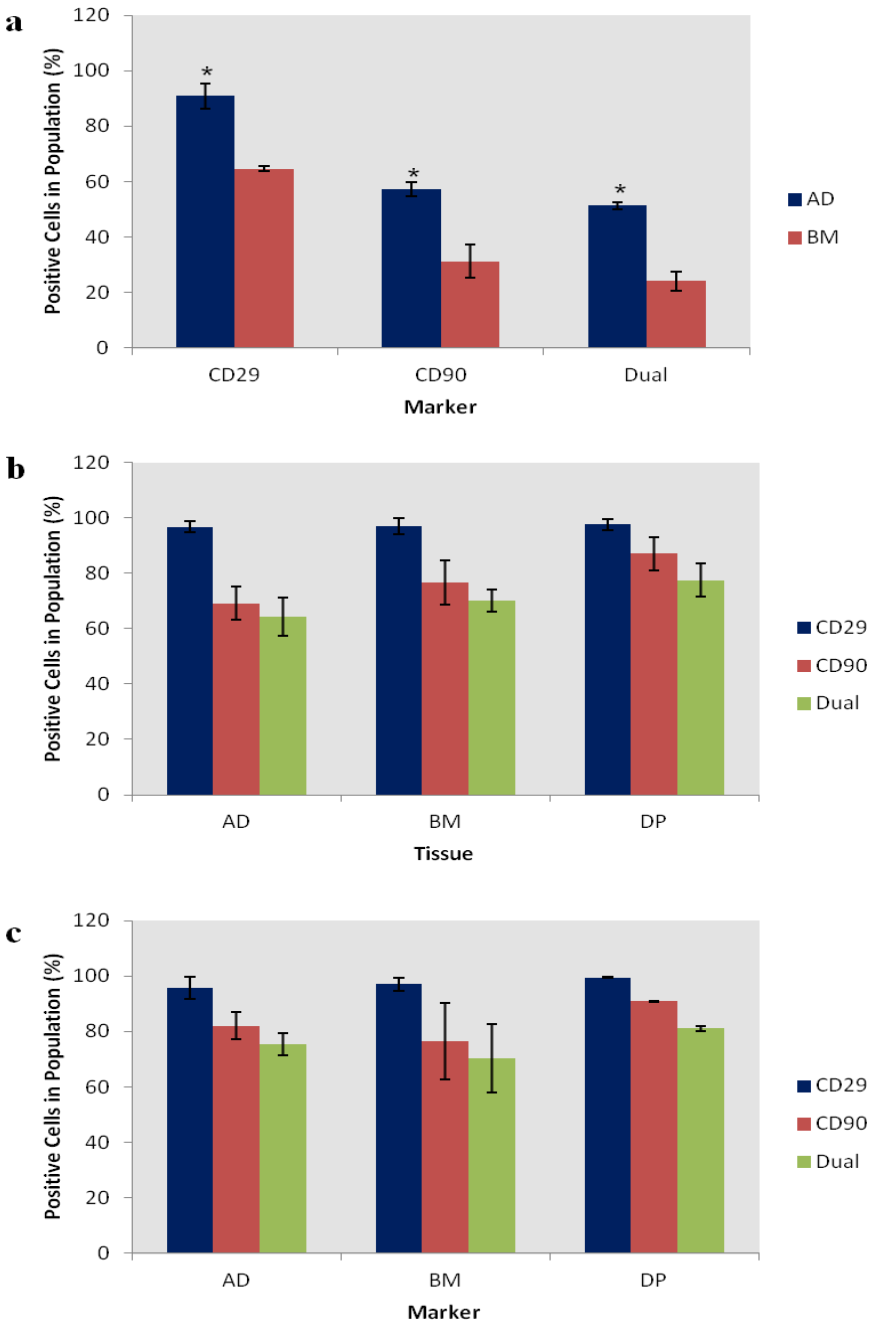

Figure 4.2.1 Graphical representation of FACS profiles displaying the percentage of CD29, CD90 and CD29/CD90 positive cells in freshly isolated ADC and BMDC cultures (a), and passage 1 (b) and 2 (c) ADC, BMDC and DPDC cultures. Y-axis represents the total number of positive cells located within a defined area. The area was selected to avoid the inclusion of dead or clumped cells (see appendix 8) (Mean ± SD, *n*=5, * = P<0.05).

4.2.2 sqRT-PCR Analysis for the Expression of Mesenchymal Stem Cell Markers

Data presented in section 4.2.1 demonstrated that ADC, BMDC and DPDC cultures contained high proportions of cells that express MSC-associated markers and that these positive populations could be expanded *in vitro*. Gene expression analyses from gel images depicted in Appendix 1 were used to provide a semi-quantitative measure of the expression of pluripotent and multipotent markers associated with MSCs over the course of 5 passages (Riekstina *et al*, 2009; Mafi *et al*, 2011; Tsai *et al*, 2012). Freshly isolated ADCs, BMDCs and DPDCs expressed pluripotent markers and continued to express these markers throughout passages 1- 4 (see figure 4.2.2a). Gene expression was most variable for freshly isolated cells from all sources but became less variable following *in vitro* culture. Primary DPDC populations demonstrated a significantly ($P<0.05$) lower expression of Nanog and Klf4 than ADC and BMDC populations, and a significantly lower expression of SOX-2 and C-myc than BMDC and ADC populations, respectively. This analysis indicated that cells isolated from all three tissues expressed stem cell markers associated with pluripotency and the expression of these markers was maintained as passage number increased.

CD34 expression was limited to freshly isolated ADC cultures and was not detected in BMDC and DPDC cultures (see figure 4.2.2b). There was also a significantly ($P<0.05$) higher expression level of CD29 and CD105 in primary ADC cultures when compared with BMDC and DPDC cultures. The levels of expression of these two markers declined when ADCs passage number increased, but the markers continued to be detected over the five passages analysed. At passage 2, the expression of multipotent markers became less variable for all three cultures, with similar trends observed for CD44, CD90, CD73 and

CD105 expression between the three cell types. Cells derived from ADC, BMDC and DPDC cultures expressed common MSC markers associated with multipotency, but the relative expression of these markers at each passage differed depending on the tissue from which they were sourced.

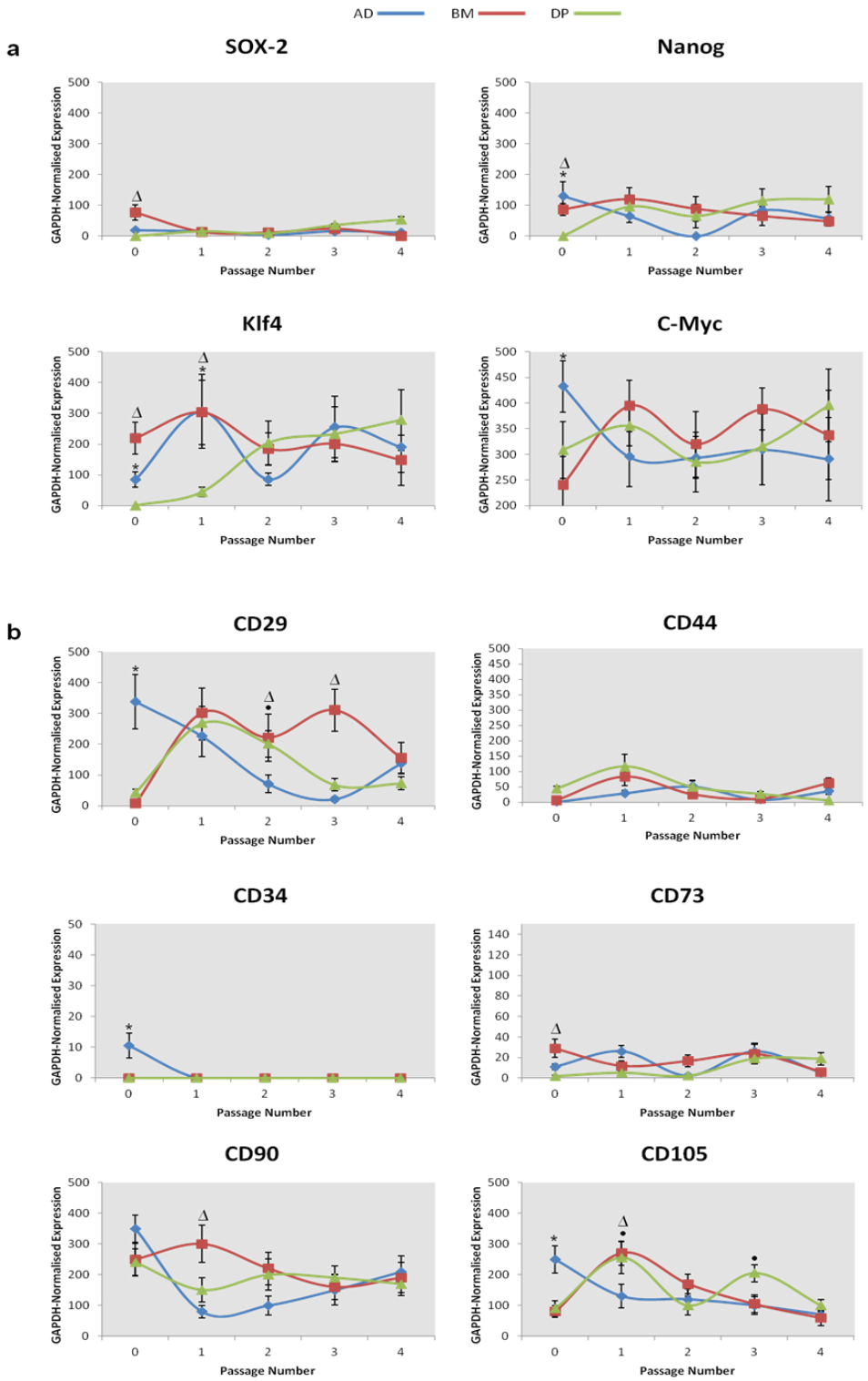

Figure 4.2.2 Gene expression profiles demonstrating the influence of passage number on the expression of (a) pluripotent and (b) multipotent genes in ADC, BMDC and DPDC cultures. Data were normalised against GAPDH and and the normalised densitometric values of PCR products represented graphically (Mean ± SD, n=5, * (ADC), Δ (BMDC) and • (DPDC) = $P<0.05$) (Information regarding primer sequences and PCR cycle numbers are provided in Table 2.7.5). For corresponding PCR gel images see appendix 1.

4.3 Multilineage Differentiation

4.3.1 The Influence of Dexamethasone on the Differentiation of ADCs towards a Mineralising Cell Phenotype

Dexamethasone is a glucocorticoid typically supplemented into osteogenic medium at varying concentrations as reported throughout the literature (Hoemann *et al*, 2009; Kyllonen *et al*, 2013). The effect of dexamethasone on mineralisation within ADC cultures was assessed by culturing them in the presence of osteogenic medium containing 10^{-7}, 10^{-8} and 10^{-9} M concentrations of dexamethasone. Images obtained using phase contrast microscopy demonstrated that dexamethasone had a dose-dependent effect on mineralisation within ADC cultures, with increasing concentrations of dexamethasone inhibiting the formation of mineralised nodules (see figure 4.3.1a). ADCs grown in the absence of dexamethasone displayed a cobblestone-like arrangement of cells that were densely packed (see figure 4.3.1I). Cultures lacking dexamethasone also lacked observable alizarin red staining (ARS). ADCs grown in the presence of 10^{-8} and 10^{-9} M dexamethasone exhibited a similar morphology and the formation of mineralised foci (see figure 4.3.1II and III). However, significant ($P<0.05$) differences were observed relating to the size and frequency of foci within the two cultures, with 10^{-9} M dexamethasone promoting a visible and quantifiable increase in nodule diameter and concentration of ARS present in these cultures (see figure 4.3.1III and b).

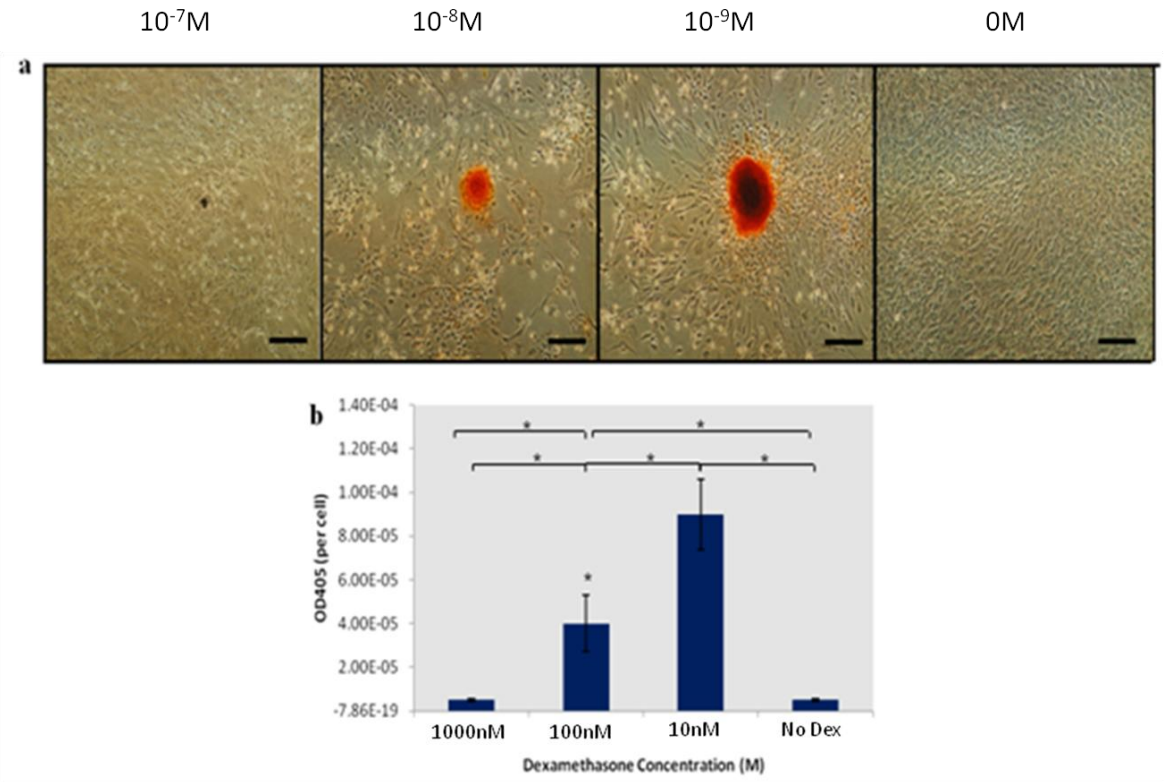

Figure 4.3.1 (a) Representative phase contrast photomicrographs demonstrating the influence of dexamethasone concentration on the mineralising capacity of ADC cultures. Cells were exposed to osteogenic medium supplemented with 10^{-7}, 10^{-8}, 10^{-9} M dexamethasone for a period of 21 days, stained and compared with osteogenic cultures lacking dexamethasone (0 M). Total ARS was then quantified (b) (Mean ± SD, n=5, * = $P<0.05$).

4.3.2 Osteogenic Induction

Phase contrast microscopy and ARS quantification were used to examine the capacity of ADC, BMDC and DPDC cultures to differentiate towards a mineralising cell phenotype (see figure 4.3.2). All three cell populations responded to osteogenic induction by forming

mineralised matrix with characteristic nodules that stained with ARS (see figure 4.3.2a). However, cells isolated from these three tissues displayed different patterns of mineralisation. ADCs exhibited the lowest level of mineralisation with fewer ARS nodules, which measured approximately ≤ 100 μm in diameter (see figure 4.3.2aII). ARS nodules in BMDC cultures were observed at a higher frequency than in ADC and DPDC cultures and typically had diameters exceeding 100 μm (see figures 4.3.2aI and aII). Microscopic examination showed that DPDC cultures demonstrated a unique pattern of mineralisation, lacking defined nodules that were visibly present in the ADC and BMDC cultures (see figure 4.3.2aI and aII). Staining in DPDC cultures resembled a sheet that covered the majority of the cell culture with no defined nodules associated with the *in vitro* mineralisation. Quantification of ARS within each culture after 7, 14 and 21 days demonstrated that DPDC cultures bound significantly ($P<0.05$) more ARS than ADC or BMDC cultures at each time point (see figure 4.3.2b). Mineralised foci were observed in BMDC populations following 14 days exposure to osteogenic medium, while ADC populations required ~21 days for mineralised nodule formation to occur. Following 14 and 21 days exposure to osteogenic medium, DPSC and BMDC populations showed significantly ($P<0.05$) greater ARS than ADC populations (as normalised for total number of cells; Figure 4.3.2b). For analysis of the relative expression of genes associated with osteogenic differentiation in ADC, BMDC and DPDC cultures see section 7.4.

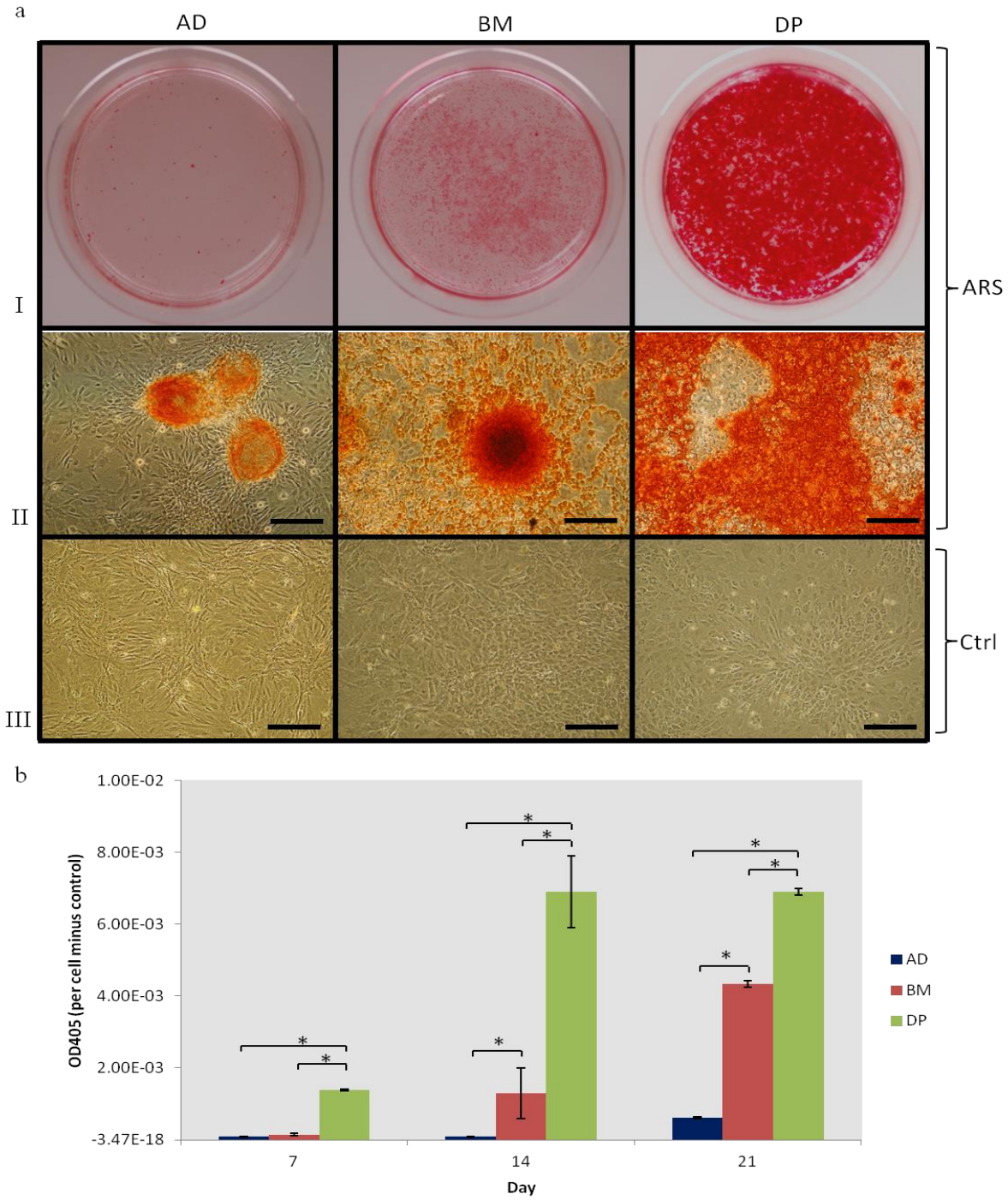

Figure 4.3.2 (a) Representative Phase contrast photomicrographs of alizarin red stained (ARS) ADC, BMDC and DPDC cultures following exposure to osteogenic (I and II) or growth medium (III) for a period of 21 days. (b) Accompanying ARS quantification normalised by the total number of cells (Mean ± SD, *n*=5, * = P<0.05). Scale bars in images represent 100 µm.

4.3.3 Quantification of the Total Inorganic Content of Osteogenic Cultures

The proportion of inorganic material present in ADC and BMDC cultures after 21 days exposure to osteogenic medium represented only 1.91 % and 2.14 % of the respective total weights (see figure 4.3.3). However, for DPDC cultures the proportion of inorganic material was significantly higher, representing 45.74 % of the total culture weight (23-fold higher than ADC and BMDC cultures). These data correlated with previous findings (Section 4.3.1) indicating that DPDC cultures were capable of producing more mineral than ADC and BMDC when exposed to osteogenic culture medium, with almost half of the weight of the final culture being represented by inorganic content.

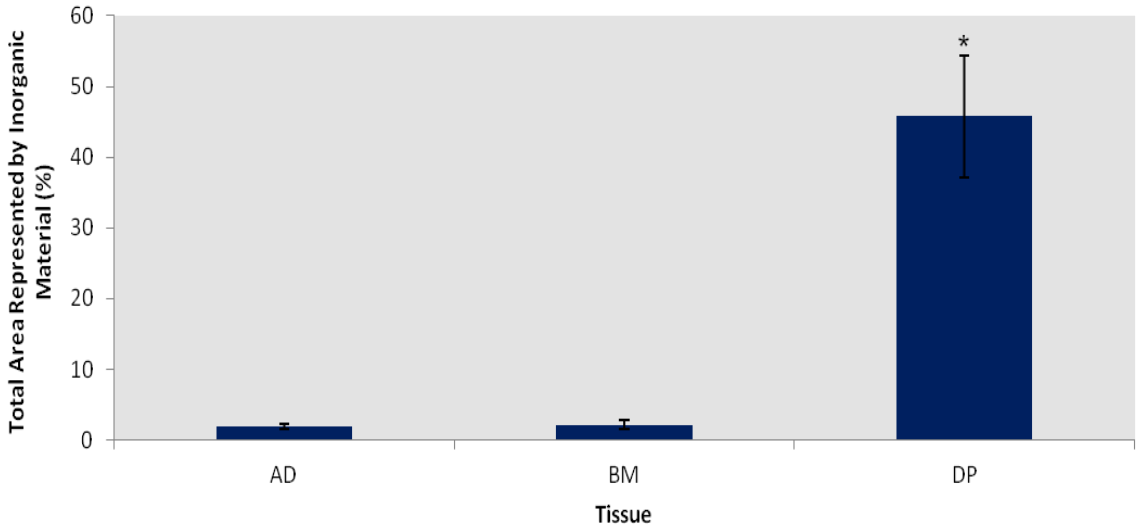

Figure 4.3.3 Quantification of the total area represented by inorganic material in osteogenic cultures. ADC, BMDC and DPDC cultures were exposed to osteogenic medium for 21 days, after which the medium was aspirated and the inorganic phase turned to ash by heating at 600 ºC for a period of 15 minutes (see section 2.8.3). Cultures were weighed before and after incubation at 600 °C and the initial and final weighs used to calculate the percentage mineral content (Mean ± SD, *n*=20).

4.4 Adipogenic Induction

Cells were cultured in adipogenic medium for a period of 21 days, after which each culture was stained with oil red O (ORO) to visualise intracellular lipid droplets. Images obtained using phase contrast microscopy demonstrated the appearance of rounded cells containing large vacuoles that stained red after the addition of ORO (see figure 4.4a). ORO staining differed between the three cell populations, with ADC cultures binding significantly ($P<0.05$) more ORO stain than BMDC or DPDC cultures following 7, 14 and 21 days exposure to adipogenic medium (see figure 4.4b). BMDC cultures bound significantly ($P<0.05$) more ORO stain than DPDC cultures after both 14 and 21 days culture. DPDC cultures demonstrated no increase in ORO uptake until day 21 with numbers of ORO positive cells remaining relatively low. Cells derived from all three tissues were able to form adipocyte-like cells after exposure to adipogenic medium. However, the relative adipogenic capacity of cells derived from the three tissues was markedly different, with ADCs displaying significantly ($P<0.05$) more ORO positive inclusions than BMDC and DPDC cultures.

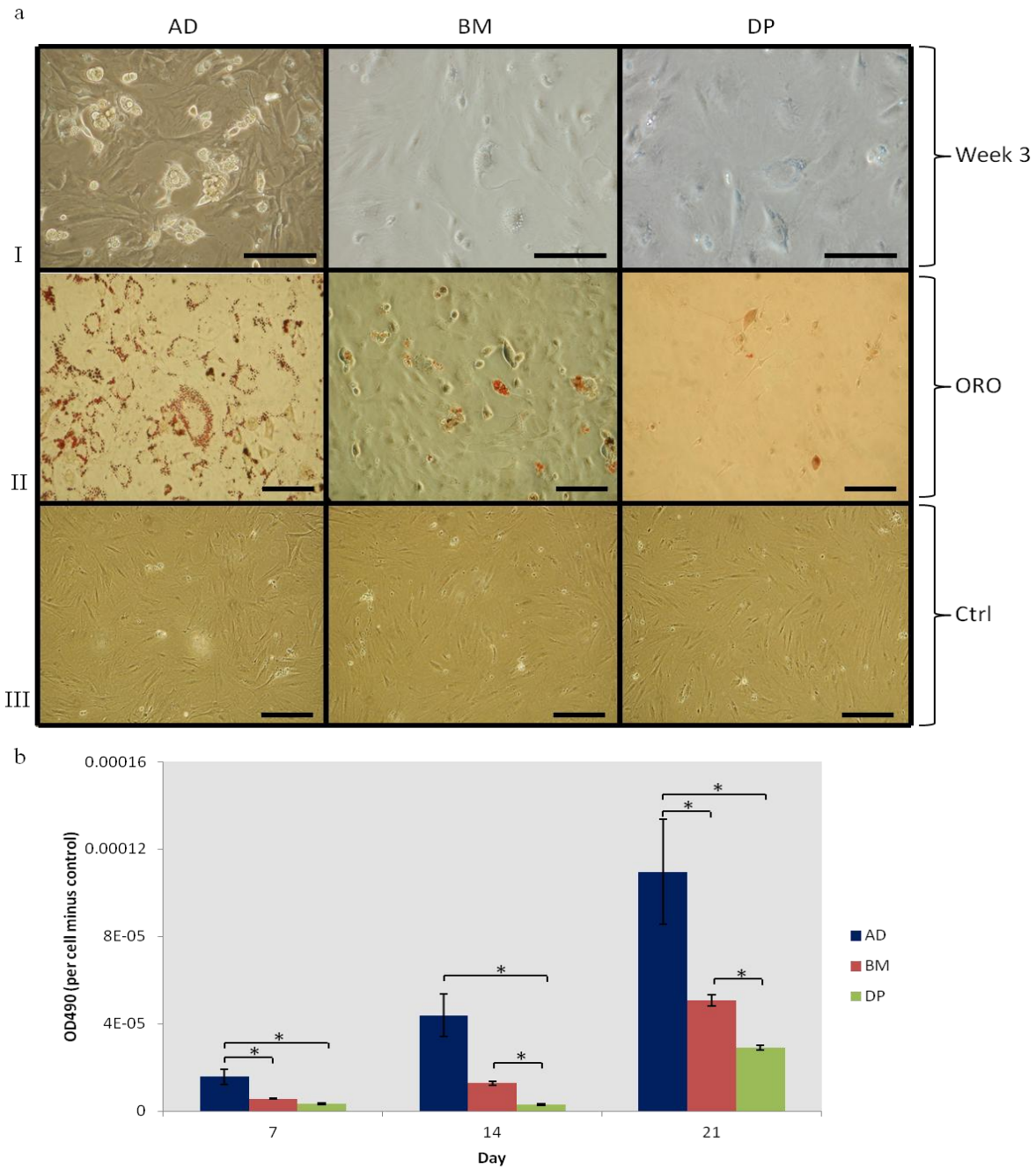

Figure 4.4 (a) Representative phase contrast photomicrographs of passage 2 ADC, BMDC and DPDC cultures exposed to adipogenic medium for a period of 21 days before (I) and after (II) ORO staining, or growth medium (III). (b) Accompanying ORO quantification normalised by the total number of cells (Mean ± SD, *n*=5, * = P<0.05). Scale bars represent 100 µm.

4.4.1 sqRT-PCR Analysis for the Expression of Genes Associated with Adipogenesis

Gene expression analysis indicated that ADCs, BMDCs and DPDCs demonstrated expression for the three adipogenic markers, LPL, aP2 and PPARγ (see figure 4.4.1) (Hu *et al*, 1995; Ding *et al*, 1999). ADC and BMDC cultures that had been exposed to adipogenic medium typically displayed highest expression of adipocytic markers after 7 days culture, with expression of LPL, PPARγ and aP2 all significantly ($P<0.05$) increased when compared with DPDC cultures. After 14 days exposure, the expression of all three adipocytic markers decreased for ADC and BMDC cultures, while remaining relatively constant in DPDC cultures. Following 21 days adipogenic culture levels of LPL expression within DPDC cultures declined and the expression of PPARγ and aP2 dropped to a basal level. ADC and BMDC cultures demonstrated slight decreases in the expression of all three markers after 21 days exposure to the adipogenic stimulus, but maintained levels significantly ($P<0.05$) higher than those observed for DPDC cultures at this time point. Cells derived from all three tissues expressed markers associated with adipogenesis but the relative expression levels of these markers were typically lower for DPDC cultures over the course of 21 days.

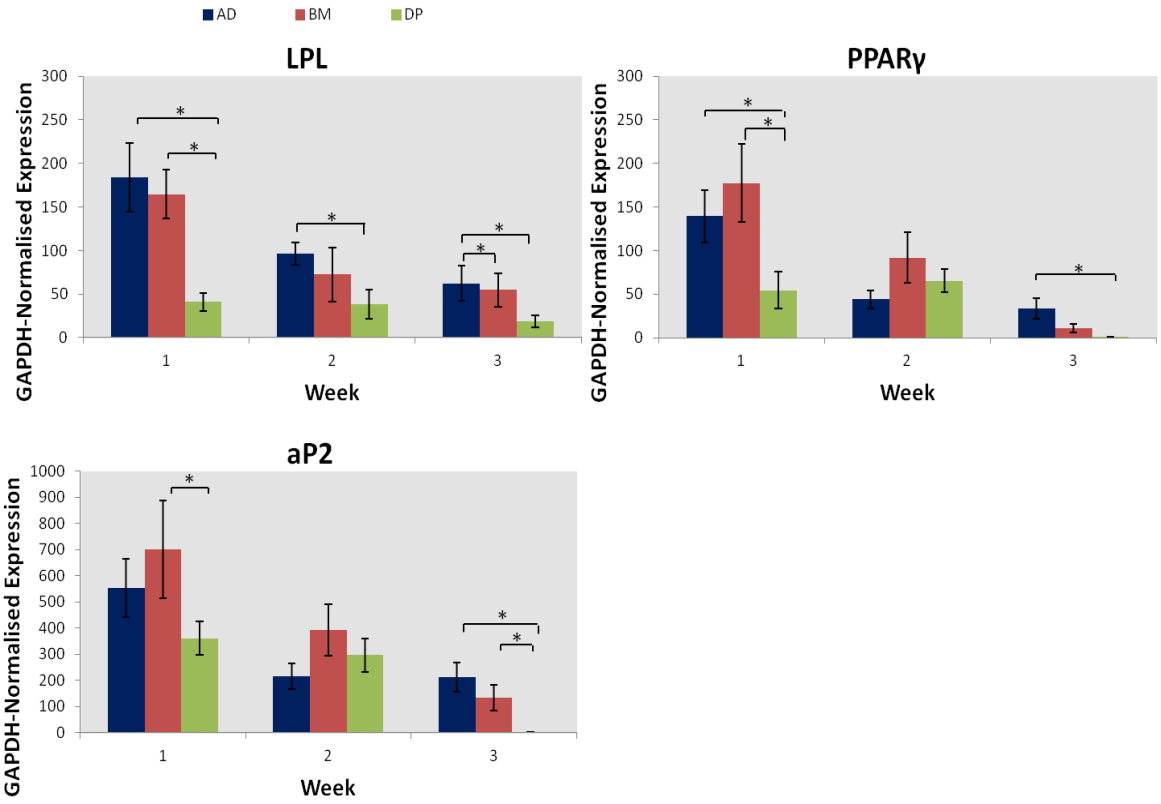

Figure 4.4.1 Graphical representation of the expression of adipogenic genes for passage 2 ADC, BMDC and DPDC cultures exposed to adipogenic medium for periods of 7, 14 and 21 days. Data were normalised against GAPDH and the normalised densitometric values of PCR products represented graphically. (Mean ± SD, *n*=3, * = P<0.05). For corresponding PCR gel images see appendix 2.

5.0 THE INFLUENCE OF FACS ON THE VIABILITY, MULTI-DIFFERENTIATION POTENTIAL AND GENE EXPRESSION PROFILES OF MSC-LIKE CELLS

Fluorescence activated cell sorting (FACS) represents a routinely applied method for the profiling and isolation of MSC-like cell populations from heterogeneous tissue isolates. However, the benefits of FACS over simple polystyrene adherence for the selection of multipotent cells, together with the influence of FACS on cell viability, are not well documented. Moreover, the commercial availability of antibodies raised against rodent MSC markers is limited. The work presented in this section utilised FACS for the antibody-based selection of cells displaying MSC-identifying surface antigens in order to evaluate if cell selection could provide a more stem-like population than simple adherence and expansion on tissue culture polystyrene (Huang *et al*, 2009; Mafi *et al*, 2011).

5.1 The Effect of FACS on Cell Viability

5.1.1 Viability of Cells Selected Using FACS

FACS utilises hydrodynamic and extensional forces for the selection of cells based on the presence of specific surface markers (Herbertson and Aubin, 1997; Zaitoun *et al*, 2010). The influence of FACS on cell viability was assessed by performing Trypan blue cells counts. Results were compared with two control groups: no primary antibody controls that were not labelled with antibody but passed through the FACS system, and no sort controls that were neither conjugated with antibody nor passed through the FACS system. Results indicated that CD29, CD90, CD29/CD90, and no primary antibody control populations

demonstrated no significant differences in viability following FACS (see figure 5.1.1). However, when sorted populations were compared with unsorted populations that had been treated identically (excluding FACS selection) and had undergone the same period of time out of culture, viability was found to be decreased. Both unsorted ADC and DPDC populations contained significantly ($P<0.05$) more viable cells than any of the corresponding FACS selected populations. Sorted populations showed a reduction in the number of viable cells in BMDC cultures, with more viable cells present in unsorted populations than CD90 and CD29/CD90 positive populations and significantly ($P <0.05$) more viable cells than CD29 selected populations and the no primary controls. This indicated that the process of cell selection significantly reduced the viability of ADC, BMDC and DPDC populations.

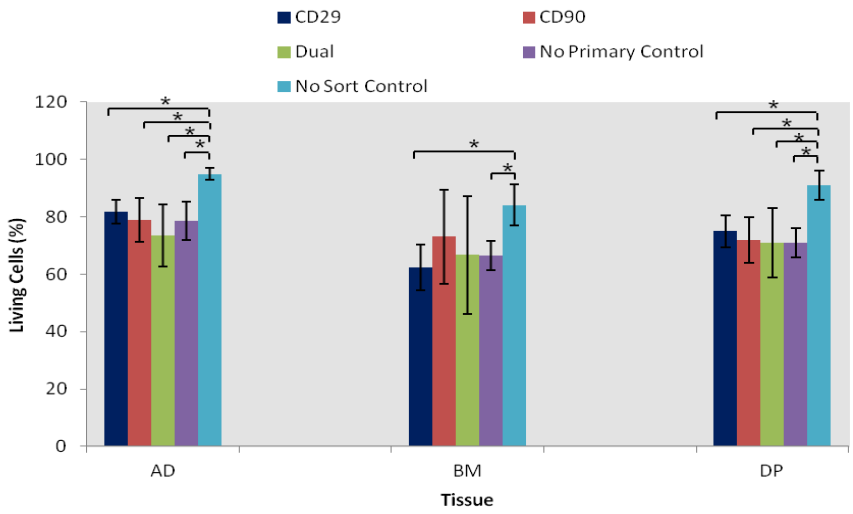

Figure 5.1.1 Viability of passage 2 cells derived from adipose (AD), bone marrow (BM) and dental pulp (DP) tissues following FACS. The viability of CD29, CD90 and CD29/CD90 sorted populations, unsorted populations and no primary controls was assessed using the Trypan blue exclusion assay. Unsorted populations underwent identical pre-FACS treatment to sorted cells. (Mean ± SD, *n*=5, * = $P<0.05$).

5.1.2 The Influence of Time Out of Standard Culture Conditions on Cell Viability

FACS requires cells to be maintained in suspension (FACS buffer, see section 2.2.1) during pre-selection labelling and during cell selection. ADCs, BMDCs and DPDCs were maintained in suspension at approximately 4 ºC for a period of 4.5 hours with Trypan blue cell counts performed every 30 minutes to assess how time spent in suspension influenced their viability. All three cell types displayed >90 % viability when removed from normal culture conditions for 60 minutes or less (see figure 5.1.2). ADCs maintained viable cell counts of >90 % over the total 270 minute incubation, while BMDCs and DPDCs maintained >90 % viability for up to 150 minutes in suspension. The number of viable cells present in both BMDC and DPDC cultures continued to decline at a similar rate following 150 minutes in suspension, with decreases of 14 % and 9 %, respectively. Following 270 minutes in suspension, BMDCs displayed the greatest decrease in viable cell numbers with a 23 % decrease. DPDC cultures displayed a 16 % reduction in the number of viable cells, whereas ADC cultures experienced only an 8 % reduction in viability. Since the time taken for FACS did not exceed 3 hours these results indicated that time spent in suspension could not solely account for the reduction in cell viability following selection (see section 5.1.1).

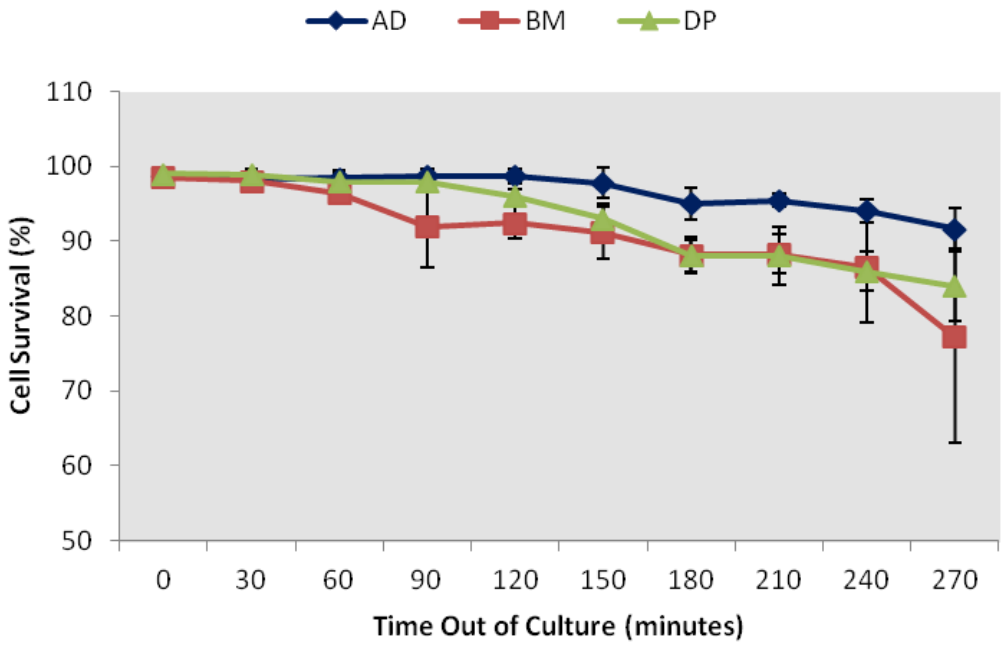

Figure 5.1.2 Graphical representation of the decline in percentage viability of ADCs, BMDCs and DPDCs maintained in suspension (FACS buffer: PBS + 1 % FCS) over a period of 270 minutes at approximately 4 ºC. (Mean ± SD, *n*=5, * = p<0.05).

5.2 The Influence of FACS on Cell Differentiation Potential

5.2.1 Differentiation towards a Mineralising Cell Phenotype

Sorting cells for CD29, CD90 or CD29/CD90 significantly reduced their ability to undergo differentiation towards a mineralising cell phenotype. Whether populations were used directly after FACS (direct) or expanded and then differentiated (indirect) (see section 2.2.1) also appeared to influence the size and number of mineralised foci formed (Figure 5.2.1a and b). Direct seeding of cells following FACS yielded more nodules when compared with indirectly seeded cells; although this increase was not significant (see

figure 5.2.1a and 5.2.1b). The diameter of ARS nodules for cells assayed directly after FACS and no primary control cultures was ≥100 μm, while cells expanded following FACS and then assayed exhibited decreased nodule sizes (<50 μm for ADC cultures and <100 μm for BMDC cultures). Nodules formed by unsorted cell cultures typically had a larger diameter (≥150 μm) and more intense ARS. Quantification of ARS confirmed the comparatively low levels of mineral deposited in CD29, CD90, or CD29/CD90 positive cultures (see figure 5.2.1c and d). Controls that had not been selected with primary antibody contained significantly ($P<0.05$) higher concentrations of ARS when compared with CD29, CD90, and CD29/CD90 sorted populations. Unsorted populations that underwent identical treatment to all sorted cell populations (excluding FACS analysis) exhibited a greater ARS, with significantly ($P<0.05$) more stain bound when compared with all sorted population and the no primary controls.

5.2.2 Adipogenic Differentiation

Phase contrast images obtained following 21 days exposure to adipogenic medium demonstrated that sorting cultures for CD29, CD90 or CD29/CD90 significantly reduced the area of intracellular lipid formed in each cell, as assessed by ORO staining (see figure 5.2.2). Direct and indirect seeding following FACS selection also appeared to have an influence on the capacity of cultures to form lipid filled adipocytes-like cells. Following the trend observed for osteogenic differentiation, no primary controls demonstrated a higher level of ORO staining (see figure 5.2.2). ORO quantification further indicated that cells not subjected to FACS selection displayed a significantly ($P<0.05$) increased ORO staining following 21 days culture when compared with cells subjected to all other sorting criteria. These results together with those obtained for osteogenic differentiation (see section 5.2.1) indicated that FACS reduced the differentiation potential of ADCs and BMDCs.

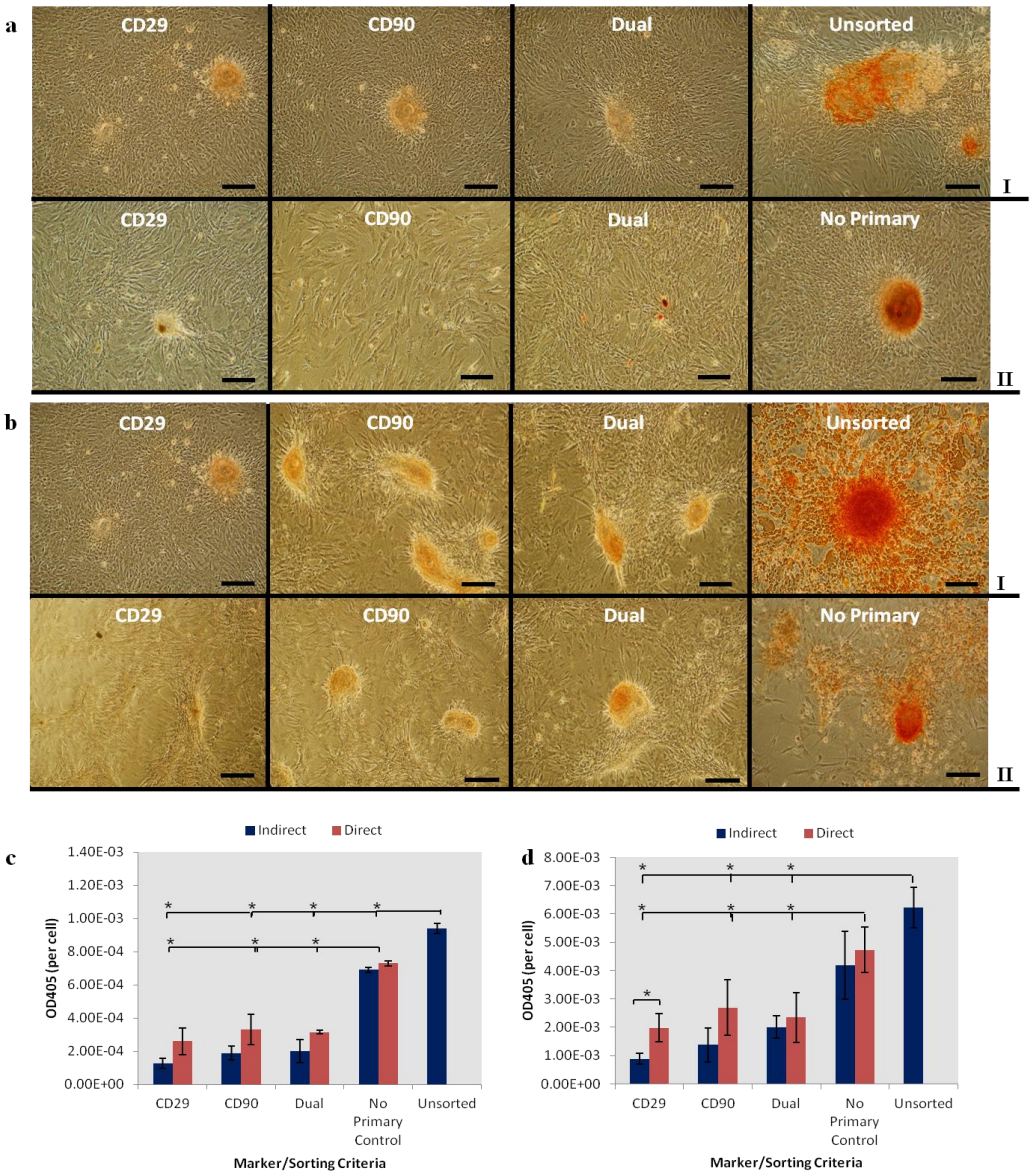

Figure 5.2.1 Representative phase contrast photomicrographs displaying FACS sorted and unsorted passage 2 ADC (a) and BMDC (b) cultures that were exposed to osteogenic medium and stained with ARS. Sorted populations were either directly assayed (I) in osteogenic medium or expanded (indirectly) in basal culture medium (II) to increase cell numbers and then assayed. ARS was quantified from both ADC (c) and BMDC (d) cultures and normalised by the total cell number (Mean ±SD, n=3, * = $p<0.05$). Scale bars in images represent 100 µm.

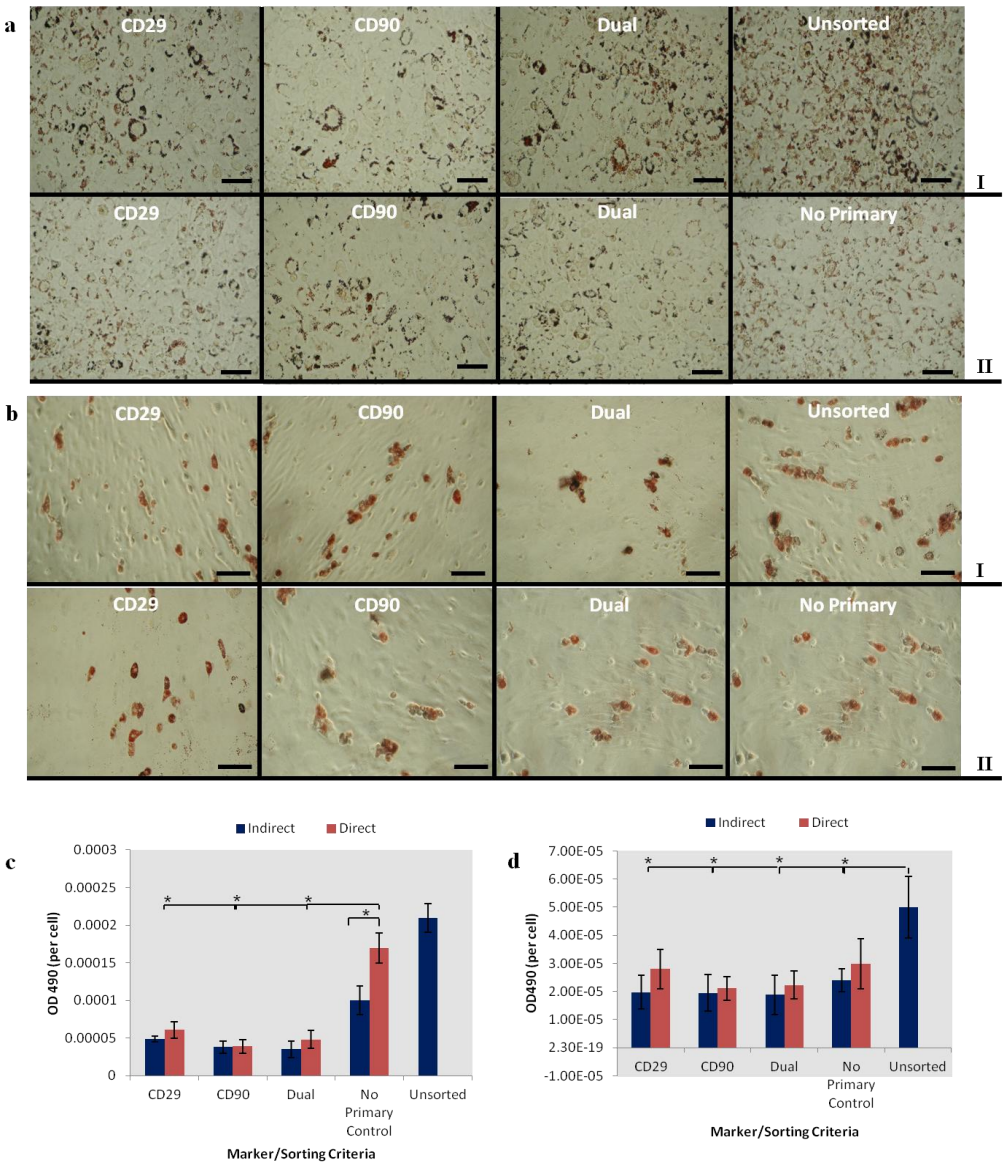

Figure 5.2.2 Representative phase contrast photomicrographs displaying FACS sorted and unsorted passage 2 ADC (a) and BMDC (b) cultures that were exposed to adipogenic medium and stained with ORO. Sorted populations were either directly assayed (I) in adipogenic medium or expanded in basal culture medium (II) to increase cell numbers and then assayed. ORO was quantified from both ADC (c) and BMDC (d) cultures and normalised by the total cell number. (Mean ±SD, n=3, * = $p<0.05$). Scale bars represent 100 µm.

5.3 FACS Analysis of CD29/CD90 Expression Following Post-FACS Culture

Following FACS selection, CD29, CD90 and CD29/CD90 positive populations were cultured in growth medium until confluent. After reaching confluence, these cells were FACS profiled to determine the influence of post-FACS expansion on the surface marker expression of each population (See section 2.2.1). ADC populations sorted using CD29 displayed a 3.4 % decline in the number of CD29 positive cells following post-FACS culture, while BMDC populations displayed a significant ($P<0.05$) 40.1 % decline (see figure 5.3). Post-FACS culture caused a significant ($P<0.05$) 17.8 % increase in the number of CD90 positive cells present within the ADC population, while only a 3.3 % increase was observed for the post-FACS cultured BMDC population. Similar proportions of CD29/CD90 positive cells were displayed in post-FACS cultured ADC and BMDC populations, with both showing significant ($P<0.05$) increases of 23.6 % and 22.9 %, respectively. These results indicated that CD90 sorted populations expanded following post-FACS culture, whilst CD29 only populations decreased.

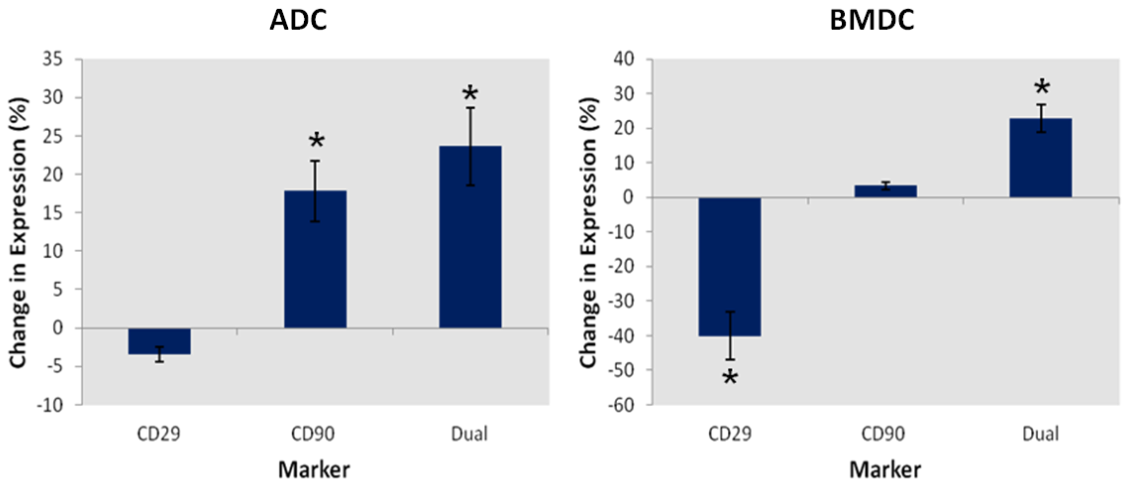

Figure 5.3 Graphical representation of the percentage change in the number of CD29, CD90 and CD29/CD90 positive cells in ADC and BMDC populations before and after post-FACS expansion. All cells were sorted, expanded in culture until confluent and then re-analysed to determine if expansion following FACS had an influence on the immunophenotype. (Mean ± SD, *n*=3, * = $p<0.05$).

5.4 The Expression of MSC Markers in FACS Cultures

5.4.1 sqRT-PCR Analysis of Stem Cell Markers

sqRT-PCR was used to evaluate the effects of expanding CD29, CD90 and CD29/CD90 populations on the expression of stem cell marker genes. FACS generally had a negative influence on the expression of pluripotent markers within ADC cultures, but had limited effects on the expression of these genes in BMDC cultures. The expression levels of Lin28, Nanog, Sox-2 and Klf4 in ADC populations were all significantly reduced following FACS when compared with unsorted populations (see figure 5.4.1aI). FACS had less of an effect on the expression of pluripotent markers in BMDC populations with

only a reduction in the expression of Sox-2 detected following CD29 selection (see figure 5.4.1bI).

The expression of multipotent markers within ADC and BMDC populations was influenced by FACS selection. Levels of CD29, CD44 and CD90 were significantly ($P<0.05$) higher following the dual selection of CD29/CD90 positive cells from ADC populations (see figure 5.4.1aII). However, the expression of CD73 and CD105 was not influenced by FACS selection. In contrast with data obtained for ADC cultures FACS had no detectable influence on the expression of CD29, CD44 or CD90 in BMDC cultures, and levels of CD73 and CD105 remained lower than in the unsorted BMDC population (see figure 5.4.1bII). These results demonstrated that CD29, CD90 and CD29/CD90 positive populations within ADC and BMDC cultures exhibited different gene expression profiles to unsorted populations.

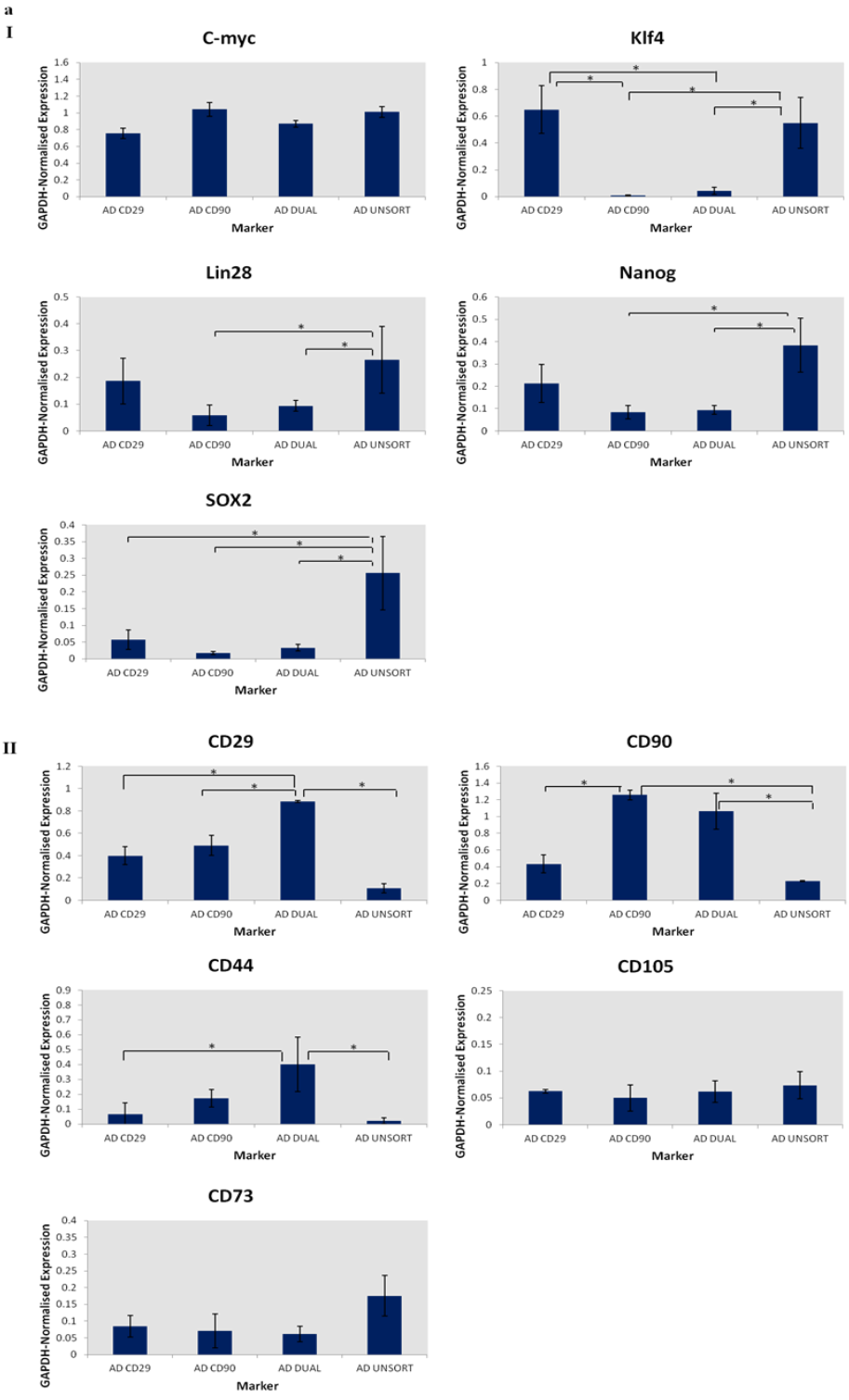

Figure 5.4.1a Gene expression profiles for pluripotent (I) and multipotent (II) stem cell marker expression in CD29, CD90 and CD29/CD90 positive ADC populations that had undergone post-FACS expansion in basal growth medium. Intensities were normalised against GAPDH and and the normalised densitometric values of PCR products represented graphically. (Mean ± SD, n=3, * = P<0.05). For corresponding PCR gel images see appendix 4 and 5.

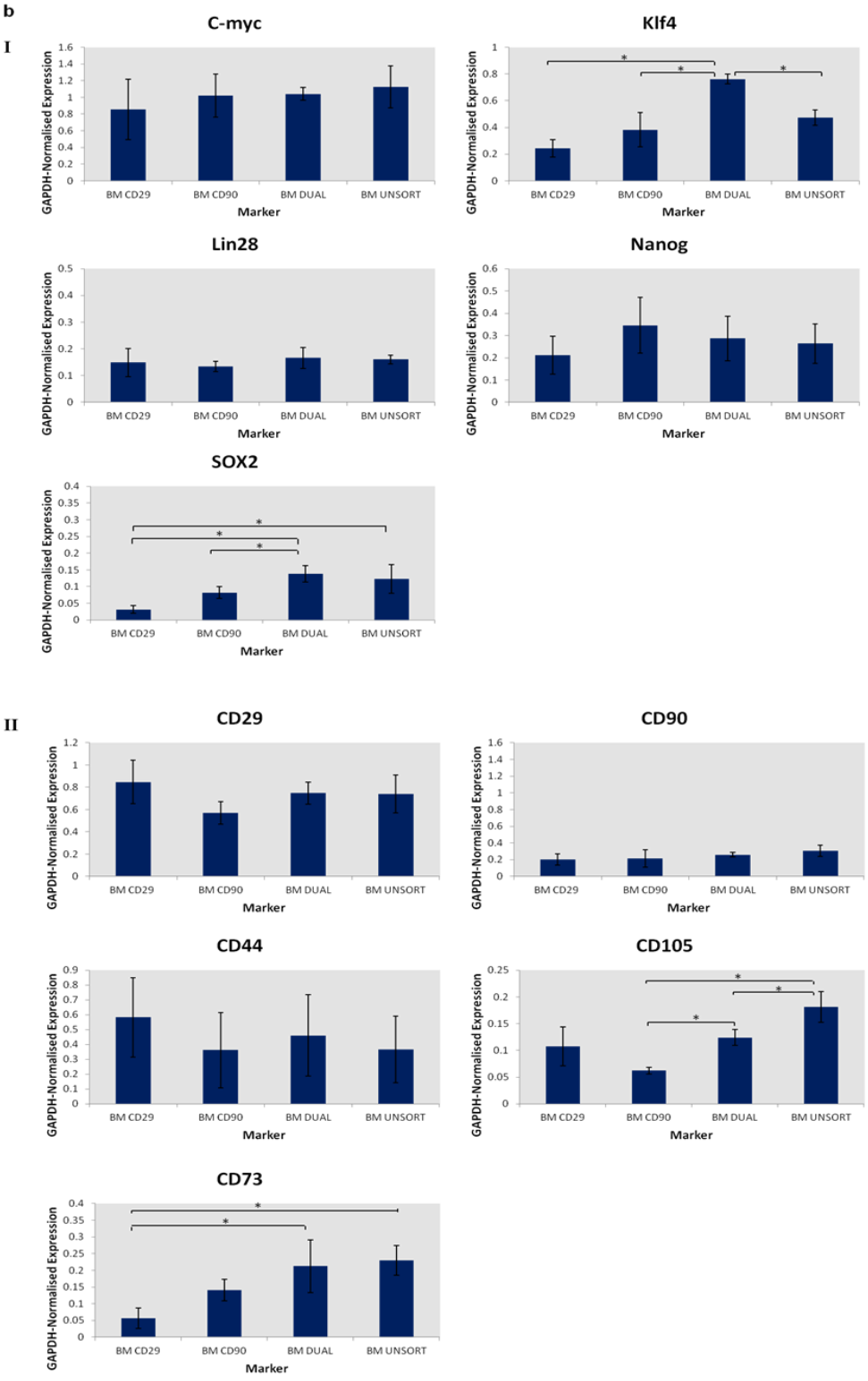

Figure 5.4.1b Gene expression profiles for pluripotent (I) and multipotent (II) stem cell marker expression in CD29, CD90 and CD29/CD90 positive BMDC populations that had undergone post-FACS expansion in basal growth medium. Intensities were normalised against GAPDH and and the normalised densitometric values of PCR products represented graphically. (Mean ± SD, n=3, * = P<0.05). For corresponding PCR gel images see appendix 4 and 5.

6.0 THE EFFECTS OF CRYO-STORAGE ON CELLS DERIVED FROM ADIPOSE, BONE MARROW AND DENTAL PULP TISSUES

A reduction in the osteogenic capacity of MSCs following liquid nitrogen storage has been previously demonstrated, as well as interspecies differences in the overall viability of stored cells (Ock and Rho, 2011; Naaldijk *et al*, 2012). However, information regarding the viability of cryo-preserved MSC populations derived from different tissues remains limited. Furthermore, it has been demonstrated that postnatal stem cells continue to express MSC markers following cryopreservation, however, information concerning the relative levels of these markers compared with freshly isolated controls is lacking (Perry *et al*, 2008). Therefore, the work in this chapter evaluates the effects of liquid nitrogen storage on the morphologies, FACS and PCR profiles, and viabilities of ADCs, BMDCs and DPDCs.

6.1 The Influence of Cryo-Storage on Cell Phenotype

6.1.1 The Influence of Cryo-Storage on Cell Morphology

Passage 2 ADC, BMDC and DPDC cultures that had been preserved for a period of 14 days using cryo-storage were thawed and grown until confluent (see section 2.1.6). Reseeded cells were observed using phase contrast microscopy and appeared morphologically similar to freshly isolated controls with both displaying a fibroblast-like appearance with thin processes extending from the cell bodies (see figure 6.1.1). The time taken for ADCs, BMDCs or DPDCs to reach confluence was also unaffected by cryo-storage with all cells becoming approximately 80 % confluent after 8 days in culture.

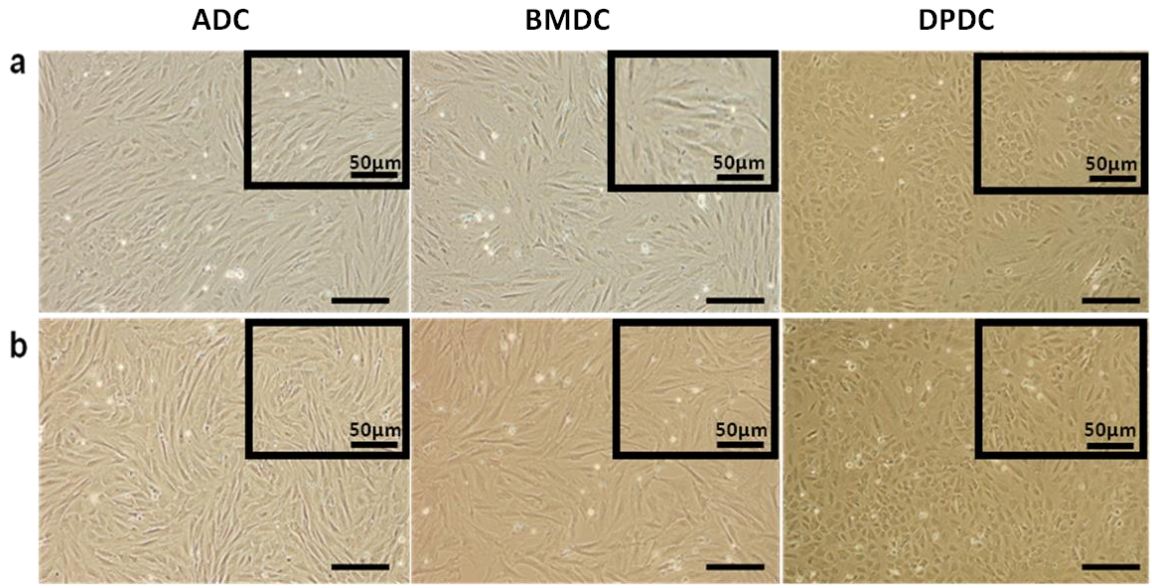

Figure 6.1.1 Representative phase contrast photomicrographs comparing the morphologies of freshly isolated (a) cyro-preserved (b) ADC, BMDC and DPDC cultures established at passage 2. Cyro-preserved cells were seeded in FCS containing 10 % DMSO and maintained in liquid nitrogen for a period of 14 days. Scale bars on unmagnified images represent 100 µm.

6.1.2 The Influence of Cryo-Storage on Cell Viability

To determine if cryo-stored cells could be used for FACS analysis (see section 5) the capacity of these cells to survive time in suspension was analysed. To evaluate the effects of cryo-storage on the viability passage 2 ADCs, BMDCs and DPDCs these cells were cryo-stored for a period of 14 days, thawed, grown to confluence and placed in a FACS buffer (see section 2.2.1) suspension for a total duration of 270 minutes. Cell viability counts were performed every 30 minutes and are displayed in figure 6.1.2. The viability of ADCs was least variable with only a 6 % decrease in the number of viable cells over the total 270 minute duration (see figure 6.1.2). DPDC cultures displayed >90 % viability

when maintained in suspension for 60 minutes. The total decline in the number of viable cells in DPDC cultures was 27 % over the entire 270 minute duration. The total number of viable cells present within BMDC cultures after 30 minutes in suspension was 37 % lower than for ADC cultures and 34% lower than for DPDC cultures. The total number of viable cells present within BMDC cultures further declined with increasing time in suspension, with significantly ($P<0.05$) fewer viable cells at all time intervals than were present in ADC and DPDC populations. After 270 minutes in suspension, BMDC cultures were significantly ($P<0.05$) less viable than respective ADC and DPDC populations indicating that MSC-like cells derived from different anatomical locations had different survival rates following cryo-storage. These results demonstrated that a reduction in the number of viable cells could partially account for the selective pressures applied by cryo-storage documented in section 6.2, particularly for BMDCs.

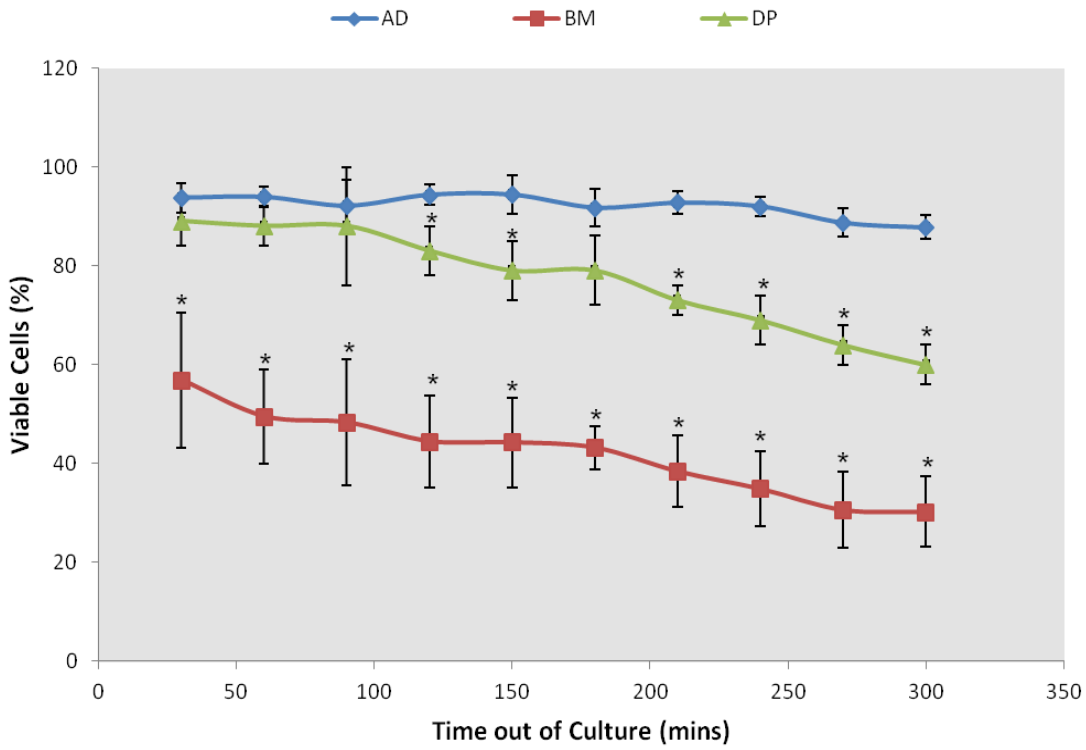

Figure 6.1.2 Measure of the number of viable cells found in ADC, BMDC and DPDC suspensions following 2 weeks in cryo-storage. Passage 2 ADC, BMDC and DPDC populations were placed in FACS buffer (PBS supplemented with 1 % FBS) and maintained out of culture for a period of 270 minutes in order to mimic the FACS procedure (Mean ± SD, *n*=5, * = P<0.05).

6.2 The Influence of Cryo-Storage on the Expression of MSC Markers

6.2.1 The Influence of Cryo-Storage on the CD29/CD90 FACS Profile

To evaluate if cryo-storage had an effect on the expression of cell surface markers, liquid nitrogen (LN) stored and freshly isolated Passage 2 adipose (ADLN), bone marrow (BMLN) and dental pulp (DPLN) cultures were FACS profiled for the expression of CD29, CD90, and CD29/CD90. The relative expression levels of CD29 between stored and freshly isolated cells from all three tissues remained similar with liquid nitrogen storage having no significant difference on the level of CD29 expression (see figure 6.2.1). Significant ($P<0.05$) increases were observed in expression of CD90 for both ADLN and BMLN cultures. The relative percentage of cells expressing CD29/CD90 was also found to be significantly ($P<0.05$) increased for ADLN, BMLN and DPLN populations.

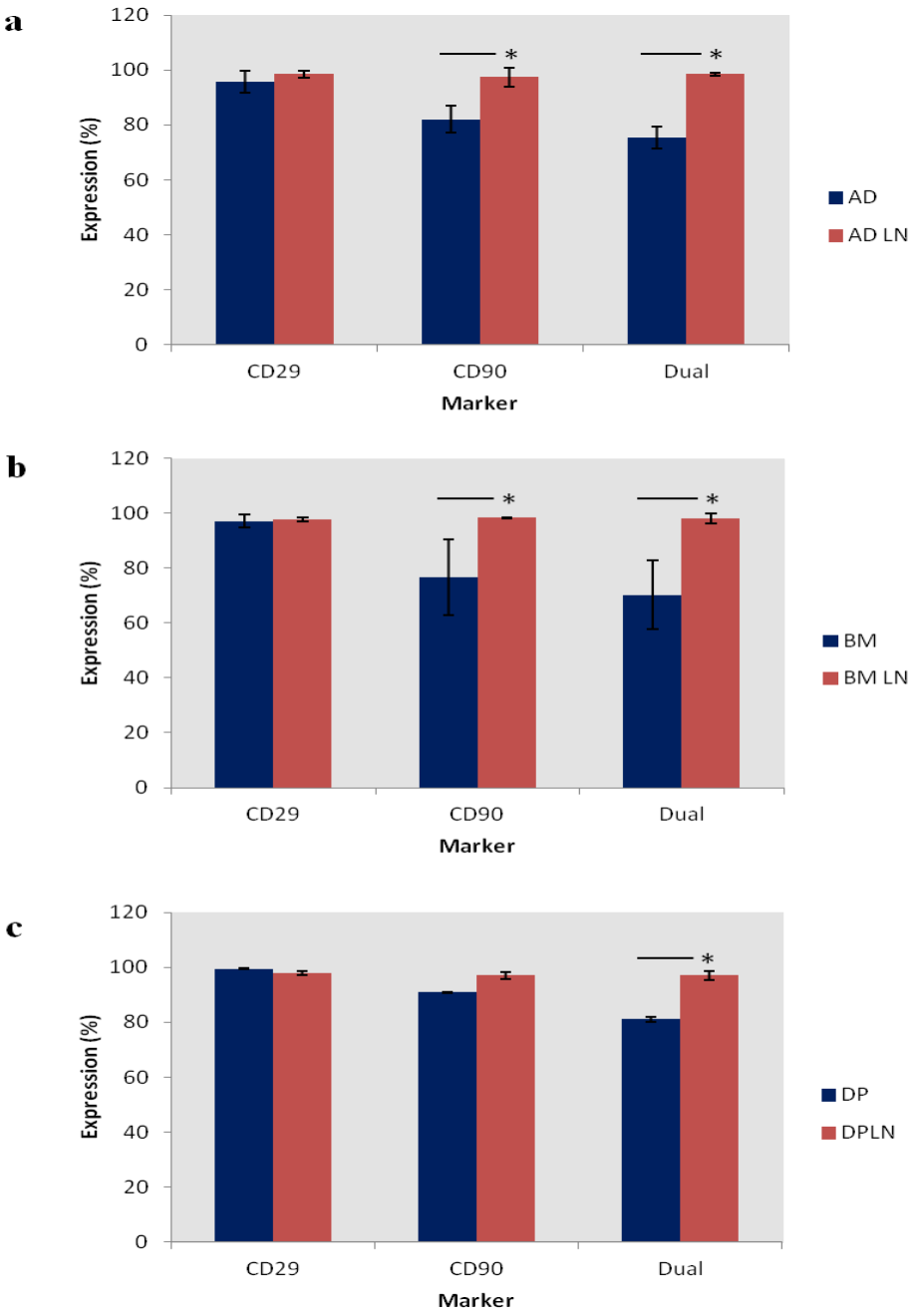

Figure 6.2.1 Comparison of the levels of CD29, CD90 and CD29/CD90 expression in cryo-stored (a) adipose (ADLN), (b) bone marrow (BMLN) and (c) dental pulp (DPLN) cultures with freshly isolated populations established at passage 2. Y-axis represents the percentage of positive cells within a manually defined area that was selected to exclude dead/clumped cells (see appendix 8) (Mean ± SD, *n*=5, * = P<0.05).

6.2.2 sqRT-PCR Analysis of the Effect of Cryo- Storage on the Expression of Stem Cell Markers Genes

Semi-quantitative RT-PCR was used to assess the effects of cryo-storage on the expression of stem cell marker genes. LN storage promoted significant ($P<0.05$) increases in the expression of the pluripotent markers Klf4, Lin28 and Nanog in ADC and DPDC cultures (see figure 6.2.2a). Cryo-storage had no detectable influence on the expression of Klf4 or Lin28 in BMDC populations when compared with freshly isolated cultures, but caused a decrease in the expression of Nanog. Cryo-storage also resulted in a significantly ($P<0.05$) higher level of c-Myc expression detected within the BMDC population (see figure 6.2.2a).

Cryo-storage led to cell-specific increases in the expression of several MSC markers. Cell selection through liquid nitrogen storage significantly ($P<0.05$) up-regulated the expression of CD73, CD90, and CD105 in ADC cultures, although it had no influence on CD44 or CD29 expression (see figure 6.2.2b). Unlike ADC cultures, both BMDC and DPDC cultures demonstrated a significantly ($P<0.05$) higher expression of CD44 following cryo-storage. BMDC cultures also demonstrated higher levels of CD105 in response to cryo-storage, while CD73 expression was significantly ($P<0.05$) higher in cryo-stored DPDC cultures. CD29 expression remained unaltered following liquid nitrogen storage in ADC, BMDC and DPDC cultures. These results demonstrated that cryo-storage resulted in an increase in the expression of stem cell markers that may be related to a selective reduction in the total number of viable cells (see section 6.1.2)

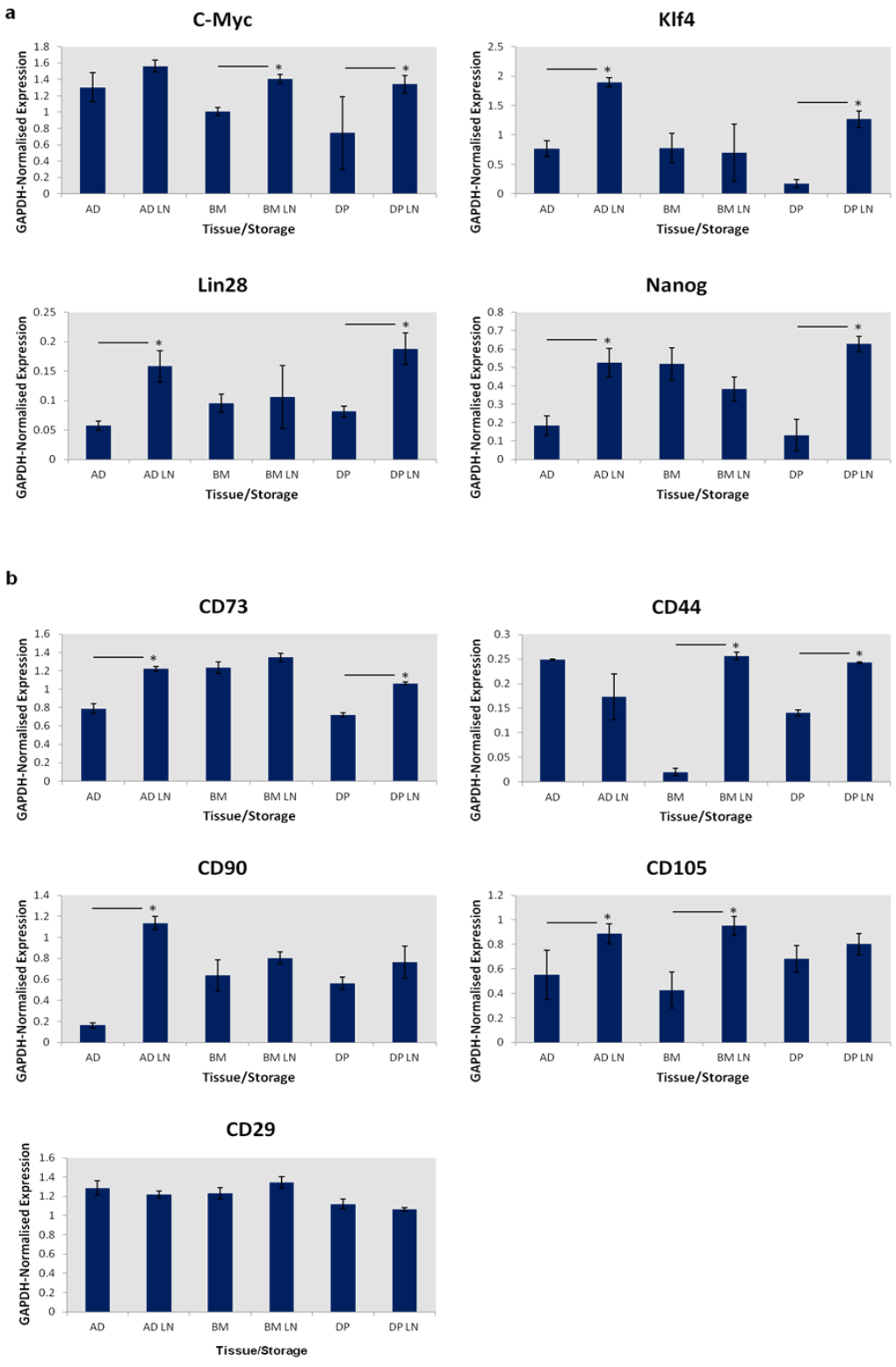

Figure 6.2.2 Effect of liquid nitrogen storage on the relative expression levels of pluripotent (a) and multipotent (b) marker genes in passage 2 adipose (AD), bone marrow (BM) and dental pulp (DP) cultures compared wtih liquid nitrogen stored adipose (ADLN), bone marrow (BMLN) and dental pulp (DPLN) cultures. GAPDH-normalised densitometric values of PCR products are represented. (Mean ± SD, *n*=3, * = P<0.05). For corresponding PCR gel images see appendix 3.

7.0 CHARACTERISATION OF DENTINE MATRIX COMPONENTS (DMCs) AND THEIR EFFECTS ON ODONTOGENIC DIFFERENTIATION

The organic dentine matrix is predominantly composed of type-I collagen, which represents 20 % of the total weight of this tissue (Goldberg *et al*, 2011). The remaining non-collagenous component is made up of non-collagenous proteins (NCPs), glycosaminoglycans (GAGs) and proteoglycans (PGs) (Butler, 1998). Dentine matrix components (DMCs) represent a heterogeneous mixture of NCPs with putative roles in tertiary dentine formation (Smith *et al*, 2012). These bioactive molecules may be released through the action of bacterial acids or physical trauma and have been implicated in the coordination of differentiation of dental pulp stem cells towards odontoblast-like cells (Liu *et al*, 2005). The addition of DMCs to MSC cultures has also been shown to promote odontoblast-like differentiation in vitro and in vivo (Liu *et al*, 2005; Lei *et al*, 2013). However, use of DMCs for the induction of adipose-derived stem cells remains unexplored.

The studies in this chapter sought to characterise EDTA-extracted DMC components (see section 7.1), define the most effective DMC concentration for the differentiation of ADCs, BMDCs and DPDCs towards a mineralising cell type (see section 7.2) and subsequently to provide a comprehensive and comparative evaluation of the effects of DMC-supplementation on odontogenic differentiation in these cultures (see sections 7.3 and 7.4).

7.1 DMC Characterisation

7.1.1 Analysis of DMC Extraction Profile

The pH and spectrophotometric absorbance values of EDTA-soluble dentine extracts were measured daily during a 14 day extraction period to monitor changes in the pH of the extraction conditions and provide a measure of the dissolution of EDTA-soluble DMCs (see figure 7.1.1). The percentage yield of DMCs extracted from dentine powder using EDTA-solubilisation was 3.06 % and the total NCP and GAG content measured using Bradford (Bradford, 1976) and Farndale (Farndale *et al*, 1986) assays was found to represent 2.5 and 2.4 mg per gram of dentine, respectively (see appendix 9). Daily measurements of the absorbance values of EDTA-soluble extracts began to decrease after the first 24 hours of extraction indicating that the dissolution of proteins from the dentine matrix began immediately (Figure 7.1.1a). Absorbance values continued to decrease over the course of the 14 day extraction period but began to stabilise around day 12. The pH of the extraction varied only by a pH of 0.5 over the course of 14 days. The pH approached neutrality as the concentration of extracted proteins decreased and reached neutrality at day 14 (Figure 7.1.1b). These results were in agreement with data previously reported (Smith and Leaver, 1979; Tomson *et al*, 2007; Graham *et al*, 2007).

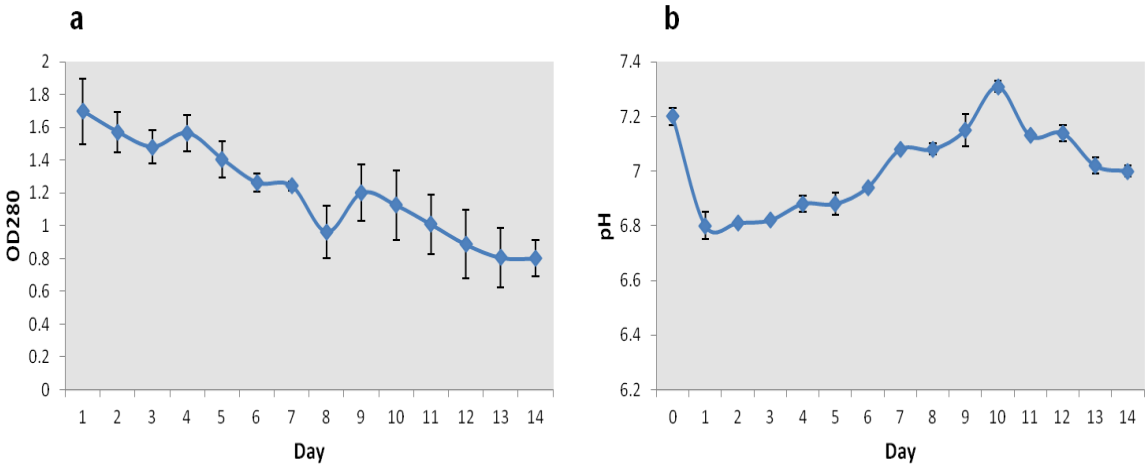

Figure 7.1.1 Characterisation of EDTA-soluble fractions over a 14 day extraction period. (a) Absorbance and (b) pH measurments were recorded daily to provide an indication of the dissolution of DMCs by EDTA and provide measure of changes in extraction conditions respectively (Mean +/- SD, *n*=3).

7.2 Examining the Effect of DMC Concentration

Previous studies have shown that DMCs can influence various aspects of the mineralisation process, including mineral crystal growth and orientation, and can promote the differentiation of stem and progenitor cells towards an odontogenic phenotype (Boskey, 1991; Begue-Kirn *et al*, 1992; Almushayt *et al*, 2006; Deque *et al*, 2006; Wu *et al*, 2008; Lei *et al*, 2013). To evaluate the inductive capacity of DMCs, three concentrations were tested for the induction of ADCs, BMDCs and DPDCs towards a mineralising cell phenotype.

7.2.1 The Influence of DMC Concentration on Cell Morphology

ADC, BMDC and DPDC cultures were exposed to growth medium containing 10 % (v/v) FBS supplemented with 0, 0.1, 1 or 10 µg/mL DMCs. Images obtained using phase contrast microscopy demonstrated no observable effects on cellular morphologies between cultures supplemented with DMCs at the concentrations tested with all cells exhibiting typical fibroblastic appearance and the presence of thin processes emanating from the cell bodies (see figure 7.2.1).

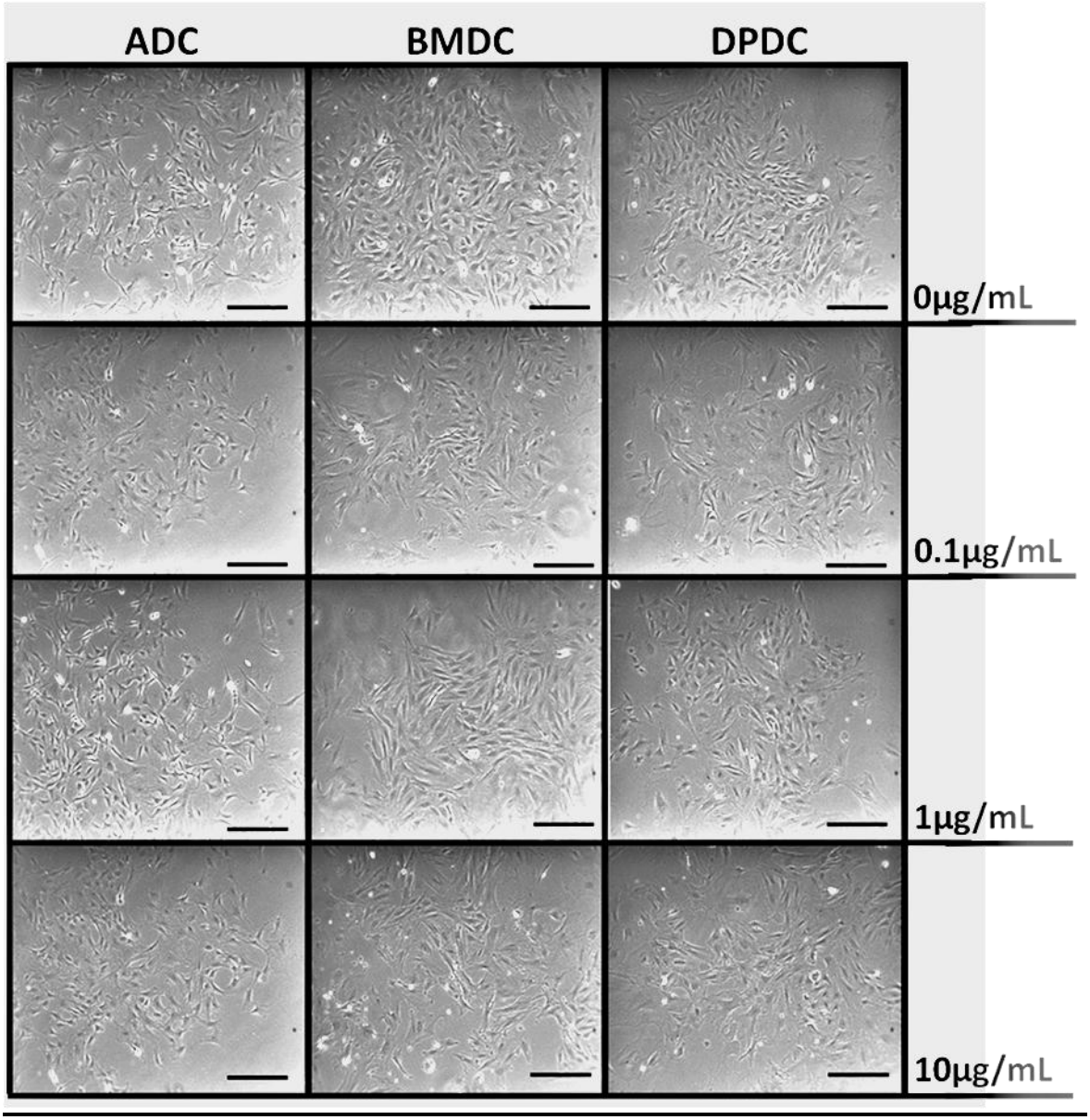

Figure 7.2.1 Representative phase contrast photomicrographs displaying the influence of dentine matrix components (DMCs) on the appearance of ADC, BMDC and DPDC cultures established at passage 2. DMCs were added to culture medium (α-MEM + 10 % FBS) at concentrations of 0, 0.1, 1 and 10 µg/mL and grown for a period of 14 days. Images were collected at day 14 to provide a representation of cell morphology (n=3). Scale bars represent 100 µm.

7.2.2 The Influence of DMC Concentration on Mineralisation in MSC Cultures

7.2.2.1 Adipose-Derived Cultures

Cells supplemented with 1 and 10 µg/mL DMCs generated more developed areas of mineralisation after 14 days culture (see figure 7.2.2.1a). All cultures exposed to osteogenic medium generated mineralised foci of varying sizes (40 µm – 150 µm) after 21 days incubation. Cultures grown in the presence of 1 µg/mL DMCs displayed the most advanced nodular arrangements (see figure 7.2.2.1a), with a darker appearance and a more intense alizarin red staining (ARS). ARS quantification indicated that cultures supplemented with 1 µg/mL DMCs stained more intensely with ARS than any other group, and significantly (<0.05) more than cultures treated with 0.1 µg/mL DMCs (see figure 7.2.2.1b). ADCs cultured in osteogenic medium supplemented with 0.1 and 10 µg/mL DMCs demonstrated less ARS than un-supplemented cultures (see section 2.6.1.1 for medium composition). This indicated that DMCs had the capacity to influence differentiation and mineralisation in ADC cultures.

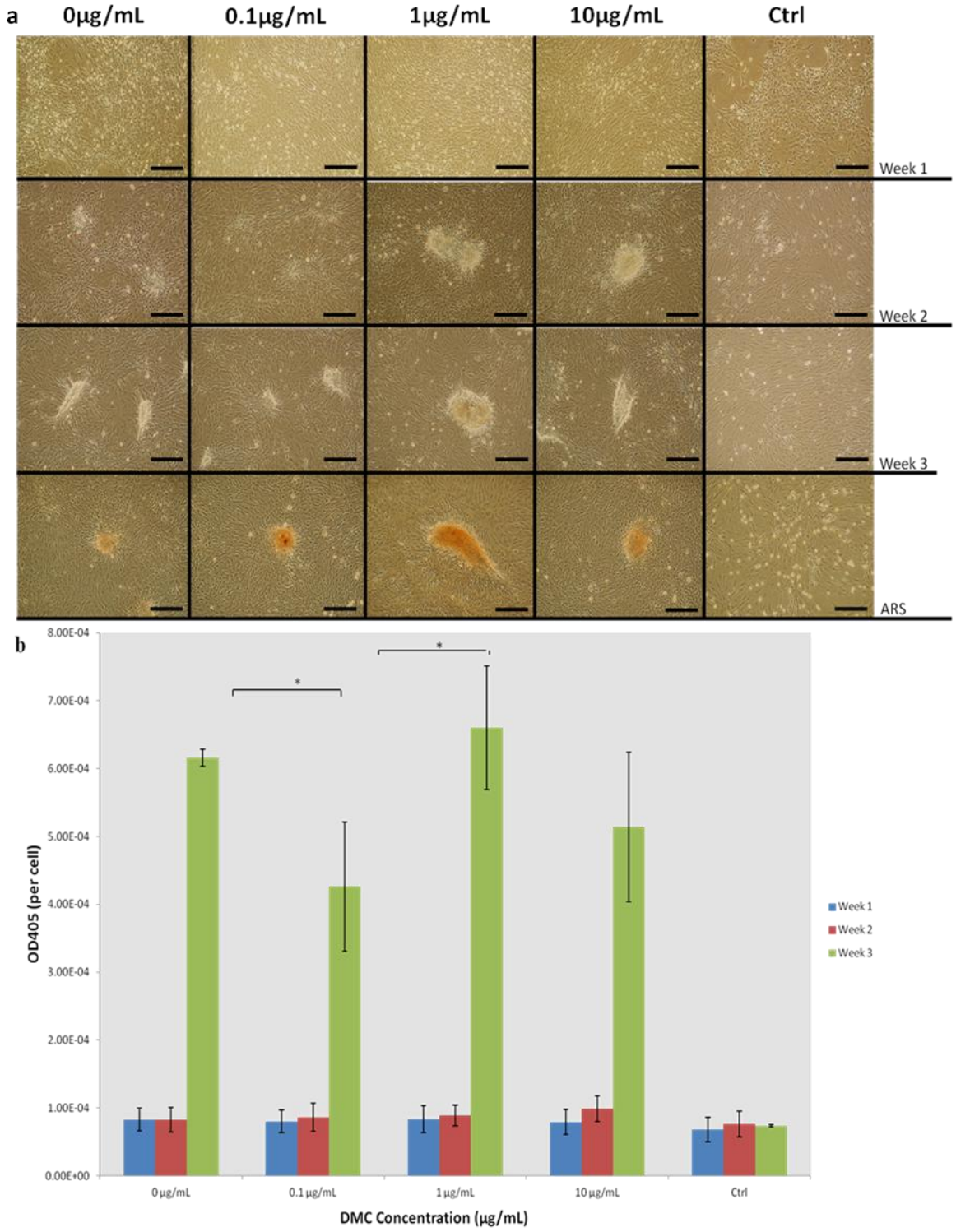

Figure 7.2.2.1 Representative phase contrast photomicrographs depicting the influence of DMC-supplementation on mineralisation in passage 2 ADC cultures (a). Cells were cultured in osteogenic medium treated with 0 , 0.1, 1 or 10 µg/mL DMCs and compared with growth medium controls over a period of 21 days. After incubation, cultures were subjected to ARS, which was then quantified and normalised by the number of cells present (b) (Mean ± SD, n=5, * = $P<0.05$). Scale bars represent 100 µm.

7.2.2.2 Bone Marrow-Derived Cultures

Observable differences in cellular morphology were evident after 7 days culture in DMC-supplemented BMDC cultures (see figure 7.2.2.2a). Small extracellular nodules were observed within a densely packed network of cells in the BMDC cultures supplemented with 1 or 10 µg/mL DMCs (see figure 7.2.2.2a). No nodules were present at this stage for cultures supplemented with 0 or 0.1 µg/mL DMCs (see figure 7.2.2.2a). After 14 days incubation, DMC-supplemented cultures displayed well developed nodular arrangements with increased diameters (> 100 µm) when compared with those cultured in untreated osteogenic medium (≤ 100 µm). Following 21 days exposure to DMC-supplemented osteogenic medium, mineralised nodules appeared darker with diameters exceeding 100 µm. Quantification of ARS indicated that significantly ($P<0.05$) more mineral was present in DMC-supplemented cultures at all concentrations when compared with the osteogenic medium controls (see figure 7.2.2.2b). The most effective concentration was 1 µg/mL (see figure 7.2.2.2b).

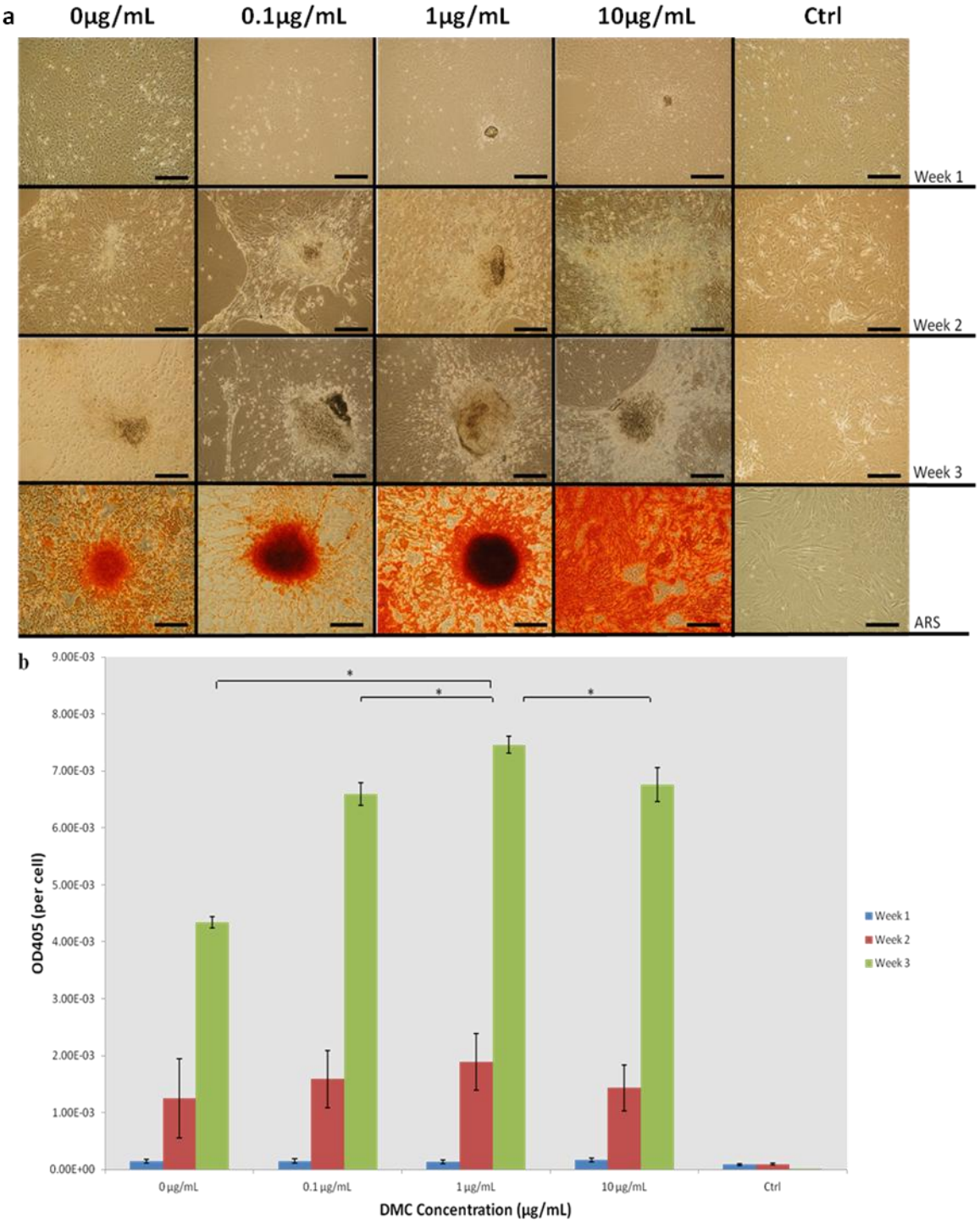

Figure 7.2.2.2 Representative phase contrast photomicrographs depicting the influence of DMC-supplementation on mineralisation in passage 2 BMDC cultures (a). Cells were cultured in osteogenic medium treated with 0, 0.1, 1 or 10 µg/mL DMCs and compared with growth medium controls over a period of 21 days. Cultures were subjected to ARS, which was quantified and normalised by cell number (b) (Mean ± SD, n=5, * = $P<0.05$). Scale bars represent 100 µm.

7.2.2.3 Dental Pulp-Derived Cultures

In DPDC cultures the appearance of dark mineralising areas became evident following 7 days culture in osteogenic medium supplemented with 1 µg/mL DMCs (see figure 7.2.2.3a). Following 14 days exposure to osteogenic medium, the deposition of mineral within all DMC-supplemented cultures significantly ($P<0.05$) increased, as indicated by phase contrast microscopy images and ARS quantification (see figures 7.2.2.3a and b). Cultures supplemented with 1 µg/mL DMCs demonstrated more intense ARS than cultures treated with 10 µg/mL DMCs or osteogenic controls (see figure 7.2.2.3b). The addition of 0.1 and 1 µg/mL DMCs to osteogenic medium led to a significantly ($P<0.05$) increased deposition of mineral (see figure 7.2.2.3b).

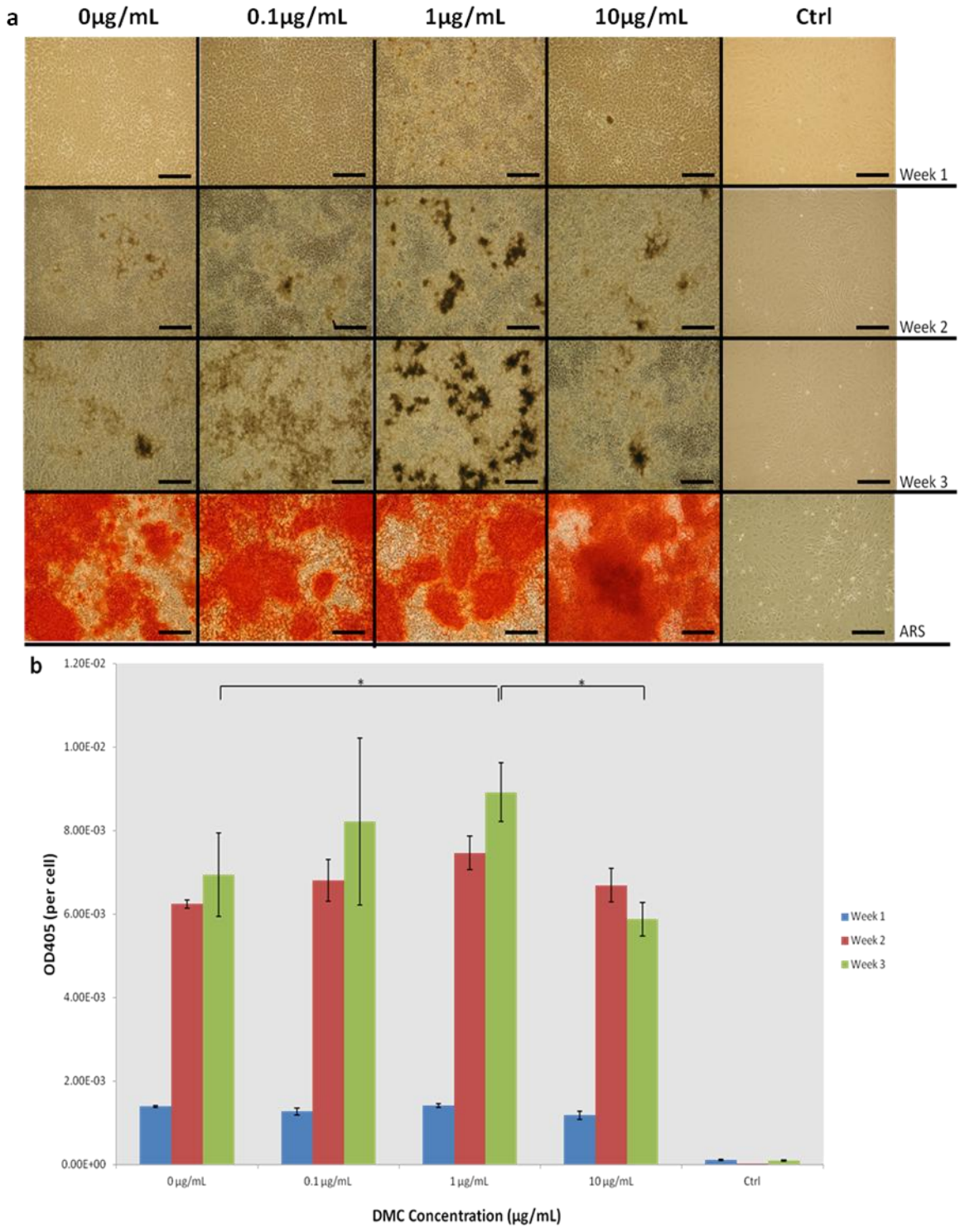

Figure 7.2.2.3 (a) Phase contrast photomicrographs depicting the influence of DMC-supplementation on mineralisation in DPDC cultures. Cells were cultured in osteogenic medium supplemented with 0, 0.1, 1 or 10 µg/mL DMCs and compared with standard growth medium controls over a period of 21 days. After incubation, cultures were subjected to ARS, which was then quantified and normalised by cell number (b) (Mean ± SD, n=5, * = $P<0.05$). Scale bars represent 100 µm.

7.2.3 The Effect of DMC Concentration on the Expression of Markers Associated with Mineralisation and Dentinogenesis: Developing an Optimal Concentration for the Induction of Odontogenic Differentiation

Semi-quantitative RT-PCR was used to examine the influence of DMC concentration on the expression of genes associated with the formation of a mineralised extracellular matrix (ECM) and differentiation towards an odontogenic phenotype. The expression of three genes associated with dentinogenic differentiation, DSPP, DMP1 and Nestin, were significantly ($P<0.05$) increased in ADC cultures supplemented with 1 µg/mL DMCs (see figure 7.2.3a). BMDC cultures also displayed a significant ($P<0.05$) increase in the expression of DSPP when treated with 1 µg/mL DMCs, whereas no concentration of DMCs had caused a relative increase in the expression of dentinogenic genes in DPDC cultures (see figure 7.2.3a). Gene expression of type-Iα1 collagen (Col1α1) was increased in ADC cultures following supplementation with 10 µg/mL DMCs, although this was not significant. The concentration of DMCs added to BMDC or DPDC cultures appeared to have minimal influence on Col1α1 and OP expression (see figure 7.2.3b). The expression of ON also appeared to not be affected by DMC-supplementation in ADC, BMDC or DPDC cultures (see figure 7.2.3b). These results indicated that DMC concentration had a significant influence on the expression of odontogenic markers within ADC and BMDC cultures, but had minimal effect on DPDC cultures. Moreover, no relationship was found between DMC concentration and the expression of common genes associated with the formation of a mineralised ECM (i.e. Col1α1, OP and ON) (see figure 7.2.3b).

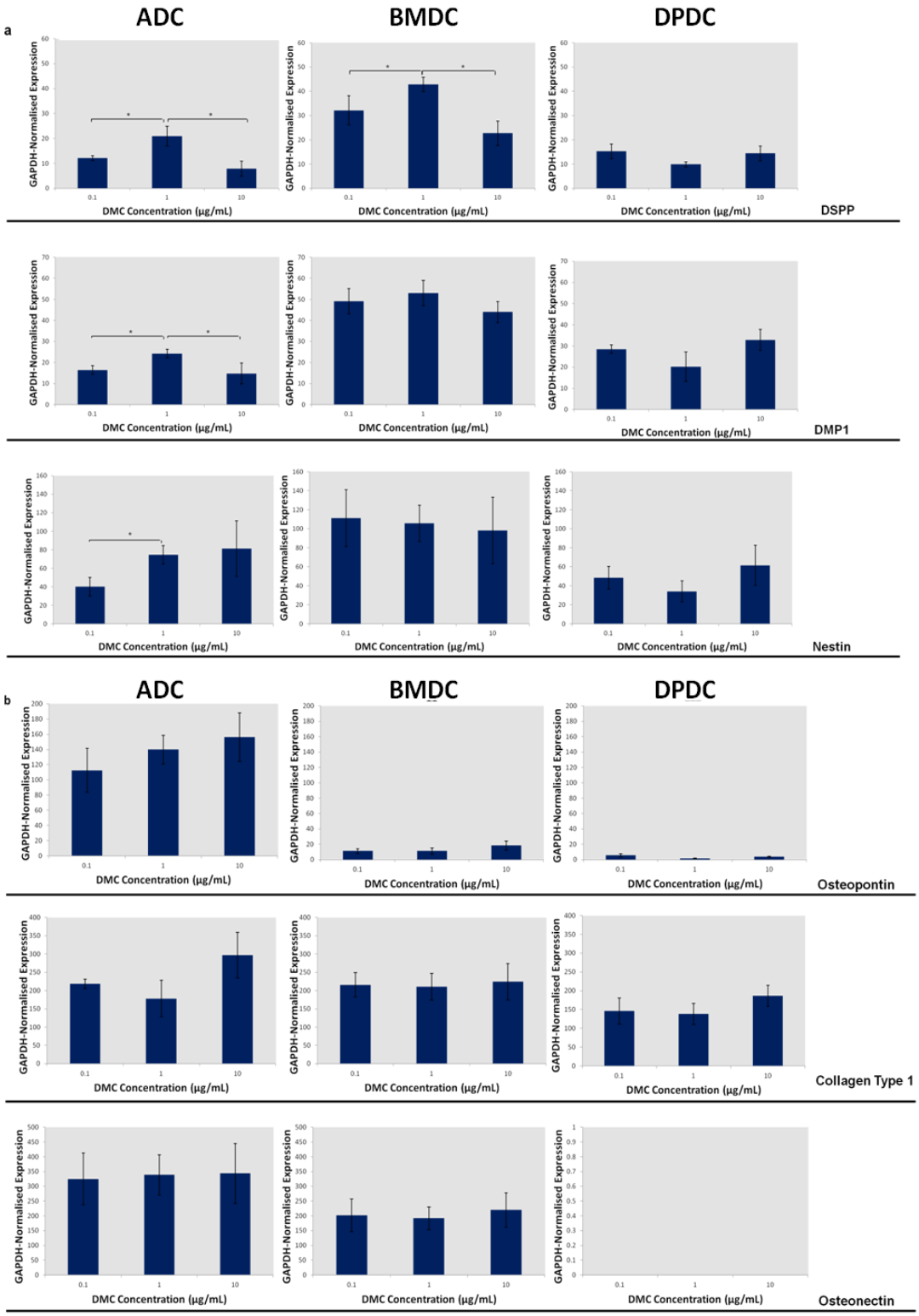

Figure 7.2.3 Graphical representation of the influence of different DMC concentrations on the expression of odontogenic (a) and mineralisation (b) marker genes in ADC, BMDC and DPDC cultures. DMCs were added to osteogenic medium at concentrations of 0.1, 1 or 10 µg/mL and cultured for 21 days. The intensity of the amplified products was normalised against GAPDH and plotted as relative gene expressions (Mean ± SD, n=3, * = $P<0.05$). For corresponding PCR gel images see appendix 6).

7.3 Effect of DMCs on Odontogenic Differentiation

Based on comparative analyses of DMC concentrations conducted in section 7.2, a concentration of 1 μg/mL DMCs was selected and added to osteogenic medium as a putative dentinogenic supplement.

7.3.1 The Influence of DMCs on Cell Growth

The influence of 1 μg/mL DMCs on ADC, BMDC and DPDC cell numbers was compared in osteogenic and basal growth (α-MEM + 10 % (v/v) FBS) media over a period of 18 days (see figure 7.3.1). Cell numbers began to decline in DMC-supplemented cultures relative to basal growth medium cultures after 3 days and remained comparatively decreased until day 18. Following 18 days culture, the addition of DMCs to basal growth medium resulted in a 5 %, 10 % and 5 % reduction in the number of cells present in ADC, BMDC and DPDC cultures, respectively (Figure 7.3.1Ia, Ib and Ic).

ADCs, BMDCs and DPDCs cultured in osteogenic medium supplemented with 1 μg/mL DMCs displayed only minor decreases in cell numbers following 6 (ADCs) and 9 days (BMDCs and DPDCs) culture (Figure 7.3.1IIa, IIb and IIc). At day 15, cell numbers plateaued within un-supplemented osteogenic cultures, causing DMC-supplemented ADC, BMDC and DPDC populations to reach similar cell numbers, with only 0.2 %, 6% and 0.9 % fewer cells, respectively.

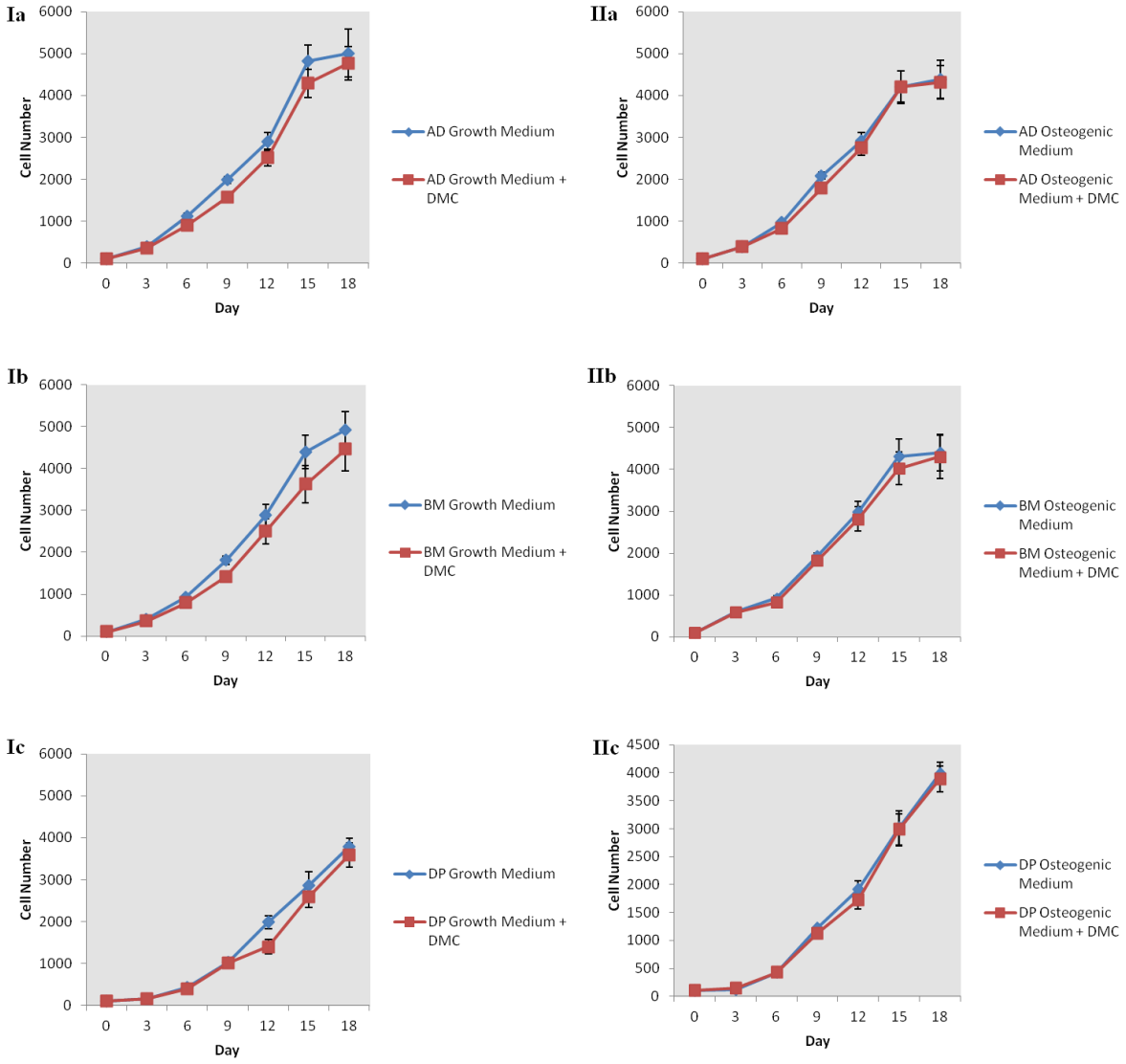

Figure 7.3.1 Growth curves displaying the influence of DMCs on cell growth in ADC (a), BMDC (b) and DPDC (c) cultures exposed to basal growth medium (I) or osteogenic medium (II) (Mean ± SD, *n*=5).

7.3.2 The Influence of DMCs on Colony Formation

Since the addition of DMCs resulted in only minor effects on cell growth (see section 7.3.1), the effects of these dentine proteins on colony formation were assessed. Passage 2 cells were cultured in the presence of basic growth medium containing 10 % (v/v) FBS or supplemented with 1 µg/mL DMCs. Colonies were defined as clusters of ≥ 50 Toluidine blue-positive cells (see figure 7.3.2a). Minor increases in colony numbers were recorded for all three populations with DMC-treated ADC, BMDC and DPDC cultures displaying increases of 17 %, 14 % and 3 %, respectively (see figure 7.3.2b). The average number of cells per colony was not affected by the addition of DMCs to ADC, BMDC or DPDC cultures (see figure 7.3.2c).

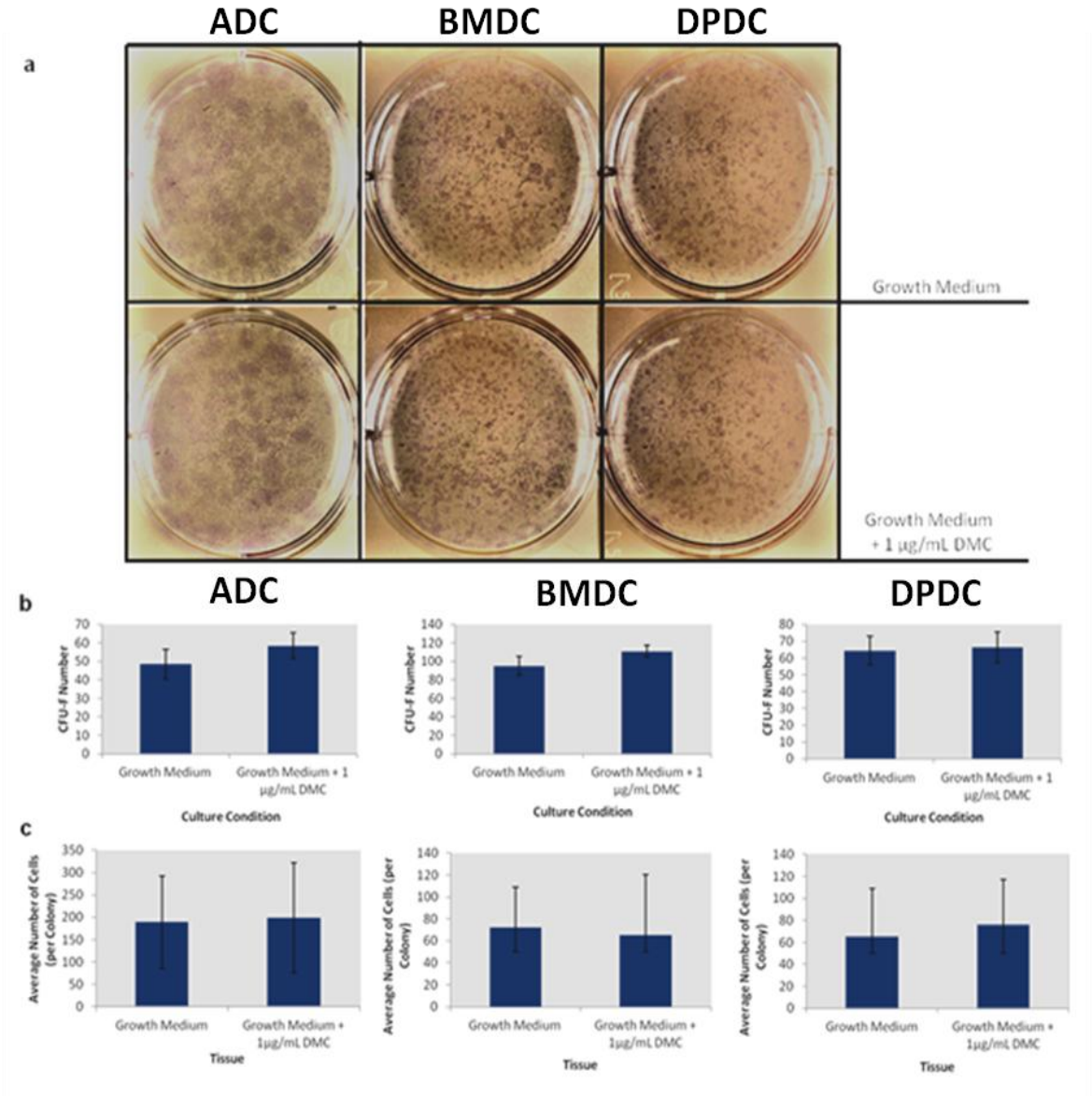

Figure 7.3.2 (a) Representative images displaying Toluidine blue-stained colonies within ADC, BMDC and DPDC cultures exposed to DMC-supplemented and un-supplemented basal growth medium. Accompanying quantification of the number of colonies present per 35 mm^2 (b) and the average number of cells per colony (c) (Mean ± SD, *n*=5).

7.3.3 The Influence of DMCs on Cellular Metabolic Activity

To determine whether the supplementation of DMCs in cultures resulted in an up-regulation in the metabolic activity per cell, MTT analysis and accompanying cell counts were performed over a period of 18 days for cells cultured in basal growth medium (see figure 7.3.3a) and osteogenic medium (see figure 7.3.3b). Metabolic activity per cell was significantly ($P<0.05$) increased after 3 days culture in DMC-supplemented BMDC populations and following 6 days culture for DMC-treated ADC and DPDC populations. The metabolic activity per cell in DPDC cultures varied from day 9 to day 18 with significant ($P<0.05$) increases on days 12, 15 and 18 relative to un-supplemented basal growth medium controls. Both ADC and BMDC cultures maintained a significantly ($P<0.05$) increased metabolic activity per cell throughout days 9-18 when compared with untreated controls. The addition of DMCs caused an up-regulation in MTT staining for all three cell types. However, this effect was more consistent for ADC and BMDC cultures over the course of the 18 day culture period. The addition of DMCs to osteogenic medium resulted in no significant ($P<0.05$) up-regulation in metabolic activity per cell in ADC, BMDC or DPDC cultures, with the exception of day 6 and 9 ADC and DPDC cultures, respectively. BMDC cultures demonstrated no increase in metabolic activity at any of the time points analysed.

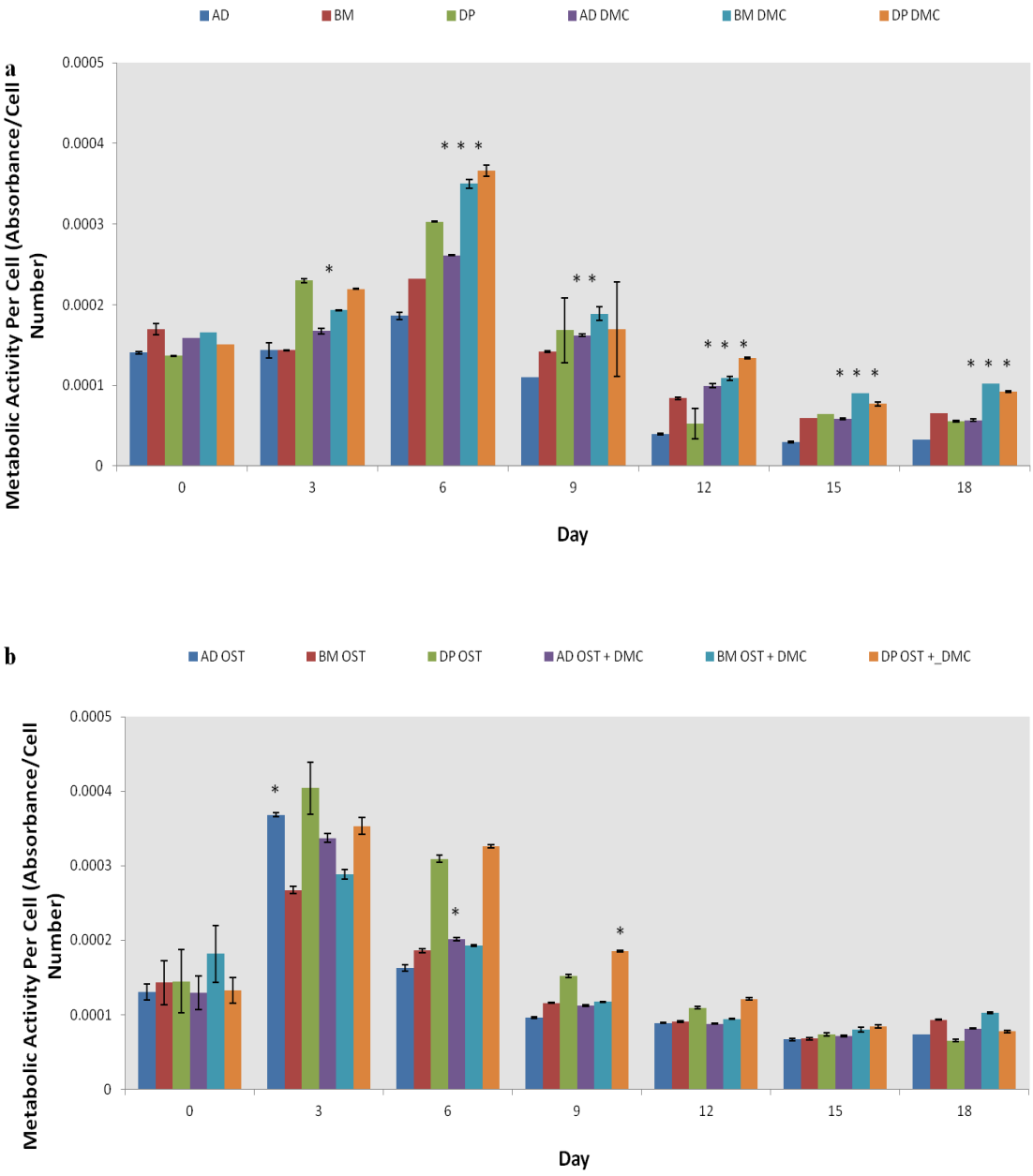

Figure 7.3.3 MTT analysis of metabolic activity per cell for passage 2 ADC, BMDC and DPDC cultures grown in basal growth medium (a) or osteogenic medium (b) in the presence or absence of DMCs. Cell counts were performed at 3 day intervals and used to normalise metabolic activity by cell number (Mean ± SD, *n*=5 * = P<0.05 and indicates significance between treated and untreated cultures at a specific day).

7.3.4 The Influence of 1 µg/mL DMCs on Mineralisation

ADC, BMDC and DPDC cultures were exposed to DMC-supplemented and un-supplemented osteogenic and basal growth media for a period of 21 days, and subjected to ARS, which was quantified per cell. The addition of DMCs to basal growth medium did not promote mineralisation (see figure 7.3.4a). Quantification of ARS per cell indicated that the amount of mineral secreted by BMDC and DPDC cultures was significantly ($P<0.05$) increased following DMC-treatment (see figure 7.3.4b). DMC-supplemented BMDC cultures contained characteristic darkly staining nodules (see figure 7.3.4a). However, these nodules were superimposed on a more prevalent sheet-like mineral deposit similar to DPDC cultures (see figure 7.3.4a). The relative concentration of ARS between BMDC and DPDC cultures exposed to osteogenic medium was also significantly ($P<0.05$) decreased by 21 % following DMC-supplementation. ADCs exposed to DMC-treated osteogenic medium appeared morphologically similar to untreated cultures with no quantitative influence on ARS (see figure 7.3.4b). DPDC cultures exposed to DMC-supplemented osteogenic medium were visually similar to those exposed to un-supplemented osteogenic medium (see figure 7.2.4a). However, quantification of ARS demonstrated a significant ($P<0.05$) 22% increase in staining for DMC-treated DPDC cultures (see figure 7.2.4b). These results demonstrated that the addition of 1 µg/mL DMCs to osteogenic medium resulted in a significant ($P<0.05$) increase in the quantity of mineral deposited in both BMDC and DPDC cultures.

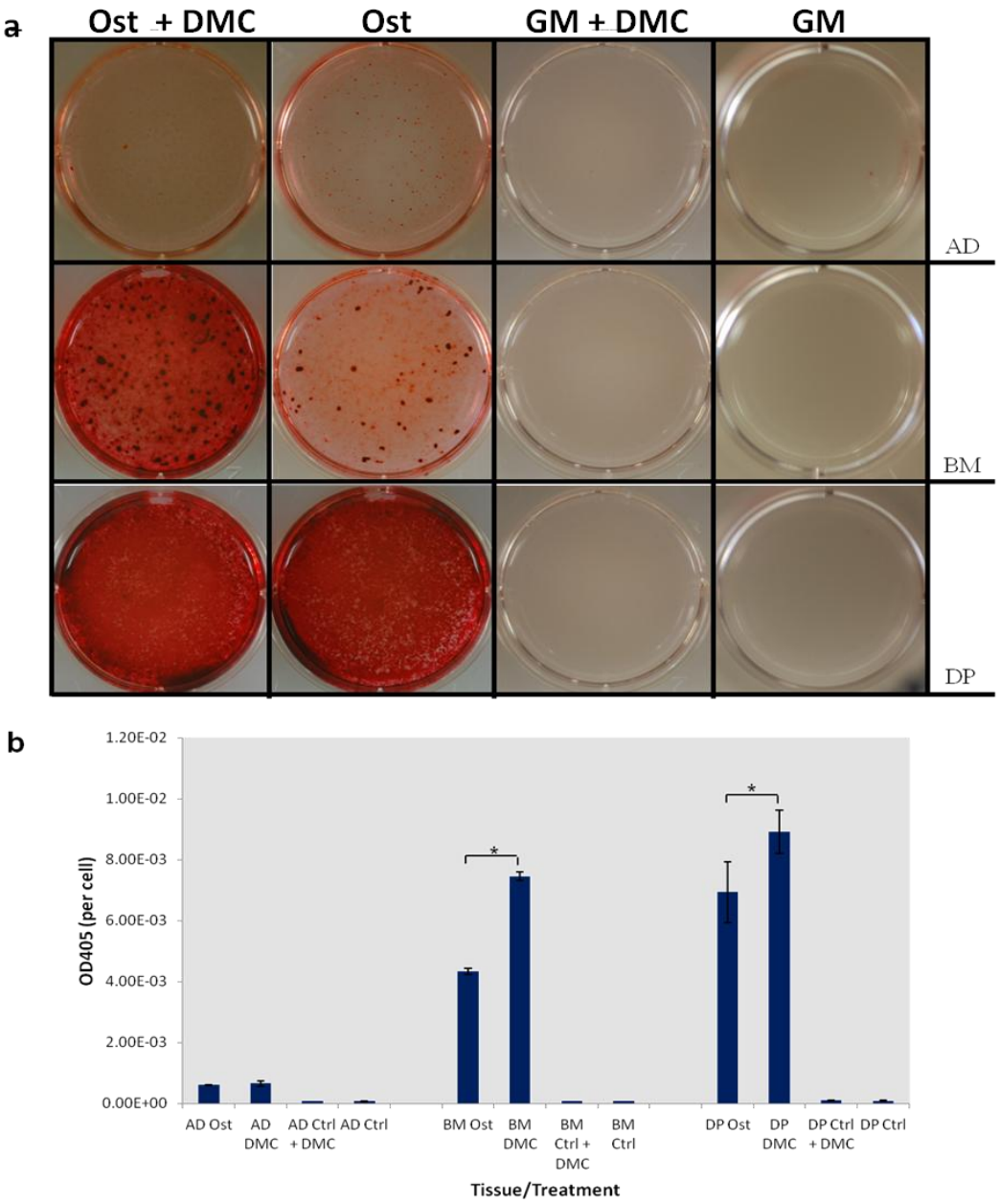

Figure 7.3.4 (a) Representative images displaying the effects of 1 µg/mL DMCs on the ARS of mineral deposits in ADC, BMDC and DPDC cultures exposed to un-supplemented osteogenic medium, DMC-supplemented osteogenic medium, DMC-supplemented growth medium (GM), or un-supplemented growth medium for a period of 21 days ARS was eluted, quantified and normalised by cell number (b) (Mean ± SD, *n*=5, * = P<0.05).

7.3.5 Analysis of Mineral using Scanning Electron Microscopy

Secondary electron and backscatter electron imaging were used to provide a more detailed examination of the effects of DMCs on the deposition of mineral within ADC, BMDC and DPDC cultures (see also sections 7.3.4, 7.3.6 and 7.3.7). After 21 days exposure, each culture was fixed and subsequently examined using a high vacuum scanning electron microscope to provide detailed morphological analysis of any mineral present. Mineralised foci formed within DMC-supplemented ADC cultures were in general larger than those formed by culture in un-supplemented osteogenic medium with average diameters of ~30 μm, approximately two thirds larger than foci formed in un-supplemented osteogenic cultures (see figure 7.3.5a). Foci formed within BMDC cultures were typically much larger than those present in ADC cultures with average diameters of approximately 80 μm for un-supplemented osteogenic cultures and 200 μm for DMC-supplemented osteogenic cultures (see figure 7.3.5b). Morphological differences in mineral deposited in DMC-supplemented and un-supplemented osteogenic cultures were also apparent. Mineral deposited in un-supplemented DPDC cultures formed a sheet that covered the underlying cells, while mineral deposited by DMC-supplemented DPDC cultures was less uniform with a large number of matrix-free areas (see figure 7.3.5c). Images obtained using backscattered electron imaging indicated the presence of high atomic number elements that confirmed the presence of mineral within these cultures. EDX and calcium/phosphate micro-determination assays (see appendix 10) further confirmed the presence of calcium and phosphate and demonstrated that these elements were present with an atomic percentage ratio of 1.25 (Ca/P). This ratio is indicative of an intermediate phase in the formation of β-tricalcium phosphate (β-TCP) within mineralising systems (Dorozhkin, 2010; Zhao *et al*, 2011).

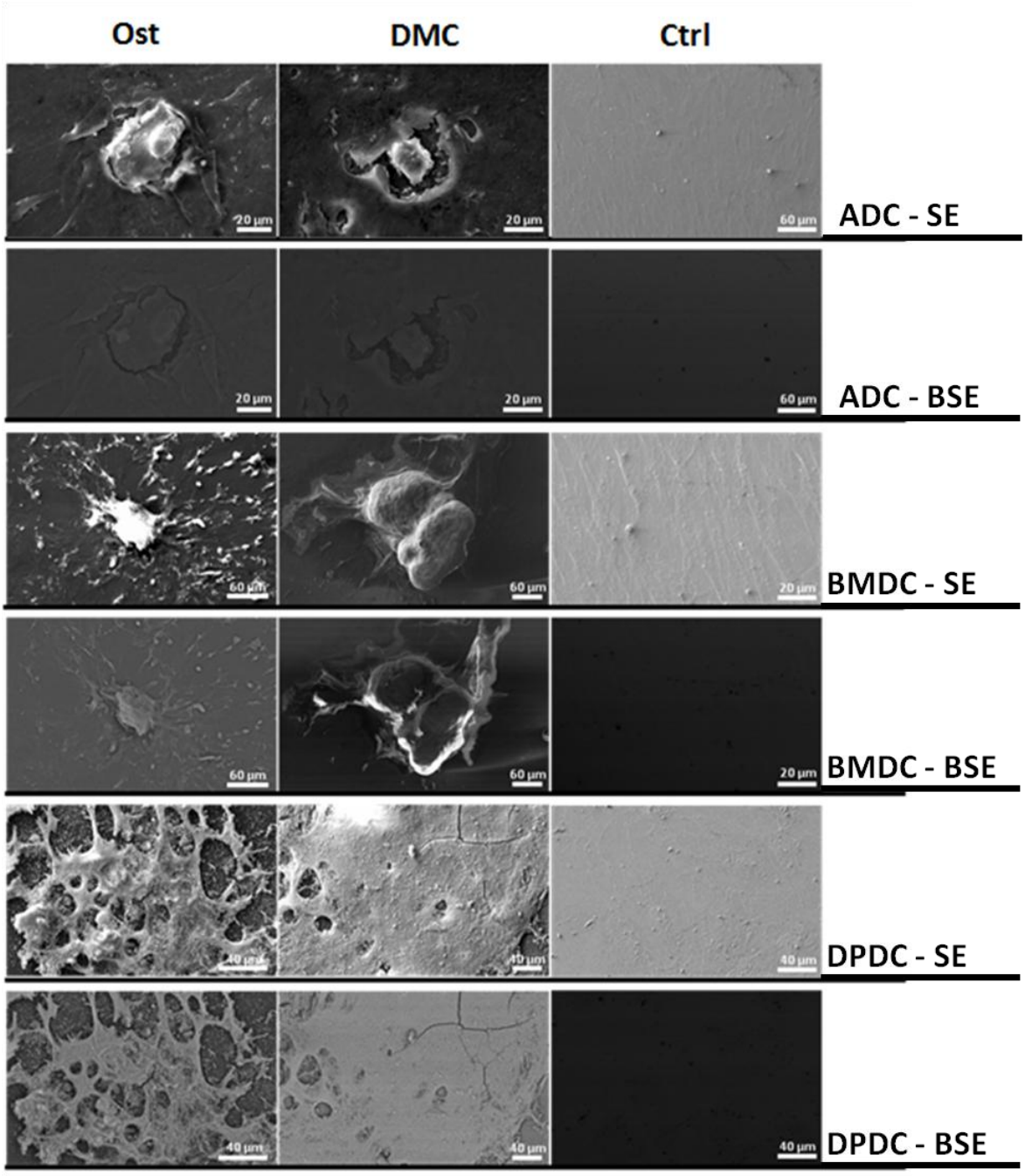

Figure 7.3.5 Secondary electron (SE) and backscatter electron (BSE) micrographs displaying the influence of DMCs on the secretion of mineral in DMC-supplemented osteogenic, un-supplemented osteogenic and control media on ADC, BMDC and DPDC cultures. All cultures were grown for a period of 21 days and analysed using secondary electron (SE) and backscatter electron (BSE) detection. (*n*=5).

7.3.6 Analysis of Mineral Deposited in DMC-Supplemented and Un-supplemented Cultures

7.3.6.1 Micro-Computed Tomography (MicroCT) Analysis of Mineralised Cell Cultures

MicroCT data were obtained for ADC, BMDC and DPDC cultures that had been grown in DMC-supplemented osteogenic medium, un-supplemented osteogenic medium or basal growth medium for a period of 21 days. These data were represented as two-dimensional surface plots of whole culture dishes (see figure 7.3.6.1a) with accompanying mineral quantification (7.3.6.1b). Surface analysis indicated an increase in the volume of mineral present in all cultures exposed to osteogenic and DMC-supplemented osteogenic medium when compared with corresponding basal growth medium controls (see figure 7.3.6.1a). Quantification based on the construction of a calibration curve using di-potassium phosphate (K_2HPO_4) phantoms indicated that the addition of DMCs promoted an increase in the volume of mineral deposited in both ADC and BMDC cultures, with this increase proving significant ($P<0.05$) for BMDC cultures (see figure 7.3.6.1b). The increase in mineral content found in the ADC cultures indicated that minor differences in the quantification of mineral could be detected using MicroCT analyses that were not detectable using ARS analysis (see section 7.3.4). However, only a minor increase in mineral deposition was found in DPDC cultures following exposure to DMC-supplemented osteogenic medium (Figure 7.3.6.1b). Quantification of mineral using a di-potassium phosphate phantom demonstrated some small differences in the total mineral content of ADC and DPDC cultures when compared with ARS quantification, possibly caused by the semi-quantitative nature of ARS (see section 7.3.4). These results

demonstrated that ADC and BMDC cultures may respond to differentiation promoting factors present within the DMCs by up-regulating the secretion of mineralised matrix and that MicroCT may provide a more sensitive method for the quantification of this mineral.

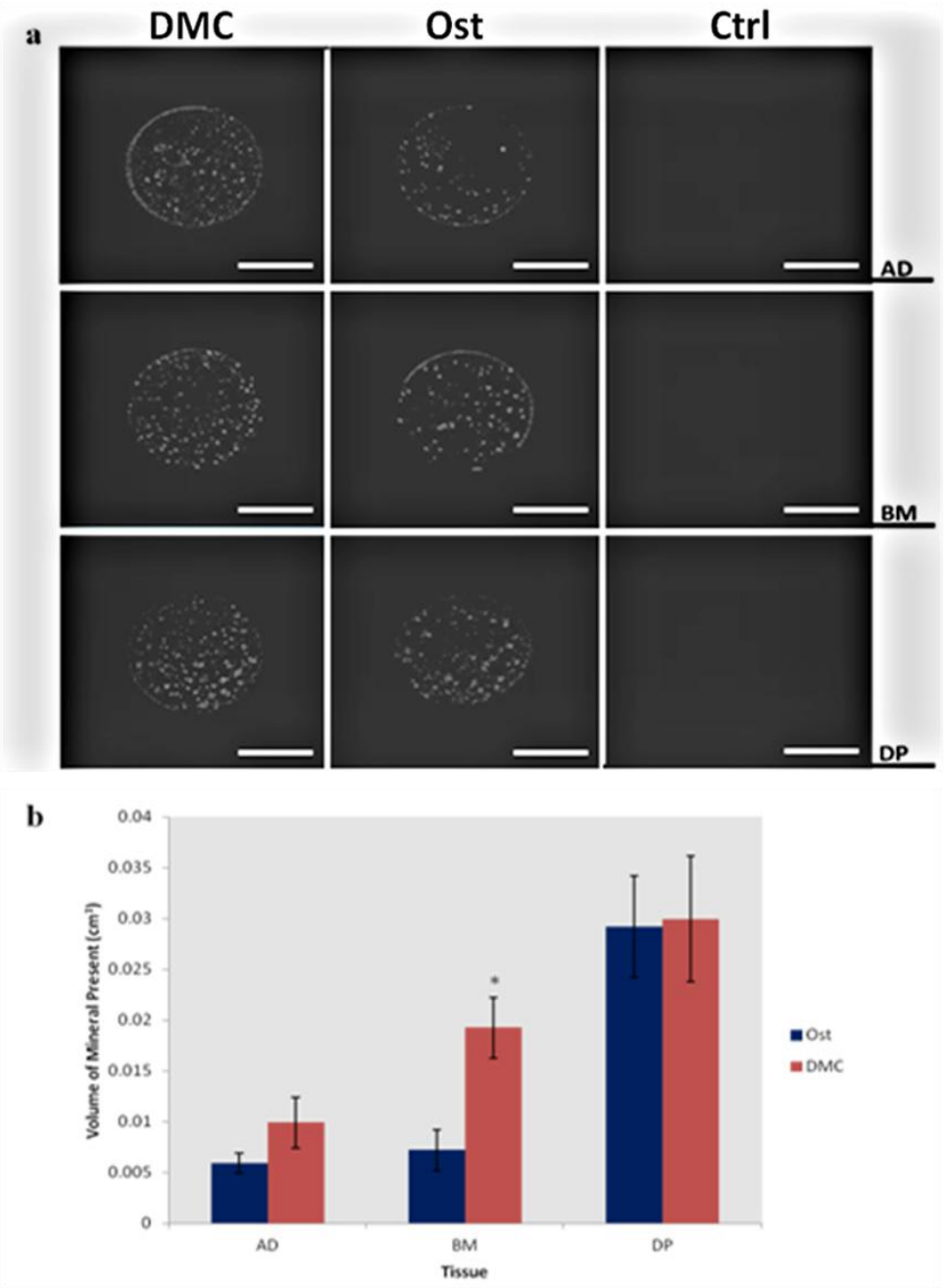

Figure 7.3.6.1 Representative Micro-CT images of cultures displaying mineral deposited in the presence and absence of DMCs (a) with accompanying quantification of the total volume of mineral present (b). Mineral volume was determined using a calibration curve constructed using di-potassium phosphate phantoms (see section 2.8.1). Images were obtained for passage 2 ADC, BMDC and DPDC cultures following 21 days exposure to DMC-treated osteogenic medium (I), un-treated osteogenic medium (II) and growth medium controls (III) (Mean ± SD, n=5, * = $P<0.05$). Scale bars represent 20 mm.

7.3.6.2 MicroCT Analysis of Mineral Density

The density of the mineral phase present in dentine and bone is important for providing the structural integrity required for optimum function, with lower densities related to dentinal caries, periodontal disease, osteoporosis and bone fractures (Kinney *et al*, 1994; Tezal *et al*, 2000). Previous studies have grouped mineral into low (200-400 mg/cm^3), medium (400-800 mg/cm^3) and high (≥ 800 mg/cm^3) density categories representing trabecular-subcortical bone, porous cortical bone and dense cortical bone respectively (Roldan *et al*, 2001). MicroCT analysis of the volumetric density of mineral secreted by ADC, BMDC and DPDC cultures demonstrated that mineral deposited following 21 days exposure to osteogenic medium was of a low density (0-250 mg/cm^3), particularly for BMDC cultures, with volumetric densities of ≥ 250 mg/cm^3 representing only 3.5 % of the total mineralised tissue (see figure 7.3.6.2). Although ADC cultures secreted a lower overall volume of mineral than BMDC cultures, a larger proportion of the mineral secreted was of a higher volumetric density than that secreted by BMDC cultures, with 50 % representing mineral of ≥ 250 mg/cm^3. DPDC cultures contained the highest volume of mineral after exposure to un-supplemented osteogenic medium with significantly ($P<0.05$) more mineral present than for ADC and BMDC cultures, 25 % and 20 % respectively. A large proportion of the mineral produced by DPDC cultures was of a low volumetric density, with 86 % of the mineral ≤ 250 mg/cm^3. High density bone has been reported to have a volumetric density of ≥ 800 mg/cm^3, representative of human cortical bone (Roldan *et al*, 2001). Cultures exposed to osteogenic medium demonstrated only a low volume of mineral with such a high volumetric density, with BMDC and DPDC cultures containing only 1 % and 1.8 % mineral ≥ 800 mg/cm^3 respectively. A comparison of mineral densities for cultures exposed to osteogenic medium demonstrated that ADC populations displayed the largest

volume of high density mineral, with mineral ≥ 750 mg/cm^3 representing 16 %. Addition of DMCs to osteogenic culture medium promoted an increase in the volume of mineral secreted by ADC, a significant ($P<0.05$) increase in the volume of mineral secreted in BMDC cultures. Exposure to DMC-supplemented osteogenic medium only resulted in a 2.4 % increase in the volume of mineral secreted by DPDC cultures. The mineral produced by MDC-supplemented DPDC cultures was of a low density (< 250 mg/cm^3), with the volume of higher density mineral (> 250 mg/cm^3) decreased by 1.2 %. ADC cultures demonstrated a significant ($P<0.05$) 40.3 % increase in mineral volume, while BMDC cultures more than doubled the volume of mineral present with an increase of 62.6 %. The increased volume of mineral secreted by DMC-supplemented ADC cultures was typically of a low density (< 250 mg/cm^3), with only a 4.5 % increase in mineral > 250 mg/cm^3. However, DMC-supplemented BMDC cultures demonstrated a significant ($P<0.05$) increase in the total volume of higher density mineral with a 98.7 % increase in the presence of mineral with a density > 250 mg/cm^3, with 14.8 % representing densities comparable with cortical bone (≥ 750 mg/cm^3). Mineral secreted by ADC, BMDC and DPDC cultures under osteogenic conditions was not uniform, but comprised of several different densities. Mineral secreted by BMDCs cultured in un-supplemented osteogenic medium was predominantly of a lower density than that secreted by un-supplemented ADC and DPDC cultures. The addition of DMCs to osteogenic medium increased the volume of mineral found in all three cultures, with an accompanying increase in mineral density for BMDC cultures. These results indicated that the application of DMCs to osteogenic culture medium could be used to increase the quantity and density of mineral deposited in ADC and BMDC cultures.

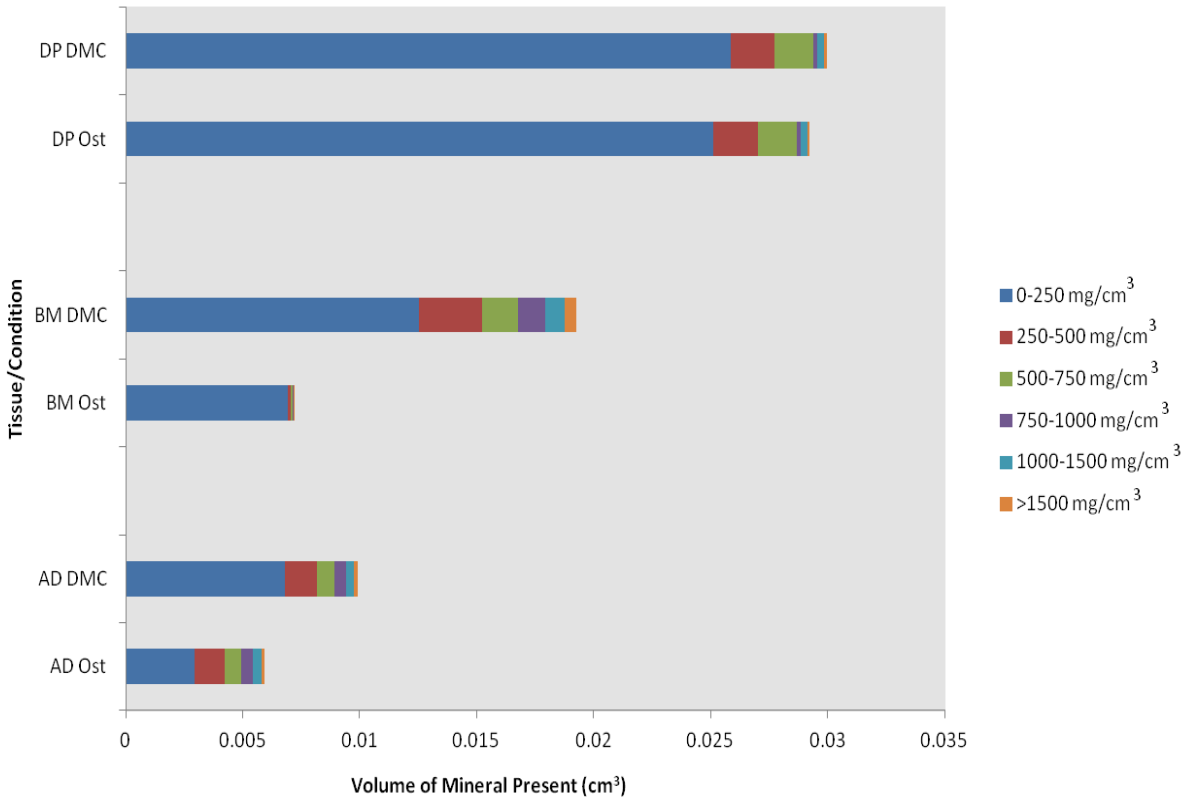

Figure 7.3.6.2 MicroCT analysis of the density of mineral secreted by cultures grown in the presence and absence of DMCs. Passage 2 ADC, BMDC and DPDC cultures were exposed to DMC-supplemented osteogenic medium for a period of 21 days and compared with un-supplemented osteogenic controls. Mineral density was determined through the use of graded di-potassium phosphate phantoms of known densities ranging from 0-1500 mg/cm^3 (Mean, *n*=5).

7.3.6.3 Analysis of Mineral Using FTIR Spectroscopy

The combined inorganic mineralised phase derived from ADC, BMDC and DPDC cultures exposed to DMC-supplemented osteogenic medium was analysed using Fourier transform infrared (FTIR) spectroscopy and compared with known dentine and hydroxyapatite (HA) standards (see section 2.8.5). Mineralised ADC, BMDC and DPDC cultures and dentine standards were burned at 600 °C for a period of 15 minutes to remove water (vaporised at 110 °C) and organic components (vaporised at 600 °C) and analysed within a spectral range of 590-2000 cm^{-1} (Holager, 1970; Lim and Liboff, 1972). Data indicated that the inorganic phase of osteogenic cultures shared a number of common functional groups with both dentine and HA standards (see figure 7.3.6.3). The presence of characteristic dentinal peaks representing amides within the spectral window of 1200-1700 cm^{-1} indicated detectable levels of collagen and matrix proteins even after heating at 600 °C for both sample and dentine standard (Sato *et al*, 2013). A corresponding band was also observed for test sample, dentine and HA standards within the 900-1200 cm^{-1} spectral range (Paschalis *et al*, 1996; Wang *et al*, 2009). This band represented the inorganic component of dentine and contained peaks indicating the presence of V_1 symmetric and V_3 anti-symmetric phosphate components present within dentinal apatite, typically seen at around 960 and 1040 cm^{-1}, respectively (Raynaud, *et al*, 2002). Further peaks representing phosphates were observed between test sample and dentine and HA standards at 605 cm^{-1}, as well as a typical carbonate peak situated at 870-880 cm^{-1} (Leventouri *et al*, 2009; Yoshihara *et al*, 2011). These data indicated that the inorganic phase secreted in response to culture in DMC-supplemented osteogenic medium shared a number of common peaks with dentine and its major inorganic constituent, HA.

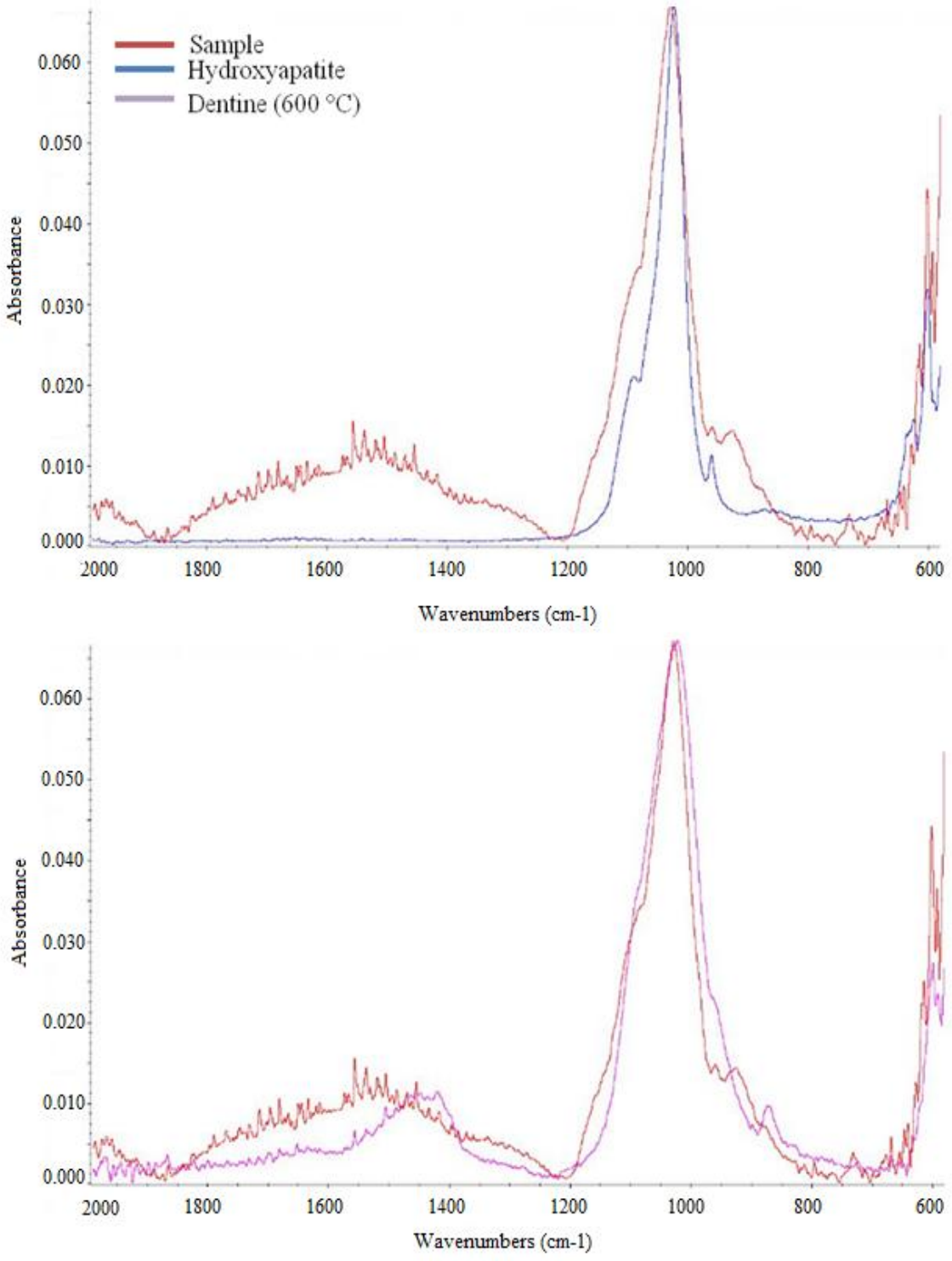

Figure 7.3.6.3 FTIR spectra of the combined inorganic component of ADC, BMDC and DPDC cultures. All three cultures were combined and heated at 600 °C to remove any organic material and compared with hydroxyapatite and dentine standards. (*n*=20).

7.4 The Influence of DMCs on the Expression of Genes Associated with Mineralisation and Odontogenesis

FTIR spectroscopy demonstrated similarities between the mineral phase of DMC-supplemented ADC, BMDC and DPDC cultures and physiological dentine (see section 7.3.6.3). However, dentine and bone are structurally and compositionally similar tissues and further evaluation of the relative expression of genes associated with osteoblast and odontoblast differentiation is commonly used to differentiate between these tissues (Hwang *et al*, 2008; Fujisawa *et al*, 1993). Therefore, semi-quantitative RT-PCR was used to provide a comparative indication of the effects of 1 μg/mL DMCs on the expression of genes related to osteogenic and odontogenic differentiation (for corresponding PCR gel images see appendix 7).

7.4.1 Early Markers of Differentiation

Cbfa1 and Osterix are commonly defined as markers of early stage mineralisation associated with osteogenic and odontogenic differentiation (Gronthos *et al*, 2003). Semi-quantitative RT-PCR analysis indicated that the addition of DMCs affected the gene expression levels of early mineralisation markers in a time and tissue-dependent manner (see figure 7.4.1). When cultures were assessed after 24 hours incubation, both DMC-supplemented ADC and BMDC populations displayed a significant ($P<0.05$) increase in the expression of Cbfa1 relative to both osteogenic medium and basal growth medium controls (see figure 7.4.1). BMDC cultures also demonstrated a significant ($P<0.05$) increase in the expression of Osterix relative to both osteogenic medium and basal growth medium controls after 24 hours culture. However, the expression of both Cbfa1 and Osterix was relatively low for DMC-supplemented DPDCs at day 1 when compared with

osteogenic medium controls. Following 7 days culture in DMC-supplemented osteogenic medium, there was a reduction in the relative expression of Cbfa1 in both ADC and BMDC cultures with a corresponding increase in Cbfa1 expression by basal growth medium controls. Levels of Osterix expression were higher for ADC and DPDC populations cultured in osteogenic medium at day 7 when compared with DMC-supplemented cultures, while DMC-treated BMDC cultures demonstrated a significantly ($P<0.05$) increased expression relative to osteogenic controls. Following 14 days culture, the relative Cbfa1 expression profiles for ADC and BMDC cultures returned to levels similar to those observed at day 1, while expression increased for DPDC populations exposed to osteogenic medium. Osterix expression remained relatively constant for BMDC cultures between days 7 and 14, while expression levels in osteogenic ADC and DPDC cultures decreased relative to DMC-supplemented cultures. These results indicated that both culture in DMC-supplemented and un-supplemented osteogenic medium resulted in an increase in the expression of early differentiation genes, but that DMC-supplementation altered the timing of these events in a cell-specific manner.

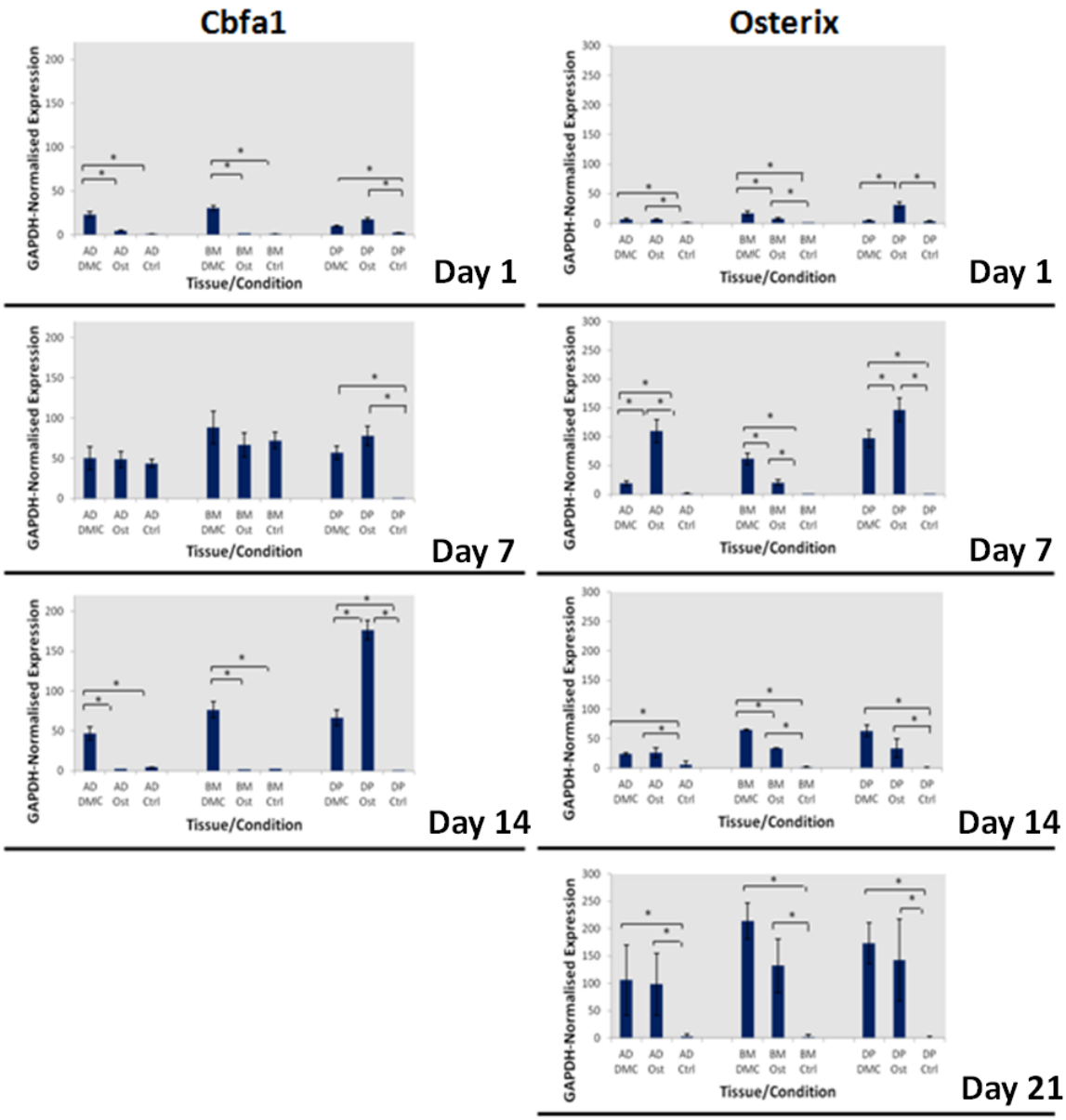

Figure 7.4.1 Representative influence of DMCs on the relative expression levels of early markers of mineralisation, Cbfa1 and Osterix. Passage 2 ADC, BMDC and DPDC cultures were exposed to DMC-supplemented and un-supplemented osteogenic media for periods of 1, 7, 14 and 21 days. PCR band intensities obtained for each gene were normalised against GAPDH and the normalised densitometric values of PCR products represented graphically. (Mean ± SD, n=3, * = $P<0.05$).

7.4.2 Differentiation Markers

Alkaline phosphatase (ALP), collagen type 1 (Col1) and osteonectin (ON) represent three markers commonly used to provide an indication of differentiation towards a mineralising cell phenotype (Gronthos *et al*, 2000). Data indicated that the addition of DMCs influenced the expression of all three markers, but that the effect was time and tissue-dependent (see figure 7.4.2). At day 1, the addition of DMCs had no significant ($P<0.05$) influence on the expression of Col1 in ADC or BMDC cultures, but promoted a significant ($P<0.05$) increase in Col1 expression for DPDC cultures. ADC, BMDC and DPDC exposed to DMC-supplemented osteogenic medium displayed a significant ($P<0.05$) reduction in the expression of ALP at day 1. The addition of DMCs appeared to have no discernible influence on ON expression at day 1 when compared with osteogenic controls. Following 7 days in culture, expression of Col1 increased for DMC-supplemented ADC cultures, while no significant ($P<0.05$) differences between DMC-supplemented and un-supplemented osteogenic cultures were observed. The expression of ON significantly ($P<0.05$) declined in both osteogenic and DMC-treated ADC and DPDC cultures relative to the basal growth medium controls at this time point. However, BMDC cultures demonstrated no decrease in ON expression at day 7 when compared with corresponding basal growth medium controls. Levels of ALP expression decreased for both ADC and DPDC populations following 7 days culture in osteogenic medium relative to DMC-treated cultures, while the expression profiles for BMDC cultures remained relatively constant. Col1 expression decreased to levels comparable with basal growth medium controls after 14 days culture, while ON and ALP expression profiles remained relatively constant for all cell populations. However, increases were observed for ON expression within DPDC osteogenic controls, and ALP expression within BMDC osteogenic controls.

No ALP expression was detected for any of the cultures following 21 days culture, while ON expression decreased significantly for ADCs and BMDCs cultured in up-supplemented osteogenic medium. These results indicated that the DMCs altered the temporal expression of markers associated with differentiation towards a mineralised cell phenotype and that the effects of DMCs are dependent on the tissue from which the multipotent cells are derived.

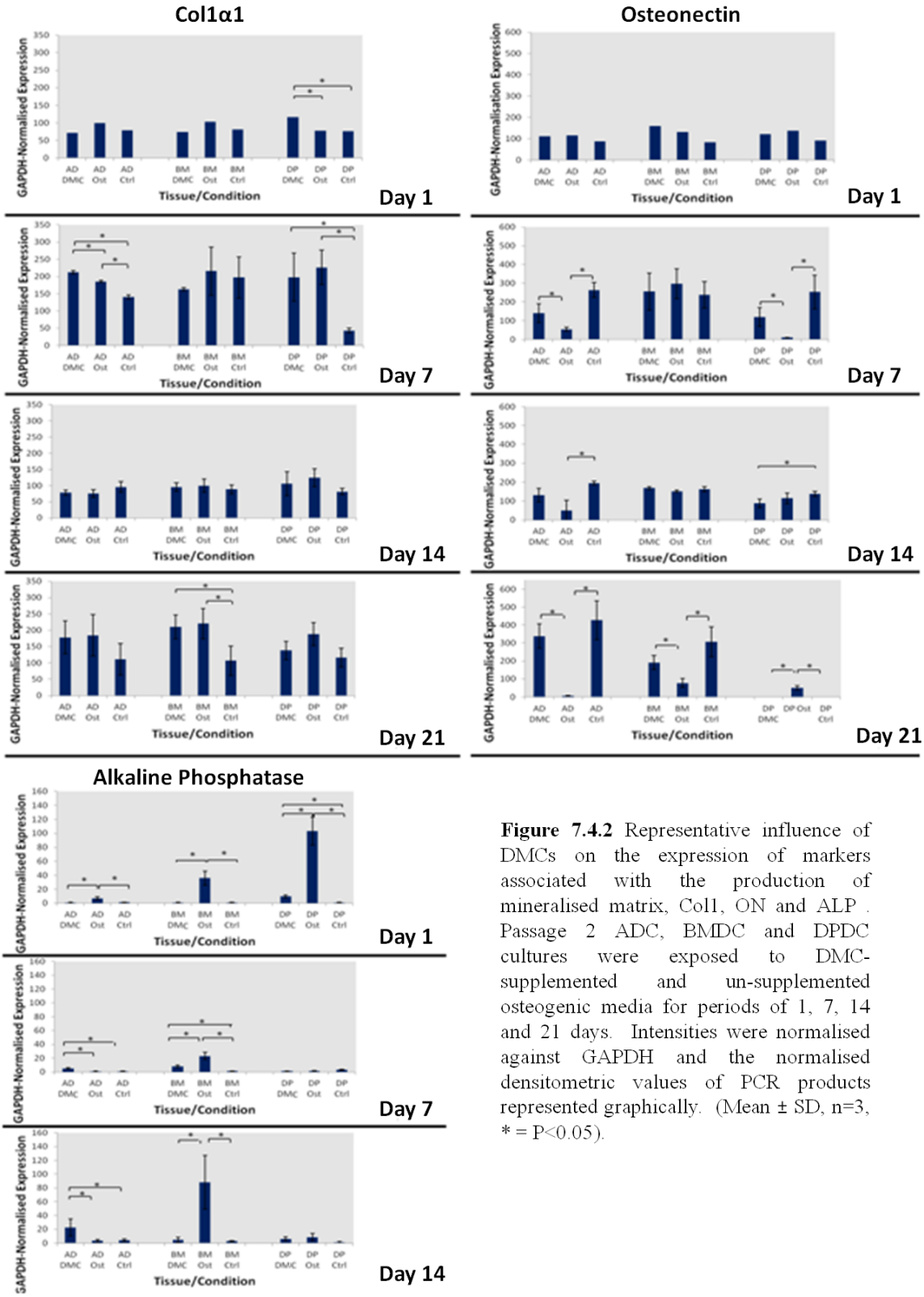

Figure 7.4.2 Representative influence of DMCs on the expression of markers associated with the production of mineralised matrix, Col1, ON and ALP . Passage 2 ADC, BMDC and DPDC cultures were exposed to DMC-supplemented and un-supplemented osteogenic media for periods of 1, 7, 14 and 21 days. Intensities were normalised against GAPDH and the normalised densitometric values of PCR products represented graphically. (Mean ± SD, n=3, * = $P<0.05$).

7.4.3 Osteogenic Markers

Bone sialoprotein (BSP) and osteopontin (OP) represent two markers frequently used to identify osteogenic differentiation (Butler, 1991). These markers are reported as being up-regulated during osteogenic differentiation whilst down-regulated during odontogenic differentiation (Fujisawa *et al*, 1993; Qin *et al*, 2001). Semi quantitative RT-PCR analysis demonstrated OP expression was significantly ($P<0.05$) down-regulated for ADC and DPDC cultures treated with DMCs at day 1 when compared with corresponding un-treated osteogenic cultures (see figure 7.4.3). This trend was also apparent for DPDC cultures at day 7, but was reduced after 14 days when expression of this marker was reduced. DMC-treated BMDC cultures displayed a significantly increased OP expression when compared with the osteogenic control at this time point. However, OP expression by DMC-treated BMDC cultures was significantly ($P<0.05$) lower than the osteogenic control at days 7, 14 and 21. The addition of DMCs to ADC cultures promoted significantly ($P<0.05$) higher OP expression at days 7 and 14 when compared with the osteogenic controls, which decreased at day 21. The expression of BSP followed a similar trend for BMDC and DPDC cultures at day 1, while expression in ADC cultures was reduced. At day 7 BSP expressions were reduced for all cultures, but increased at day 14 for osteogenic BMDC and DPDC cultures when compared with DMC-treated cultures and the basal growth medium controls. BSP expression was reduced for all ADC treatments over the entire 14 day culture period. These results demonstrated that the addition of DMCs caused significant reductions in the expression of genes related to osteogenic differentiation.

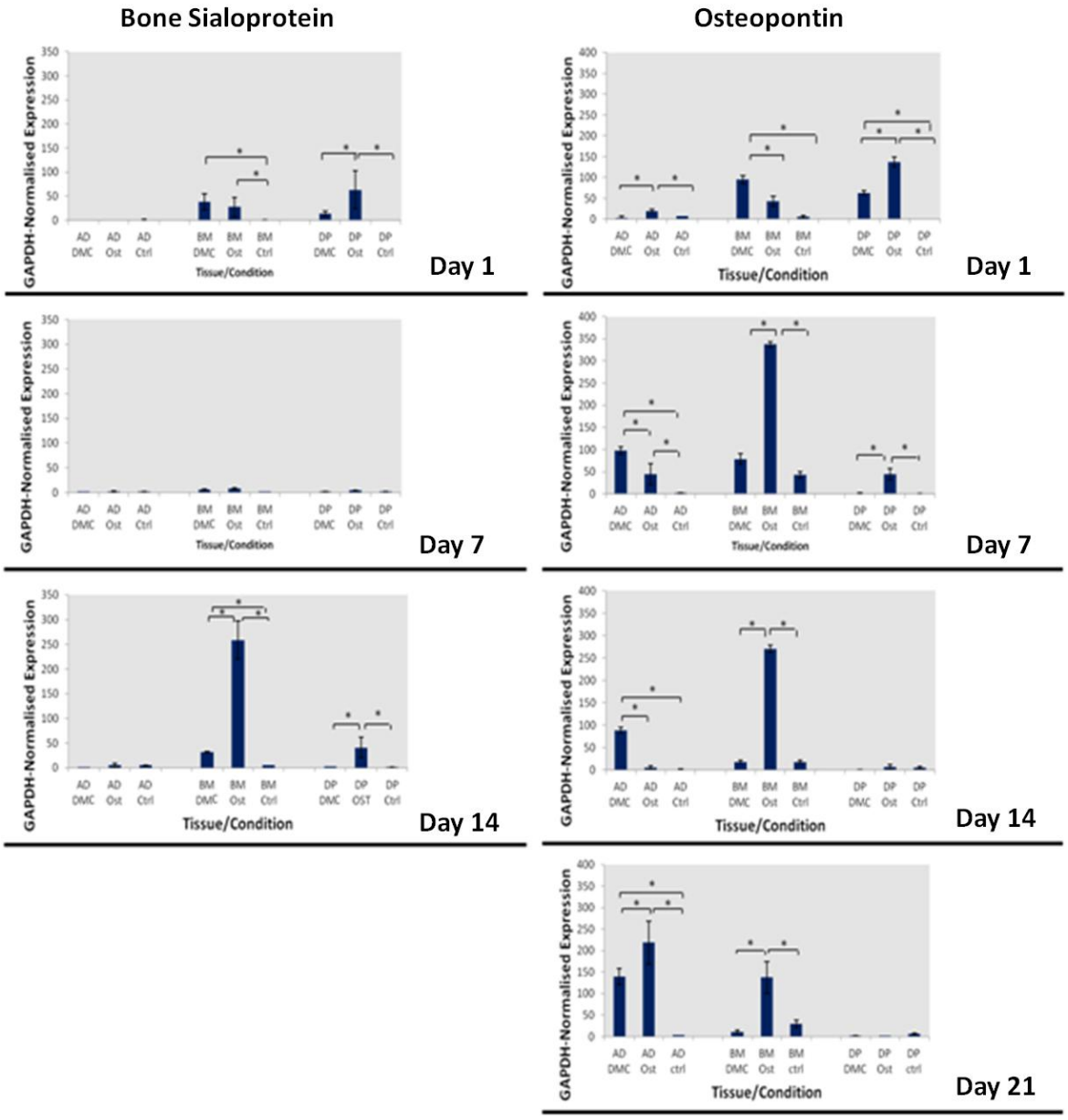

Figure 7.4.3 Representative influence of DMCs on the expression of markers associated with osteogenic differentiation, BSP and OP. Passage 2 ADC, BMDC and DPDC cultures were exposed to DMC-supplemented and un-supplemented osteogenic medium for periods of 1, 7, 14 and 21 days. Intensities were normalised against GAPDH and and the normalised densitometric values of PCR products represented graphically. (Mean ± SD, n=3, * = P<0.05).

7.4.4 Odontogenic Markers

Dentine matrix protein 1 (DMP1), dentine sialophosphoprotein (DSPP) and Nestin represent three markers more commonly used to identify odontogenic differentiation (Narayanan et al, 2006; Li *et al*, 2011). These markers are expressed during osteogenic and odontogenic differentiation, but the relative levels are reportedly greater for cultures undergoing odontogenic induction (Zhao *et al*, 2007; Hwang *et al*, 2008). Semi-quantitative RT-PCR analysis demonstrated that DSPP and Nestin shared a common expression profile for all cell types (see figure 7.4.4). Expression of these two markers by ADC and DPDC cultures was significantly ($P<0.05$) reduced when compared with osteogenic controls at days 7 and 14, but significantly ($P<0.05$) increased at day 21 for ADCs. In comparison, DMC-treated BMDC cultures demonstrated a significantly increased expression for both of these genes at days 14 and 21 when compared with BMDCs cultured in un-supplemented osteogenic medium. Expression levels of DMP1 was relatively similar for DMC-treated and untreated osteogenic cultures at day 1 for ADC and BMDC populations, while expression was significantly ($P<0.05$) increased for un-supplemented osteogenic BMDC cultures. At day 7, ADC and DPDC cultures displayed a similar trend with increased DMP1 expression in un-supplemented osteogenic cultures. DMP1 expression decreased for all cultures at days 14 and 21, although levels of expression were significantly ($P<0.05$) higher for DMC-treated ADC and BMDC cultures when compared with osteogenic and growth medium controls. The addition of DMCs altered the temporal expression of markers strongly associated with odontogenic differentiation and caused the most consistent increases ($P <0.05$) in the expression of these genes in BMDC cultures. These results together with those described in section

7.4.3 demonstrated the addition of DMCs promoted relative increases in the expression of odontogenic genes with corresponding decreases in the expression of osteogenic genes.

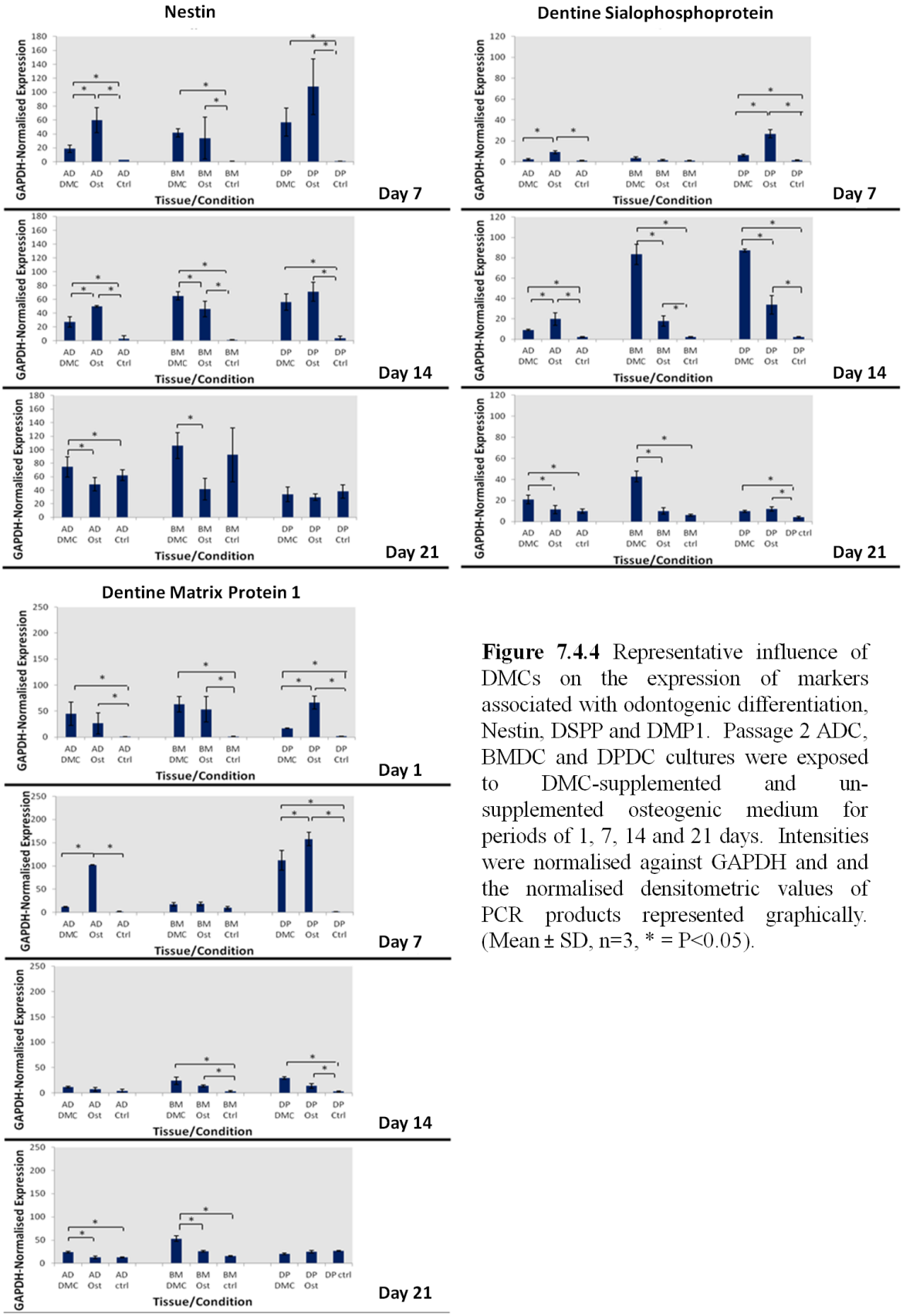

Figure 7.4.4 Representative influence of DMCs on the expression of markers associated with odontogenic differentiation, Nestin, DSPP and DMP1. Passage 2 ADC, BMDC and DPDC cultures were exposed to DMC-supplemented and un-supplemented osteogenic medium for periods of 1, 7, 14 and 21 days. Intensities were normalised against GAPDH and and the normalised densitometric values of PCR products represented graphically. (Mean ± SD, n=3, * = P<0.05).

9.0 DISCUSSION

Adipose tissue can be isolated in large volumes and contains a source of post-natal stem cells that can be abundantly harvested in a relatively painless manner. Additionally, the proportion of MSCs present in adipose tissue (~10 %) is greater than within more commonly used stem cell sources such as bone marrow (0.001-0.1 %) (Aust *et al*, 2004; Oedayrajsingh-Varma et al, 2006; Fraser *et al*, 2007; Zhu *et al*, 2012). Adipose-derived stem cells (ADSCs) are routinely used throughout many areas of tissue engineering research and their popularity is continuously growing. However, there is much debate concerning the properties of ADSCs when compared with other commonly used MSCs such as bone marrow stem cells (BMSCs), with studies reporting variations in the MSC marker profile and differentiation capacity of these cells (Gronthos *et al*, 2000; De Ugarte *et al*, 2003; Mafi *et al*, 2011). Variations in MSC profiles are primarily thought to arise due to non-standardised isolation protocols, donor-mismatched comparisons between ADSCs and other MSCs, and a lack of data relating to the consistency of MSC characteristics between species. The use of these cells for regenerative dental research also remains relatively unexplored with only a small number of studies investigating the dentinogenic potential of these multipotent cells (Wu *et al*, 2008; Jing *et al*, 2008; Ferro *et al*, 2011; Tobita *et al*, 2013).

9.1 Adipose-Derived Cell (ADC) Isolation

Previous studies have shown that a population of multipotent stem cells reside in the stromal vascular fraction of adipose tissue (Zuk et al, 2001). These adipose-derived cells (ADCs) can be readily isolated in large numbers and represent a highly proliferative cell type that have been utilised for tissue engineering applications. The original method employed for the dissociation of ADCs was introduced in the mid 1960s and required digestion with type I collagenase followed by centrifugal isolation of the stromal vascular fraction (SVF) (Rodbell, 1966; Rodbell and Jones, 1966; Bourin *et al*, 2013). This procedure has since been modified, but still requires a lengthy extraction process averaging several hours with no commonly defined period of enzymatic digestion (Zuk *et al*, 2001). Typical digestion periods used for the isolation of ADCs vary between 30 and 90 minutes depending on the study, while type I collagenase generally remains the most frequently used enzyme for the release of ADCs (Gimble and Guilak, 2003; Eom *et al*, 2011; Yu *et al*, 2010). Furthermore, most studies evaluating the release of ADCs from adipose tissue are conducted using human or mouse adipose tissue (Safford *et al*, 2002; Aust *et al*, 2004; Fraser *et al*, 2007). Therefore, comparative analysis of digestive enzymes and incubation times for the release of rat-derived ADCs are required. One of the objectives of the present study was to compare several methods of ADC isolation to establish the most optimal and reproducible protocol for the isolation of ADCs from rat inguinal adipose tissue. The present study found that 30 minute digestion using 1 g/L type I collagenase was most effective for the dissociation of ADCs; releasing approximately 1.8 million cells per gram of adipose tissue (see section 3.1). This is in agreement with the method presented by Zuk et al. (2001) for the dissociation of ADCs from human adipose tissue. Moreover, the protocol used in the present study uses a shorter digestion period than many

employed throughout the literature and is able to yield equivalent numbers of colony-forming cells (Yoshimura *et al*, 2007; Kingham *et al*, 2007; Wang *et al*, 2012). Varying cell harvesting protocols employing a range of digestive enzymes and incubation periods are generally thought to be partly responsible for discrepancies within the literature concerning the expression of MSC-associated markers, as well as contributing to conflicting information regarding the multi-lineage differentiation potential of ADCs (Oedayrajsingh-Varma *et al*, 2006; Baptista *et al*, 2009; Strioga et al, 2012; Priva *et al*, 2012). Therefore, it is important that protocols are optimised for dissociation of ADCs in different species and standardised to reduce the amount of variation in MSC profiling and tissue engineering research.

9.2 Expansion of Tissue Culture Polystyrene Adherent Cells Derived from Adipose, Bone Marrow and Dental Pulp Tissues

Previous studies have shown that mesenchymal stem cells can be isolated from virtually every tissue in the body (da Silva Meirelles *et al*, 2006; Hoogduijin *et al*, 2011; De Miguel *et al*, 2012). These cells are defined by their adherence to tissue culture polystyrene (TCP), their colony-forming capacities, the expression of a defined profile of surface markers, such as CD73, CD90 and CD105, and the potential to differentiate towards osteogenic, adipogenic and chondrogenic lineages (Dominici *et al*, 2006). However, thus far no experimental studies have been conducted to directly compare the "stem-like" properties of adipose (ADCs), bone marrow (BMDCs) or dental pulp-derived cells (DPDCs) (Huang *et al*, 2009; Peng *et al*, 2009). In this study, ADCs, BMDCs and DPDCs isolated from 6 week old male rats were compared using cells at the same passage number

to eliminate any discrepancies relating to donor-specific variation and alterations in MSC characteristics due to differential expansion (Siddappa *et al*, 2007; Alves *et al*, 2012). Minor differences were observed between the morphologies of cells derived from each tissue (see section 4.1.1). All cells displayed characteristic fibroblast "stromal" cell morphology, but ADCs appeared to possess slightly smaller cell bodies with thinner processes, as reported in previous studies (Monaco *et al*, 2009; Hematti, 2012). Colonies formed within ADC cultures were larger than those formed within BMDC and DPDC cultures, although their frequency was comparatively low (see section 4.1.3). A relatively low number of colonies containing higher numbers of cells within ADC cultures have previously been reported (Rider *et al*, 2008; Tawonsawatruk *et al*, 2012; Al-Nbaheen *et al*, 2013). Variations in the number of colony forming cells present in ADC, BMDC and DPDC cultures may be due to differences in composition of these tissues, since dental pulp and adipose tissues represent more fibrous environments than the bone marrow (Gronthos *et al*, 2000; Al-Nbaheen *et al*, 2013).

Comparison of growth kinetics of ADC and BMDC populations established at passage 1 and 2 indicated that these two cell types shared similar growth rates (see section 4.1.2). Comparison of the growth kinetics of ADCs and BMDCs within the literature is conflicting with some studies claiming that BMDCs display a slightly elevated growth rate when compared with ADCs, while other studies report no discernible differences (Kisiel *et al*, 2012; Tawonsawatruk *et al*, 2012). Differences in the growth kinetics of DPDC and ADC cultures are likely due to the varying methods employed for dissociation of these cells from the surrounding tissue, with different studies using collagenase I, dispase, trypsin or even a mixture of these enzymes for the isolation of DPDCs from the pulp (Huang *et al*, 2008; Alge *et al*, 2010; Smith *et al*, 2012). DPDC populations demonstrated

an initial lag phase not present in ADC or BMDC populations and as a consequence, demonstrated significantly lower cell numbers following an identical period of cell culture (see section 4.1.2). Conflicting data have been reported regarding the presence of a lag phase in DPDC cultures (Gronthos *et al*, 2000; Eslaminejad *et al*, 2010; Alge *et al*, 2010). This may in part be due to the method of isolation, the concentration of FBS added to the culture medium or the cell seeding density used for the analysis of DPDC growth kinetics (Eslaminejad *et al*, 2010). A previous study has proposed that this lag phase may represent a beneficial period of adaptation during which these cells readjust to the new culture conditions and may be characterised by the presence of small agranular cells called recycling stem cells, which give rise to larger proliferating cells during the log phase of cell growth (Ferro *et al*, 2012). It has been further demonstrated that the exponential growth phase is lengthened if preceded by an initial lag phase, potentially due to the higher availability of nutrients per cell (Neuhuber *et al*, 2008). Therefore, these results indicated that although the number of cells isolated from adipose, bone marrow and dental pulp differed, each cell type represents a potential source of multipotent cells for dental tissue engineering.

9.3 Mesenchymal Stem Cell Marker Profiles

The International Society for Cellular Therapy (ISCT) defined CD73, CD90 and CD105 as the three key cell surface markers required for MSC characterisation (Dominici *et al*, 2006). Other markers cited include CD29 and CD44 (Maurer, 2011), whilst a recent review reported that the five most commonly used markers for MSC selection were CD105, CD90, CD44, CD73 and CD29 (in descending order) (Mafi *et al*, 2011). In the

present study, the effect of *in vitro* expansion on the MSC-associated marker profiles of rat-derived ADCs, BMDCs and DPDCs was evaluated using FACS and sqRT-PCR gene expression analysis. The work presented here is novel since studies focussing on the profiling of several anatomically distinct rat-derived MSCs do not exist since the majority of studies are devoted to human and mouse cell lines (Zangi et al, 2006; Harting *et al*, 2008). The present investigation found that both pluripotent and multipotent markers were expressed in primary cell populations and continued to be expressed until passage 4, but that the relative levels of expression were not consistent between populations isolated from different tissues, particularly for primary and passage 1 cultures (see section 4.2.2). The level of expression of these markers became more stable with successive sub-culture indicating that the MSC population had expanded and become more homogeneous. This correlated with previous evidence indicating that expression of MSC-associated genes increased and became more stable with adherence to tissue culture polystyrene (TCP) and *in vitro* expansion until passage 5 (Mitchell *et al*, 2006).

Analysis of MSC-associated cell surface markers using fluorescence activated cell sorting (FACS) and sqRT-PCR demonstrated the presence of MSC markers such as CD29, CD44, CD73, CD90 and CD105 in ADC, BMDC and DPDC cultures (see section 4.2). Quantification of cell surface markers using FACS indicated primary ADC cultures displayed a 26 % higher level of CD29 and CD90 expression when compared with primary BMDC cultures, while passage 1 and 2 ADC, BMDC and DPDC populations all demonstrated > 90 % CD29 positivity and ≥ 70 % CD90 positivity, which is consistent with other reports in the literature (see section 4.2.1) (Mitchell *et al*, 2006; Zhu *et al*, 2008). A 26 % higher expression of CD29 and CD90 in primary ADCs could be related to the heterogeneity of primary adipose populations, since these markers are present on

MSCs as well as lymphocytes, fibroblasts, activated endothelial cells and differentiating neuronal cells that are reduced following *in vitro* expansion on TCP (Seymour *et al*, 1997; Pruszak *et al*, 2009; Kisselback *et al*, 2009; Mafi *et al*, 2011). In fact, reports have indicated that a largely homogeneous population of MSCs exists in BMDC cultures obtained from rat after just three passages (Harting *et al*, 2008).

One of the main issues faced when attempting to define MSCs within newly isolated tissues is that stromal progenitors within primary isolates are likely to exist at various stages of differentiation, thereby influencing the expression of surface markers, renewal capacity and multi-lineage potential (Niemela *et al*, 2008; Lin *et al*, 2010). This may provide some rationale for the divergence in the expression of MSC-associated genes displayed by primary ADC, BMDC and DPDC populations in the present study (see section 4.2.2). Relative differences in the expression of MSC-associated markers in primary cultures can also be explained by the relative differences in stem/progenitor cell content or the comparative cellular heterogeneity of each tissue, with the proportion of mesenchymal stem cells found in different tissues typically representing between 0.001-10 % of the entire cell population, with bone marrow tissue shown to contain the lowest proportion of MSCs (0.001-0.1 %) (Pittenger *et al*, 1999; Bourin *et al*, 2013). The different proportions of stem cells and various other tissue-dependent cell types is likely to influence the relative expression levels of single genes used for the profiling of stem cell populations. This is particularly applicable to primary cultures, but may even account for the comparatively low CD90 positive fraction in FACS analysed passage 1 and 2 BMDC cultures when compared with ADC and DPDC cultures (see section 4.2.1) (Halfon *et al*, 2011). A second reason for the divergence in MSC marker expression is that the characterisation of MSCs has primarily been conducted using stem cells derived from

humans or mice with comparatively few studies focussing on rat stem cells (Pittenger *et al*, 1999; Jiang *et al*, 2002; Harting *et al*, 2008). This means that the majority of research relies on the comparability of features such as stem cell markers and multi-differentiation capacities common to human and murine MSCs and then attempts to apply them to MSCs derived from rats (Harting *et al*, 2008). Previous research has indicated that such assumptions may not be entirely accurate with BMDCs derived from rats demonstrating a much faster selection of predominantly pure MSCs on TCP (by passage 3) than BMDCs derived from mice (over 10 passages required) (Harting *et al*, 2008).

Genes such as Sox2, Nanog, Klf4, Lin28 and C-myc function in the maintenance of embryonic stem cell self-renewal and regulate the expression of loci involved in pluripotency and differentiation (Kashyap *et al*, 2009; Varlakhanova *et al*, 2010; Shyh-Chang, 2013). Recent evidence has indicated that these stem cell maintenance factors may play a very similar role within the adult-derived stem cell population (Greco *et al*, 2007; Saulnier *et al*, 2011; Tsai *et al*, 2012; Seo *et al*, 2013). The transfection of combinations of these factors has been shown to induce pluripotency in unipotent cell types, such as fibroblasts, and has also been shown to increase the proliferation and differentiation potentials of MSCs (Go *et al*, 2008; Kashvap *et al*, 2009; Chambers and Tomlinson, 2009). Semi-quantitative RT-PCR analysis of the expression of MSC-associated genes presented in this study demonstrated the presence of such markers within ADC, BMDC and DPDC populations, with the expression of Sox-2, Nanog, Klf4 and C-myc varying most between ADCs, BMDCs and DPDCs in primary and passage 1 cultures (see section 4.2.2a). Differences in the expression of pluripotent markers within primary populations may be explained by the fact that the combination of markers expressed by MSCs derived from different anatomical locations is not universal. In fact, the presence and absence of certain

pluripotent markers may provide an indication of functional and compositional tissue-specific differences that are maintained during early cell culture but lost as populations become more homogeneous following adherence and expansion on TCP (Riekstina *et al*, 2009). This proposal is based on the fact that the present study identified differences in the expression of markers of pluripotency between primary MSC cultures isolated from different tissue sources, with Sox-2, Nanog and Klf4 expression not detected in primary DPDC cultures but detected in primary ADC and BMDC cultures (see section 4.2.2a). The lack of Nestin and SOX-2 expression in primary DPDC cultures has been previously documented and may be related to differences in the embryological derivation of the three MSCs, with both ADCs and BMDCs being of mesodermal origin and DPDCs being of ecto-mesenchymal origin (Lengler *et al*, 2005; Greco *et al*, 2007; Riekstina *et al*, 2009).

The presence of CD34 on ADCs remains a controversial topic within the literature (Yu *et al*, 2010; Lin *et al*, 2010; Mafi *et al*, 2011). The present study detected CD34 positivity among primary ADC populations, which became reduced to un-detectable levels following culture on TCP at passage 1 (see section 4.2.2b). Various hypotheses have been put forward to explain the ambiguous nature of CD34 expression within ADC populations, with some reports indicating that CD34 positive members of the stromal vascular fraction (SVF) represent a distinct group of cells that also express other MSC-defining markers, such as CD73, CD90 and CD105 (Zvaifler *et al*, 2000). Interestingly, the presence of CD34 has been previously demonstrated within Stro-1 positive dental pulp stem cell isolates and may indicate the presence of a pericytic fraction with a multipotent capacity or the association of stem cells with a perivascular niche (Shi and Gronthos, 2003; Laino *et al*, 2006; Stokowski *et al*, 2007; Martens *et al*, 2012). The present study indicated that the expression of CD34 was limited to primary ADC isolates and would support previous

studies demonstrating that CD34 positivity is present only in freshly isolated adipose digests after which expression of this marker decreased rapidly (Mitchell *et al*, 2006; Maumus *et al*, 2011).

The results presented in this study indicated that even though the expression of MSC markers differed between primary populations of ADCs, BMDCs and DPDCs, once established in culture at passage 2-4 the levels of these markers reached similar levels. Therefore, ADCs represent an accessible and rapidly proliferating population of cells that have an expression profile common with MSC-like cells isolated from the dental pulp and bone marrow.

9.4 Differentiation Capacity of ADCs, BMDCs and DPDCs

The reported ability of ADCs, BMDCs and DPDCs to differentiate towards different lineages commonly varies within the literature (Im *et al*, 2005; Rosenbaum *et al*, 2008; Pachon-Pena *et al*, 2011; Shafiee *et al*, 2011). This study only compared the differentiation of MSCs towards osteogenic and adipogenic lineages since a standardised protocol for the differentiation of ADCs towards a chondrogenic phenotype does not exist. Data presented in section 4.3 demonstrated that even though all three cell types were capable of bi-lineal differentiation, the relative capacity for adipo- and osteogenic differentiation differed considerably between these cells. These findings are in agreement with other published work indicating that ADCs have a low osteogenic potential as compared with BMDCs or DPDCs, while DPDCs, have a reduced adipogenic potential when compared with ADCs (Gronthos *et al*, 2000; De Ugarte *et al*, 2003; Kern *et al*, 2006; Izadpanah *et al*, 2006; Liu *et al*, 2007; Zaminy *et al*, 2008; Hayashi *et al*, 2008; Pachon *et*

al, 2011). These differences were confirmed at the RNA level, with DPDC cultures displaying comparatively low levels of lipoprotein lipase (LPL), peroxisome proliferator-activated receptor γ (PPARγ) and adipocyte protein 2 (aP2) when compared with ADC and BMDC cultures (see section 4.4.1).

The appearance of mineral in different MSC cultures varies considerably; with some groups claiming that BMDCs are able to form a continuous osteogenic sheet of small mineralised nodules, while others indicate this is a characteristic of DPSCs (Yu *et al*, 2007; Monaco *et al*, 2009). The present study indicated that mineral present in DPDC cultures had a different appearance to that found in ADC and DPDC cultures. The mineral present in DPDC cultures lacked defined nodules typically observed in mineralising cell cultures and instead formed a sheet of mineral that covered the entire culture dish (see sections 4.3.2, 7.3.4 and 7.3.5) (Gronthos *et al*, 2000; Monaco *et al*, 2009; Balic *et al*, 2009). Comparison of the total amount of mineral present in these cultures using alizarin red staining (ARS) confirmed that DPDC cultures mineralised at a faster rate than ADC and BMDC cultures with significantly more mineral present after 7, 14 and 21 days culture in osteogenic medium (see section 4.3.2). Evaluation of the density of the mineral present in all three cultures using micro-computed tomography indicated that it was predominantly ≤ 250 mg/cm^3 (see section 7.3.6.2), while dissociation of the organic and inorganic phases revealed that almost half of the weight of DPDC cultures was represented by inorganic material, while this only represented approximately 2 % of ADC and BMDC cultures (see section 4.3.3). Previous studies using microarrays to analyse the differential expression of genes between MSCs on a genome-wide level have shown differences in the expression of ECM components, cell adhesion molecules, and growth and transcription factors (Yamada *et al*, 2006). It is likely that such variations in the level of growth and transcription factors

within the developing extracellular matrix could lead to differences in the pattern of mineralisation observed in the present study (Sapir-Koren and Livshits, 2011).

Differences in the capacity of ADCs, BMDCs and DPDCs to differentiate towards osteo- and adipogenic lineages may also be explained by the relative proportions of MSCs residing within each tissue. For instance, several authors have suggested that in spite of morphological and immunophenotypic similarities, ADCs may represent a heterogeneous population of cells containing a relatively small proportion of multipotent cells capable of osteogenic differentiation (Im *et al*, 2005; Mochizuki *et al*, 2006; Yoshimura *et al*, 2007). There is evidence to suggest that although ADSCs and BMDCs express an array of common genes allowing differentiation towards early stage osteoblasts or adipocytes, various late-stage differentiation factors are required for the onset of maturation, with these late stage factors potentially influenced by MSC origin (Lin *et al*, 2005; Liu *et al*, 2007). This hypothesis was supported by the identification of late stage factors that have been associated with osteogenic and adipogenic lineage commitment that include osteomodulin (OMD), apolipoprotein (APOD), adiponectin (ACDC) and fatty acid binding-protein 5 (FABP5), which have been shown to be differentially expressed by ADCs and BMDCs (Liu *et al*, 2007). Furthermore, there is evidence to suggest that it is likely that progenitors localised within tissues are likely not to exist as a single multipotent population but as an uncharacterised mixture of pluripotent, tripotent, bipotent, and unipotent progenitor cells of varying proportions (Pittenger *et al*, 1999; Zuk *et al*, 2002; Guilak *et al*, 2006). Therefore, the comparatively low osteogenic capacity of ADCs may be due to the fact that the ratio of true multipotent cells capable of osteogenic differentiation in ADC cultures may be relatively low when compared with unipotent adipogenic progenitors, while in BMSC and DPSC populations the proportion of mineralising progenitors may be higher

(Liu *et al*, 2007). In fact, a study investigating BMDC clonal expansion revealed that only one third of the cells present were capable of trilineage differentiation, with approximately 70% demonstrating osteo- and chondrogenic potential (bipotent), and nearly all displaying osteogenic potential (Murgalia *et al*, 2000).

The osteogenic potential of ADCs can also be significantly influenced by culture conditions (concentration/use of dexamethasone), the species from which the ADCs are isolated, the harvesting technique employed, whether cells are cultured *in vitro* or *in vivo* and whether the cells were cultivated in a 2D or 3D (tissue engineering scaffold) environment (Gronthos *et al*, 2001; Cowan *et al*, 2004; Gabbay *et al*, 2006; Wan *et al*, 2006; Mitchell *et al*, 2006; Touomura *et al*, 2007). Currently, there is no consensus regarding induction factors required for the osteogenic differentiation of ADCs with investigators typically using osteogenic medium originally formulated for use with BMSCs, not knowing whether this medium is best suited to promote ADC differentiation (Kyllonen *et al*, 2013). For instance, dexamethasone is routinely added to osteogenic medium at concentrations of 10^{-7} M (Gronthos *et al*, 1994; Zhang *et al*, 2006). The addition of dexamethasone at concentrations of 10^{-7} and 10^{-8} has been shown to lead to a reduction in alkaline phosphatase (ALP) activity, the expression of genes associated with mineralisation such as osteopontin or osteocalcin (OC), or the induction of adipogenesis in ADC cultures (de Girolamo *et al*, 2007; Hoemann *et al*, 2009; Mostafa *et al*, 2012). Examination of the influence of dexamethasone on mineralisation in ADC cultures in the present study demonstrated that 10^{-9} M doses of dexamethasone were optimal for promoting mineralisation in ADC cultures (see section 4.3.1). Recent evidence has indicated that the osteogenic differentiation of these cells may be further improved by culturing ADCs in a xeno-free medium or a medium containing human serum rather than

standard culture medium containing FBS (Kyllonen *et al*, 2013). This recent study has also demonstrated that differentiation of ADCs to a mineralising cell phenotype may also be enhanced by increasing the ratio of ascorbic acid to β-glycerophosphate (Kyllonen *et al*, 2013).

Elemental analysis of the inorganic phase present in mineralising cell cultures following exposure to osteogenic medium demonstrated a calcium to phosphate ratio of 1.25 indicative of an amorphous (immature) calcium phosphate (ACP) (see section 7.3.5) (Zhao et al, 2011) The majority of inorganic matter within hard tissue is composed of a non-stoichiometric calcium deficient - biological – apatite, with calcium to phosphate ratios for bone and dentine representing 1.71 and 1.61, respectively (Dorozhkin and Epple, 2002). The formation of calcium-based biomineral is thought to progress from the formation of stable prenucleation clusters, which aggregate to form an amorphous precursor (Termine and Posner, 1966). Therefore, the presence of amorphous calcium phosphate (ACP) characterises a transient stage in a kinetically driven process leading to the formation of octacalcium phosphate, and finally crystallised hydroxyapatite (HA) (Skrtic *et al*, 1996; Penn and Banfield, 1999; Takano *et al*, 2000; Banfield *et al*, 2000; Zhao *et al*, 2011). Thus, it can be postulated that the presence of ACP-like inorganic matter may represent an early, transitory phase in mineralisation that can be correlated with the early stages of bone and tooth mineralisation during which ACP functions as an important intermediate phase (Bodier-Houlle *et al*, 2000; Tsuji *et al*, 2008).

Data collected in this study demonstrated that ADCs, BMDCs and DPDCs demonstrated a bi-lineal differentiation potential. Together with the adherence of these cells to TCP and their expression of MSC markers these results indicated that the cells isolated in this study

could be characterised as MSC-like. However, the comparatively limited capacity of ADCs to differentiate to a mineral-secreting cell type casts some doubts on the suitability of this cells type for dental tissue engineering.

9.5 The Effects of Cryo-Storage on ADCs, BMDCs and DPDCs

In order to determine whether the data presented in this study could be obtained using both cryo-preserved and freshly isolated cells the influence of cryo-storage on MSC viability and marker expression needed to be determined. Furthermore, the ability of MSCs to survive long term storage and maintain their phenotype upon revival is critical if they are to be banked and used for future therapeutic purposes. The majority of published cryo-preservation protocols rely on the use of dimethyl sulfoxide (DMSO), to prevent the formation of intra and extra-cellular crystals during the freezing process (Thirumala *et al*, 2009; Chin *et al*, 2010). However, recent studies have confirmed that the survival and number of colonies formed by MSCs is significantly decreased following cryopreservation and that the severity of this decline is inversely proportional to DMSO content (Ock and Rho, 2011). The present study indicated cryo-storage with 10 % DMSO had no observable influence on the morphology of ADCs, BMDCs or DPDCs following culture (see section 6.1.1), while the effects of cryo-storage on viability were found to be cell-type specific, with the average number of viable cells following expansion in culture being 94 % for ADCs, 89 % for DPDCs and 60 % for BMDCs (see section 6.1.2). Intriguingly, recent high magnification cell surface analysis of cryo-stored and un-stored ADCs has revealed changes in cell shape from broad and flat to a more stellate morphology following cryo-preservation, which could not be detected by low magnification phase contrast

imaging used in the present study (James *et al*, 2011). The reduction in cellular viability following cyro-storage may be explained by cell damage occurring due to hyperosmotic stress, differences in the concentrations of intracellular salts, membrane alterations or the toxic effects of DMSO (Lovelock, 1954; Meryman, 2007). Previous studies have also noted that although DMSO acts as a hydroxyl-free radical scavenger, damage can still occur due to the action of oxygen radicals formed during the freeze-thaw process (Kruuv and Glofcheski, 1993; Limaye, 1997).

The influence of cryo-storage on the expression of MSC-associated markers was also evaluated using sqRT-PCR gene expression analysis and FACS profiling. These analyses showed higher levels of expression of MSC-associated markers CD73, CD90 and CD105 for ADC cultures, CD44 and CD105 for BMDC cultures, and CD73 and CD44 for DPDC cultures following cryo-storage (see section 6.2). Genes associated with cell maintenance and pluripotency such as Klf4, Lin28 and Nanog were also increased following cryo-storage of ADC and DPDC cultures (see section 6.4). Loss of viability coupled with an increase in the expression of MSC-associated markers indicated that cryo-storage may reduce the viability of certain non-MSCs and consequently cause a proportional increase in the number of cells expressing MSC markers within these heterogeneous populations. The fact that no increase in the expression of pluripotent markers in BMDC cultures was detected following cryo-storage, together with the comparatively large reduction in BMDC viability may indicate that the population of stem cells present within bone marrow isolates decreased following cryo-storage. These results suggest that MSCs present within the bone marrow may be more susceptible to hyperosmotic damage resulting from cryo-preservation. Increases in the expression of transcription factors first identified with the maintenance of multi-potency and cell renewal in embryonic stem cells, such as Nanog,

Lin28, C-myc and Klf4 indicate that cryo-storage may have an effect on the relative "stemness" of heterogeneous MSC-containing cultures.

The effect of cryo-storage on cell viability and MSC marker expression led to the sole use of freshly isolated cells in the present study. This choice aimed to minimise variation in MSC characteristics and provide an accurate and reproducible evaluation of the odontogenic capacity of MSCs.

9.6 The Influence of the Fluorescence-Activated Cell Sorting (FACS) Procedure on Cell Viability, Differentiation and Immunophenotype

This study was designed to determine if selecting cells for markers associated with MSCs had an influence on their viability and multi-differentiation capacity. It has been postulated that MSCs must be sorted from heterogeneous populations since the heterogeneity of a culture may compromise the proliferation and/or differentiation potential of the MSCs contained (Lennon *et al*, 2000; Schrepfer et al, 2007; Rada *et al*, 2011). As nutrient limitation and the action of endogenous enzymes are known to have a detrimental effect on cells when kept outside of normal culture conditions, the present experiments were carried out at 4 ºC to limit reductions in cell viability, and sorted into polypropylene collection tubes containing a high percentage FBS medium to ensure maximum cell recovery (Laiho and Penttila, 1981; McNally and Brockbank, 1992). Section 5.2 presented results demonstrating the osteogenic and adipogenic differentiation of ADC, BMDC and DPDC cultures following FACS on the basis of MSC-associated markers CD29 and CD90. The findings indicated that selecting for MSC markers significantly reduced the osteogenic and adipogenic capacity of TCP adherent cells.

Moreover, the FACS procedure was also shown to significantly reduce the viability of these cells (see section 5.1.1). These data are consistent with previous reports suggesting that hydrodynamic and extensional forces encountered by cells during FACS have a negative influence on cell viability, osteogenic differentiation, as well as on gene expression and a variety of signalling pathways (Herbertson and Aubin, 1997; Alenghat and Ingber, 2002; Mollet *et al*, 2008; Zaitoun *et al*, 2010).

FACS preparation and analysis required maintenance of cells in suspension for up to 3 hours. Therefore, time spent out of culture was assessed over a 5 hour period mimicking the conditions experienced when preparing for FACS (see section 2.2). Data indicated that ADCs could survive for long periods out of culture with a minimal loss of cell viability (<10 % after 5 hours out of culture), while DPDCs (~16 %), and particularly BMDCs (~23 %) underwent greater reductions (see section 5.1.2). These data demonstrated differences in the ability of MSC-like cells isolated from different tissues to survive prolonged periods out of culture, indicating that ADCs may represent a more robust cell type than other MSC sources (Wu *et al*, 2013). The comparative robustness of ADCs was also corroborated by a relatively high level of viability following FACS and cryo-storage, also documented in the present study (see sections 5.1.1 and 6.1.2), as well as the documented ability of these cells to maintain their phenotype with age and increasing time spent in culture (Wu *et al*, 2013). However, since the entire FACS procedure consistently took no longer than 3 hours, loss of viability due to time out of culture could not solely account for the decline experienced following sorting. The ability of ADCs to survive out of culture for longer periods than either DPDCs or BMSCs was also interesting given that this tissue has recently been shown to contain a population of multi-lineage differentiating stress-enduring (Muse) cells that are able to endure extreme stresses such as hypoxia, serum

deprivation, long term exposure to proteolytic enzymes such as collagenase, and low temperatures (Heneidi *et al*, 2013; Kuroda *et al*, 2013). Similar cells with the ability to survive extreme stress within bone marrow and dental pulp tissues have not yet been described. However, measuring the percentage of viable cells present following FACS gave no indication as to whether the remaining population were capable of differentiation or rather comprised of a population of pre-apoptotic or senescent cells (Tomlinson *et al*, 2013). Therefore, the capacity of sorted cells to undergo differentiation towards osteogenic and adipogenic lineages was assessed.

The differentiation capacity of cells selected using FACS was compared with no primary controls and unsorted populations to determine whether sorting for characteristic MSC markers had a positive influence on the differentiation capacity of these cells. Previous studies have suggested that CD90 can be used as a selective agent to increase the osteogenic capacity of heterogeneous ADC populations (Chung *et al*, 2013). However, in this example, the CD90 positive cell population was removed directly from the SVF with no prior selection on TCP. Interestingly, data obtained in the present investigation indicating that any enhanced osteogenic capacity resulting from CD90 selection becomes lost following the *in vitro* expansion of these cells (see section 5.2.1). A previous study has demonstrated that independent sorting for CD29 and CD90 positivity resulted in an up-regulation in some MSC-associated genes and altered the differentiation capacity of these cells (Rada *et al*, 2011). However, these results were obtained using adipose tissue derived from female donors and sorted using immunomagnetic beads within the first 24 hours of isolation. Immunomagnetic sorting is unable to provide the purity of selection associated with FACS but this technique may provide a more delicate method for the selection of immuno-positive cell populations due to the reduced hydrodynamic forces

involved in this method (de Wynter *et al*, 1995; Dainiak *et al*, 2007; Pruszak *et al*, 2007; Zhu et al, 2013).

Since reportedly 0.001-10 % of primary populations are represented by MSCs, the selection of cells based on the presence of MSC markers often results in the recovery of a very small proportion of the original population (Pittenger *et al*, 1999; Zuk, 2010; Bourin *et al*, 2013). This study indicated that the proportion of CD90 and CD29/CD90 positive cells was increased following the *in vitro* culture of FACS selected populations, but the proportion of CD29 positive cells was decreased (see section 5.3). This may be related to the reduction in cell viability as a consequence of FACS (see section 5.1.1). Interestingly, corresponding significant increases were observed in Klf4 gene expression within CD29 positive fractions (see section 5.4.1a). Klf4, as well as having a role in the self-renewal and differentiation capacities of stem cells, has been associated with inflammation and the differentiation of monocytes. Therefore the high level of Klf4 expression within CD29 positive populations may result as a consequence of cell damage following FACS (Feinberg *et al*, 2005; Alder *et al*, 2008). Alternatively, since CD29 is known to be important for the adhesion and migration of MSCs, it could be postulated that the impact of labelling and the shearing forces imparted during FACS may cause damage to this cell surface integrin and limit the ability of sorted cells to adhere and proliferate in culture (Konakahara *et al*, 2004). This capacity appears to be at least in part restored by the presence of CD90, potentially due to its role as cell-cell and cell-matrix adhesion protein (see section 5.3) (Rege and Hagood, 2006; Kisselbach *et al*, 2009).

To assess how selecting for cells based on the presence of MSC surface markers CD29 and CD90 affected the expression of other defined MSC markers ADC, BMDC and DPDC

gene expression was compared before and after FACS. The results demonstrated tissue-specific changes in the levels of various pluripotent markers in FACS selected ADC and BMDC populations (see section 5.4.1). Sorted ADC populations typically displayed a reduction in the expression of pluripotent genes associated with cell renewal and maintenance, while CD29/CD90 sorted populations displayed increases in certain multipotent markers such as CD44, CD29 and CD90 (see section 5.4.1a). BMDC populations demonstrated a very different profile of pluripotent gene expression following FACS with only minor decreases observed in the expression of the pluripotent marker SOX-2 and multipotent marker CD105 (see section 5.4.1b). Changes in gene expression have been shown to result from nuclear distortions resulting from the application of pressure to cells, with stem cells being particularly susceptible due to the presence of large nuclei and relatively little cytoplasm (Dahl *et al*, 2008; Karaoz *et al*, 2009; Bray *et al*, 2010). Previous studies have shown such stimulation can influence the expression of various pluripotent genes such as Nanog and SOX-2 through rearrangement of the actin cytoskeleton (Teramura *et al*, 2012; Horiuchi *et al*, 2012). Therefore, it could be hypothesised that the hydrodynamic shearing forces encountered during FACS may cause distortions in nuclear shape and that these changes may be associated with alterations in the gene expression profiles of ADCs and BMDCs. It was also evident that TCP adherent ADCs and BMDCs did not respond to forces experienced during FACS in an identical manner with ADCs displaying a greater number of significant reductions in the expression of pluripotent and multipotent genes following selection (see section 5.4.1).

This study suggested that FACS selection for CD29 and CD90 MSC markers negatively affected the viability of ADCs, BMDCs and DPDCs and did not improve the osteogenic/adipogenic potential of these cells. This effect may be due to cell disruption by

hydrodynamic forces encountered during the FACS procedure or the fact that the markers used did not select for clones with osteogenic/adipogenic potential. This informed the choice to carry out all subsequent experiments using TCP adherence alone for the selection of multipotent cell types for odontogenic differentiation.

9.7 Odontogenic Differentiation

Various local and systemic factors, such as TGF-β, BMP-2, FGF-2, platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF) are associated with early odontoblast and osteoblast lineage commitment (Canalis *et al*, 1993; Canalis, 1993; Centrella *et al*, 1994). Many of these factors have been documented within EDTA-extracted dentine fractions and their ability to promote the differentiation of multipotent cells towards an odontoblast-like phenotype has been reported (Begue-Kirn *et al*, 1992; Martin *et al*, 1998; Hao *et al*, 2004). The present study found that the supplementation of DMCs at a concentration of 1 µg/mg promoted the most significant increase in mineral deposition, as well as in the expression of odontogenic genes (see sections 7.2.2 and 7.2.3) for ADC and BMDC cultures, while DPDC cultures demonstrated limited response to any of the DMC concentrations studied. Previous studies have demonstrated that DPDCs can be induced to differentiate towards an odontogenic cell type through exposure to growth media containing dexamethasone, by supplementing media with EDTA-extracted DMCs, or through transfection with vectors expressing proteins common to the dentine matrix, such as BMP-2 or FGF-2 (Tziafas *et al*, 1995; Smith *et al*, 2001; Alliot-Licht et al, 2005; Liu *et al*, 2005; Lopez-Cazaux *et al*, 2006; Prescott *et al*, 2008; Galler *et al*, 2011). However, other *in vitro* studies have indicated that the addition of total EDTA-extracted

DMCs only had a limited influence on the differentiation capacity of odontoblast precursors, with the addition of DMCs only able to promote odontoblast-like differentiation when combined with TGF-β1 or BMP-2, or when purified for the active TGF-β1 fragment using heparin or DEAE-celluose chromatography (Begue-Kirn *et al*, 1992; Liu *et al*, 2005). This may be related to the dental pulp niche, with the presence of factors in the pulp resembling those present within the dentine matrix (Garcia *et al*, 2003). The present study may also highlight differences in the capacity of DPSCs isolated from rodent incisors to respond to inductive dentine components, potentially due to the continuous growth of these teeth (Ohshima *et al*, 2005).

The addition of DMCs has been shown to reduce cell growth and promote the deposition of mineral in MSC cultures (Smith and Leaver, 1981; Goldberg and Smith, 2004; Wu *et al*, 2008; Ma *et al*, 2008). DMCs may act as a differentiation-inducing agent, whilst down-regulating proliferation and promoting entry to the G_0/G_1 phase of the cell cycle (Zhu and Skoultchi, 2001; Wu *et al*, 2008). This inverse relationship between cell growth and differentiation was corroborated in the present study by quantitative analyses demonstrating that culture with DMC stimulated *in vitro* minearalisation while causing a reduction in overall cell growth (see sections 7.3.4 and 7.3.1). Analysis of the mineral deposited by ADC and BMDC cultures indicated that mineralised nodules formed following exposure to DMCs were larger and more developed than those formed after osteogenic culture (see sections 7.3.4 and 7.3.5). The addition of DMCs to DPDC cultures also altered the appearance of mineral, causing the formation of a continuous mineralised sheet over the culture dish (see sections 7.3.4 and 7.3.5). Results documented in section 7.4 demonstrated that the expression of DMP1 and Cbfa1 within ADC and BMDC cultures was increased by the addition of DMCs, with corresponding increases in the volume and

density of mineral present in these cultures (see section 7.3.6.2). Interestingly, DMP1 and Cbfa1 gene expression was not increased within DMC-supplemented DPDC cultures, nor was the density of the mineral formed in these cultures increased (see sections 7.4 and 7.3.6.2) potentially highlighting an association between Cbfa1 and DMP1 previously reported by Fen *et al*. (2002) in the developing skeleton. Analysis of the composition of mineral present in all three cultures using FTIR spectroscopy indicated that it displayed many similarities with non-stoichiometric apatite's that make up physiological bone and dentine, with the presence of characteristic phosphate (960, 1024 and 1095 cm^{-1}) carbonate (872 $cm^{-}1$) and amide (1550 and 1640-1650 cm^{-1}) peaks (Lazarev *et al*, 1984; Rey *et al*, 1989; Rey *et al*, 1991; Gadaleta *et al*, 1996; Boskey, 2007).

It has been hypothesised that levels of DSPP and DMP1 in comparison with BSP and OP may provide an indication as to whether the mineralising matrix formed in the presence of various inductive factors is bone or dentine-like (Fujisawa *et al*, 1993; Zhao *et al*, 2007; Hwang *et al*, 2008). This hypothesis is based on the fact that the expression of DMP1 and DSPP is significantly higher within dentine, while BSP and OP expression is higher in bone (Fujisawa *et al*, 1993). Gene expression analysis using sqRT-PCR demonstrated that BSP and OP levels were higher for BMDCs and DPDCs cultured in un-supplemented osteogenic medium (see section 7.4.3), while the addition of DMCs to ADC and BMDC cultures increased the expression of genes associated with odontogenesis, DSPP and DMP1 (see section 7.4.4). Both DSPP and DMP1 have been described as key markers of odontoblast-like differentiation and over-expression of the DSPP gene within ADC and BMDC cultures, and the DMP1 gene within embryonic stem cell cultures, has previously been shown to induce differentiation towards an odontoblast-like cell type, as characterised by the expression of characteristic markers of early- and late-stage

odontogenesis (Narayanan *et al*, 2001; Goldberg and Smith, 2004; Almushayt *et al*, 2006; Zhu *et al*, 2008). Such increases in the expression of odontogenic genes together with accompanying reductions in the expression of osteogenic genes indicated that the addition of 1 μg/mL DMCs to osteogenic culture medium promoted a more odontogenic pattern of differentiation, particularly for BMDC cultures.

Expression of OP, a well characterised regulator of mineralisation, is known to be reliant on the presence of alkaline phosphatase (ALP), with OP acting to regulate levels of phosphate homeostasis during the post-proliferative phases of osteo- and odonto-genesis (Owen *et al*, 1990; Beck *et al*, 2000; Beck and Knecht, 2003). In the present study, levels of ALP gene expression within DMC-treated cultures were consistently lower than those cultured in un-supplemented osteogenic medium (see section 7.4.2). Both the expression of OP and ALP have been shown to be influenced by the the concentration of dexamethasone added to osteogenic medium, (Leboy *et al*, 1991; Beresford *et al*, 1994; Rickard *et al*, 1994). Different concentrations of dexamethasone can have a major influence on the level and timing of ALP gene expression, as well as influencing levels of other stage-specific osteogenic markers such as osteocalcin (OC), bone sialoprotein (BSP) and osteonectin (ON) (Kim *et al*, 1999; Jorgensen *et al*, 2004). The expression of ON, a non-collagenous protein shown to have a role in the regulation of collagen deposition within the ECM and the remodelling of mineralised tissues, was reduced following exposure to osteogenic medium and increased through the addition of DMCs (see section 7.4.2) (Gundberg *et al*, 1984; Boskey *et al*, 2003; Kawasaki *et al*, 2006; Martinek *et al*, 2007; Delany and Hankenson, 2009). Previous evidence indicates that the addition of TGF-β superfamily members, such as TGF-β1, a common dentine matrix protein, or members of the BMP family such as BMP-2 or 4 have been shown to increase the

expression of ON but repress the activity of ALP (Nakashima *et al*, 1990, 1994; Shiba *et al*, 2001). Therefore, it could be proposed that the presence of these TGF-β superfamily members within the DMC fraction may be responsible for the related changes in expression of both ALP and ON observed in this study.

Finally, the effect of DMCs on the expression of genes involved in the regulation of epithelial-mesenchymal interactions during tooth morphogenesis and the terminal differentiation of odontoblasts were investigated (Hirata *et al*, 2009). Semi-quantitative RT-PCR analysis indicated that DMC-supplementation of osteogenic medium caused a significant increase in the expression of Cbfa1 and Osterix in ADC and BMDC cultures (see section 7.4.1) (Komori *et al*, 1997; Ducy *et al*, 1997; Disouza *et al*, 1999). However, the addition of DMCs had only a negligible effect on DPDC cultures with expression of these genes reduced when compared with the osteogenic controls. Previous studies have demonstrated that the forced expression of Cbfa1 in non-osteoblast cells promoted the expression of key osteoblast/odontoblast-specific markers, while the introduction of Cbfa1 antisense DNA resulted in the supression of DSPP and DMP1 gene expression (Ducy *et al*, 1997; Otto *et al*, 1997; Komori *et al*, 1997; Chen *et al*, 2009; Kobayashi *et al*, 2006). It has also been demonstrated that by supplementing osteogenic medium with 10 μg/mL DMCs in the absence of dexamethasone, the expression of both Cbfa1 and DSPP were increased, perhaps indicating that the addition of dexamethasone to DPDC cultures may have limited the odontogenic differentiation of these cells (Liu *et al*, 2005). These findings, together with a correlation between the late-stage expression of Cbfa1 and corresponding increases in DMP1 and DSPP found in the present study, suggest that odontogenic differentiation can be induced by the addition of DMCs. However, the capacity of DMC-

supplementation to induce the mineralisation and the expression of dentinogenic genes in MSCs isolated from different tissues was was not equal.

10.0 CONCLUSIONS

For MSCs to be used for regenerative medicine they need to represent an abundant cell type that can be isolated using minimally invasive procedures, and differentiate towards multiple lineages in a reproducible manner (Gimble, 2002). Based on these criteria, ADCs could potentially provide a valuable source of MSCs since they can be isolated in large quantities under local anaesthesia, causing the patient relatively minimal discomfort (Zuk *et al*, 2001). However, at present the comparative characterisation of ADCs with other commonly used MSCs, such as BMDCs and DPDCS remains relatively unexplored. There also remains a lack of published research comparing the benefits of cell selection methods, such as FACS, with simple adherence to tissue culture polystyrene for the expansion of stem cell populations. Furthermore, if ADCs and other MSCs are to be clinically and commercially advantageous, the relative MSC marker profile of these cells before and after cryo-storage needs to be examined. Finally, the use of ADCs for regenerative dentistry is as yet relatively unexplored with the majority of research focussing on the use of dental and bone marrow stem cells. Therefore, the work conducted in this study aimed to develop an optimal methodology for the isolation of ADCs from adipose tissue, evaluate the influence of cell selection and cryo-storage on MSC phenotype and compare the relative "stemness" and odontogenic potential of ADCs with BMSCs and DPDCs.

This study compared the relative effectiveness of type I collagenase digestion for the release of ADCs with other digestive enzymes such as trypsin, Accutase and a combination of type I collagenase/trypsin. The results confirmed that incubating adipose tissue with type-I collagenase for a period of 30 minutes at 37 ºC was the most effective

approach for the isolation of ADCs. These ADCs demonstrated increased CFU-F formation and proliferation when compared with ADCs obtained using all other enzymes and digestion periods. These data highlighted the need for a consistent method of ADC dissociation and demonstrated that collagenase digestion should be limited to 30 minutes for the release of the most highly proliferative ADCs.

This study next compared the MSC marker profile and differentiation capacity of ADCs with BMDCs and DPDCs. All three cells types expressed similar levels of MSC markers up to passage 4 and demonstrated a bi-lineal differentiation potential. However, this study indicated for the first time using rat-derived donor-matched MSCs that using a standard osteogenic protocol, ADCs had a limited capacity for osteogenic differentiation when compared with BMDCs and DPDCs. Moreover, the patterns of mineralisation between these three cell types differed with DPDCs depositing a mineralised layer throughout the culture while ADCs and BMDCs deposited mineral in a discrete nodular arrangement. These data may reflect the lasting effect of stem cell niche on the differentiation capacity of its MSCs, possibly resulting from epigenetic modifications such as DNA methylation and histone modifications (Teven *et al*, 2011). This study further highlights the need for a comprehensive evaluation of rat-derived MSCs in order to determine any species-specific differences in the marker profiles and differentiation capacities of these cells.

Cryo-storage is a commonly used method for the long term preservation of MSCs. Therefore, the influence of cryo-storage on MSC viability and the expression of stem cell markers needed to be evaluated. This study demonstrated for the first time that cryo-storage had a cell-specific effect on viability, with BMDCs demonstrating a greater decrease in viability compared with ADCs and DPDCs. Furthermore, cryo-storage also

caused a significant increase in the expression of MSC markers when compared with freshly isolated cells. These results suggest that ADCs and DPDCs represent a more robust cell type than BMDCs and that cryo-storage might provide a means of increasing the proportion of MSCs in a heterogeneous population.

The establishment of homogeneous MSC populations often relies on the FACS selection of cells presenting defined stem cells markers, such as CD29, CD73, CD90 or CD105 (Dominici *et al*, 2006). Previous studies have shown that FACS can have a negative influence on cell viability due to the high shearing and hydrodynamic forces experienced by the cells during selection (Mollet *et al*, 2008). This study presented novel data demonstrating that the selection of CD29/CD90 positive cells from heterogeneous adipose, bone marrow and dental pulp isolates using FACS significantly reduced the viability and ability of these cells to differentiate towards osteogenic and adipogenic lineages. Moreover, cells that were not antibody labelled, but were FACS processed, also demonstrated reductions in viability and differentiation. This indicated that the FACS procedure was responsible for these negative effects, rather than the selection criteria. This study indicated that FACS represents a relatively harsh method of selecting MSCs that is not as effective as simple adherence to tissue culture polystyrene for the selection of bi-potent cells. It's plausible that a more "gentle" cell sorting procedure such as MACS may be better suited for the selection of MSCs.

This study demonstrated that the addition of DMCs as a putative dentinogenic supplement promoted an increase in the mineralisation of ADC, BMDC and DPDC cultures. Data also indicated that the addition of DMCs significantly increased the density of mineral present in BMDC cultures. Furthermore, DMC-supplementation resulted in the increased

expression of the dentinogenic genes, DMP1 and DSPP, in ADC and BMDC cultures. These novel results demonstrated that DMCs could be used to promote dentinogenic differentiation of non-dental MSCs.

Ultimately, the data compiled in this thesis have shown that although ADCs represent a convenient source of MSCs, the potential of these cells to produce mineral and differentiate to an odontogenic cell type remains limited when compared with DPDCs and BMDCs. However, due to the relative abundance of ADCs throughout the body, their comparative ease of isolation and the increasing number of liposuction surgeries carried out each year, it is important to further optimise the potential of these cells for regenerative mineralised tissue research.

11.0 FUTURE WORK

The work presented in this study has provided a detailed account of the similarities and differences between rat-derived MSCs isolated from different tissue sources and the potential of these cells for dental tissue engineering. This study has demonstrated that ADCs can be isolated in relatively large numbers, proliferate at a similar rate to BMDCs and express key markers associated with odontogenic differentiation following supplementation with DMCs. Data presented in this study have demonstrated the negative effects of FACS on cell viability and differentiation. Furthermore, the study has also highlighted that ADCs possess a comparatively limited potential to form a mineral-secreting cell type when compared with BMDCs, and particularly DPDCs, and that the need to develop an optimal culture medium that improves the odontogenic potential of these cells is paramount if they are to be used for regenerative dentistry.

Different types of culture serum (e.g. human or xeno-free serum) could be used in the composition of mineralising medium to determine if these improve the differentiation of ADCs towards a mineral-secreting cell type. Recently, the application of human or xeno-free serum to mineralising culture medium has been shown to increase the capacity of ADCs to differentiate towards a mineralising cell type (Kyllonen *et al*, 2013). The application of growth factors may also be appropriate, since the addition of members of the BMP family has been shown to improve the mineralising potential of MSC cultures (Luu et al, 2007).

It would be beneficial to further evaluate the differentiation potential of tissue isolates sorted using other available MSC markers, such as CD73 and CD105, to better understand

the effects of cell sorting on the differentiation potential of these cells. Different methods of cell sorting (e.g. MACS) could also be explored for their ability to isolate viable and multipotent cell populations. However, this approach is dependent and potentially limited by the commercial availability of antibodies that react with MSC antigens in the rat species.

In the present study the capacity of ADCs to differentiate towards a mineral-secreting cell type was comparatively limited when compared with BMDCs and DPDCs. Previous studies have indicated that the differentiation capacity of ADCs is increased when cultured in three-dimensions (Shen *et al*, 2013). This approach could be used to assess the effect on the mineralisation potential of ADCs and their capacity to differentiate towards an odontoblast-like cell.

Finally, the heterogeneous population of proteins extracted using EDTA solubilisation could be fractionated using techniques such as affinity chromatography to identify the constituents which may contribute to the odontogenic differentiation process. Such a study could provide better understanding as to how DMC constituents contribute to the signalling mechanisms involved in the odontogenic differentiation of ADCs.

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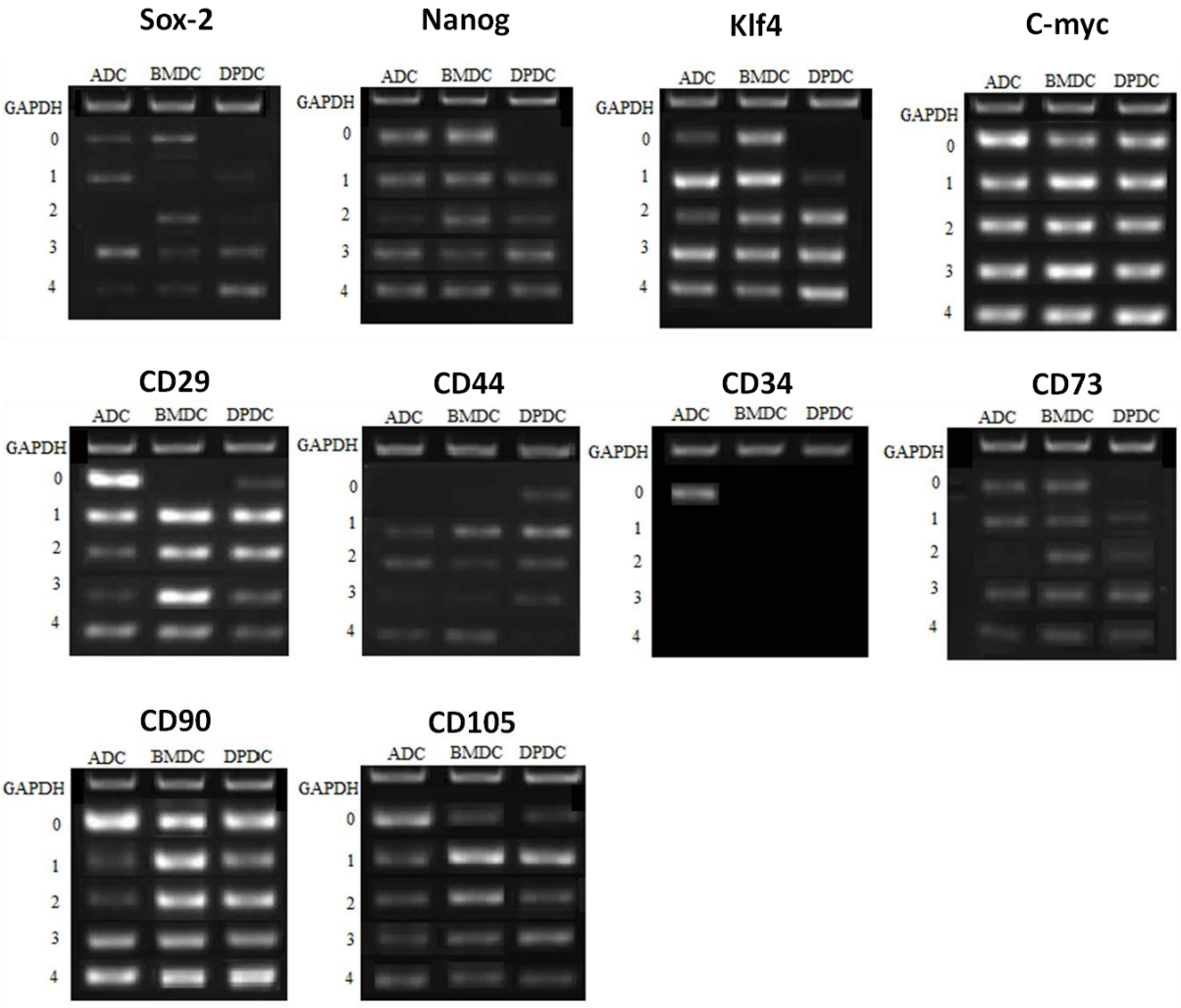
APPENDIX 1

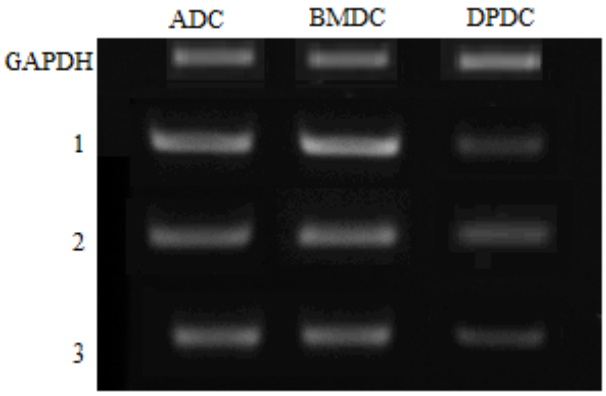
Gel images corresponding to Figure 4.2.2



APPENDIX 2

Gel images corresponding to Figure 4.4.1

LPL



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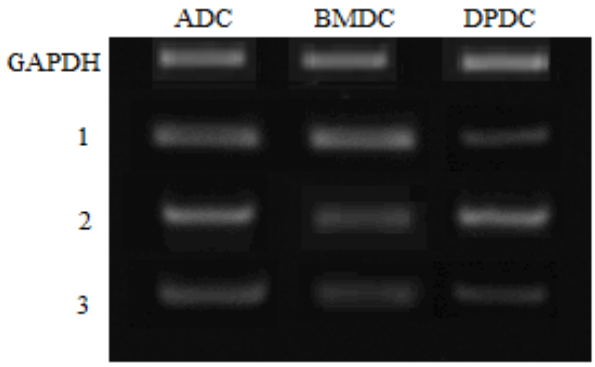

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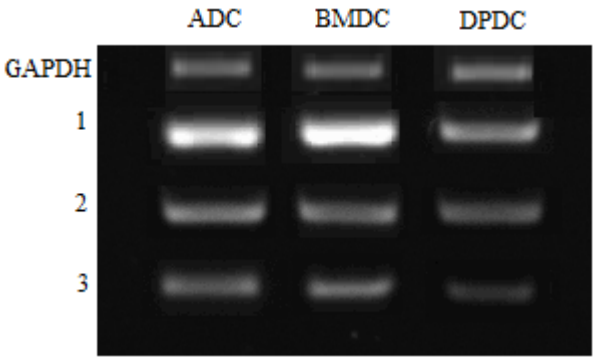
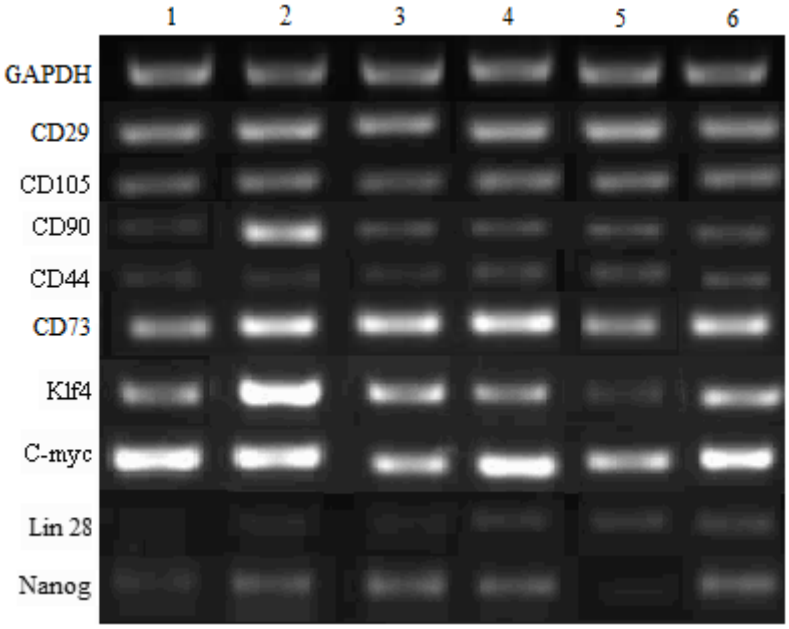


1, 2 and 3 corresponds to weeks 1, 2 and 3

APPENDIX 3

Gel images corresponding to Figure 6.2.2



1 = ADC

2 = Cryo-Stored ADC

3 = BMDC

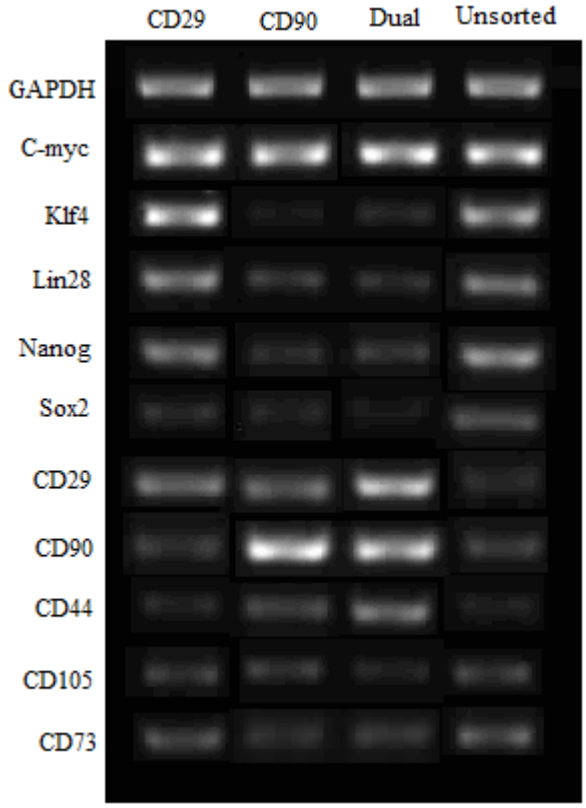
4 = Cryo-Stored BMDC

5 = DPDC

6 = Cyro-Stored DPDC

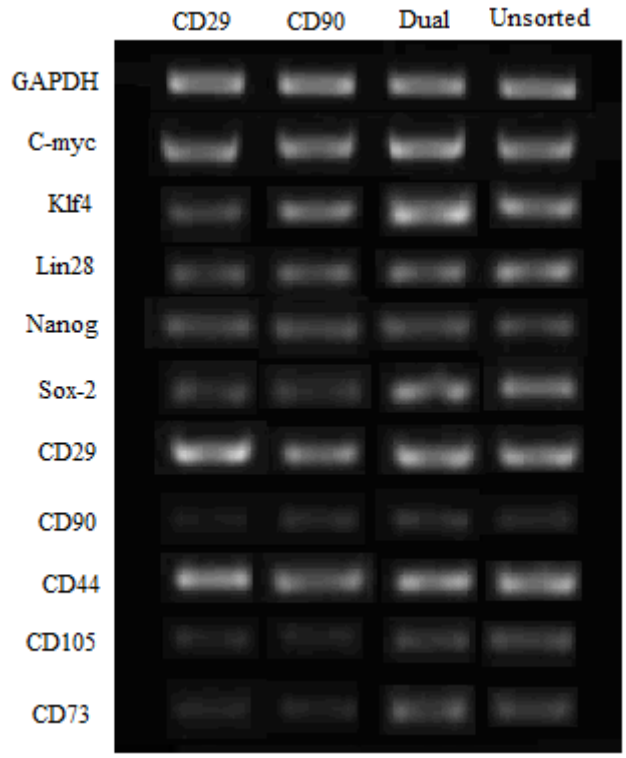
APPENDIX 4

Gel images corresponding to Figure 5.4.1a



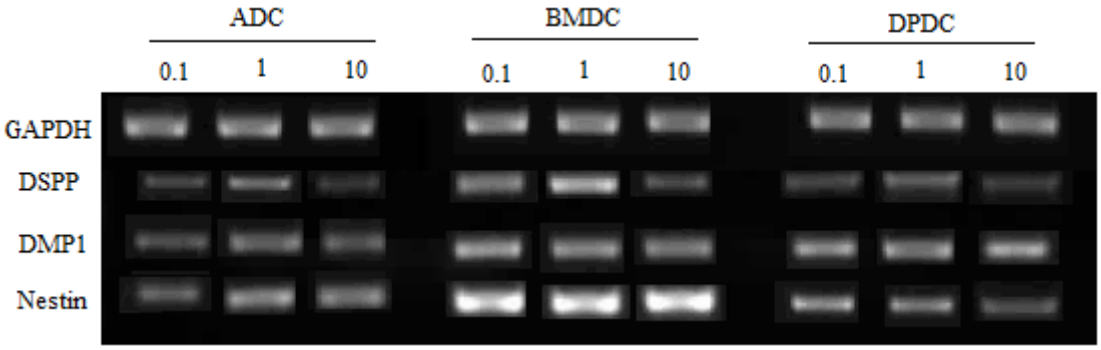
APPENDIX 5

Gel images corresponding to Figure 5.4.1b

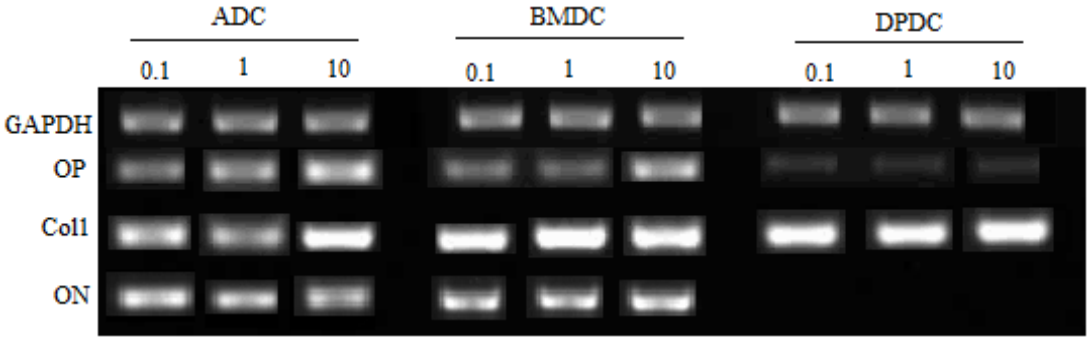


APPENDIX 6

Gel image corresponding to Figure 7.2.3a



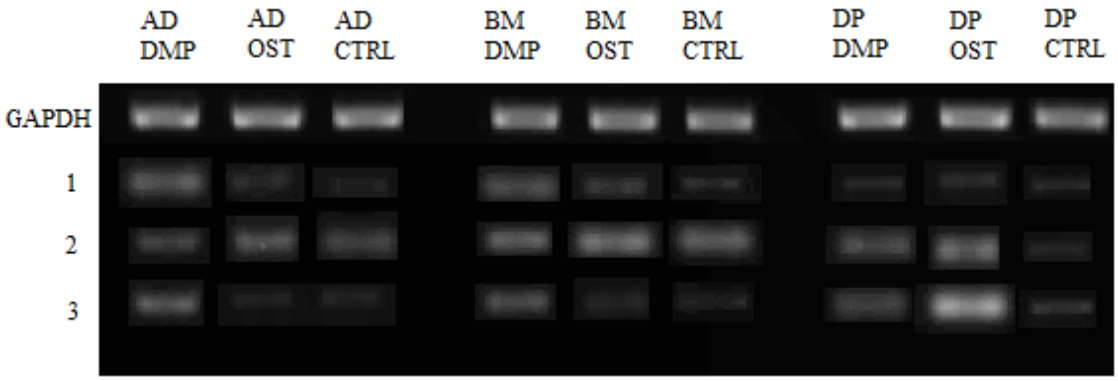
Gel image corresponding to Figure 7.2.3b



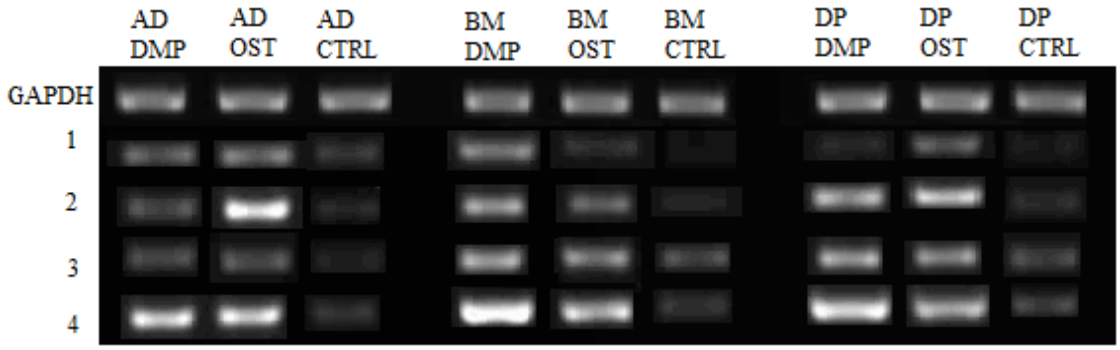
APPENDIX 7

Gel images corresponding to Figure 7.4

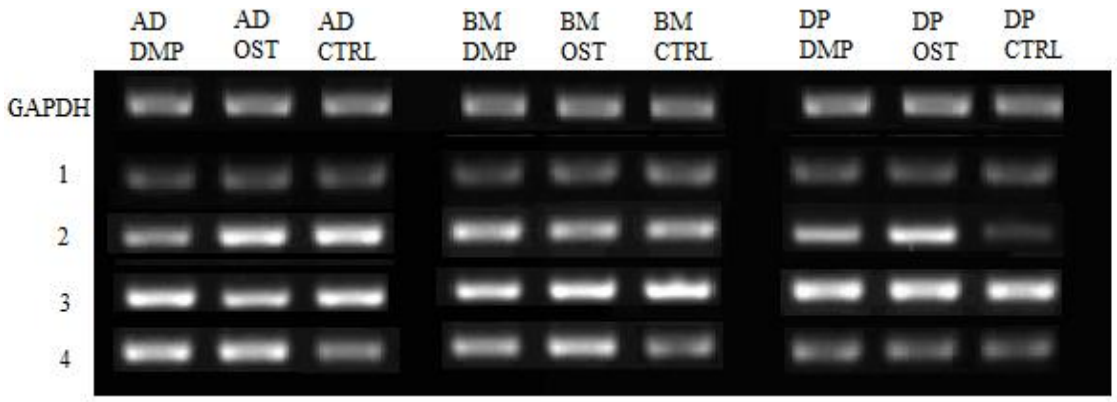
Cbfa1 (Figure 7.4.1a)



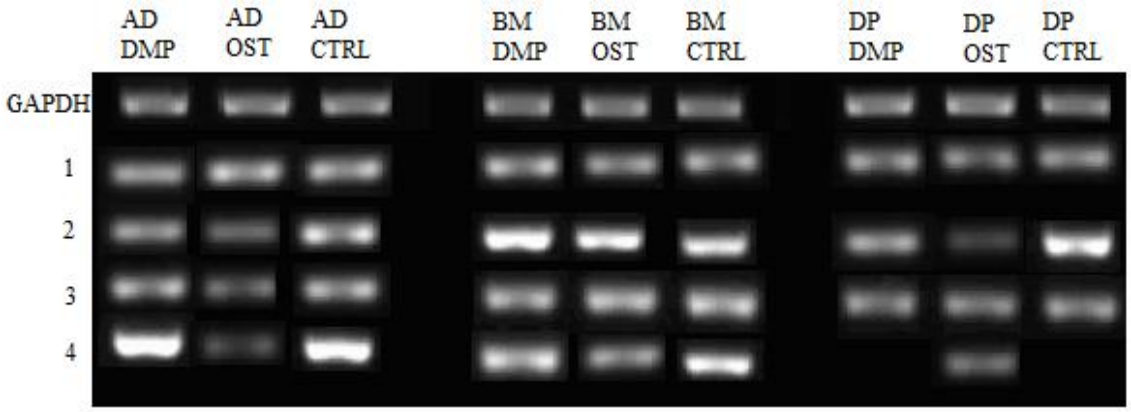
Osterix (Figure 7.4.1b)



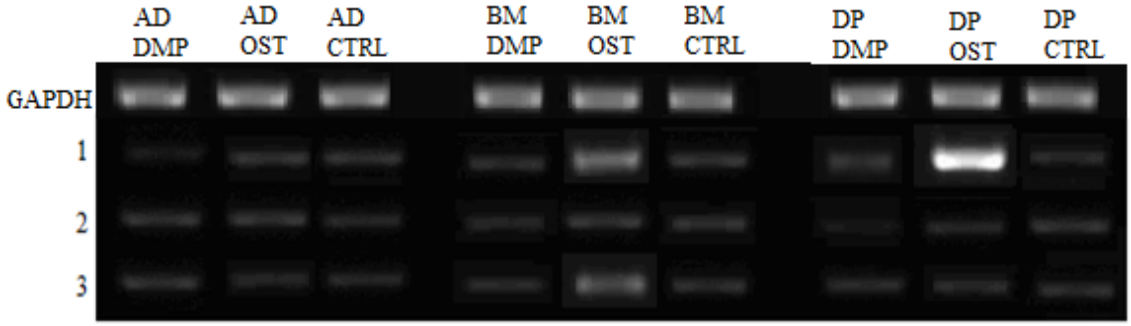
COL1 (Figure 7.4.2a)



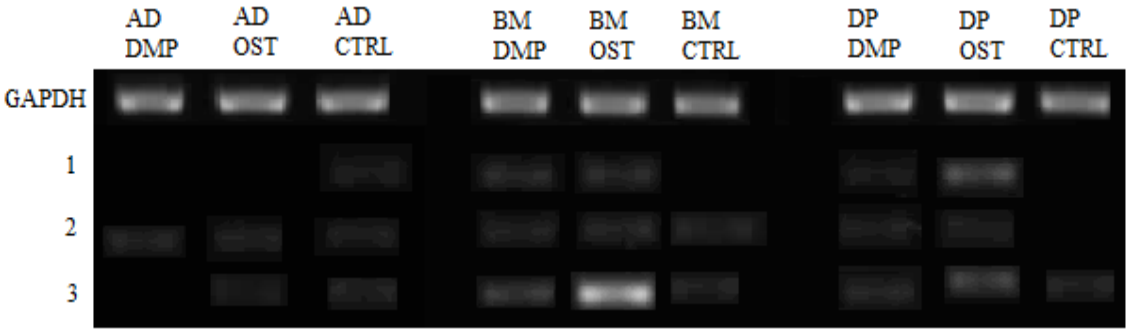
ON (Figure 7.4.2b)



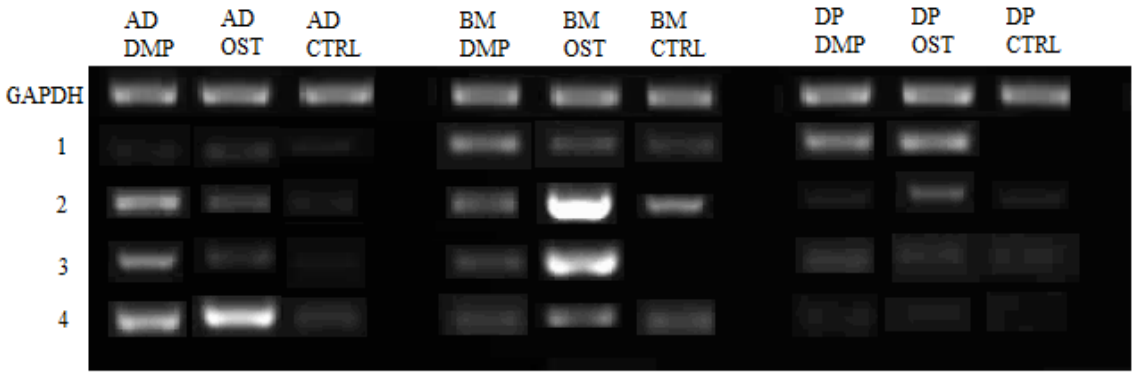
ALP (Figure 7.4.2c)



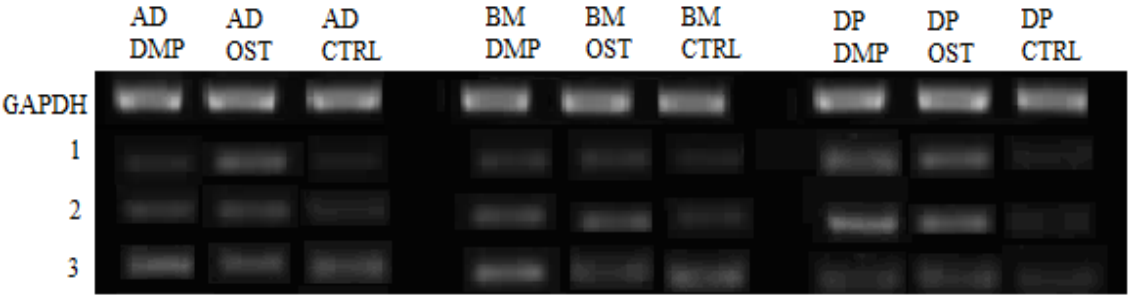
BSP (Figure 7.4.3a)



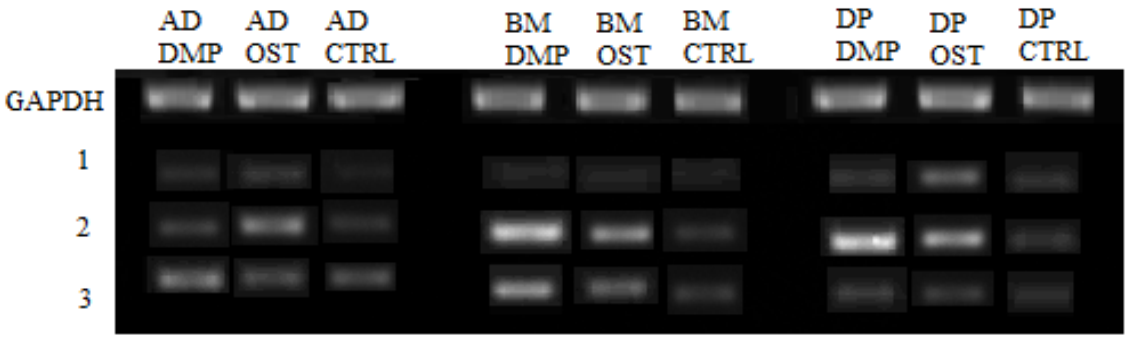
OP (Figure 7.4.3b)



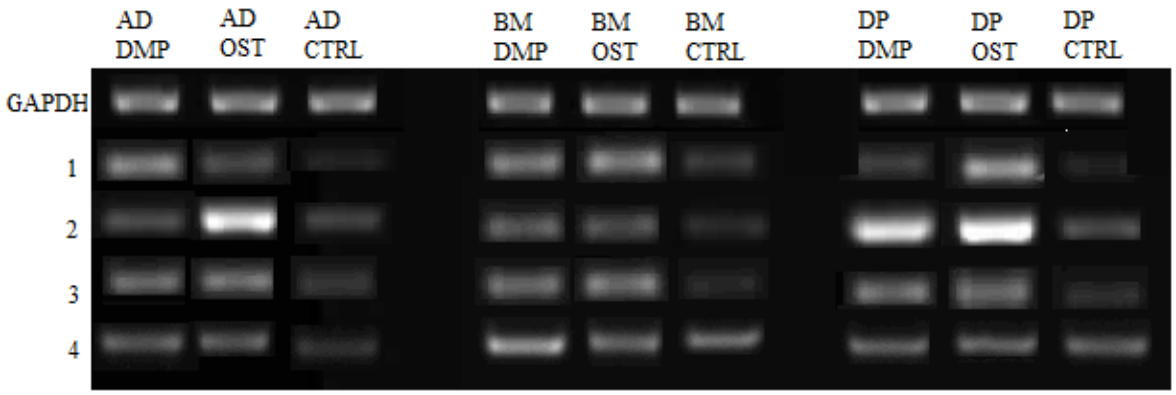
Nestin (Figure 7.4.4a)



DSPP (Figure 7.4.4b)

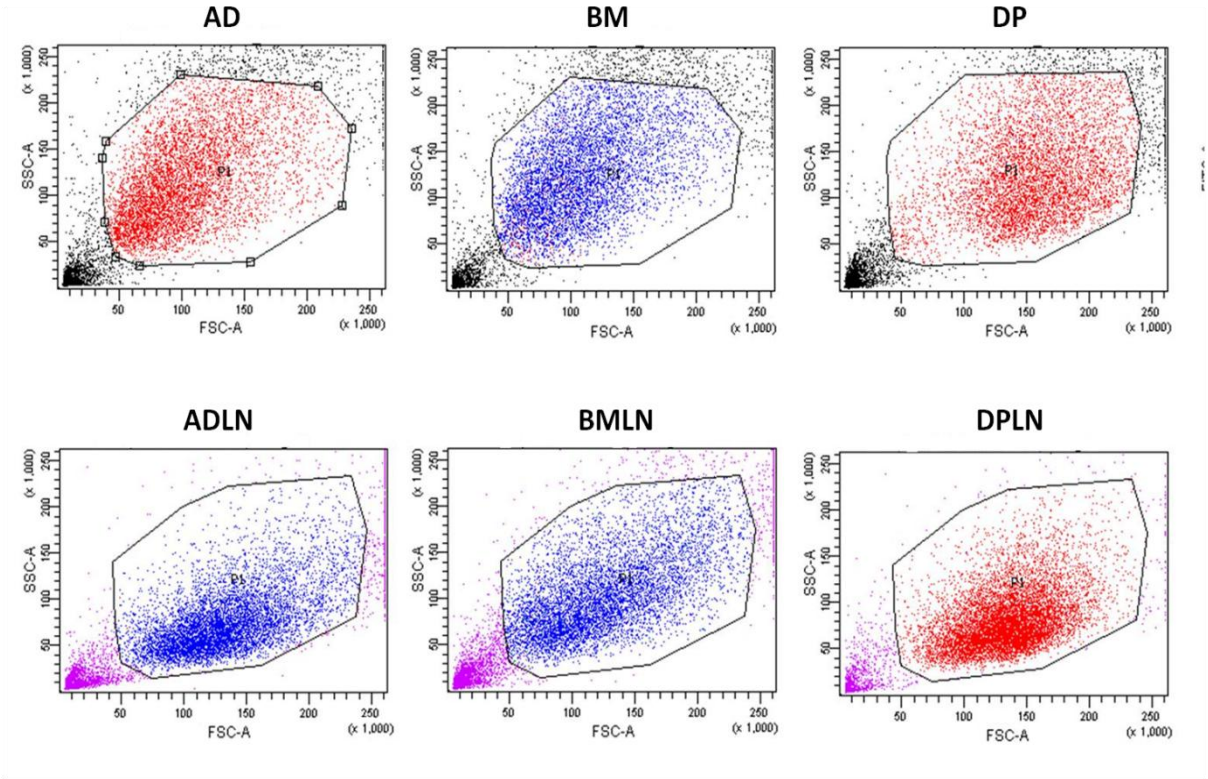


DMP1 (Figure 7.4.4c)



APPENDIX 8

FACS images corresponding to Figures 4.2.1 and 6.2.1



AD = Adipose

BM = Bone Marrow

DP = Dental Pulp

LN = Liquid Nitrogen

APPENDIX 9

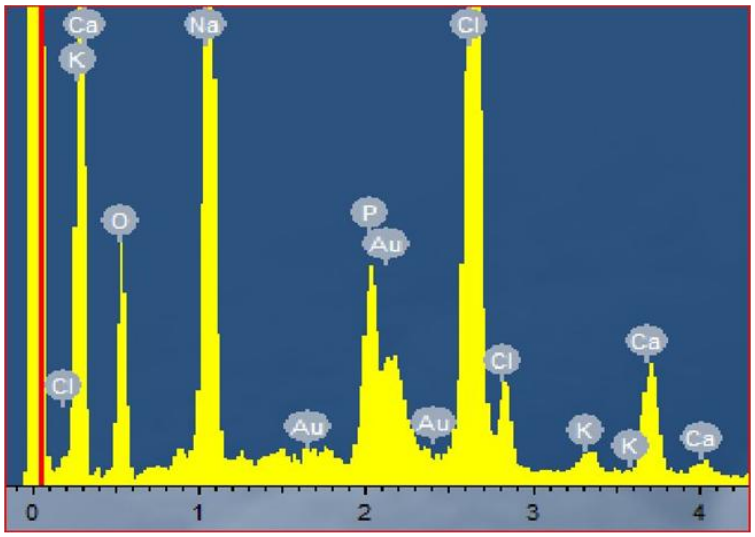
Dentine analyses corresponding to figure 7.1.1

Powdered Dentine (g)	Extract Recovered (g)	Percentage Yield (%)
53.2	1.63	3.06

	µg per mg of Extract	µg per gram of Dentine
Bradford Assay (NCP)	46.45	2471
Farndale Assay (GAG)	45.95	2444

APPENDIX 10

EDS and calcium/phosphate micro-determination analyses corresponding to figure 7.3.5



	Sample	HA Standard
EDS	1.25 ± 0.04	1.66 ± 0.031
Micro-determination Assay	1.25 ± 0.026	1.65 ± 0.011

Table 2.7.5 DNA sequences, annealing temperatures, and cycle numbers for primers used in the RT-PCR reaction. All primers were designed using Primer Blast software (http://ncbi.nlm.nih.gov/tools/primer-blast/) and manufactured by Invitrogen, UK.