Characterisation of Dental Pulp Cells Derived from Carious Teeth

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others

Declaration

"The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others"

In introduction chapter:

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In Result chapter:

Conferences papers

1- IGF Axis Expression during Osteogenic Differentiation of Dental Pulp Cells

School of Dentistry, University of Leeds, United Kingdom

2- IGF Axis Expression during Osteogenic Differentiation of Dental Pulp Cells

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3- IGF Axis Expression during Osteogenic Differentiation of Dental Pulp Cells

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International Association of Dental research (IADR) +American Association of Dental Research (AADR), Boston, United States

5- Cross Talk Between Inflammation And Regeneration In Dental Pulp Cells

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Abstract

This study investigated some characteristics of dental pulp stromal/stem cells (DPSCs) isolated from healthy teeth (hDPSCs) compared to DPSCs isolated from teeth with shallow carious lesions (cDPSCs) with a view to the use of both cell types in hard tissue engineering strategies. Osteogenic differentiation was investigated using appropriate histochemical staining and osteogenic marker expression (*ALPL, OC, RUNX-2*). In addition, angiogenic (*VEGFR-2, PECAM-1*) and inflammatory gene markers (*TLR-2, TLR-4*) were investigated together with the secretion of the pro-inflammatory cytokines (IL-6 and IL-8). The activity of the pro-osteogenic IGF axis was also investigated in hDPSCs and cDPSC cultures. cDPSCs exhibited significantly higher clonogenic potential, and possess a higher proportion of cell that express mesenchymal stem cell markers (CD146+, CD90+ and CD105+, CD45-, CD31-) compared with hDPSCs. Evidence also suggested that cDPSCs had a greater osteoblastic differentiation potential than hDPSCs. cDPSCs expressed higher levels of inflammatory markers than hDPSCs together with higher concentrations of IL-6 and IL-8 in conditioned medium indicative of retention of a carious phenotype following cell isolation and culture. Finally functional examination of the IGF axis suggested a role for insulin-like growth factor binding proteins-2 and -3 (IGFBP-2 and -3) in the osteogenic differentiation of DPSCs. Therefore cDPSCs are comparable to hDPSCs in terms of their osteogenic potential and the inflammatory environment of cDPSCs may offer a promising cell source for future mineralised tissue repair and regeneration.

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List of Abbreviations

ALP: alkaline phosphatase (protein)

ALPL: alkaline phosphatase (gene)

BMP: bone morphogenic protein

BSA: bovine serum albumin

BSP: bone sialoprotein

CBA: cytometric bead array

CD: cluster of differentiation

cDNA: complementary DNA

cDPSCs: carious human dental pulp stromal/stem cells

COL1A1: type I collagen α1

DCs : dendritic cells

DNA: deoxy ribonucleic acid

DSPP: dentine sialophosphoprotein

DSP: dentine sialoprotein

DMP-1: Dentine matrix acidic phosphoprotein 1

ECL: enhanced chemiluminescence

EGF: epidermal growth factor

ECM: extra cellular matrix

ELISA: enzyme-linked immunosorbent assay

FACS: fluorescence-activated cell sorting

FBS: Foetal bovine serum

FCAP: flow cytometry analysis programme

FGFs: Fibroblast growth factors

GAGs: glycosoaminoglycans

Gdf11: Growth differentiation factor 11

GH: growth hormone

HA: hydroxyapatite

HBD: heparin binding domain

hDPSCs: healthy human dental pulp stromal/stem cells

HRP: horseradish peroxidase

IGF: insulin-like growth factor

IGF-1: insulin-like growth factor-1

IGF-2: insulin-like growth factor-2

IGFBP-1: insulin-like growth factor binding protein -1

IGFBP-2: insulin-like growth factor binding protein -2

IGFBP-3: insulin-like growth factor binding protein -3

IGFBP-4: insulin-like growth factor binding protein -4

IGFBP-5: insulin-like growth factor binding protein -5

IGFBP-6: insulin-like growth factor binding protein -6

IL-6: interleukin -6

IL-8: interleukin -8

IR: insulin receptor

kDa: Kilo Dalton

LPS: lipopolysaccharide

LTA: lipoteichoic acid

Mab: monoclonal antibody

MAPK: mitogen activated protein kinase

MCP-1: Monocyte chemotactic protein 1

mRNA: messenger ribonucleic acid

MSCs: mesenchymal stem cells

OC: osteocalcin (gene)

OCN: osteocalcin (protein)

PAPP-A: pregnancy-associated plasma protein-A (PAPP-A)

PTHRP: Parathyroid hormone-related protein

PBS: phosphate buffered saline

PDGF: platelet-derived growth factor

Pen/Strep: penicillin/streptomycin

PECAM -1: platelet endothelial cell adhesion molecule 1(gene)

PI3K: phosphatidylinositol-3-kinase

qRT-PCR: quantitative real time polymerase chain reaction

RUNX-2: runt-related transcription factor (gene)

RT: room temperature

SHED: stem / stromal cells from exfoliated deciduous teeth

shRNA: short or small hairpin RNA

siRNA: short inhibitory RNA

TBS: Tris buffered saline

TGF-β: Transforming growth factor-beta

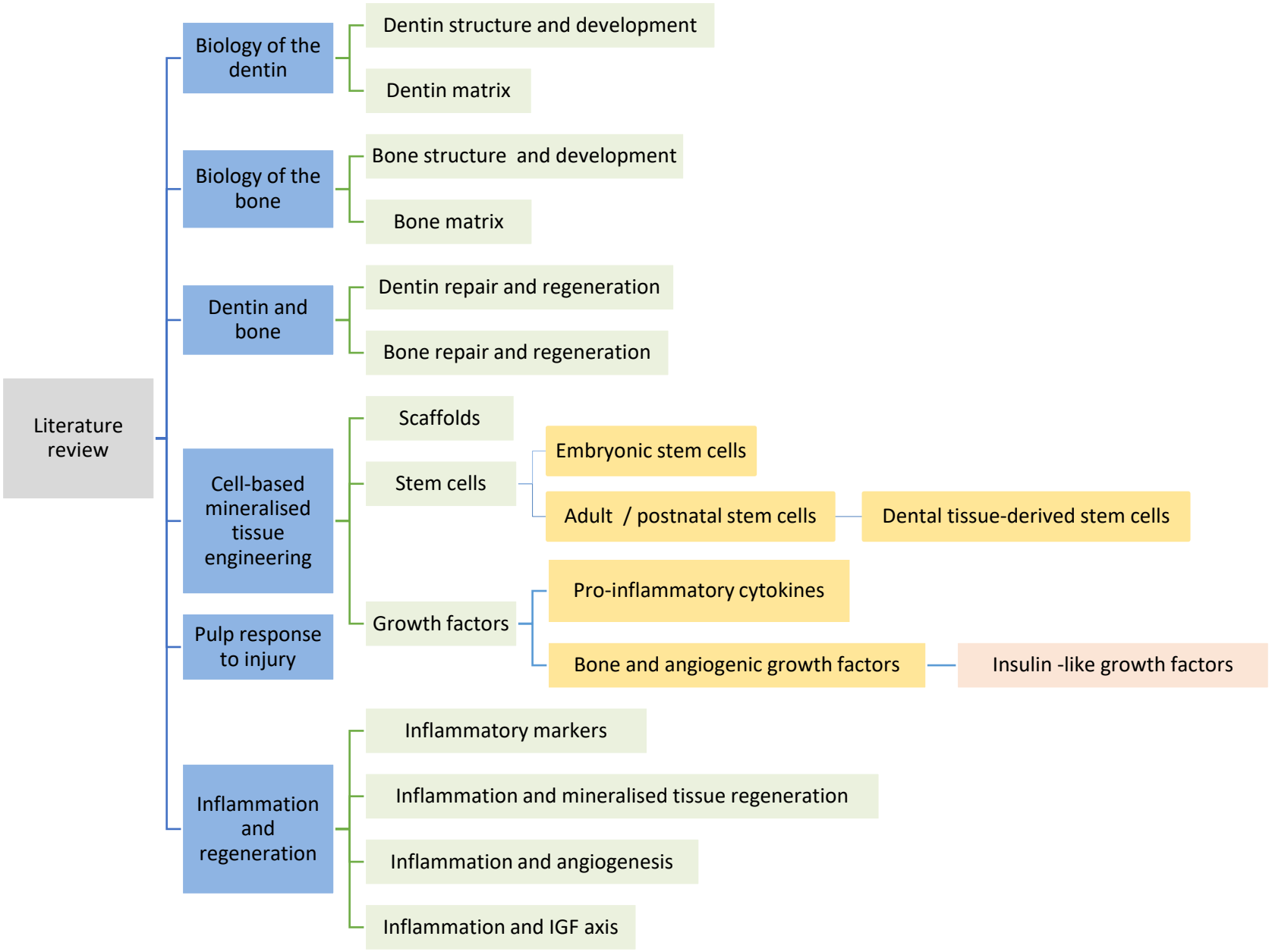
TNF-α: tumour necrosis factor alpha

TLR-2: Toll-like receptor-2 (gene)

TLR-4: Toll-like receptor-4 (gene)

VEGF: vascular endothelial growth factor

VEGFR-2: vascular endothelial growth factor receptor 2 (gene)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Chapter 1: Literature Review

1.1 General introduction

Tissue engineering using the triad; scaffolds, stem cells and signals may provide an alternative method for pulp capping and root therapy, as well as for maxillofacial bone reconstruction [1-3]. Defects in the jaw bone (mandible or maxilla) happen due to trauma, inflammation or tumour and they remain a major clinical challenge [4]. The terms “tissue repair” and “tissue regeneration” have been used to describe the process of restoration of tissue morphology, structure and function after injury [5]. *In vivo* such processes are controlled by the differentiation of remaining vital cells or trans-differentiation of stem/progenitor cells located in or around the damaged tissue [5-7]. Mineralised tissue formation following noxious stimuli affecting the dentine-pulp complex, indicates that this distinctive tissue can adapt itself in early disease conditions to maintain vitality and essentially replace lost structure and function [8].

1.2 Biology of dentine

Dentine is thick mineralised tissue that forms the bulk of the tooth to protect the underlying non-mineralised pulp tissue. It is capped with the enamel (highly mineralised protective tissue in the crown part of the tooth, and in the root, it is covered by the cementum (a mineralised structure contributing to the attachment of the teeth to the surrounding bony socket). Generally, dentine is comprised of minerals (70% in weight), organic matrix (20% in weight) and water (10% in weight) [9].

1.2.1 Dentine structure and development

The initial stage of tooth development starts with invagination of epithelial cells from stomodeum. These cells form the enamel organ, which consists of enamel epithelium, stratum intermedium, stellate reticulum and enamel matrix-secreting ameloblasts [10]. The enamel organ is surrounded by ectomesenchymal tissue that are derived from the neural crest, which later forms the dental papilla [11]. The outermost layer of dental papilla opposes the inner enamel epithelium (IEE) and is induced by it to differentiate into odontoblast. Odontoblasts are the principle cells involved in the synthesis of dentine matrix [9]. These cells produce dentine, and their cell bodies are located outside the pre-dentin/dentine layer; at the dentine-pulp interface. Odontoblastic processes cross the dentine layer and are located inside the dentinal tubules. The number of these tubules is higher in the inner third layer compared with the outer third layer of dentine [12]. In the outer layer of the tooth, dentine structure is atubular or contains thin and curved tubules; called mantle dentine (in the coronal region), and Tomes granular layer and hyaline Hopewell-Smith layer (in the root region). These peripheral layers are less mineralised and resilient to adapt for dissipating pressures or forces transported from the enamel, which otherwise would cause enamel cracks and consequently detachment of the fragmented enamel [9]. Again in the root region, the elasticity of peripheral dentine layer is crucial to resist the axial and lateral pressures [13]. The largest part of the dentine layer is formed by the circumpulpal dentine. This layer is mainly composed of intertubular dentine (prominent part) and peritubular (around the lumen of the tubules). Differences in the composition (non-collagenous protein),

structure and some crystallo-chemical specificities between the two types of dentine are well documented [14-17].

Dentine deposition is a continuous function of odontoblasts and occurs at a consistent rate throughout the life of the tooth, although it is considerably diminished in rapidly progressing carious lesions [18]. Primary dentine is the earliest dentine secreted during tooth development [8] (Figure 1), and its deposition rate is approximately 4µm/day until the tooth becomes functional (antagonistic cusps become in contact) and the roots become completely formed [9].Then the formation of secondary dentine starts but the deposition rate decreases to approximately 0.4µm/day and it continues throughout the life of the tooth. Primary and secondary dentine share the same chemical composition and histological structure [8], however, they are different in their morphology [9]. Secondary dentine is physiologically secreted following tooth eruption and apical closure. It is responsible for narrowing the pulp canal as it is deposited on the roof and lateral walls of the pulp chamber [8]. Tertiary dentine is secreted to protect the underlying pulp from possible injury in response to external stimuli such as abrasion, erosion and bacterial infection [8, 19]. Its secretion is regulated by bioactive molecules, which are sequestered in dentine matrix during tooth development [20-22]. In the early stages of pulp response to moderate external stimuli, pre-existing odontoblasts produce reactionary dentine (tertiary dentine) which becomes continuous with primary and secondary dentine structures [23, 24]. However, in the case of more intense external stimuli, localized odontoblastic damage occurs and odontoblast-like cells differentiate from dental pulp stem cells, which secrete reparative dentine (tertiary dentin) to form dentine bridging in

the area of pulp exposure. Many factors may be involved in localized odontoblasts damage including bacterial toxins or high levels of pro-inflammatory mediators, which are secreted locally as a response to severe dental injury [25]. During inflammation, stem/progenitor cells are recruited to the site of injury where they differentiate into odontoblast-like cells and secrete tertiary reparative dentine. This protects the underlying pulp tissue from further bacterial attack [20].

Odontoblast gene expression profile :

Odontoblasts are formed as a layer of palisade cells at the dentine pulp interface (Figure 2). They are post-mitotic cells, which play a crucial role during dentine formation by secreting the organic matrix macromolecules (pre-dentine) and are actively involved in the mineralisation processes [26, 27]. In earlier studies, a unique *in vitro* culture system of human dental pulp cells has been successfully investigated, which allow differentiation of these cells into odontoblast-like cells with comparable morphological and functional properties to that of native odontoblasts [28, 29]. Cultured odontoblast-like cells have been used to investigate the biological functions of these cells *in vitro* [30-32]. Moreover, successful isolation of mature native human odontoblasts from the pulp chamber allow researchers to profile the gene expression of these cells [33]. Interestingly, it has been observed that both cultured odontoblasts and mature native odotoblasts expressed the odontoblastic markers including DSPP, DSP, Hsp25 and nestin [34]. However, DSPP and DSP specificity to odontoblasts has been argued [33, 35, 36]. As odontoblasts are hard tissue-forming cells contributing to dentine

formation, the expression of COL1A1 and Matrilin 4 has been observed by these cells [33].

1.2.2 Dentine matrix

Odontoblast are the key cells for extracellular matrix (ECM) formation. Depending on dentine type and location, dentinogenesis comprises three different mineralisation processes as follows: 1) dentine outer layer resulting from the cell-derived events involving the presence of matrix vesicles and their enzymatic equipment and 2) intertubular dentine formed as a result of active transformation of pre-dentine to dentine, which is a matrix controlled process, and 3) the peritubular dentine resulting from passive deposit of serum-derived molecules along the walls of the dentinal tubules [9].

Biomineralisation is defined as a process by which hydroxyapatite (HA) is secreted in the extracellular matrix. Initiation and regulation of the mineralisation process are mediated by the extracellular matrix [37-39]. Table 1 summarizes the molecules that play a role in dentinogenesis processes.

During dentine mineralisation; three components are needed to achieve proper mineralisation including collagen that forms the scaffold, non-collagenous proteins that bind to the collagen scaffold and act as a mineral nucleator, and crystalline calcium phosphate that is deposited in a controlled manner [40]. The collagen fibrils and their associated proteoglycans are synthesized by odontoblasts in the proximal pre-dentine. These fibrils increase in diameter by end to end elongation and lateral aggregation. Then

they migrate throughout the pre-dentine, reaching the place where they undergo mineralisation [41-43]. This is followed by discharge of non-collagenous phosphorylated proteins and mineral associated proteoglycans, which are secreted distally in the pre-dentine (near pre-dentine/dentine junction), where the mineralisation occurs [9]. Some matrix components follow the intercellular pathway and migrate directly from the serum to the dentine; albumin and phospholipids were observed to be involved in this process [44].

Table 1: The key player components of ECM and their role in dentin formation and mineralization.

ECM components	Notes[9]

Type I collagen	↓ Major protein found in dentine (~90% of the organic matrix) ↓ Synthesized by odontoblasts ↓ Provide an organised scaffold ↓ Has self-aggregating properties that lead to the formation of calcospherites.
Dentine Sialo Phospho Protein (DSPP)	↓ SIBLINGs (small integrin-building ligand N-linked glycoproteins) ↓ Immediately cleaved after secretion into DSP, DGP and DPP ↓ Its mutation leads to different forms of dentinogenesis imperfecta
Dentine Sialo Protein (DSP)	↓ SIBLINGs (small integrin building ligand N-linked glycoproteins) ↓ It is suggested to be less effective mineralisation mediator relative to other ECM molecules. ↓ Inhibits the formation and growth of calcium phosphate mineral crystal
Dentine Phospho Proteins (DPP)	↓ SIBLINGs (small integrin building ligand N-linked glycoproteins) ↓ Form more than 50% of the non-collagenous protein in dentine ↓ Binds to calcium, hydroxyapatite and collagen ↓ At low concentration, it causes nucleation ↓ At high concentration it inhibits the crystal growth
Dentine Matrix Protein-1 (DMP-1)	↓ SIBLINGs (small integrin building ligand N-linked glycoproteins) ↓ Interacts with other molecules and regulates the transcription of DSPP gene ↓ Has a calcium binding capacity and great affinity to collagen fibrils ↓ Enhances the nucleation of calcium phosphate crystals and regulate their growth
Bone Sialo Protein (BSP)	↓ SIBLINGs (small integrin building ligand N-linked glycoproteins) ↓ Initiate the formation of mineral crystals ↓ Marker of osteogenic differentiation, it is also expressed in dentine but at lower level. It is intensifies collagen fibrillation ↓ Initiates crystal nucleation and induce cell adhesion to the ECM
Osteopontin	↓ SIBLINGs (small integrin building ligand N-linked glycoproteins) ↓ Mediates hydroxyapatite binding ↓ Mediates cell attachments/signalling ↓ Its phosphorylation is important in enhancing mineralisation
Matrix Extracellular Phosphoglyco Protein (MEPE)	↓ SIBLINGs (small integrin building ligand N-linked glycoproteins) ↓ The central protein of MEPE (Dentoin) plays a role in initiating the differentiation of pulp cells into odontoblasts/osteoblasts.
Osteoclacin (OCN)	↓ Highly expressed in differentiating odontoblasts as it might be involved in glucose metabolism needed by these active cells
Glycosaminoglycans (GAGs)	↓ Identified as mineralisation inhibitor, need to be enzymatically modified at place where mineralisation should be initiated ↓ Two distinct group; a) First group observed in the pre-dentine and involved in collagen fibrils moving from the proximal to the distal part of the pre-dentine b) Second group is secreted in dentin associated with mineralisation and they are stable, participated in dentine formation and become dentine component

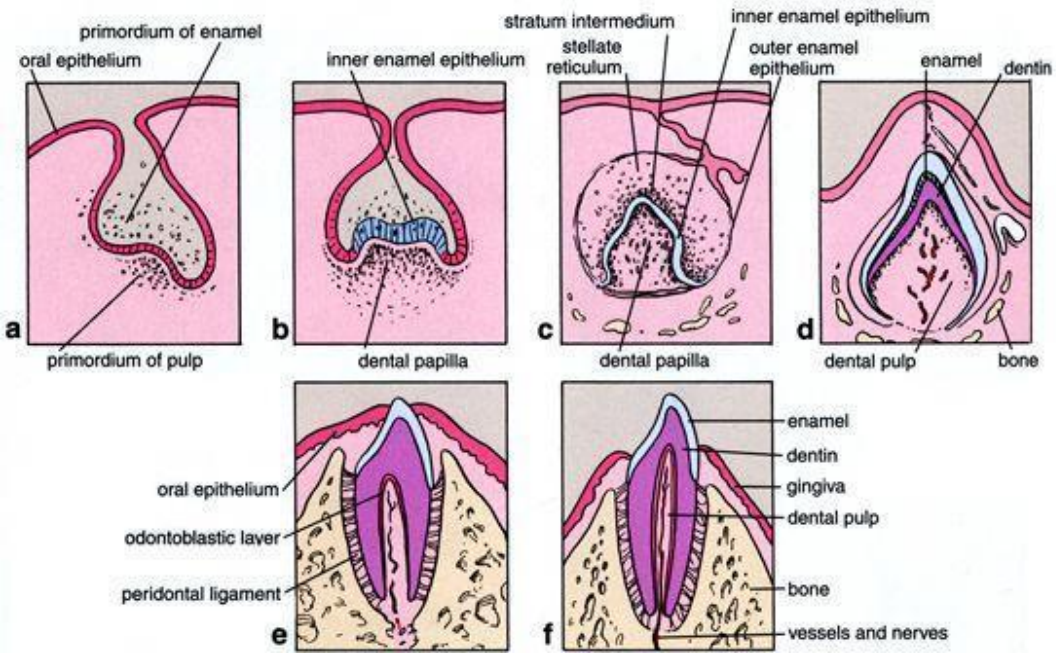


Figure 1: Tooth development

Teeth develop from both ectoderm and mesoderm. Enamel develops from ectoderm of the primordial oral cavity, and all other tooth associated tissues develop from the associated mesenchyme. **(a) Bud stage:** localized growth of epithelial cells surrounded by proliferating mesenchymal cells. **(b) Cap stage:** the inner surface of each ectodermal tooth bud becomes invaginated by mesenchymal tissue called dental papilla, which gives rise to dentine and dental pulp. The ectodermal cap-shaped covering over the papilla is called an enamel organ and will produce the future enamel of the tooth. As the enamel organ and dental papilla form, the surrounding mesenchyme condenses forming the dental sac, which later forms the cementum and periodontal ligament. **(c) Bell stage:** The mesenchymal cells in the dental papilla, adjacent to the inner enamel epithelium, differentiate into odontoblasts, which produce pre-dentine, and deposit it adjacent to the inner enamel epithelium. The pre-dentin later calcifies to form dentine. As the dentin thickens, the odontoblasts regress toward the centre of the dental papilla but odontoblastic processes remain embedded in the dentine. Cells of the inner enamel epithelium near the dentine form ameloblasts, which produce enamel in the form of prisms or rods over the dentine layer and forms the outer layer of the tooth or the crown. As enamel increases, the ameloblasts regress. **(d)** Both enamel and dentine help to create the crown. **(e)** Root development occurs during the later stages of enamel and dentine development. **(f)** As the teeth develop, the jaws ossify and the outer cells of the dental sac also become active in bone formation. Each tooth is surrounded by bone, except at its crown, and is held in its bony socket or alveolus by the periodontal ligament.
(Adapted from http://dentallecnotes.blogspot.co.uk/2011/08/note-on-tooth-developmentodontogenesisw.html)

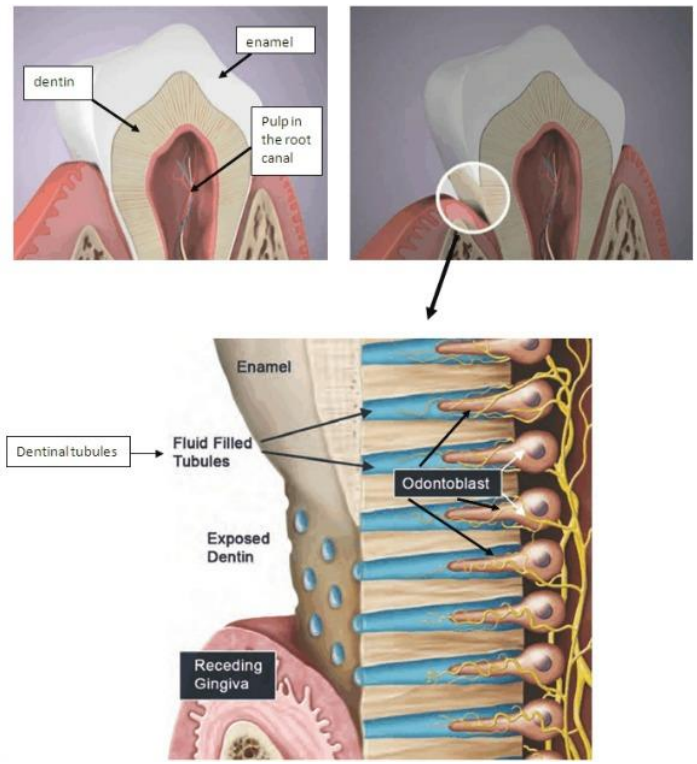


Figure 2: Anatomical representation of enamel, dentine, pulp and odontoblasts

Enamel: Tooth enamel is the hardest substance in the human body. It consists mainly of calcium phosphate and calcium carbonate. Enamel covers the crown of each tooth and is important because its hard structure protects the underlying dentine structure.

Dentine: The main structure of the tooth, which is a calcified connective tissue. It is underlying the enamel and slightly softer than enamel, contains millions of dentinal tubules which filled with fluid and odontoblastic processes.

Pulp: The soft connective tissue that includes blood vessels, nerves and lymphatic vessels. It is contained within the central part of the tooth called the "pulp cavity".

(adapted from: http://www.dentist-charlotte-north-carolina-nc.com/tooth-pain-mechanism.html)

1.3 Biology of bone

Bone is a highly vascularized and mineralised tissue, and it is back bone of the musculoskeletal system. It supports and protects the internal vital organs and acts as a storage area for minerals (such as calcium), and provides a protective environment for marrow tissue (where blood cells are produced). In the context of dental tissue physiology, important bone structures include the bone of the mandible, maxilla, zygoma, frontal and nasal bones, other facial bones and base of the skull [45].

Bone tissue regeneration has been a major research topic for decades. Minimal damage in any of the facial bones, can lead to considerable deformities. Therefore, the tissue engineering of such bony defects, in particular the mandible and maxilla remains an area of great interest [4].

1.3.1 Bone structure and development

There are two distinct pathways of bone development; intra-membranous bone ossification and endochondral cartilaginous bone ossification, both of which are dependent on mesenchymal cellular condensation prior to subsequent bone formation [46]. Although, the two types of bone ossification are different, they share the same key regulator molecules. These include, parathyroid hormone related protein (PTHRP), bone morphogenic proteins (BMPs), vascular endothelial growth factor (VEGF), and fibroblastic growth factors (FGFs) [47-52].

- **Intra-membranous ossification**

The intra-membranous ossification occurs by condensation of mesenchymal tissue and differentiation of stem cells into osteoblasts, which is associated with vascular invasion. The mandible and many cranial bones are formed by this process [46].

- **Endochondral/cartilaginous ossification**

Endochondral/cartilaginous ossification occurs in regions of low vasculature at the site of bone formation. Cartilaginous precursor is first established and this is reformed into bone. Chondrocytes undergo apoptotic death followed by resorption of cartilage which is replaced by bone tissue [52]. All long bones of the body and spinal vertebrae formed through this type of ossification [46].

Bone is also composed of bone lining cells (resting osteoblast or surface osteocytes), bone forming cells (osteoblasts), bone resorbing cells (osteoclasts), and bone matrix (organic and inorganic matrix components).

Bone Cells

- **Bone lining cells**

These cells are flattened and located in the periosteum and endosteum. These cells may act to remove the bone matrix covering (osteoid), as a preparative stage before osteoclastic resorption. Other evidence suggests that these cells are pre-osteoblasts and contribute to crystal growth in bone development [47, 53].

- **Osteoblasts**

These cells are derived from multipotent mesenchymal stem cells [54], and are regulated by various signalling pathways and physiological stimuli [55]. Particularly important in this respect is the transcription factor RUNX-2 which influences the expression of other genes responsible for matrix protein assembly such as osteocalcin, type 1 collagen and bone sialoproteins [56, 57]. Osteoblasts themselves contribute to the production and secretion of bone matrix proteins [58], along with cytokines and other signalling proteins [48, 59].

Osteoblast gene expression profile :

Osteoblasts have three stages in their development cycle; differentiation, proliferation, and matrix formation/mineralisation, and a specific gene expression profile is associated with each of these stages [60]. The *COL1* gene is expressed very early by osteoblasts during the proliferation phase [61], and down-regulated when the mineralisation begins [62]. Alkaline phosphatase (*ALP*L) is an early post proliferative osteoblast differentiation gene and is up-regulated as mineralisation begins. It is down-regulated in highly mineralised mature or maturing bone matrix [61, 63, 64]. As indicated above, *RUNX-2* encodes a crucial transcription factor for early stage osteoblasts differentiation [65] and function[66]. RUNX-2 binds to promotor regions of the genes, which encode other bone marker proteins such as osteopontin (OP), osteocalcin (OC) and type 1 collagen alpha chain (COL1A1) [67]. Osteocalcin is expressed post proliferatively [62, 64] and is

associated with mineralised nodule formation and bone mineralisation [60]. It is as a marker for osteoblast maturation and its expression is associated with advanced stages of differentiation [61, 68].

- **Osteocytes**

When osteoblasts become entrapped within the mineralised bone matrix, they are defined as osteocytes and they are connected to each other by actin-rich cellular processes [48, 58]. These cells are sensitive to mechanical pressure and contribute to bone remodelling and metabolism [47, 48, 58].

- **Osteoclasts**

These cells are characterized as giant multi nucleated bone resorbing cells, and play an important role in bone remodelling. Osteoclasts are derived from undifferentiated mesenchymal stem cells and monocytes [47, 48, 53, 58]. Osteoclasts are involved in demineralisation and degradation of inorganic and organic matrix of bone through their specialized morphology and enzymes secretion profile. The balance between osteoclastic and osteoblastic function is important for bone remodelling [47, 69, 70].

1.3.2 Bone matrix

Bone matrix comprises 70% inorganic component, 20% organic component and 10% water. The inorganic component of bone extracellular matrix is composed mainly of carbonated calcium phosphate mineral in the form of extremely small hydroxyapatite crystals. These crystals are found to align in parallel layers in a collagenous organic framework. Collagen and hydroxyapatite together are the essential bone matrix building blocks [71-

73]. The organic component of bone extracellular matrix is composed of 90% collagens and 10% non-collagenous proteins (Figure 3 and Table 2).

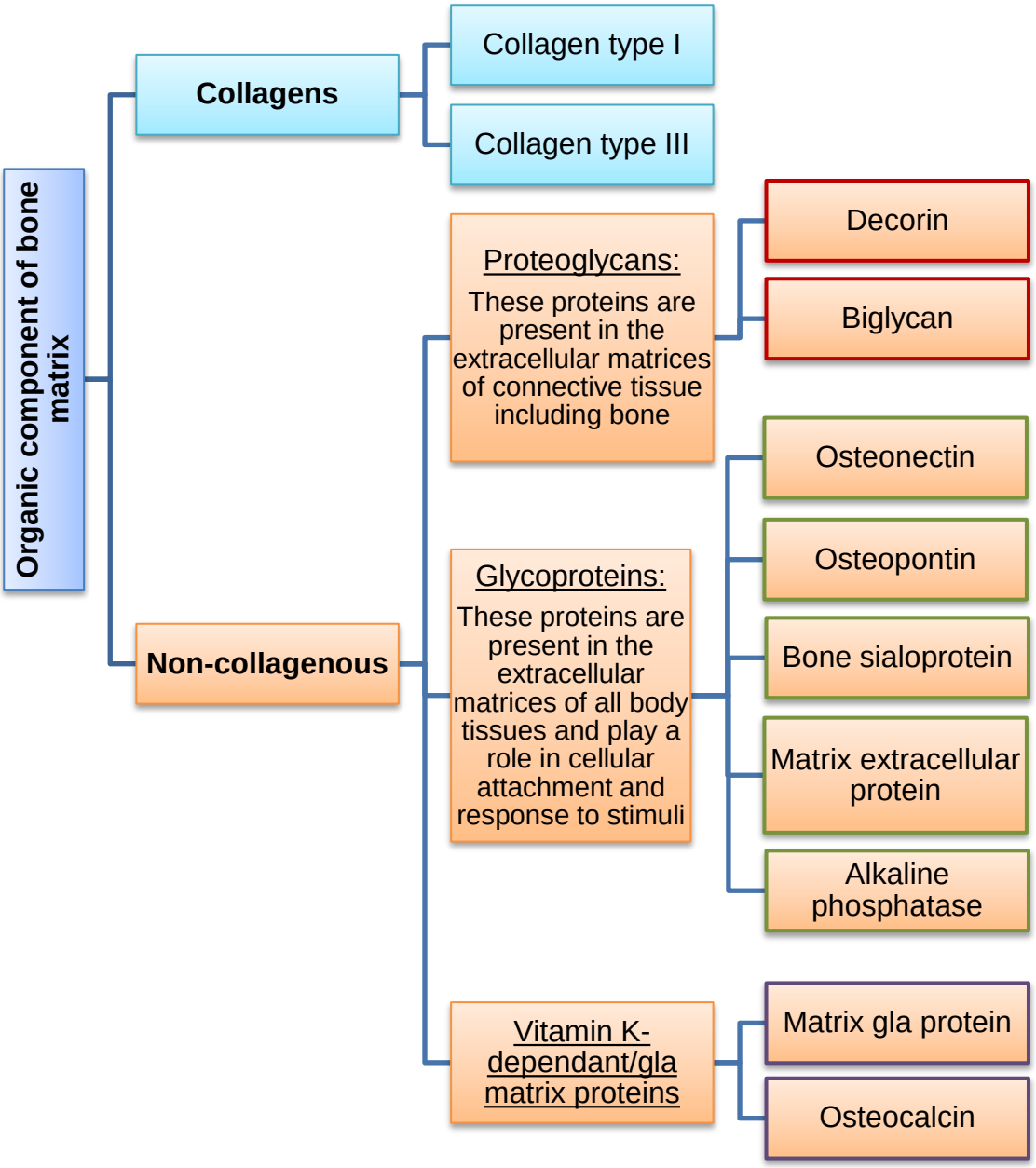


Figure 3: Classification of the main organic component found in the bone extracellular matrix [74]

* Next table summarise the main role of ECM components in bone formation and mineralization

Table 2: A summary of the key organic components of bone ECM and their role in bone formation and mineralization [74]

Protein type	*Notes*
Collagen type I	Main structural protein in organic matrix Forms organic scaffold of the bone
Collagen type III	Structural protein Co-expressed with collagen type I Associated with the walls of blood vessels and peripheral nerves
Decorin	Highly expressed in bone matrix Its expression is associated with osteoblast proliferation, during early matrix formation and bone mineralisation Member of small leucine-rich proteoglycan family (SLRPs) Binds collagen type I and growth factors Regulate fibrillogenesis and mineralisation
Biglycan	Highly expressed in bone matrix Its expression is associated with osteoblasts proliferation, during late matrix formation and bone mineralisation Member of small leucine-rich proteoglycan family (SLRPs) Binds collagen type I and growth factors Regulate fibrillogeneis and mineralisation
Osteonectin	Binds to collagen, hydroxyapatite and growth factors Regulate cell proliferation and angiogenesis Has a regulatory role in bone turnover
Osteopontin	SIBLING (small integrin binding ligand N-linked glycoprotein) Expressed in kidney, liver, bone, vascular and immune system. Has mineral binding sites Regulate cell attachment and proliferation Initiate intracellular signalling Promote osteoclast migration
Bone sialoprotein	Has mineral binding sites Associated with initiation of mineralisation Act as crystal nucleator
Matrix extracellular protein	SIBLING (small integrin binding ligand N-linked glycoprotein) Associated with mineralisation and bone remodelling
Alkaline phosphatase	Present in body tissues (liver, intestine) and known as non-specific alkaline phosphatase In bone, it is known as bone specific and found within vesicles on the extracellular membrane of osteoblasts Act as calcium binding protein Play a role in mineralisation Considered as an early osteoblastic marker
Matrix gla protein	Act as calcium binding protein Play a role in regulation and inhibition of mineralisation
Osteocalcin	One of the most abundant proteins in bone Frequently used as a biochemical marker for bone formation Act as a calcium binding protein Play a role in mineralisation and bone turnover Has direct effect on the growth and maturation of hydroxyapatite crystals in bone

1.4 Dentine and bone

There are similarities between bone and dentine but there are also some specific differences in detailed structure and molecular profile. Both tissues have mesodermal embryonic origin. In bone, osteoblasts are responsible for matrix formation and become osteocytes when embedded in bone canaliculi. However, odontoblasts are responsible for dentin formation then remain at the dentine-pulp interface with cell processes extending through dentin and occupying the lumen of dentinal tubules (Figure 2). In contrast with bone, dentine is not vascularized. In addition, bone formation also undergoes a constant remodelling, while dentine is a quite a stable structure. Despite this both osteoblasts and odontoblasts show similarities in behaviour at various stages of their life cycles [8, 9].

1.4.1 Dentine repair and regeneration

As indicated above, signalling cues from the enamel epithelium induce dentine formation by specialized odontoblasts [75]. In addition, demineralised dentine as well as bone extracts, enhance mineralisation when applied directly at sites of pulp exposure [76, 77]. Bone morphogenetic proteins (BMPs), combined with collagen-based matrices, bone sialoprotein (BSP) and growth/differentiation factor 11 (Gdf11) stimulate reparative dentine formation and dentine regeneration [77-83], and this occurs when these agents are in direct contact with dental pulp [84]. However in the case of inflamed pulp, these mechanisms are compromised [85]. On the other hand, autologous transplantation of stem cells derived from human-extracted

teeth into dentine and periodontal defects, with existence of appropriate signals was observed to partially regenerate dentine and periodontal tissues [86]. Therapeutic agents including Trioxide aggregates and Calcium hydroxide induce dentine deposition at the pulp/dentine interface, and this protects the pulp from further bacterial attack [87, 88]; however, this treatments did not show 100% successful clinical outcome. New therapeutic agents targeting the cells and molecules responsible for dental pulp inflammation are therefore necessary to increase healing capacity, counteract inflammation and maintain tissue vitality [89]. The reparative tissue induced by therapeutic reagents may on occasion lack appropriate structural integrity [87, 88], although they are able to enhance dentinogenesis under non-inflammatory conditions [85]. The control of the inflammatory context is essential for dental pulp healing . Such control needs to be exerted as early as possible as pulp tissue can quickly and irreversibly be damaged as a result of inflammation [90].

1.4.2 Bone repair and regeneration

Following bone fracture, repair occurs by similar processes and molecules that are involved in both intramembranous and endochondral bone formation (Section1.3.1). It is exclusively heals without formation of any scar tissue [91, 92]. Bone injury leads to hematoma formation which is associated with the inflammatory response and leads to recruitment of essential cells and signalling molecules that regulate bone repair. These include pro-inflammatory cytokines (TNF-α, interleukins) and other essential growth factors (FGFs, PDGF, VEGF) that will be discussed further in Section1.5.3.

For many years, simple autogenic, allogenic or xenogeneic bone grafts have been used for general tissue regeneration [93]. These include techniques such as free tissue transfer with microvasculature re-anastomosis of vascularized flaps from various body sites [94, 95]. Although these procedures have proven reliable and show effective outcomes, they need extended hospitalization and the donor site is associated with high rates of morbidity and complications. Tissue engineering can be considered as an alternative as it offers various potential advantages over other approaches including; decreased technical complexity and morbidity , as well as an ability to mimic the *in vivo* microenvironment [93]. However, more advanced approaches are needed for large bone defects to regenerate functionally sensitive tissue like maxillofacial tissue. Current regenerative approaches for bone reconstruction are summarized in (Table 3).

In summary, mineralised tissue repair and regeneration are largely dependent on the extent of the inflammatory response, which influences the release of essential signalling molecules to achieve new mineralised tissue formation. It also depends on the balance between the damage and the regeneration induced by inflammation. Clinical intervention (mainly transplantation) in critical bone defects has shown a wide range of success. However, these techniques are not ideal because of their associated complications, and improved techniques and methodologies are required [46]. Hence cell-based tissue engineering therapies show great promise.

Table 3: Summery of commonly used regenerative approaches for bone repair and reconstruction

Regenerative approach	Overview
Autologous bone reconstruction	↓ Current gold standard approach [46, 96] ↓ Contains essential components to achieve osteoinduction , osteoconduction and osteogenesis[97] ↓ Use bone from rib, iliac crest of the same patient [96] ↓ Its availability is limited with considerable postoperative complications [96, 98, 99] ↓ Vascularized grafts have higher osteogenic potential. ↓ Easily incorporated without immunogenic responses [100]
Allogenic/Xenogenic bone reconstruction	↓ It is available in various forms; demineralised bone matrix, cancellous chips, cortico-cancellous, cortical grafts, osteochondral, and whole-bone segments [46, 101] ↓ Lower osteoinductivity with no cellular component as grafts are devitalized during preparation [46] ↓ Immunoreactions issues and transmissions of infections [46, 102]
Synthetic scaffolds	↓ Wide range of alloplastic scaffolds used clinically such as ceramics and polymers [100] ↓ Some synthetic polymers such as polycaprolactone, polylactic, polyglycolic acid and polylactic-co-glycolic acid scaffolds has been approved for use in craniofacial applications or as bone pins/screws [103]
Gene therapy	↓ Application of exogenous cytokines and growth factors, essential factors for bone regeneration such as bone morophogenic proteins, fibroblast growth factor, insulin-like growth factors, vascular endothelial growth factor and platelet derived growth factor [103, 104] ↓ Can enhance cell adhesion, proliferation, migration, as well as osteogenic differentiation [104] ↓ Combinations of growth factors have been demonstrated considerably greater bone healing compared to single growth factor treatment [105]
Prefabricated bone engineering	↓ Construction of tissue with similar characteristic as possible to the defect to be restored [106] ↓ Well- established procedure in plastic reconstructive surgery that adapts to complex geometry of the defect [107]
Cellular approach	Section 1.5
Autologous cells and others [46]	Bone marrow aspirate concentrate Platelet-rich plasma Bioreactors Vascularization techniques

1.5 Cell-based mineralised tissue engineering

Tissue engineering is a multidisciplinary field that holds hope and promise for tissue regeneration therapies. In order to understand the tissue engineering approach for regeneration therapy, three main pillars need to be investigated (scaffold, stem cells, and growth factors) to allow growth, differentiation and regeneration of tissues in 3D structures [108].

1.5.1 Scaffold

Three-dimensional scaffolds offer the necessary platform for cell growth, proliferation, and maintenance of differentiation. Scaffolds physically support deposited extracellular matrix and vascular ingrowth until the entire bone function is restored [109, 110]. Scaffold architecture determines the eventual shape of the new bone and cartilage. Several reviews have been published on the general properties and design features of biodegradable polymers and scaffolds [46, 111-116].

1.5.2 Cells used in mineralised tissue engineering

Early implantation of an effective cell type in the area where mineralised tissue regeneration is required leads to secretion of essential osteogenic and vasculogenic regulators as well as other key growth factors. Transplanted cells are able to recruit host cells and together form the vascularized tissue [46]. One of the most common challenges facing mineralised tissue regeneration is to define an appropriate source of cells that can be

successfully differentiated into mineralised tissue as well as neo-vasculature. [117, 118].

1.5.2.1 Stem cells

Stem cell identification, function and therapeutic applications is an area of ongoing interest to many researchers. Stem cells were discovered in the early 1900s, and described as undifferentiated, primitive cells which can be used as building blocks for different tissues due to their ability to differentiate into other lineages and produce various cell types. During embryonic life, stem cells are described as totipotent and can differentiate to become any other cell type. With subsequent embryogenesis, cells are restricted by lineage and adopt a phenotype usually described as pluripotent [119] (Figure 4).

One of the well characterised adult somatic stem cells is the mesenchymal stem cell population which has been isolated from bone marrow and specific dental tissues [119]. Stem cells are often present together in special tissue compartments called niches and contain cells that may be used in the development and repair of body tissues. In these stem cell niches, stem cell proliferation, division, survival, migration and aging are regulated by cell-cell interaction and specific bioactive molecules. Stimulation of the stem cell niche(s) can occur during pathological conditions to repair tissue damage [120, 121].

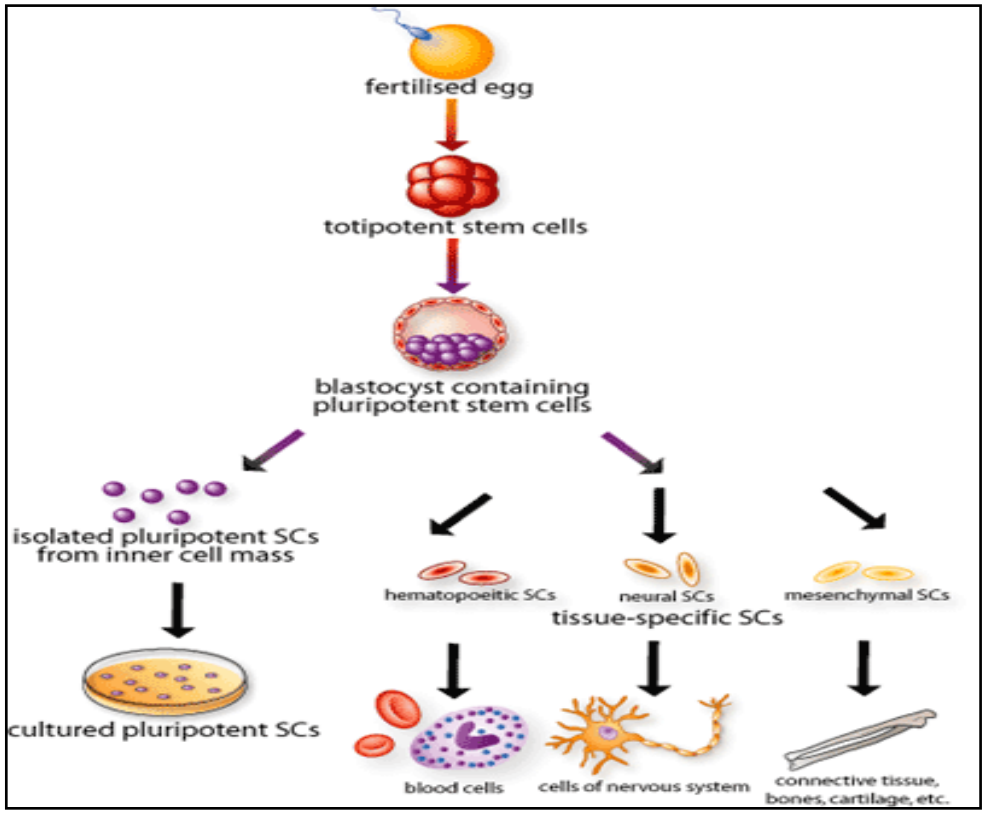


Figure 4: Embryonic and adult stem cell differentiation

Stem cells can be differentiated into different types of tissue such as: adipose, cartilage, bone, cardiac, and neural tissues.
(Adapted from wateverailsyou.blogspot.com)

1.5.2.1.1 Embryonic stem cells (ESCs)

ESCs are formed following the fertilization up until the ninth week of gestation. ESCs are pluripotent with a high proliferative capacity and are used as a multi-lineages cell source. They can differentiate into vascular cells, nerve cells and mineralised tissue regeneration cells [122], and have therefore found application in bone tissue engineering [46]. ESCs are located in the inner cell mass of blastocytes and are distinct from adult stem cells which usually reside in the various adult somatic tissues. Research and clinical therapies using embryonic stem cells have been limited due to ethical and political issues; for example, issues around the destruction of human embryos for the sake of regenerative medicine, cloning of human embryos and violation of human dignity and privacy have all been raised [123]. Another cell type of comparable versatility is the induced pluripotency stem cells (iPSCs), which are pluripotent stem cells generated from non-pluripotent somatic cells by transfection of appropriate genes artificially. iPSCs were first described in 2006, and were created via retroviral delivery of four transcription factors (Oct4, Sox2, Klf4, and Myc) into mouse fibroblasts [124]. iPSCs are similar to ESCs in morphology, gene expression profile, surface antigen presentation, and differentiation potential [125]. However, the clinical use of ESCs and iPSCs is still considered a potential risk due to their potential to form teratomas and other tumours [126]. In comparison and as an alternative to these cells, adult stem cells have been

investigated, which are present in many parts of the human body and their isolation does not involve the destruction of life or tumorigenesis [126].

1.5.2.1.2 Adult / postnatal stem cells

The identification of adult / postnatal stem cells has opened up new opportunities for stem cell research. These cells were investigated in a variety of postnatal tissues; for example, bone marrow, adipose tissue, cord blood, peripheral blood, and most important for the current project, they were also investigated in various dental tissues [127-136].

Dental tissue-derived stem cells:

More relevant to our studies, stem cells have been isolated from dental structures including periodontal tissue (PDLSCs) [137], apical papilla (SCAP) [138], dental follicle (DFSCs) [139], and dental pulp stem cells isolated from adult (DPSCs) and deciduous teeth (SHED) [140] (Figure 5).

As dental tissues developed from oral ectoderm and neural crest derived mesenchyme, they contain pluripotent stem cell populations which display a developmental potential similar to ESCs and are able to differentiate into different lineages [141, 142]. Different cell types from different portions of the tooth germ (dental pulp, periodontal ligament, apical papilla, and dental follicles) share special features related to their neural crest origin; they also express mesenchymal stem cell-markers along with their pluripotent differentiation capacity. Typically they display a fibroblast-like morphology with associated high efficiency for adherent colony formation and high proliferative potential [143].

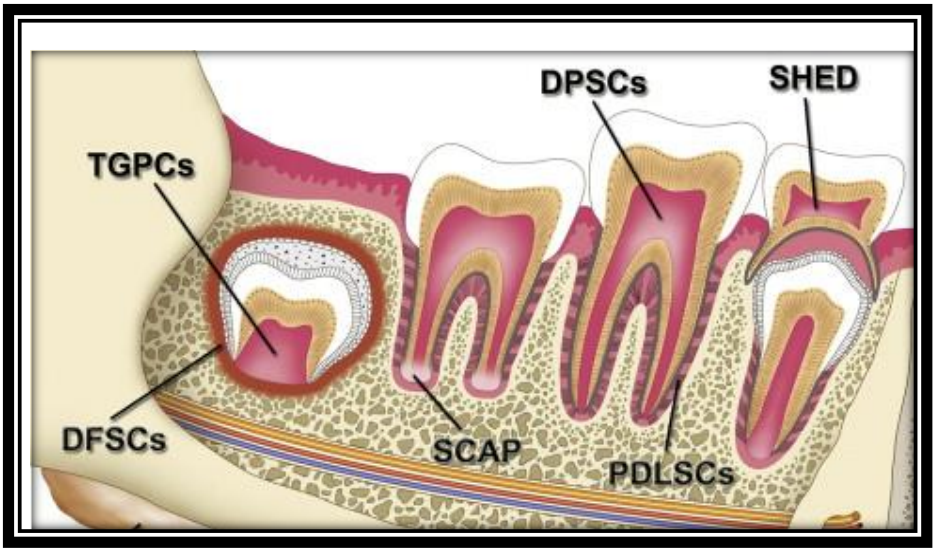


Figure 5: Location of Dental Stem Cells

TGPCs: Tooth Germ Progenitor Cells; DFSCs: Dental Follicle Stem Cells; SCAP: Stem Cells of Apical Papilla; DPSCs: Dental Pulp Stem Cell; PDLSCs: Periodontal Ligament Stem Cells; SHED: Stem cells from Human Exfoliated Deciduous teeth.
(Adapted from: http://www.slideshare.net/hamedgholami104/dental-stem-cells-18007596)

❖ **Periodontal ligament stem cells (PDLSC):**

Periodontal ligament contributes to tooth anchorage, nutrition, homeostasis and repair. PDLSCs can differentiate into cementoblasts, osteoblasts, chondrocytes and adipocytes and are characterised by a high rate of proliferation [137, 144].

❖ **Stem cells derived from apical papilla (SCAP):**

SCAP are a distinct type of dental stem cells which are positioned at the root tips during development before the tooth appears in the oral cavity [145]. These cells can undergo odontogenic, osteogenic, adipogenic, chondrogenic, and neurogenic differentiation [146].

❖ **Dental follicle-derived stem cells (DFSCs):**

The dental follicle defines the sac that surrounds the un-erupted tooth, and controls bone remodelling during the process of tooth eruption [147]. DFSCs are usually isolated from impacted third molars and studies showed the potential of DFSCs to differentiate into osteogenic, adipogenic, and neurogenic lineages [139] .

❖ **Dental pulp stem cells (DPSCs)**

These are the cells which were used exclusively in our studies and therefore a short description of the biology of this tissue is provided.

Biology of the dental pulp

The dental pulp organ originates mainly from the neural crest and first branchial arch mesoderm [148, 149]. Human dental pulp is the core connective tissue that occupies the centre of the tooth, surrounded by

dentine in the crown area and by cementum in radicular area. The condensed connective tissue of the pulp contains a mixed population of cells that are embedded in a fibrous vascular stroma [150]. Dental pulp has an inherent regenerative ability as it is a highly vascularized tissue containing stem/progenitor cells [108, 151]. Dental pulp contains many different types of cells including fibroblasts, lymphocytes, macrophages, dendritic cells, nerve cells, pericytes, endothelial cells and undifferentiated mesenchymal cells, each of which has unique functions [8, 150]. Odontoblast progenitor cells differentiate to odontoblasts, which are mesenchymal cells that are densely packed at dentine/pulp interface and which participate in synthesis and deposition of type I collagen-rich matrix known as pre-dentine which is subsequently mineralised to form dentine [152]. Odontoblast cytoplasmic cell processes run through the dentinal tubules, which interconnect the dentine pulp complex (Figure 2). Mature odontoblasts are not mitotically active [153, 154] and as they are situated at pulp-dentine interface, they act as first line of defence against bacterial infection [89, 155, 156].

Dental pulp possesses stem/progenitor cells that are involved in dentine repair following damage by noxious stimuli [151, 157]. DPSCs undergo self-renewal and multi-lineage differentiation but also interact appropriately with engineered scaffold. They are easily accessible and easily banked, and can therefore be used for autologous regenerative therapies including the generation of mineralised tissue [83, 151, 157-163]. In this respect they have proved of value for maxillofacial reconstruction and periodontal ligament tissue regeneration [2, 3, 164].

DPSCs isolation and characterization:

DPSCs express various stem cell markers such as mesenchymal stem cell marker CD146, CD105, CD90 and STRO-1, as well as OCT4, which is an embryonic stem cell marker [157]. DPSCs proved to be a generic source of mesenchymal stem cells [159], retaining the characteristic stem cell feature of high proliferative ability after prolonged culture [140, 165]. Transplantation of DPSCs into immune-suppressed mice resulted in formation of functioning dentine/pulp-like tissue architecture [157]. Moreover, these cells were able to differentiate into odontoblasts, adipocytes, chondrocytes, and osteoblasts *in vitro* [166, 167]. They can also differentiate into functioning neurons, and have been considered a source of material for treatment of various neural diseases [168, 169]. Third molars are often extracted for clinical reasons and subsequently discarded. However, they provide an excellent source of dental stem cells [170]. Development of these teeth starts at 5-6 years with calcification between 7-10 years and complete root development by 18- 25 years. Stem cells from this source proliferate well [157], form pulp-like tissue with well-established vascularization [171].

Dental pulp stem cells from adults (DPSCs) versus deciduous (SHED) teeth:

In the context of tissue engineering, stem cells from deciduous dental pulp tissue are less affected by genetic and/or environmental factors, and may have advantages for cell-repair and regeneration. In general, the anatomical and functional differences between adult and deciduous teeth affect stem cell differentiation potential [143]. Although the isolation technique for

DPSCs and SHED are similar, SHED tend to grow in clusters with higher proliferation rates and higher number of colony forming units (CFUs) compared with DPSCs [140, 172].

DPSCs isolated from healthy versus carious teeth:

Dental caries is one of the most prevalent chronic diseases worldwide [173]. It is a result of complex interaction over time between acid-producing bacteria and fermentable carbohydrate, and other host factors including saliva [173]. Penetration of oral bacteria into the dentine layer trigger inflammatory responses in the dental pulp [25], which responds to injury or inflammation according to the severity of infection (mild versus deep caries). The inflammatory process is characterized by inflammatory cell infiltrate, and immature progenitor cells recruitment [174]. Dental pulp cell biology is known to be affected by caries, but further studies are needed to determine in details the causative molecular mechanisms. DPSCs themselves have immunomodulatory effects and they have been successfully isolated from inflamed dental pulps [175]. Interestingly these cells showed high expression levels of some MSCs markers [176] although there is some controversy in relation to their differentiation potential in comparison with cells isolated from normal pulps [177, 178]. The rate of dentine repair/regeneration is closely related to the population size of remaining vital odontoblasts or newly differentiated odontoblast-like cells. If the inflammation is not too severe and/or is rapidly controlled, then innate pulp repair mechanisms can generally suffice for regeneration of the tissue(s) [25]. Carious teeth are usually extracted and discarded; however, as indicated above these cells are also a potential source of DPSCs [175, 179, 180].

1.5.3 Growth factors

The third factor which plays a role in mineralised tissue engineering is signalling molecules, which control cellular activity. Growth factors are amongst the most important of these agents. They are proteins secreted extracellularly, which bind to specific cell receptors to influence cell function. Growth factors regulate cell adhesion, proliferation, migration and differentiation, all of which are involved in tissue regeneration. In mineralised tissue healing and regeneration amongst the most important factors are the pro-inflammatory cytokines, bone growth factors, angiogenic growth factors and insulin-like growth factors [181, 182].

- **Pro-inflammatory cytokines**

This includes interleukins-1 (IL-1), IL-6, IL-7 and IL-8 and TNF-α. They are released by inflammatory cells and cells of mesenchymal origin. In mineralised tissue regeneration, they stimulate cell recruitment (chemotaxis), extracellular matrix synthesis, angiogenesis, and fibrinogenesis at the site of injury. At a later stage, they also contribute to bone resorption and remodelling [181, 182]**.**

- **Bone and angiogenic growth factors**

Osteoblasts and endothelial cells secrete growth factors that influence growth, migration and differentiation of both cell types [183, 184]. These growth factors are released during blood vessel formation and are expressed during angiogenic response to the injury although they also play a key role during bone repair/regeneration [101, 185]. These growth factors

included members of TGF-β, BMP, FGF, PDGF, and VEGF families. The contribution of these various growth factors is summarised in Table 4.

Table 4: Key player bone and angiogenic growth factors

Growth factors	Notes
TGF-β	↓ Produced from degranulating platelet in hematoma and extracellular matrix at the site of injury ↓ Enhances recruitment, proliferation and differentiation of mesenchymal cells into bone forming cells [186-188]
BMPs	↓ Osteoinductive molecules [189, 190] ↓ Enhance the differentiation of mesenchymal stem cells [191] ↓ BMP-2, BMP-4, BMP-7 have been investigated widely for osteogenic applications [192-194] ↓ BMP induce the production of VEGF from osteoblasts [195]
FGF	↓ Stimulate mitogenesis of endothelial cells and osteoblasts [196, 197] ↓ Basic FGF (bFGF) influences secretion of Matrix Metalloproteinases and VEGF [198] ↓ Initiate expression of bone markers (ALP, RUNX-2 and OC) [199] ↓ Stimulates the production of VEGF from osteoblasts
PDGF	↓ Secreted by platelets [200] ↓ Angiogenic factor that modulate the expression of VEGF [201]
VEGF	↓ Has five isoforms; VEGF-A, -B, -C, D, E [202] ↓ Major angiogenic regulator stimulating blood vessel formation [202] ↓ Enhances the proliferation and migration of endothelial cells [202] ↓ Plays a crucial role in recruitment, migration and differentiation of osteoblasts [203-205] ↓ Mediates the osteoinductive factors (BMP-2, FGF-2 and IGF) [206, 207] ↓ A key regulator between angiogenesis and osteogenesis during bone tissue repair [208, 209]
IGF	See Section “Insulin-like growth factor (IGF) axis”

Insulin-like growth factor (IGF) axis

As a significant portion of the experimental work, this thesis describes the potential role of the IGF axis in the osteogenic differentiation of DPSCs.The IGF axis plays an important role in tissue regeneration. It comprises two peptide hormones IGF-1 and IGF-2, with corresponding cell surface receptors; IGF-1R and IGF-2R together with six circulating high affinity binding proteins (IGFBP 1-6) [210, 211]. Most components of the IGF axis are expressed in human dental pulp stem cells [212].

❖ IGF axis components

➢ Insulin-like growth factor -1 and -2

IGF-1 is a 70 amino acid polypeptide (7.5 KDa) hormone that has similar structure to human pro-insulin. It is present in serum largely bound to IGFBPs, the free form representing only 1% of total IGF-1. Total concentration in plasma is of the order of 150-400ng/ml. IGF-1 is secreted mainly from the liver under the control of pituitary growth hormone (GH), but it is also secreted by extra-hepatic tissues including skeletal muscle, bone, and cartilage. IGF-1 acts to control cell development, proliferation, differentiation and migration as well as exhibiting anti-apoptotic actions [213, 214]. Many IGFs actions are considered to operate via autocrine and paracrine mechanisms [215]. IGF-1 is reported to regulate the differentiation of osteoblasts and subsequent bone formation [119], and is also involved in the differentiation and proliferation of osteoblasts from mesenchymal cells during the process of fracture healing [216, 217]. IGF-1 transcription in

osteoblasts is modulated by oestrogen and glucocorticoids [177, 216]. IGF-1 acts mainly through IGF-1R, although it also binds the insulin receptor (IR) but with lower affinity, a consequence of homology between IGF-1R and IR [218].

IGF-2 is a 67-amino acid polypeptide hormone. IGF-2 concentrations peak during foetal growth and then decline in adult life. IGF-2 expression is not dependent on pituitary growth hormone [219, 220]. IGF-2 stimulates osteotypical matrix deposition and osteodentine formation [221]. Growth hormone and both IGFs act in concert to regulate skeletal growth, maturation, and maintenance [119].

- **Insulin-like growth factor-1 and -2 receptors**

IGF activity is mediated via their respective cell surface receptors; IGF-1R and IGF-2R [222]. IGF-1R is a heterotetrameric transmembrane tyrosine kinase receptor [223]. It is composed of two extracellular α-subunits that contain the growth factor binding domain and which are connected by disulphide bonds to two transmembrane β-subunits that contain tyrosine kinase catalytic activity [221, 224, 225]. When the receptors are occupied by ligand, they undergo conformational changes that trigger the tyrosine kinase activity, leading to activation of downstream signalling molecules by protein phosphorylation [215]. Among these are the SRC homology 2 domain-containing protein (SHC) and insulin receptor substrate family of proteins (IRS1-4). These signalling intermediates play an important role in activation of the phosphoinositide 3 kinase (PI3K) and mitogen-1 activated protein (MAPK) kinase pathways [225]**.** IGF-1R has higher binding affinity for IGF-1

than IGF-2. IGF-2R is a mannose- 6- phosphate receptor and binds both IGF-1 and IGF-2 although with a higher affinity for IGF-2 compared with IGF-1 [220]. IGF-2R is a key player in IGF-2 turnover and degradation [226].

- **Insulin-like growth factor binding proteins**

Insulin-like growth factor binding proteins (IGFBPs) are secreted by several tissues and are soluble proteins [215]. IGFBP-3 and -5 are commonly found in a ternary complex with an acid labile subunit (ALS) [227, 228]. IGFBPs have higher affinity for IGFs than the cell surface receptors. IGFBPs are considered as a reservoir for IGFs as 99% of circulating IGFs are bound to IGFBPs [229]. IGFBPs regulate the circulating IGFs levels either by sequestering IGFs from IGF-IR and IGF-2R or by releasing them into the circulation to bind and activate their receptors [215, 230]. IGFBPs affinity for IGFs can be affected by post-translational modifications of these proteins and particularly by proteolysis [222, 230].

IGFBP-1 stimulates cell motility, adhesion and migration. It binds specifically to the $\alpha_5\beta_1$ integrins [230, 231], and is present at very high concentrations in placenta and there are higher levels in female plasma compared to male plasma [232]. IGFBP-1 expression is inversely regulated by insulin and phosphorylation of IGFBP-1 reduces IGF affinity [230].

IGFBP-2 levels increase with age [233]. IGFBP-2 is crucial to transport IGFs, and IGFBP-2 serum levels correlate with normal bone formation and bone remodelling [234]. IGFBP-2 has been considered as a negative regulator of bone formation induced by IGF-1, including IGF-1 stimulation of osteoblast proliferation, collagen secretion and bone formation [235].

However, growing evidence indicates the anabolic effect of IGFBP-2 on bone density [236, 237]. Recently, a unique heparin-biding domain (HBD) has been identified in IGFBP-2, which is suggested to mediate the anabolic effect of IGFBP-2 [238]

IGFBP-3 is the most abundant IGFBP in serum and reaches highest levels during the puberty. IGFBP-3 provides 75-80% of the IGF carrying capacity in serum, and binds IGFs in high affinity complexes. The IGF1-IGFBP3 complex forms a tripartite complex with a third protein known as acid labile subunit (ALS) and this prolongs the circulating half-life (~16 hours) of bound IGF-1 compared to free growth factor (<15 minutes) [215] . Around 90% of IGFBP-3 and 55% of IGFBP-5 circulate in a trimeric complex with ALS during adult life [239]. Interestingly and pertinent to the current study, IGF activity on osteoblasts is enhanced by IGFBP-3 [240].

IGF-1 can regulate IGFBP-4 and IGFBP-5 expression and can also activate the IGFBP-4 specific protease. This forms a positive feedback loop releasing IGFs from IGFBP-4 to act locally on tissues. However in some tissues, IGF-1 increases IGFBP-5 expression and this may serve as a negative feedback mechanism regulating growth factor activity. Clearly regulation of IGF action is a complex process and it remains an area of intense study [218, 241, 242]. In bone, IGFBP-5 potentiates the proliferative action of IGF-2 and during bone remodelling processes IGFs are released from IGFBP-5. IGFBP-5 is therefore considered to have an enhancing effect on IGF action in bone tissue [243]. IGFBP-5 affinity for IGF-1 is decreased by association with extracellular components including collagen, laminin, and fibronectin

and this may lead to IGF release into the local tissue environment [231]. Although the studies described above suggested an osteogenic function for IGFBP-5, this area remains controversial and IGFBP-5 may also inhibit bone formation by sequestration of IGFs from their receptors on the osteoblasts surface [244].There are relatively few studies on the function of IGFBP-6. IGFBP-6 showed the ability to inhibit the IGF-2 actions as it has higher affinity for IGF-2 than IGF-1 [245]. A diagrammatic representation of the IGF axis is shown in Figure 6.

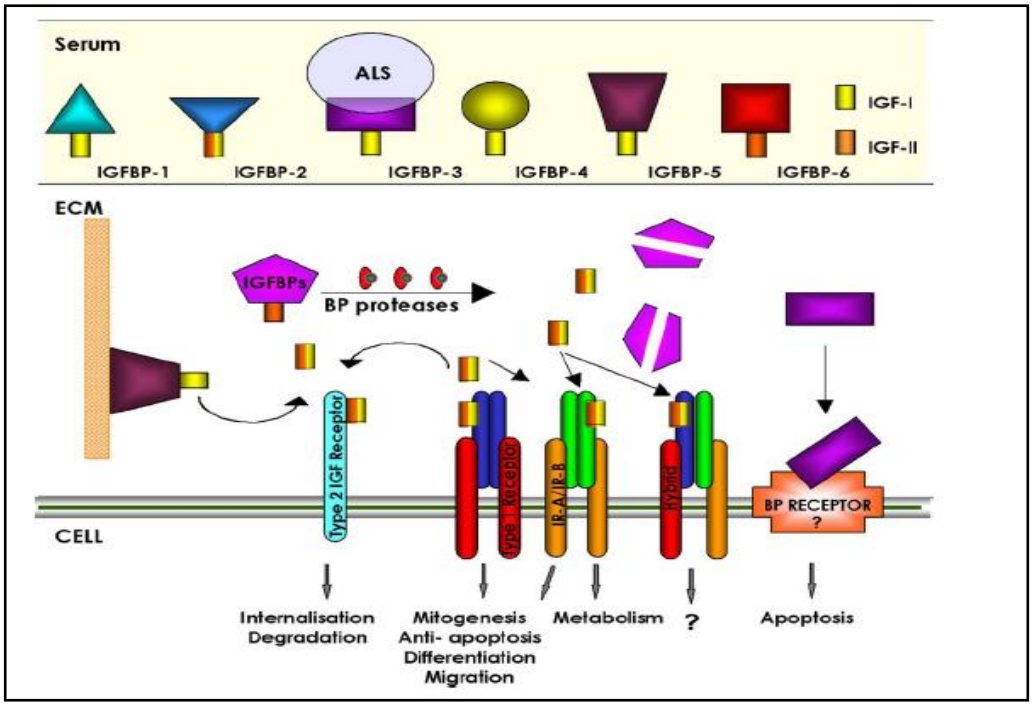


Figure 6: Insulin-like growth factor (IGF) axis

IGF axis comprises two related polypeptide growth factors IGF-1 and IGF-2, two cell surface receptors IGF-1R and IGF-2R, and six soluble binding proteins (IGFBP1-6). Some IGFBPs can bind to extracellular matrix (ECM) and IGFBP-3 circulates in serum bound to an acid labile subunit (ALS). IGFBPs show similar affinity for IGF-1 and IGF-2 with the exception of IGFBP-6 which has a 20-fold higher affinity for IGF-2 than IGF-1. Most IGFBPs can be proteolysed by specific IGFBP proteases. IGF-1 and IGF-2 can also bind to both A and B isoforms of the insulin receptor and to the hybrid IGF-1R/IR receptors. Finally IGFBPs have direct effects independently of IGFs and these may be mediated by specific IGFBP receptors (adapted from Igf-society.org).

❖ IGF axis in dental tissue

IGF-1 is suggested to have a role in pulp repair/regeneration as it induces proliferation and differentiation of dental pulp cells to form odontoblast-like cells. There is evidence that IGFs are trapped in the matrix during dentine formation and that soluble growth factor can be released into dental pulp following demineralisation of dentine [20-22]. IGF-1 has been used as a pulp capping material in rat molars and enhanced reparative dentinogenesis in this experimental model. Caviedes *et.al* (2004), demonstrated higher expression of IGF-1R in dental pulp derived from human teeth having incomplete roots compared with complete roots. This might be as a result of increased cell proliferation during root formation and suggests a role for IGFs in this process [212]. Gotz *et al.* (2001), demonstrated the expression of IGF axis components during reparative processes in periodontal connective tissue with IGF axis proteins found in both the cellular and acellular compartments of extrinsic fibres of cementum. Cementoblasts and periodontal ligament fibroblasts express IGF-1R. IGF-1 induces the accumulation of amelogenin and ameloblastin suggesting that this growth factor plays a role in enamel bio-mineralisation as ameloblasts also express IGF-1R [246].

❖ *IGF axis* during angiogenesis

Endothelial cells (ECs) express several components of the IGF axis including IGF-1, IGFBP-2, -3, -4 and -6 [247-249]. Expression of the IGF axis in ECs is modulated by several factors including cell density [250], hypoxia [251], TGF-β, VEGF [252], and IGF-1 itself [253]. IGF-1 stimulates

endothelial cell migration and angiogenesis, as well as inflammatory and vasodilatory responses in these cells [254-256]. IGF-1 acts via the PI3K pathway and cross talk with other growth factors and hormones (e.g. ostradiol), also occurs through this signalling pathway to regulate responses such as vasodilation [257, 258]. IGF-1 is important for initiating angiogenesis and neovascularization. An earlier study demonstrated that an IGF-1R antagonist inhibits retinal neovascularization *in vivo* [259, 260]. IGF-1 also act as a chemo-attractant to increase cytokine release further stimulating angiogenesis with associated production of extracellular matrix [261]. In addition, IGF-1 administration increases circulating levels of VEGF and TGF-β [262-265].

1.6 Pulp response to injury

Cariogenic bacterial populations release acids that progressively demineralize enamel, which normally protects the dentine/pulp complex [89, 266], and dentine layer of the tooth [267]. Dentine demineralisation releases bioactive molecules from the dentine matrix, which diffuse towards the dental pulp where they can stimulate and signal the repair processes, and trigger appropriate inflammatory and immune responses in the dental pulp [90, 267, 268]. DPSCs respond to the noxious stimuli by many different routes, and carious lesions may cause localized odontoblastic damage and activate DPSCs to differentiate into odontoblast-like cells that contribute to tertiary/reparative dentine formation [269]. Odontoblast differentiation during development and repair appeared to be similar processes [270]; however, a

more complex and variable signalling environment modulated by infection may exist following severe dental injury [271].

In the case of mild inflammation, bacterial insult initiates dentine repair accompanied by mineralised tissue formation at the pulp/dentine interface. Untreated severe and rapidly progressing inflammation reaches the pulp and cause irreversible damage, pulp necrosis, infection of the root canal system and periapical disease [90, 267]. In shallow and moderate dentine carious lesions, Gram positive bacteria are the predominant microorganisms [267]. While in deep carious lesion, the loads of Gram positive aerobic bacteria decrease and the Gram negative anaerobic species increases; including Fusobacterium, Prevotella, and Tannerella SPP [266].

1.7 Inflammation and regeneration

Most tissue injury is followed by inflammatory processes as a fundamental part of early healing events. These modulate the final repair/regeneration outcome according to severity of trauma/insult [272]. Two of the most important molecular axes involved in the response to dental pulp infection and tissue damage are the Toll-like receptor (TLR) family and the interleukin (IL) family. As a part of the experimental work subsequently reported in this thesis describes the expression and activities of these inflammatory markers in carious dental pulps, a short description of the members of these molecular axes along with a brief synopsis of their activities is provided.

1.7.1 Inflammatory markers

- **Toll-like receptors (TLRs)**

Innate immunity depends on the recognition of microbial pattern products by a set of germ-line encoded receptors termed pattern recognition receptors (PRRs) [273]. The PRRs are responsible for recognition of pathogen-associated molecular patterns (PAMPs), found in the bacterial cell wall components including lipopolysaccharide (LPS), peptidoglycan, Lipoteichoic acid (LTA), as well as bacterial and viral nucleic acids including single- and double-stranded RNA and DNA. The TLR family constitutes an important class of PAMP recognition receptors [274, 275]. TLRs are type I integral transmembrane glycoproteins with an extracellular domain containing numbers of leucine-rich repeat (LRR) motifs responsible for agonist recognition and a cytoplasmic domain homologous to the interleukin-1 receptor responsible for intracellular signal transduction [274, 276]. The TLR family comprises ten functional TLRs (TLR-1-10) with various critical roles in the recognition of different PAMPs and subsequent activation of innate immune responses through the NF-kB pathway [277-280]. Depending on TLR cellular localisation and PAMP ligands, they are classified into extra-cellular and intra-cellular receptors. Extra-cellular receptors essentially recognise microbial membrane components including lipids, lipoproteins (TLR-1, TLR-2, TLR-6), lipopolysaccharide (TLR-4) and flagellin (TLR-5). The intra-cellular TLRs recognise double-stranded RNA (TLR-3), single-stranded viral RNA (TLR-7 and TLR-8), in addition to un-methylated CPG-DNA of viruses and bacteria (TLR-9) [275]. TLRs regulate the migration, proliferation, differentiation and immunosuppressive activities of MSCs [281].

They also activate the effector phase of the innate immune response including release of pro-inflammatory cytokines that recruit inflammatory cells [282-284], and maturation of antigen presenting dendritic cells (DCs) [285]. Maturation of DCs leads to initiation of an adaptive immune responses and therefore TLRs have a pivotal role in coordinating innate and adaptive immunity [277].

TLR-4 is expressed on various cell types including endothelial cells [286], cardiac myocytes [287] and cells of the central nervous system (CNS) [288]. TLR-2, -3, and -4 are expressed on stem and progenitor cells in various tissues [289-293], including dental follicle-derived stem cells [272, 294]. TLR-4 is expressed in human stem cells isolated from apical papilla [295], and all TLRs except TLR-7 are expressed in periodontal ligament stem cells [291, 296]. All TLR family members are also expressed on dental pulp stem cells [272, 297, 298]. Such wide range of expression suggests a broad range of activities not limited to immune responses. Indeed recent research demonstrated that TLR-4 function in central nervous system homeostasis [299], learning and memory [300], and neurodegeneration as well as neuroprotection after ischemic preconditioning [301]. TLR-4 signalling may also be involved in cancer development and progression [302]. TLR-4 activity is mediated by two main signalling pathways: (1) MyD88-dependent and NF-kB-driven, and (2) MyD88-independent and IRF3-driven pathways [303]. The NF-kB pathway is also activated independently of MyD88, although this is a minor pathway and exhibits different dynamics and kinetics [304]. The balance between MyD88-dependent and MyD88-independent pathways may regulates apoptosis, proliferation, differentiation and

migration of cells, although data is not conclusive and further research is required [304]. Activation of NF-kB signalling by the TLR-4 complex (containing a complex of TLR-4, MD-2, CD14) involves degradation of inhibitor kB allowing the subsequent nuclear translocation of NF-kB and regulation of transcription of target genes [305, 306]. Responses to PAMPs are dependent not only on TLRs but also the presence of potential co-factors as well as the levels of each PAMP [307]. Some TLRs act in combination to stimulate or inhibit the cellular response to selective agonists [308, 309].

Different dental pulp cells express a range of TLRs [89, 155, 298]. TLR-2 is expressed on odontoblast and can be activated by LTA to stimulate the nuclear translocation of NF-kB [155]. This signalling pathway can also be stimulated by LPS in DPSCs or odontoblast like-cells [297, 310, 311], possibly via crosstalk with the MAPK signalling pathway [310, 312, 313]. Both of these pathways have crucial roles in innate immunity [314, 315]. Various factors modulate the immunostimulatory or immunotolerance function of TLRs. These can include the nature and load of bacterial invasion, and degree of penetration into the dental tissue (shallow or deep caries). The developmental stage of the tooth (un-erupted or erupted) may also be important, other sources of contamination from the resident oral bacteria may also be involved [316]. TLR expression is low in non-inflammatory situations and this prevents unnecessary inflammatory responses [317, 318].

- **Interleukins**

Carious pulp tissue shows up-regulation of several interleukins; including IL-4, IL-6, IL-8, and IL-10 [319-321]. IL-8 plays a crucial role in immune responses, particularly in the activation of neutrophils, which are usually the first immune cell type to appear at the site of injury [322]. Most cytokines also use p38 MAPK and NF-kB signalling pathways to activate responsive cells [323]. During bacterial infection of dental pulp, many cytokines show pleiotropic actions; for example, IL-6 has antibacterial and immunomodulatory properties but also plays a stimulatory role in angiogenesis and the mineralised tissue reparative process [324]. IL-6 and IL-8 in addition to chemokines are secreted mainly as a result of TLRs activation and ligation [274, 285, 325]. IL-6 is secreted by a variety of immune and non-immune cells and mediates various aspects of the immune response [326]. IL-6 and IL-10 levels increase following bacterial stimulation of TLR-2 in odontoblasts [327, 328], and this provides a mechanism for regulating the intensity of the immune response as the immunosuppressive cytokine IL-10 regulates the immune response through negative feedback of IL-6 and IL-8 expression [327], to inhibit inflammation-associated immune responses and minimize host damage [329]. Also, relevant to these observations is the biphasic response of dental pulp cells to pro-inflammatory signalling molecules. Therefore, low levels of cytokines and growth factors show stimulatory effects on these cells whilst higher levels of these molecules appeared to cause cell damage [330-333].

1.7.2 Inflammation and mineralised tissue regeneration

During the initial phase of dental pulp immune responses, various coordinated mechanisms are stimulated to down-regulate both pre-dentine matrix secretion and mineralisation [89]. TLRs expressed by odontoblasts influence the production of extracellular matrix proteins [89, 334], and increase the synthesis of dentine, decorin and initiate odontoblast differentiation [334]. LPS-induced TLR-4 activity accelerates synthesis of ameloblastin mRNA [335]. The role of TLRs in osteogenic differentiation of human MSCs is controversial and may depend on the tissue of origin of MSCs [336]. Although TLR-4 stimulates the production of tertiary dentine, the TLR-4 receptor expression is decreased during the mineralisation of murine odontoblast-like cells [156, 337, 338], and this may be mediated by inhibition of ALP activity [339]. In a similar vein, LTA down-regulates type I collagen, the major pre-dentine structural component and activation of the NF-kB pathway inhibits the expression of α 1 (I) and α 2 (I) collagen [340, 341]. Moreover, LPS significantly down-regulates DSPP, a glycoprotein that plays a critical role in the pre-dentine mineralisation process [342]. Many of the TLR activated signalling pathways are also involved in the BMP mediated osteogenic differentiation of rat dental follicle stem cells [343]. Critically as outlined above the activation of NF-kB signalling pathway is important in regulating the immune response to infection but also is involved in differentiation of stem cells from various sources [344, 345], and this provides a focus for cross talk between inflammatory and differentiative pathways. There are also issues associated with dosage effects. Thus LPS (10µg/ml) treatment showed a significant inhibitory effect on the human

DPSCs growth (after 14 days) and proliferation (after 24h), although at 0.1 µg/ml LPS showed a significant stimulatory effect after the same period of time [297]. Such effects may reflect different responses dependant on severity of bacterial challenge as indicated previously. LPS has been reported to promote the odontogenic differentiation of human DPSCs via the TLR-4, ERK and p38 MAPK signalling pathways. While the NF-kB signalling pathway does not appear to have a role in LPS-induced odontogenic differentiation [297]. LPS treatment of human DPSCs enhanced mineralised nodule formation in a time-dependent manner. Thus DSPP and DMP-1 were increased on day 7 and day 14 after LPS treatment but ALP and OCN expression was up-regulated on day 14 and 21 [297]. Conversely in a separate study, LPS inhibited the expression of ALP, DSPP and RUNX-2, and supressed mineralisation nodule formation in a rat dental papilla-derived cell line [342].

1.7.3 Inflammation and angiogenesis

Vasculogenesis is defined as the formation of the primary vascular plexus from pre-existing vascular precursor cells in the new embryo while angiogenesis is the formation of new blood vessels from pre-existing vessels. The latter is accountable for the greater part of blood vessel formation in normal physiological tooth development and healing pulp injury [346-349]. Angiogenesis is stimulated by hypoxia and limited nutrient supply, and is controlled by growth factors, cytokines, endogenous angiogenesis inhibitors, transcription factors, adhesion molecules and components of extracellular matrix [350-354]. Generally, inflammation induces the

expression of mitogenic and proangiogenic factors including VEGF, FGF and PDGF in human pulp and gingival fibroblast [347, 355, 356]. The integrity of the vital pulp tissue is dependent on the process of angiogenesis at the site of injury [357], and up-regulation of angiogenic signalling during inflammation may also aid survival and differentiation of DPSCs into mature odontoblast-like cells at the site of trauma and angiogenic signalling pathways together with inflammatory cytokines secreted during bacterial infection are crucial for reparative dentinogenesis [358]. Thus angiogenesis provides blood supply, oxygen nutrition and stem/progenitor cells essential for healing processes [359], and indeed VEGF directly stimulates the proliferation and differentiation of DPSCs [360]. In the case of caries-induced inflammation, the number of capillaries in the pulp under the lesion increases and these capillaries extend into the odontoblast layer [90]. Chronic exposure of dental pulp tissue to an inflammatory environment results in vasodilation and increased vascular permeability with increases in the blood flow [154, 361]. LPS stimulates VEGF expression in DPSCs via NF-kB [310, 362], and as is evident from discussions above NF-kB acts as a master transcription factor modulating a range of pro-inflammatory cytokines and angiogenic factors in dental pulp cells and oral epithelial cells [85, 363, 364]. LTA stimulates the production of the pro-angiogenic chemokine CXCL2 that binds its receptor CXCR2. This receptor is abundantly expressed on endothelial cells and increases vascularization under inflammatory conditions [365]. IL-6 is implicated in oedema following continuous intra-dentinal diffusion of Gram positive bacteria also resulting in increased

vascular permeability [327]. Chemokines in addition to their role in cell locomotion also induce angiogenesis [366, 367].

TLR-4 expression has been reported in adult pericytes located close to vasculature [304], and activated TLR-4 was reported to stimulate proliferation of endothelial progenitor cells [290]. Stem cells express proangiogenic factors and differentiate directly into endothelial cells. DPSCs express VEGF, monocyte chemotactic protein-1 (MCP-1) [368], FGF-2, PDGF, IGFs, TGF-β and IL-8. These factors regulate endothelial cell migration via PI3K/AKT and MEK/ERK signalling pathways and initiate the tubulogenesis seen in these cells [347, 356, 360, 369, 370].

1.7.4 Inflammation and IGF axis

Many growth factors are sequestered within the dentine matrix as it is secreted by odontoblasts during tooth development [25]. During dentine demineralisation these factors may be released and modulate both repair and regenerative processes [25]. In general, inflammatory cells are the source of most growth factors/cytokines that contribute to wound healing [371]. In the area of inflammation, all types of wound cells including macrophages, fibroblast and endothelial cells express IGF-1 [372]. IGF-1 plays an important role in healing and typically establishes responsive conditions in which other growth signals undertake reparative roles. In cystic fibrosis, inflammation acts as an important modulator of the IGF axis causing a decrease in IGF bioactivity and there may be a reciprocal relationship between cytokines and the IGF axis in cystic fibrosis. Patients

have been described with high serum concentration of IL-1β, TNF-α, and IGFBP-2 compared to significantly low concentrations of IGF-1 and IGF-2 [373]. In non-diabetic patients with cardio-metabolic risk factors, plasma IGF-1 and IL-6 are inversely related to each other. Clinical and experimental data showed that IGF-1 acts as an anti-inflammatory molecule inhibiting IL-6 expression. Also IL-6 decreases IGF-1 levels by enhancing its clearance [374]. This is also seen in chronic inflammation where high IL-6 concentrations are correlated with significant decrease in IGF-1 and IGFBP-3 levels [375]. On the other hand, IGF-1 stimulates IL-17-induced expression of inflammatory cytokines and chemokines. Insulin/IGF-1 signalling pathway crosstalk with IL-17 occurs via activated NF-kB [376]. IGF-1 expression in intestinal epithelial cells stimulates the expression of IL-10 and inhibited IFN-ɣ and TNF-α in immunosuppressive monocytes [377].

TLR-2 deficiency is associated with a significant decrease in IGF-1 levels, and compromised innate immune responses in the brain resulted from TLR-2 deficiency leading to reduced IGF-1 levels [378]. In skeletal muscle injury, the expression of IGF-1 affects the expression of the inflammatory cytokines involved in the recruitment of monocytes/macrophages. In general, it appears that following appropriate modulation of inflammatory responses, IGF-1 expression provides a suitable environment for effective repair and regeneration of damaged tissue response [379].

Chapter 2: Aim and Objectives

The main aim of this thesis was to characterise the dental pulp stromal/stem cells isolated from teeth with shallow caries (cDPSCs) for the purpose of future use in mineralised tissue (bone/dentine) regeneration.

This aim was addressed via a series of specific experimental objectives, summarized as follows:

1- Isolation, characterisation and comparison of dental pulp stromal/stem cells from healthy (hDPSCs) and carious teeth with shallow caries (cDPSCs). This included investigation of colony forming ability and identification of stem cell surface markers.
2- Comparing the osteogenic potential of cDPSCs to those of hDPSCs in monolayer cultures.
3- Comparing the changes in the expression of osteogenic, angiogenic and inflammatory markers under basal and osteogenic conditions in hDPSCs and cDPSCs in monolayer cultures, in order to examine the effect of inflammatory environment on the expression of these regenerative molecules.
4- Investigating the expression of insulin-like growth factor (IGF) axis during the osteogenic differentiation of hDPSCs and cDPSCs.
5- Investigating the potential role of insulin-like growth factor axis during osteogenic differentiation of hDPSCs

Clinical Rational:

1- cDPSCs can be banked and used as candidates for cell based hard tissue regeneration therapies.

2- Investigating low-grade inflammation and regeneration for future development of endodontic and conservative dental regeneration therapies.

Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Cell culture

Alpha-Modified Minimum Essential Medium (α-MEM) catalogue number (#)BE12-169F and phosphate buffered saline (PBS) # BE17-516F were from Lonza BioWhittaker, UK. Penicillin/Streptomycin (Pen Strp) #P4333, Foetal bovine serum (FBS) #F9665, L-glutamine #G7513, 0.25% (w/v) Trypsin-EDTA solution #T4049, Trypan blue solution, 0.4% (w/v) #T8154, dimethyl sulfoxide (DMSO) #276855, dexamethasone #31375, L-ascorbic acid #A4403, Naphthol AS-MX phosphatase solution 0.25% (w/v) #855-20mL, fast violet B salt #F1631, Alizarin red stain #A5533 were from Sigma-Aldrich, UK. Collagenase type I #17100-017 was from GIBCO™. Dispase II neutral protease, grade II #114662200 was from Roche, Germany. Tissue culture plastic including 15mL centrifuge tubes #430790, 50mL centrifuge tubes #430828, T-25 cm^2 tissue culture flasks #430639, T-75 cm^2 tissue culture flasks #430641, T-175 cm^2 tissue culture flasks #431080, 10cm Petri dishes #353803, 6 well tissue culture plates #18341 and Stripette were from Corning®, UK. Additional items included pipette tips (Starlab, Tip one, UK) Syringes #SS* 05SE1 (TERUMO, UK) ,syringe filters #16532 (Sartorius - UK) 70µm strainer #352350 (Falcon, USA) and Pasteur pipettes #PIP4105 (SS Scientific lab supply, UK).

3.1.2 Flow cytometry

Human umbilical vein endothelial cells (HUVECs) were generously provided by Gary Grant from Leeds Institute of Cancer and Pathology and the U937 myeloid cell line was generously provided by Dr Gina Doody from Leeds Institute of Cancer and Pathology and Sophie Stephenson from section of Experimental Haematology, Leeds Institute of Cancer and Pathology. Human large vessel endothelial cell growth medium package #ZHM-2953 was from Cellworks, UK. RPMI 1640 medium for U937 culture #R0883 was from Sigma-Aldrich

Fluorochrome-labeled mouse anti-human monoclonal antibodies against cell surface markers of interest were from BD Biosciences, UK (Table 5). Fixable Viability Stain #564406, human BD Fc block™ #564220, Stain Buffer BSA #554657, Brilliant Stain Buffer #563794, BD™ CompBeads #552843 were also from BD Biosciences, UK. 5mL polystyrene round-bottom tubes for flow cytometry (12x75mm) #352054 were from Falcon, UK.

Data was acquired on an LSRII FACS analyser (BD Biosciences) using 405nm, 488nm and 640nm laser excitations. Analysis of acquired data was performed using both FACS DivA software (BD Biosciences) and FlowJoV10 (Tree Star).

Table 5: List of antibodies and isotypes used in flow cytometry

Antibody against	Description	Isotype
CD146	PE-Cy7 Mouse Anti-Human (Cat# 562135)	PE-CY7 Mouse IgG1,k Isotype Control (Cat# 557872)
CD90	PerCP-Cy™5.5 Mouse Anti-Human (Cat# 561557)	PerCP-Cy™5.5 Mouse IgG1,k Isotype Control (Cat# 550795)
CD105	BV421 Mouse Anti-Human (Cat# 563920)	BV421 Mouse IgG1, k Isotype Control (Cat# 562438)
CD45	APC-Cy7 Mouse Anti-Human (Cat# 557833)	APC-Cy7 Mouse IgG1,k Isotype Control (Cat# 557873)
CD31	FITC Mouse Anti-Human (Cat# 555445)	FITC Mouse IgG1,k Isotype Control (Cat# 555748)
Fixable Viability dye BV510 (FVS 510)	(Cat# 564406)	----------

3.1.3 Quantitative real time polymerase chain reaction (qRT-PCR)

RNeasy® mini kit #74104 was from (Qiagen, UK. β-mercaptoethanol #A4338,0100 was from Applichem panreac, UK, absolute ethanol (200 Proof, Molecular Bi-ology Grade) #BP2818- 500 was from Scientific Laboratory Supplies Ltd, UK. Ultrapure DNAse/RNase-free distilled water #10977035, RNase-free tubes #AM12400 and lens cleaning tissue #FB13067 were from Thermo Fisher Scientific, UK. DNase I Amplification Grade #18068015 was from Invitrogen, UK. Optical adhesive seal #P3-0300 was from Geneflow Ltd, UK. High Capacity RNA to cDNA kit #4387406, master mix #4369016 and TaqMan probes (see Table **6** for further information) were from Applied Biosystems (ABI), UK. PCR tubes (0.2mL flat cap) #TFI0201 were from Bio-Rad, UK. Non-stick RNA-free 1.5mL microfuge tubes #AM12450 were from Ambion ®, Mexico. 96 well PCR microplates (LightCycler type) #I1402-9909 were from Starlab, USA. Thermal cycler (PTC-100 Peltier -version 9) Roche LC480 Light Cycler was used to generate data.

Table 6: Details of Taqman® gene expression assays used in qRT-PCR

Gene Name	Gene Description	Taqman ® gene expression assay number
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	House-keeping gene (Control)	Hs99999905-m1
Alkaline phosphatase, Liver / kidney / bone (*ALPL*)	Bone marker (Gene of interest)	Hs01029144-m1
Runt-related transcription factor-2 (*RUNX-2*)	Transcription factor, bone marker (Gene of interest)	Hs00231692-m1
Bone gamma-carboxyglutamic acid-containing protein (BGLAP)/ Osteocalcin (*OC*)	Select calcium binding protein, bone marker (Gene of interest)	Hs00609452-g1
Insulin like growth factor-1 (*IGF-1*)	Naturally-occurring polypeptide protein hormone (Gene of interest)	Hs01547656-m1

Insulin like growth factor-2 (*IGF-2*)	Naturally-occurring polypeptide protein hormone (Gene of interest)	Hs04188276-m1
Insulin like growth factor-1receptor (*IGF-1R*)	Protein found on the surface of human cells (Gene of interest)	Hs00609566-m1
Insulin like growth factor-2 receptor (*IGF-2 R*)	Protein found on the surface of human cells (Gene of interest)	Hs00974474-m1
Insulin like growth factor binding protein-1 (*IGFBP-1*)	Carrier protein for IGFs (Gene of interest)	Hs00236877-m1
Insulin like growth factor binding protein-2 (*IGFBP-2*)	Carrier protein for IGFs (Gene of interest)	Hs01040719-m1
Insulin like growth factor binding protein-3 (*IGFBP-3*)	Carrier protein for IGFs (Gene of interest)	Hs00426289-m1
Insulin like growth factor binding protein-4 (*IGFBP-4*)	Carrier protein for IGFs (Gene of interest)	Hs01057900-m1
Insulin like growth factor binding protein-5 (*IGFBP-5*)	Carrier protein for IGFs (Gene of interest)	Hs00181213-m1

Insulin like growth factor binding protein-6 (*IGFBP-6*)	Carrier protein for IGFs (Gene of interest)	Hs00181853-m1
Vascular Endothelial Growth Factor Receptor (*VEGFR-2*)	Angiogenic marker (Gene of interest)	Hs00911700-m1
Platelet/ endothelial Cell Adhesion Molecule (*PECAM-1*)	Angiogenic marker (Gene of interest)	Hs01065279-m1
Toll- Like Receptor-2 (*TLR-2*)	Inflammatory marker (Gene of interest)	Hs01014511-m1
Toll- Like Receptor-4 (*TLR-4*)	Inflammatory marker (Gene of interest)	Hs00152937-m1

3.1.4 Western and ligand blot

Precision Plus protein dual colour standards #1610374, 10x Tris-buffered saline (TBS) #1706435, Mini-protean® 4 -15% (30 µl) TGX stain-free™ gels # 456-8083, Mini-protean® 12% (50µL) TGX stain-free™ gels #456-8044, Trans-Blot Turbo transfer pack 7x 8.5cm #1704156, Trans-Blot® Turbo transfer system (transfer pack mini format 69 BR007547) and ChemiDoc imager were all from BioRad, UK. Electrophoresis sample buffer 2% #Sc-45085 was from Insight Biotechnology, UK. β-mercaptoethanol #A4338 was from Application Panreac, UK. Tween® 20 #BPE337-500 was from Fisher Scientific Ltd, UK. Bovine albumin Faction V #160069 was from MP Biomedicals, UK. Tergitol solution type NP-40 70% solution #NP40S-1 and Corning gel-loading tips (0.2MM) #CLS4884-400EA were from Sigma-Aldrich, UK. Monoclonal mouse antibodies to hIGFBP-2 #MAB6741, hIGFBP-3 #MAB305, hIGFBP-4 #MAB8041, hIGFBP-5 #MAB8751 and hIGFBP-6 #MAB8761 were from R&D Systems, UK. Anti-mouse HRP conjugate secondary antibody #ab97046 was from Abcam, UK. Super-Signal® West Femto Maximum Sensitivity Substrates #34095 was from Fisher scientific, UK. Mono-biotinylated human IGF-2 receptor Grade #ABB-AM01 was from GroPep, Australia. Round gel-loading tips #I1022-0810 were from Starlab, UK.

3.1.5 Enzyme linked immunosorbent assay (ELISA)

Human IGFBP-3 Quantikine ELISA kit #DGB300, IGFBP-2 Duo Set #DY674 wash buffer #WA126, Reagent Diluent #DY004, normal goat serum #DY005, streptavidin- horseradish peroxidase (HRP) #890803, Substrate Solution: colour reagent A (H_2O_2), colour reagent B (Tetramethylbenzidine) #DY999 and stop solution -2 N H_2SO_4 #DY994 were from R&D Systems, UK. The 96 well ELISA plates #S1837-9600 were from Starlab, UK. Absorbance was determined with a Thermo-Scientific Varioskan Flash type 300 spectrophotometer.

3.1.6 Human inflammatory cytokines quantification

CBA Human Inflammatory Cytokines Kit #551811 was from BD Biosciences, UK.

A 4 Laser LSRII flow cytometer, (BD Biosciences) using 405nm violet laser, 488nm blue laser, 355nm UV and 640nm red laser excitations was used a high through-put plate arm (HTS) to run the cytokine bead array. Flow cytometry analysis software: Flow Cytometric Analysis Program (FCAP) Array software (BD Biosciences)

3.1.7 *In vitro* bioassay

p-Nitrophenol #100-02-7, Alkaline buffer solution #A9226-100mL, and p-Nitrophenyl Phosphate Liquid Substrate System # N7653 were from Sigma-Aldrich, UK.

3.1.8 Gene knockdown

IGFBP-2 shRNA plasmid #sc-37195-SH, control shRNA plasmid-A #sc-108060, copGFP control plasmid #sc-108083, Puromycin #CAY13884-25, shRNA plasmid transfection medium #sc-108062, shRNA plasmid transfection reagent #sc-108061 were all from Santa Cruz Biotechnology, UK. Electroporation cuvettes (0.2mm) #Ep-102 were from Cell Projects, UK. A Gene Pulser Xcell™ electroporation system was from Bio-Rad, UK. Axio observer research microscope was from ZEISS.

3.2 Methods

3.2.1 Isolation of dental pulp stromal cells

Healthy and carious third molar teeth were used to study the expression of osteogenic, angiogenic and inflammatory markers in addition to IGF axis components under basal conditions and during differentiation of dental pulp stromal/stem cells (DPSCs) into osteogenic lineages. Freshly extracted healthy and carious fully erupted third molars were collected from adult patients (20-40 years of age) at the out patients dental clinic of Leeds Dental Institute. Teeth were obtained through Leeds Dental and Skeletal tissue bank (LDI Research Tissue Bank; 130111/AH/75), with patients' informed consent. The age and gender of patients were recorded (Table 7). Carious lesions in this study was chosen based on the depth of the decay in the dentine layer, and assessment of this group was made during the sectioning of the teeth. Teeth with more than 2mm of sound dentine measured from the edge of carious lesion to the pulp tissue, were included in this study and

categorized as shallow caries [380, 381] (Figure 7A). This was assessed visually and by the use of a WHO periodontal probe (Figure 7B). External tooth surfaces were washed using sterile PBS and surrounding soft tissue attachments were removed using a sterile scalpel. External tooth surfaces were washed again with sterile PBS and were cracked open using a decontaminated vice to access the pulp tissue. Pulp tissue was gently separated by sterilized tweezers from the crown and root chambers, avoiding the apical third of the pulp tissue to prevent cross contamination with periodontal tissues. Isolated tissues were minced using a sterile scalpel before being digested in a solution of 5mL α-MEM, 3mg/mL collagenase type I and 4mg/mL dispase. The tissue- enzyme mixture was incubated for 1 h at 37° C and was continuously mixed using a shaker inside the tissue culture incubator. The tissue-enzyme mixture was regularly checked at 15 mins intervals to avoid over-digestion. The enzymatic reaction was stopped with 20% (v/v) FBS after complete dissociation of the pulp tissue. Cell pellets were obtained by centrifugation at 1000 g for 10 minutes. The supernatant was carefully aspirated and discarded. The pelleted cells were re-suspended in proliferation medium consisting of α-MEM, supplied with 20% (v/v) FBS, 100 unit/mL Pen Strep, and 200mM L-glutamine. The cell suspension was passed through a 70µm strainer and seeded into T-25 cm^2 flasks and 15cm Petri dishes. The cultures were incubated at 37°C and 5% CO_2 in proliferation medium.

Table 7: Details of all donors isolated during the current study

*** donors included and investigated in the current study**

Dental pulp stromal/stem cells isolated from healthy teeth (hDPSCs)

Dental pulp stromal/stem cells isolated from carious teeth (cDPSCs)

Cell type	Age	Gender	tooth Type
hDPSCs	11	Female	First molar
hDPSCs	11	Female	First molar
hDPSCs	9	Male	First molar
hDPSCs	40	Male	Third molar
hDPSCs	31	Female	Third molar
hDPSCs	31	Female	Third molar
hDPSCs	14	Female	Second premolar
hDPSCs	14	Female	Second premolar
hDPSCs	14	Male	Second premolar
***hDPSCs** (H2)	35	Female	Third molar
hDPSCs	35	Female	Third molar
hDPSCs	23	Male	Third molar
hDPSCs	9	Female	First molar
hDPSCs	19	Female	Second premolar

hDPSCs	19	Female	First premolar
hDPSCs	19	Female	First premolar
hDPSCs	18	Male	Third molar
hDPSCs	18	Male	Third molar
hDPSCs	18	Male	Third molar
hDPSCs	34	Male	Third molar
hDPSCs	23	Male	Third molar
hDPSCs	31	Female	Third molar
hDPSCs	22	Female	Third molar
***hDPSCs** (H1)	20	Female	Third molar
***hDPSCs** (H3)	24	Female	Third molar
***cDPSCs** (C1)	40	Male	Third molar
cDPSCs	40	Male	Third molar
cDPSCs	8	Female	First molar
cDPSCs	10	Male	First molar
cDPSCs	10	Male	First molar
cDPSCs	9	Male	First molar
***cDPSCs** (C3)	24	Female	Third molar

cDPSCs	24	Female	Third molar
cDPSCs	17	Female	First molar
cDPSCs	17	Male	First molar
cDPSCs	34	Male	Third molar
cDPSCs	34	Male	Third molar
***cDPSCs** (C2)	36	Female	Third molar
cDPSCs	36	Female	Third molar

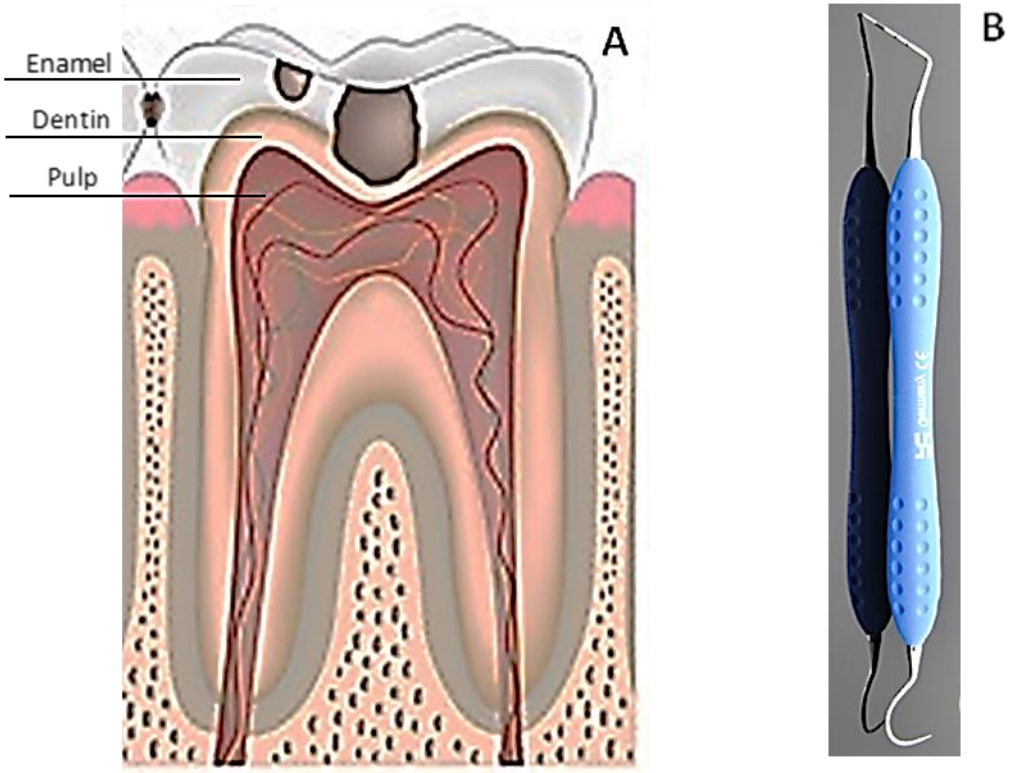


Figure 7 : Assessment of Dental Caries

Carious lesions in this study were chosen based on the depth of the decay in the dentine layer, and assessment of this group was made during the sectioning of the teeth. Teeth with more than 2mm of sound dentine measured from the edge of carious lesion to the pulp tissue, were included in this study and categorized as shallow caries [380, 381] .This was assessed visually and by the use of a WHO periodontal probe

A:Illustrates carious lesion affecting enamel and superficial dentine (Adapted from: http://patient.info/health/tooth-decay)

B: WHO probe used to measure the caries depth
(Adapted from: intelligentdental.com).

3.2.2 Cell culture and expansion

Isolated DPSCs from healthy (hDPSCs) and carious teeth (cDPSCs) were sub-cultured in T-175 cm^2 flasks, and media were changed every 5 days until cells reached 80% confluence. Monolayers were then washed with sterile cold PBS, and detached using 0.25% (w/v) Trypsin-EDTA solution for 5 minutes. Twenty percent FBS was added to neutralize the trypsin, then the cell suspension was transferred to a 50mL universal tube and centrifuged at 1100 g for 5 minutes [63]. The supernatant was discarded and the cell pellet was re-suspended in fresh proliferation media and cultured in the tissue culture flasks or plates according the experimental requirements. Cells were counted using a haemocytometer after staining with 0.01% (w/v) Trypan blue to exclude dead cells. Cells were seeded with the required densities according to the experimental plan.

3.2.3 Stem cell characterization

3.2.3.1 Colony Forming Unit Fibroblast (CFU-F) Assay

hDPSCs (n=3) and cDPSCs (n=3) were isolated and seeded in 15cm Petri dishes for 14 days, then fixed with absolute ethanol for 20 minutes after washing with PBS. Cells were stained with 10% (v/v) Trypan blue for 5 minutes and washed gently with distilled water to remove any unbound stains. Aggregates of 50 cells or more were defined as a colony. All colonies were counted and recorded under the light microscope.

3.2.3.2 Flow cytometric analysis of cell surface epitopes

Flow cytometric analysis was used to characterise the surface phenotypic profile of cDPSCs versus hDPSCs. For this purpose, hDPSCs (n=3) and cDPSCs (n=3) were expanded to passage 4, then $1X10^6$ cells were stained following the manufacturer's instructions. Required antibody concentration for each marker was determined by titration (Section 3.2.3.2.1). Cells were stained with Fixable Viability Stain (FVS 510) in sodium azide and protein-free Dulbecco's Phosphate Buffered Saline (1x DPBS) in 5mL FACS tube at 4^0C for 30-60 minutes in dark. One µL of FVS510 stock solution for each mL of cell suspension (1:1000) was then added. Cells were washed twice with 2mL of staining buffer (BSA) and centrifuged at 400 g for 5 minutes. After washing, 5µL of human Fc block was added per $1x10^6$ cells, which blocked the Fc receptors (receptors of immunoglobulin) to minimize non-specific binding. Fc receptors are found on the surface of some immune cells; the fragment crystallisable region (Fc region) is the tail region of an antibody that interacts with these receptors [382]. After this, cells were incubated for 10 minutes at room temperature and stained with the following pre-titrated fluorochrome-conjugated antibodies (Table 8): CD146/PE-Cy7, CD90/PerCP-Cy5.5, CD105/BV421, CD45/APC-Cy7, CD31/FITC, in 100µL of staining buffer (BSA) and incubated at 4º C for 30-60 minutes. Brilliant stain buffer was added (50µL/$1x10^6$ cells) to reduce the reagent interactions between the polymer based brilliant violet dyes, and to improve the staining quality when two or more dyes were used in the same experiment. Finally, the cells were washed twice in 2mL staining buffer and centrifuged (4°C, 400 g for 5 minutes). The cells were re-suspended gently in 500µL staining

buffer, and analysed on an LSRII digital flow cytometer. Single colour stained CompBeads were used for purpose of compensation (Section 3.2.3.2.2). Unstained cells, cells labelled with mouse IgG1 Isotype (Table 8) and fluorescence minus one (FMO) was used as a control for each stain (Section 3.2.3.2.3).

3.2.3.2.1 Antibody /Isotype titration

An important step in optimization of flow cytometry experiments is titration of antibodies and isotype controls to use both of them at a level that saturates all the binding sites for that specific antibody. This is to ensure appropriate enumeration of protein expression and to maximise resolution between differentially stained fractions. The isotype controls are antibodies (immunoglobulin, Ig) available in different classes (IgA, IgG, IgD, IgE, or IgM), and these are used as one of the control measures to show the degree of non-specific binding of the antibody of interest (test antibody). Both test antibody and its isotype control should be used with the same class of immunoglobulin, matched with the host species, conjugated to the same fluorochrome and used at the same concentration in the staining procedure. Following the standard protocol for the flow cytometry experiment including the use of viability dye and blocking steps, we stained a known amount of cells ($1X10^6$) with serial antibody and isotype control dilutions. Cells recommended by the company were used for the titration and they were known to express the surface markers that were included in this study (Figure 8). The degree of binding is defined by the relative median fluorescence shift of the given antibody concentration compared to the same

concentration of its corresponding isotype control (Figure 8).The antibody and isotype control dilutions that were used in this study are listed in (Table 8).

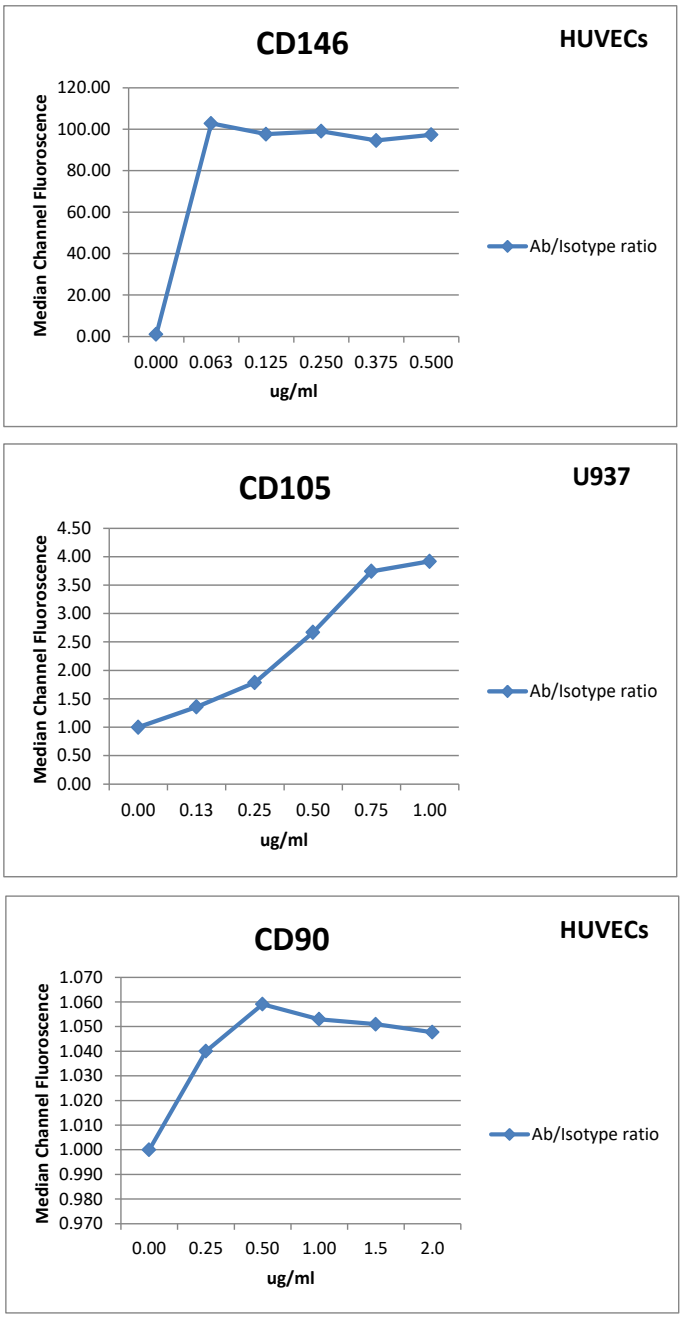


Figure 8: Antibody/Isotype control titration of positive stem cells markers

Saturation occurs at the point where the ratio of the median intensities of antibody and its isotype (antibody/isotype) is the greatest; known as the Relative Median Fluorescent Shift. HUVECS: human umbilical vein endothelial cord cells, U937: model cell line were isolated from the histiocytic lymphoma of a 37-year-old male patient

Table 8: List of titrated test antibodies and their corresponding isotype antibodies that were used in this study

Test Antibody	Dilutions	Isotype control	Dilutions	Supplier
PE-Cy7 Mouse Anti-Human CD146	1:40	PE-CY7 Mouse IgG1, κ	1:80	BD Biosciences
PerCP-Cy™5.5 Mouse Anti-Human CD90	1:20	PerCP-Cy™5.5 Mouse IgG1, κ	1:40	BD Biosciences
BV421 Mouse Anti-Human CD105	1:5	BV421 Mouse IgG1, κ	1:6	BD Biosciences
APC-Cy7 Mouse Antihuman CD45	1:20	APC-Cy7 Mouse IgG1, κ	1:20	BD Biosciences
FITC Mouse Anti-Human CD31	1:4	FITC Mouse IgG1, κ	1:4	BD Biosciences

3.2.3.2.2 Compensation controls

Compensation is as a mathematical process by which we correct multi-parameter flow cytometric data for spectral overlap (spill over). Spill over occurs whenever the fluorescence emission of one fluorochrome is detected in a detector designed to measure signals from another fluorochrome [383]. To correct this spectral overlap, a single-stained (SS) sample was run on the flow cytometer for the viability dye and CompBeads were run for other fluorochrome-labelled antibody (Table 9). CompBeads set provides two populations of polystyrene micro particles, which are used to optimize fluorescence compensation setting for multicolour flow cytometric analysis; the CompBeads anti-Mouse Ig, κ particles which bind any mouse κ light chain-bearing immunoglobulin, and the CompBeads negative control, which has no binding capacity. When mixed together with fluorchrome-conjugated mouse antibody, the CompBeads provide a clear bright and negative stained populations to suitably determine the spectral overlap for each given antibody marker without using valuable tissue samples. Compensation controls allow to investigate the bleeding of one dye on all other dyes. Compensation settings were adjusted until only a single colour was seen in each channel and no spill over of one colour into another colour's channel was detected .

Table 9: Single-stained (SS) compensation controls for flow cytometry

	PE-Cy7 CD146	PerCP-Cy™5.5 CD90	BV421 CD105	APC-Cy7 CD45	FITC CD31	Fixable viability dye 510
Unstained	X	X	X	X	X	X
SS-CompBeads	✓	X	X	X	X	X
SS-CompBeads	X	✓	X	X	X	X
SS-CompBeads	X	X	✓	X	X	X
SS-CompBeads	X	X	X	✓	X	X
SS-CompBeads	X	X	X	X	✓	X
SS- DPSCs	X	X	X	X	X	✓

✓ : Included in the experiment

X : not included in the experiment

3.2.3.2.3 Fluorescence minus one (FMO) controls

Fluorescence minus one (FMO) is one of the control measures used to optimise flow cytometry experiments. In this method, cells were stained with all fluorochromes that were used in the experiment except one (Table 10), in order to determine the exact range of the negative population for this particular fluorochrome [383]. This identifies the gate where the negative data spread due to the multiple fluorochromes in the panel used in this study. In the FMO controls, one specific fluorochrome from the panel was excluded each time and replaced it by its corresponding fluorochrome-conjugated isotype control (Table 10). The corresponding isotype control was used to determine the background of non-specific binding in the presence of other cell phenotype markers which allows accurate determination of cell populations of interest.

Table 10: Fluorescence minus one (FMO) controls for flow cytometry

FMO control	PE-Cy7 CD146	BV421 CD105	PerCP-Cy™5.5 CD90	APC-Cy7 CD45	FITC CD31	Fixable Viability Stain 510
Tube 1 Unstained	**-------**	**-------**	**----------**	**---------**	**-------**	**---------**
Tube 2 PE-Cy7 FMO	**PE-Cy7 relative isotype**	CD105	CD90	CD45	CD31	FVS 510
Tube 3 BV421 FMO	CD146	**BV421 relative isotype**	CD90	CD45	CD31	FVS 510
Tube 4 PerCP-Cy5.5 FMO	CD146	CD105	**PerCP-Cy5.5 relative isotype**	CD45	CD31	FVS 510
Tube 5 APC-Cy7 FMO	CD146	CD105	CD90	**APC-Cy7 relative isotype**	CD31	FVS 510
Tube 6 FITC FMO	CD146	CD105	CD90	CD45	**FITC relative isotype**	FVS 510
Tube 7 Fixable viability stain 510 FMO	CD146	CD105	CD90	CD45	CD31	**---------**

3.2.4 Osteogenic differentiation of DPSCs

hDPSCs and cDPSCs at passage 4 were cultured in 6-well plates at $1X10^5$ cells/well under basal conditions (α-MEM supplemented with 20% (v/v) FBS, 200mM L-glutamine, and 100unit/mL Pen Strep). When the cells reached 80% confluence, they were cultured in triplicate under basal or osteogenic conditions (basal medium + 10nM dexamethasone and 100μM of L-ascorbic acid). Cultures were terminated at 1 and 3 weeks for further investigation of changes in gene expression using qRT-PCR for detection of relative changes in the expression of osteogenic, angiogenic, and inflammatory markers, as well as changes in the IGF axis gene expression. Histological staining (Alkaline Phosphatase (ALP) and Alizarin red stains), as well as IGF protein expression were also investigated. Experiments were performed on cells derived from three healthy and three carious donors and triplicate wells were used for each time point and each culture condition; basal and osteogenic.

3.2.5 Quantification of gene expression using quantitative real time polymerase chain reaction (qRT-PCR)

3.2.5.1 Extraction of mRNA from hDPSCs and cDPSCs cultured in monolayers under basal and osteogenic conditions

hDPSCs and cDPSCs cultured for 1 and 3 weeks under basal and osteogenic conditions were detached as described above and counted to determine the amount of cell lysis buffer required. mRNA extraction was performed according to manufacturer's instructions using Qiagen's RNAeasy® min kit summarised in Table 11.

3.2.5.2 mRNA quantification

A NanoDrop spectrophotometer (ND 1000) was used to quantify the yield and purity of mRNA. Two µL of the extracted mRNA was used and quantities were recorded as ng/µL. A260/280 ratios were also recorded as an indication of mRNA purity and were typically 1.8 - 2.0.

Table 11: Steps of RNA extraction using RNAeasy mini kit

<table>
<tr><th>Reagents</th><th colspan="2">The amount needed to perform the reaction</th></tr>
<tr><td rowspan="4">Buffer RLT
1- Add sufficient buffer RLT and mix.
2- Transfer lysed cells to nuclease free Eppendorf and mix on the vortex for 1 min.
3- Add 70% ethanol at 1:1 (v/v) with buffer RLT
4- Transfer up to 700µL of the mix to RNeasy spin column placed in 2mL collection tube</td><td>Number of pelleted cells</td><td>Volume of buffer RLT (µL)</td></tr>
<tr><td>< 5 X 10^6</td><td>350</td></tr>
<tr><td>5 X 10^6 to 1 X 10^7</td><td>600</td></tr>
<tr><td>More than 1 X 10^7</td><td>Not suitable</td></tr>
<tr><td>5- Add RW1, centrifuge, and discard the flow through.</td><td colspan="2">700µL</td></tr>
<tr><td>6- Add buffer RPE, centrifuge, and discard the flow through.
7- Repeat step no. 6, and use new collection tube.</td><td colspan="2">500µL</td></tr>
<tr><td>8- Add nuclease free water, centrifuge and collect the RNA elutes in a new tube.</td><td colspan="2">30µL</td></tr>
</table>

~~3.2.5.2~~3.2.5.3 DNase treatment

To ensure that the mRNA was pure and clear of any genomic DNA, DNase I amplification grade kit was used according to the manufacturer's instructions. Briefly, a mixture of DNase I buffer, enzyme, and mRNA sample (up to 1µg) were mixed in a 10µL reaction volume and incubated at room temperature for 15 minutes. The reaction was stopped by adding 1µL EDTA (25 mM) to the mix, then the mix is incubated at 65°C for 10 minutes in the PTC-100 thermal cycler.

~~3.2.5.3~~3.2.5.4 Reverse transcription

Reverse transcription was carried out to generate single stranded cDNA from the mRNA by preparing 20µL reaction volume using the ABI high capacity RNA to c-DNA kit. Briefly, 10µL of buffer were added to 1µL of enzyme, and 9µL of mRNA sample, and then incubated in the PTC-100 thermal cycler for 1 hour at 37°C followed by 5 minutes at 95°C. Negative controls for the experiment were generated during this step, by removing the mRNA template in one reaction and reverse transcriptase enzyme in the other.

~~3.2.5.4~~3.2.5.5 qRT- PCR

Quantitative real time PCR was performed using a Roche LC480 light cycler. The experiment was performed in a 20µL reaction volume composed of 10µL gene expression master mix, 1µL Taqman gene expression assay specific for each gene, 8µL nuclease free water and 1µL c-DNA sample. The

20µL mix was added into each well of 96 well PCR reaction plate in triplicate. In addition, triplicates of non-template negative control and RT negative control were included in each plate. The plate was sealed securely and centrifuged for 10 second before starting the amplification procedure using the light cycler. Amplification was carried out according to Applied Biosystems' (AB) recommendations for Taqman® probes (Figure 9).

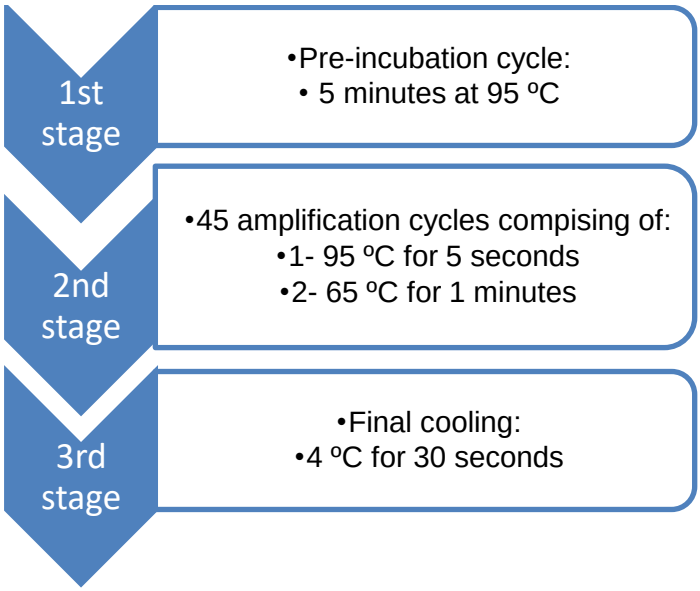


Figure 9: qRT-PCR amplification program

recommended by Applied Biosystems for the Taqman® probes

~~3.2.5.5~~3.2.5.6 Data analysis

For each gene, relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [384, 385]. In brief, the threshold cycle (Ct) value was determined for each gene of interest in triplicate. Technical replicates were averaged, then normalized to that of the house-keeping gene GAPDH. This is referred to as the ΔCt value. The ΔΔ Ct values were determined by normalizing the ΔCt value for each treated sample (under osteogenic conditions) to the appropriate control samples (under basal conditions) at each time point to be able to determine the relative changes in gene expression of interest (osteogenic, angiogenic, inflammatory and IGF axis) following osteogenic induction of hDPSCs and cDPSCs. The relative changes in gene expression were calculated using the $2^{-\Delta\Delta ct}$ equation. In all cases, the $2^{-\Delta\Delta ct}$ values for all markers were compared in both hDPSCs and cDPSCs. Changes in the gene expression levels were plotted as $2^{-\Delta\Delta ct} \pm SD$.

~~3.2.5.6~~3.2.5.7 House-keeping gene optimization

The selected house-keeping gene (HKG) should have constant expression regardless the changes in cell culture conditions. Expression of GAPDH, a commonly used HKG, was measured under basal and osteogenic culture conditions at different time points. Data were analysed using Students' t-test and P-values were determined (Figure 10). No statistically significant differences were found between all samples at different time points and under different culture conditions, which indicated that GAPDH was a

suitable HKG for this study; because it did not undergo any changes in response to different culture conditions or time points (Figure 10).

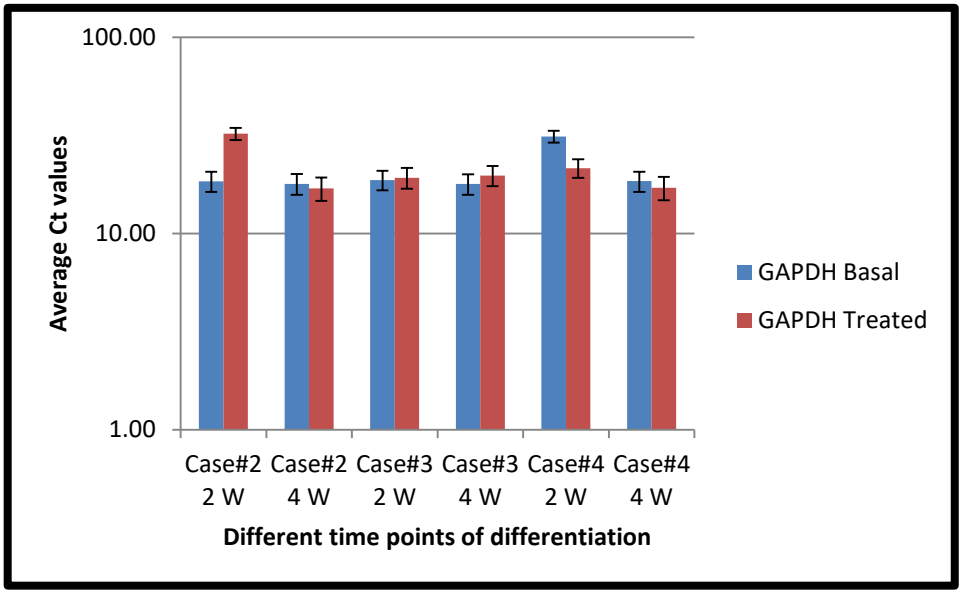


Figure 10: Validation of GAPDH as house-keeping gene

GAPDH expression was analysed under basal and osteogenic conditions for different samples; no significant difference in GAPDH expression was apparent after osteogenic differentiation. P-value = 0.83

3.2.6 Protein expression

hDPSCs and cDPSCs were cultured under both basal and osteogenic conditions for 1 and 3 weeks as described previously, changing media was performed once every week. One mL of conditioned medium was collected from each medium change, and was either freeze-dried and stored at -20ºC or directly stored at -80ºC without freeze-drying.

3.2.6.1 Optimization of protein detection method:

Western blotting

In western blotting, 1mL of medium conditioned by hDPSCs cultured under basal and osteogenic conditions was collected, and freeze-dried. Freeze-drying is a process used to remove the water from the sample by freezing then drying it under a vacuum at very low temperatures. Powdered media were then dissolved in 1X SDS-polyacrylamide (SDS-PAGE) sample buffer containing β-mercaptoethanol (1:20, v/v) and loaded onto 15% (w/v) SDS-polyacrylamide pre cast gels (30µL /well). Suitable molecular weight markers (5µL of dual colour standards, 10 -250 kDa) were loaded in a lane next to the samples to be able to determine molecular weight of the target proteins. Gels were run at 120V for 1 hour then proteins were transferred to PVDF semi dry membranes using the Trans-Blot Turbo device (BioRad) for 10 minutes. After blotting, the membrane was rinsed three times with distilled water (dH_2O) and incubated for 1hour with gentle agitation at room temperature in blocking solution, which comprised 5% bovine serum albumin (BSA) in Tris-buffered saline containing Tween-20 (TBS-T, 0.05%, v/v). After

blocking, the membrane was washed three times for 5 minutes using 0.05% (v/v) TBS-T then incubated overnight at room temperature with one of the following primary antibodies: anti- IGFBP-2, anti- IGFBP-3, anti- IGFBP-4, anti-IGFBP-5 or anti-IGFBP-6 reconstituted (1:1000, v/v) in 0.05% (v/v) TBS-T. Next day, the membrane was washed three times for 5 minutes using 0.05% (v/v) TBS-T then incubated with the appropriate streptavidin horseradish peroxidase (HRP) conjugated secondary antibody reconstituted (1:10000, v/v) in 5% (w/v) BSA in 0.05% (v/v) TBS-T for 1 hour with gentle agitation at room temperature. The membrane was washed three times, each for 15 minutes using 0.05% TBS-T, and was developed with ultra-sensitive enhanced chemiluminescent (ECL) substrate (A and B, 1:1 v/v) and images were obtained and recorded on the ChemiDoc imager.

Ligand blotting

For ligand blotting, 1mL of medium conditioned by hDPSCs cultured under basal and osteogenic conditions was collected, and freeze-dried. Freeze-dried medium was re-suspended in non-reducing SDS-PAGE sample buffer. Electrophoresis and blotting were performed as described in the previous section for Western blot analysis. After the blotting step, the membrane was incubated; first in 3% Tergitol solution type NP40 in TBS for 30 minutes, and incubated in blocking solution (3% (w/v) BSA in TBS) for 2 hours. Lastly, the membrane was incubated in 0.1% (v/v) TBS-T for 10 minutes with gentle agitation at room temperature. After that, the membrane was incubated overnight at 4°C with 20ng mono-biotinylated IGF-2 in 1mL of 1% (w/v) BSA and 0.1% (v/v) TBS-T. Next day, the membrane was washed three times,

each for 15 minutes in 1% (w/v) BSA in 0.1% (v/v) TBS-T. The membrane was then incubated in streptavidin-HRP reconstituted (1:2000, v/v) in 1% (w/v) BSA in 0.1% (v/v) TBS-T for 1 hour with gentle agitation at room temperature. Finally, the membrane was washed three times, each for 15 minutes in 0.1% (v/v) TBS-T then developed using ECL substrate (A and B, 1:1 v/v) and images were obtained and recorded on the ChemiDoc imager.

Enzyme-linked immunosorbent assay (ELISA)

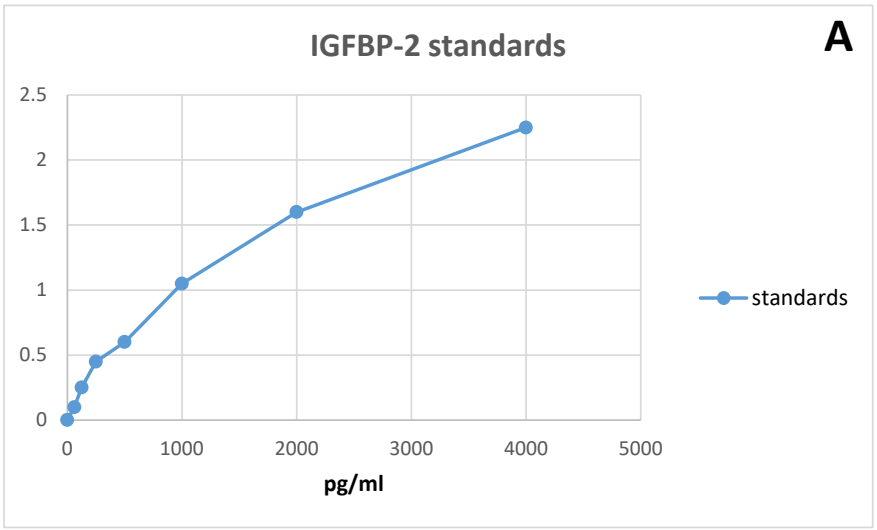
hDPSCs and cDPSCs were grown under basal and osteogenic conditions for 1 and 3 weeks. Conditioned medium (1mL) was collected from each culture at 1 and 3 week time points under basal and osteogenic conditions. IGFBP-2 and IGFBP-3 concentrations in conditioned media were determined by ELISA using human IGFBP-2 Duo Set ELISA kit and human IGFBP-3 Quantikine ELISA Kit according to the manufacturer's protocol. Briefly, for IGFBP-2, diluted capture antibody (mouse anti-human IGFBP-2) at 2µg/mL in PBS was plated in 96 well microplate (100µL/well) and incubated overnight at room temperature. On the following day, the plate was washed with 300µL of 1:25 (v/v) diluted washing buffer (0.05% (v/v) Tween-20 in PBS) four times. Plates were blocked using 300µL of the 1:5 (v/v) reagent diluent (5% (v/v)Tween -20 in PBS, 0.2µm filtered) and incubated for 1 hour at room temperature. After washing four times with 400µL washing buffer, 100µL of the samples, and appropriately diluted standards (representative standard curve is shown in Figure 11A) were added to the 96 well plate and incubated for 2 hours at room temperature. Plates were washed as above and 100µL of the detection antibody (biotinylated goat anti-human IGFBP-2)

were added at 200ng/mL in reagent diluent with 2% (v/v) heat activated goat serum for 2 hours at room temperature. After washing, 100µL of working dilution of streptavidin-HRP (1:200, v/v) was added for 20 minutes and plates were incubated at room temperature in the dark. Finally the plates were washed and 100µL of substrate solution (colour reagent A: H_2O_2 and colour reagent B: Tetramethylbenzidine, 1:1 v/v) were added and the plate was incubated for 20 minutes at room temperature in the dark. 50µL of stop solution (2 N H_2SO_4) was added and absorbance was determined immediately using a microplate reader (Thermo-Scientific Varioskan Flash type 300 spectrophotometer) at 450nm.

For IGFBP-3, the Quantikine ELISA kit was used. The assay was performed following the manufacturer's instructions. In brief, 100µL of assay diluent RD1-62 (buffer with blue dye) was plated in a 96 well plate. Next, 100µL of the samples, and appropriately diluted standards (recombinant human IGFBP-3 in buffer) in calibrator diluent RD5P (buffered protein base) were added to the assay diluent RD1-62 and incubated for 2 hours at 2-8ºC, representative standard curve is shown in (Figure 11B). The plate was washed with 400µL of 1:25 (v/v) washing buffer (buffered surfactant) three times. Then 200µL of chilled IGFBP-3 polyclonal anti-IGFBP-3 HRP conjugate was added per well and incubated for 2 hours at 2-8ºC. The plate was washed as above and 200µL of substrate solution were added per well (colour reagent A: stabilized H_2O_2, colour reagent B: stabilized Tetramethylbenzidine, 1:1, v/v) and incubated for 30 minutes at room temperature in the dark. Finally, 50µL of stop solution (2 N H_2SO_4) was

added and absorbance was determined immediately using a microplate reader at 450nm.

There was evidence of serum interference in immunoblotting techniques, which reflects the relative lack of sensitivity of such techniques in this study. Therefore, ELISA was used for any further investigation of protein level, to be able to calculate the accurate protein concentration as ELISA was more accurate with high throughput.



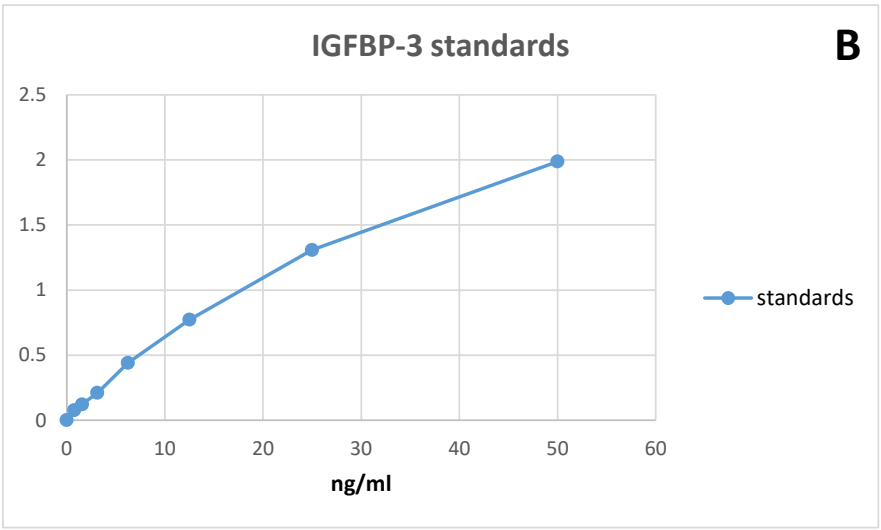


Figure 11: Representative standard curves for standards used in ELISA quantitative analysis of IGFBPs

(A) IGFBP-2 standard curve

(B) IGFBP-3 standard curve

3.2.6.2 Inflammatory cytokine assay

The BD ™ CBA Human Inflammatory cytokines kit was used to quantify the following cytokines; interleukin-8 (IL-8), interleukin- 1β(IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), tumour necrosis factor (TNF), and interleukin-12p70 (IL-12p70) protein levels in media conditioned by hDPSCs and cDPSCs grown under basal and osteogenic conditions for 1 and 3 weeks. This assay was performed in 96 well plates following the manufacturer's protocol. Briefly, the mixed capture beads were vortexed, and then 50µL were added to each well. The standard dilutions were added (50µL) to the control wells, representative standard curves are shown in Figure 12. Likewise, 50µL of each of our samples (basal and osteogenic conditioned media) from both hDPSCs and cDPSCs were added to the 96 well plate followed by 50µL of the PE detection reagent. The plate was incubated for three hours, at room temperature in the dark. After that, the plate was washed twice using 250µL of washing buffer (centrifuged at 400 g for 2 minutes). Finally, 120µL of washing buffer was added to re-suspend the beads and the plate was ready for acquisition on the flow cytometer (LSR II 4 Laser). The data were analysed using flow cytometry analysis program (FCAP) array software.

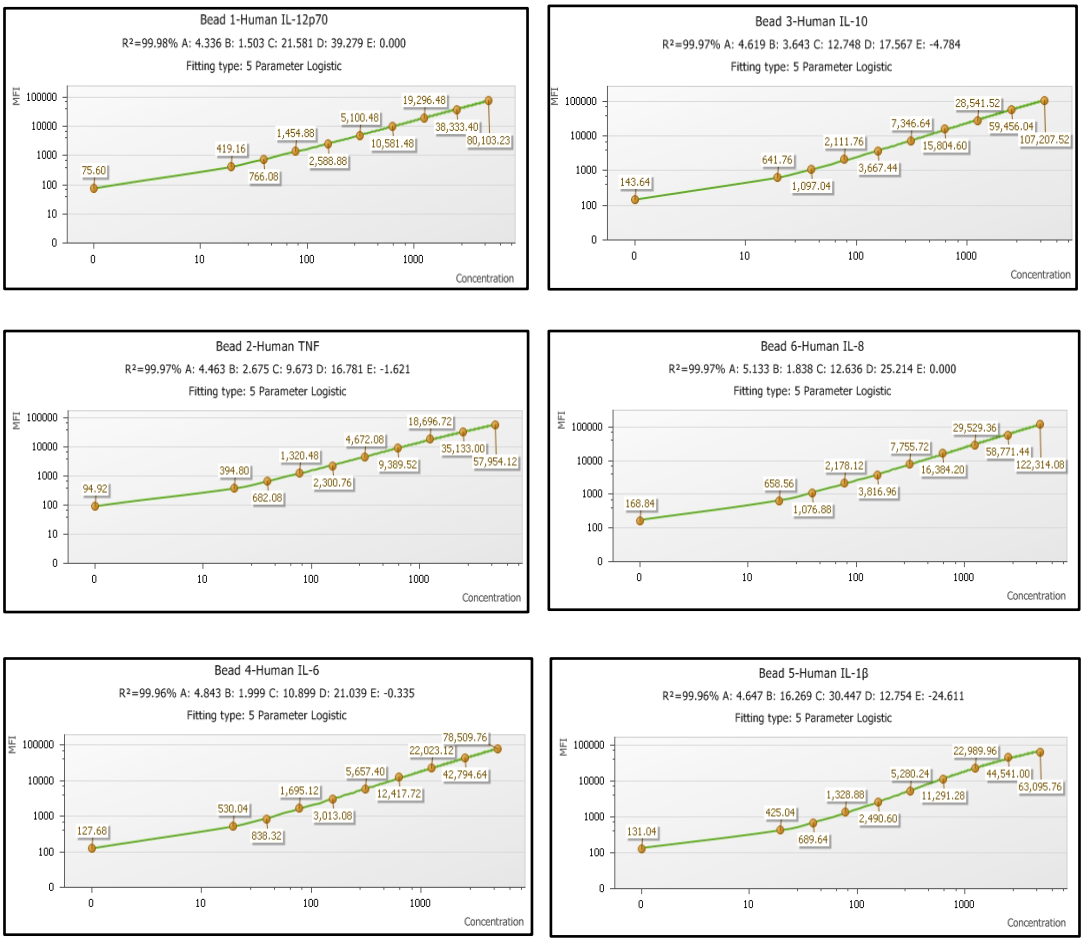


Figure 12: Representative standard curves for standards used in CBA quantitative analysis of (IL12p70, TNF, IL-10, IL-6, IL-8, IL-1β) as labelled

Data were generated using flow cytometer LSRII 4 Lasers, BD Biosciences equipped with 4 lasers (405nm violet laser, 488nm blue laser, 355nm UV laser and 640nm red laser) and analysed using flow cytometric analysis program (FCAP) Array software (BD Biosciences)

3.2.7 *In vitro* bioassay

hDPSCs were grown to 80% confluence and incubated in osteogenic medium (Section 3.2.4) with a fixed concentration of IGFBP-2 and IGFBP-3 at (10nM), and varying concentrations (0nM, 1nM, 10nM, 100nM) of IGF-1 or IGF-2. Medium was changed at day 4, 7, 10 and cultures were terminated at day 14. ALP enzyme activity has been examined as an osteogenic marker. Alkaline phosphatase activity was measured by the ability of alkaline phosphatase enzyme to convert the colourless substrate p-nitrophenylphosphate (pNPP) into yellow p-nitrophenyl (pNP) as previously described [386]. Briefly, cells were washed twice with PBS, and lysed by 200µL of 0.1% (v/v) Triton™x-100 followed by three cycles of freezing and thawing. Lysates were centrifuged (1000g) for 5 minutes and 20µL of the supernatants were used in ALP activity assay. Standards were prepared by diluting pNP in buffer, provided with the pNPP substrate as a kit, at concentrations of 5, 25, 50, 100, 150 and 200 nmol/mL. A 100µL of each standard was added to the wells of a 96 well plate (n=3). A 90µL of pNPP substrate was then added to 10µL of sample lysates in triplicates in 96 well plates. The plate was then incubated at 37 ºC for 30 minutes in the dark. The reaction was stopped by adding 100µL of 1M NaOH. The sample absorbance was measured at 405nm using a microplate reader. Alkaline phosphatase specific activity was expressed as nmoles of p-Nitrophenol/µg DNA of the sample.

3.2.8 Cell transfection

3.2.8.1 Puromycin optimisation

A puromycin selection system was used to select stably transfected hDPSCs. hDPSCs were seeded at a concentration of $1x10^5$ cells/well in 6 well plate in antibiotic free growth medium one day prior to addition of puromycin (experiments were done in duplicate). Freshly prepared medium containing puromycin at 0-10 µg/mL was added to 80% confluent cells in duplicate wells. Medium was replaced every three days for up to a week. Cultures were examined by light microscopy for signs of cell death to determine the lowest puromycin concentration, which resulted in 100% cell death. This concentration was subsequently used in selection of stably transfected cells.

3.2.8.2 Lipid- based transfection

Small or short hairpin RNA (shRNA)- based strategy has been used attempting to knock down the IGFBP-2 expression in hDPSCs, which will be discussed later in this study (Chapter 7). Dental pulp stromal/stem cells were isolated from healthy pulp tissue, seeded at passage 4 at $1x10^5$ cells/mL in 6 well plates in proliferation medium (α-MEM, 20% (v/v) FBS, 200mM L-glutamine, 100 unit/mL Pen Strep) and grown under basal conditions until cells reached 70% confluence. Cells were transfected according to the manufacturer's protocol (Santa Cruz Biotechnology, Inc). Briefly, cells were washed with 1mL of transfection medium and incubated for 6 hours with

either negative control (control shRNA plasmid) or IGFBP-2 shRNA plasmid in the presence of 0.5-3% (v/v) lipid based transfection reagents and transfection medium in a final volume of 1mL. After 6 hours, 1mL of antibiotic free- proliferation medium was added to each well and incubation continued overnight. Next day, media were replaced with 2mL of antibiotic free-proliferation medium containing the selected concentration of puromycin. Media were changed every 2 days. Dead cells were removed with medium changing, and puromycin resistant cells should have been able to grow for 3-4 weeks. Unfortunately, successful transfection for hDPSCs was not achieved and no viable living cells were observed even after 4 weeks.

3.2.8.3 Electroporation- based transfection

As an alternative to the lipid based transfection, electroporation based methodology was used. Electroporation creates transient pores in the cellular membrane that allow nucleic acid to pass into the cells [387]. The protocol was adapted from a previous study, which optimised the electroporation conditions for transfection of dental pulp stem cells [388]. Briefly, hDPSCs were isolated from healthy pulp tissue, seeded at passage 4 in a T-175 flask in proliferation medium under basal conditions until cells reached 70% confluence. Cells were passaged, counted and $1X10^6$ cells/mL were brought together with either positive control (copGFP control plasmid), negative control (control shRNA Plasmid) or IGFBP-2 shRNA plasmid in electroporation buffer (α-MEM medium without any additives). The transfection mix was transferred to the 0.2mm electroporation cuvettes then exposed to 100V for 20msec using a one-pulse square- wave method.

Electroporation was performed using a Gene Pulser Xcell main unit. After 24 and 48 hours, the positive control cells (copGFP control plasmid) were imaged using an Axio Observer Research microscope.

3.3 Statistical analysis

All experiments were carried out in triplicate from three different donors in each group; hDPSCs and cDPSCs. Flow cytometry, qRT- PCR, and ELISA results were analysed using Students' t-test and one way analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests. P-values were determined, and $P < 0.05$ was considered significant. The statistical analyses were carried out using the Graph Pad Prism software (v 6).

Chapter 4: Results

Dental pulp stem cells in healthy and carious teeth

4.1 Introduction

Stem/progenitor cells have been identified and isolated from dental pulp. However, it is not clear if pulp cells isolated from carious teeth (cDPSCs) express stem cell markers and whether they retain the same regenerative abilities as the well characterised dental pulp stem cells isolated from healthy teeth (hDPSCs) [3, 151, 157, 389-392]. A number of mesenchymal stem cell (MSC) surface markers have been reported in the literature (Table 12) and the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy have suggested a set of standards to define human MSCs for both laboratory-based scientific investigations and pre-clinical studies. They defined the MSCs as *cells able to grow as adherent cells, differentiate into bone, cartilage and adipose cells, and express the stem cell surface markers including CD105, CD73 and CD90, but not hematopoietic or endothelial markers such as CD45, CD34, CD11b and CD19* [393-395]. Putative MSCs were first isolated from dental pulp tissue and partially characterized in 2000 [151]. Subsequent studies demonstrated that dental pulp cells possessed stem cell properties, including the expression of known stem cell surface markers, adhering to plastic and the ability to differentiate into multiple lineages [3, 393, 396]. Interestingly, studies also noted the high proliferative capacity of such cells isolated from third molars (also the source of dental pulp cells described in

this current study) with the ability to undergo osteogenic and chondrogenic differentiation. However, no cartilage tissue formation has been reported [390]. Adipogenic differentiation of hDPSCs has also been observed under appropriate conditions [160, 390, 391] although some reports suggested that this effect is less pronounced [151, 389]. Dental pulp stem cell populations showed comparable properties to those of MSCs, including the ability to self-renew and multi-lineage differentiation capability although with higher potential toward odontogenic lineages and dental pulp regeneration [397]. The broad capacity for differentiation of DPSCs could be related to their developmental origin, which includes neural crest derived cells [398], and complete pulp regeneration has been reported in pulpectomised adult canine teeth after transplantation of autologous pulp stem/progenitor ($CD105^{+}$) cells with stromal cell-derived factor-1 (SDF-1) into a root canal [399].

Dental caries is one of the most common oral diseases and is characterized by pulp inflammation due to bacterial infection. In contrast to healthy dental pulp, there are few data regarding the properties of stem cells isolated from cDPSCs. However, the dental repair process occurring following carious lesions confirm the presence of a stem cell population in the pulp tissue [400]. This has led to an ongoing interest in cDPSCs especially with regard to distinctive stem cell characteristics and whether or not they retain tissue regeneration potential. Previous studies have demonstrated that DPSCs isolated from deeply carious teeth retain the characteristics of mesenchymal stem cells, including self-renewal proliferation and multi-lineage differentiation capability [175, 177]. Alongi *et al.* (2010), isolated DPSCs from teeth affected with irreversible pulpitis which showed higher levels of stem

cell markers STRO-1, CD90 and CD146 compared to DPSCs isolated from unaffected teeth using immunohistochemical analysis, while CD146 demonstrated moderate to high expression levels in both DPSCs isolated from non-inflamed and inflamed dental pulps using flow cytometry analysis [175]. In a later study, DPSCs were isolated from third molars affected by deep caries and the phenotype pattern was compared with DPSCs isolated from unaffected third molars using flow cytometry. Flow cytometry analysis indicated higher expressions of STRO-1, CD90, CD105 and CD29 in cDPSCs when compared to hDPSCs [177].

The present study was designed to investigate the changes in stem cell characteristics including expressed stem-cell markers, clonogenic ability and osteogenic differentiation of dental pulp stromal/stem cells isolated from teeth with shallow caries. Investigations of the characteristics of cDPSCs will give us information about the ability of cDPSCs to be involved in regeneration therapies. Three donors were studied in each group. hDPSCs were isolated from fully erupted third molars without caries or pulp disease, while the cDPSCs were isolated from teeth with shallow caries.

Table 12: Selection markers for mesenchymal stem cells

Positive	Biological role	References
CD146	Stem cell marker including MSCs derived from periodontal ligament, bone marrow, placenta and adipose tissue. Correlates with multi-potency and clonogenic ability.	[401], [402], [403], [404], [405], [392], [406], [3], [151]
CD90/Thy1	Wound repair, cell-cell and cell-matrix interactions.	[407], [408], [393], [392], [3]
CD105/Endoglin	Cell adhesion molecule, vascular homeostasis.	[407], [408], [393], [392], [3]
STRO-1	Putative stem cell marker. Successfully used to enrich CFU-Fs from human bone marrow	[407], [392], [406], [3]
CD73	Cell adhesion molecule. Expressed in mesenchymal stem cells	[408], [393], [409], [3]
Negative	**Used to exclude**	**References**
CD45	Leukocytes	[407], [408], [393], [392], [406], [3], [151]
CD31	Endothelial cells	[407], [408],
CD34	Primitive hematopoietic cells and endothelial cells	[408], [393], [392], [151]
CD11b and CD14	Monocytes and macrophages	[408], [393]
CD79 alpha and CD19 alpha	B cells	[393]

4.2 Results

4.2.1 Colony forming unit fibroblast assay (CFU-F) in dental pulp cells isolated from carious versus healthy teeth

Colony forming units (CFUs) were counted in primary basal cultures of hDPSCs (n=3) and cDPSCs (n=3) isolated from third molars. Colonies were identified as clusters of 50 cells or more. DPSCs derived from healthy and carious teeth showed the ability to form colonies. hDPSCs showed an average of 60 ±10 colonies, whereas cDPSCs showed an average of 100 ±7.6. cDPSCs displayed a significant increase in CFUs when compared to hDPSCs (Figure 13) suggesting an increased colony forming efficiency in these cells.

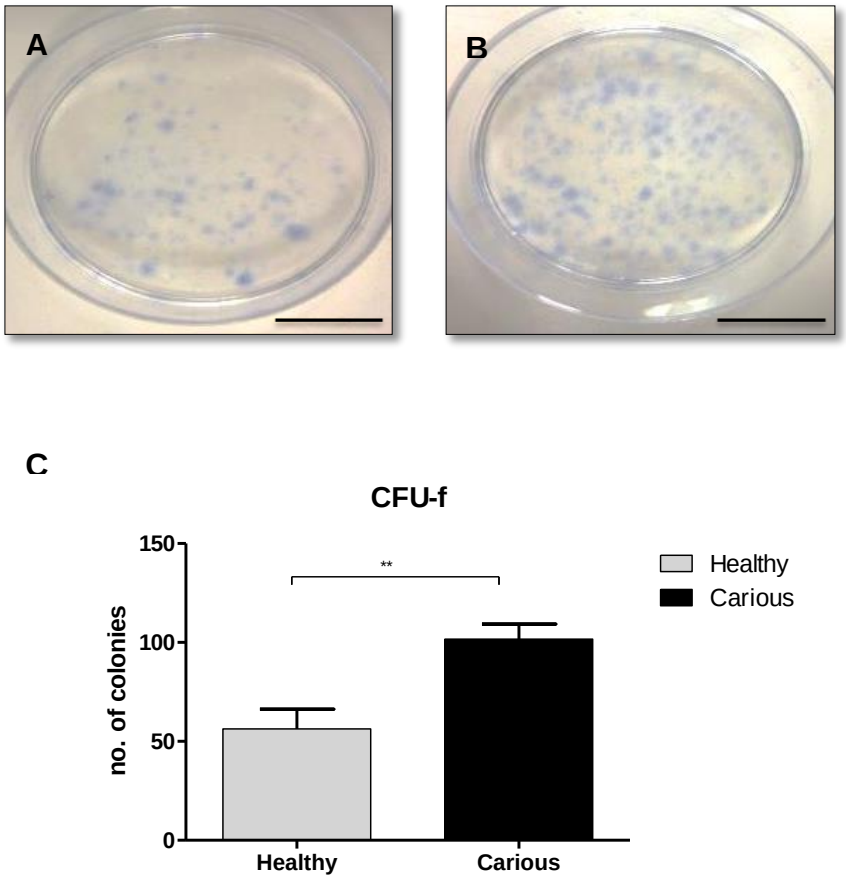


Figure 13: Colony Forming Unit Fibroblast assay (CFU-F)

An image showing colony forming units arising from hDPSCs (A) and cDPSCs; (B) after 14 days of basal culture, cells were stained with 10% Toluidine blue. Scale bar = 5 cm. (C) Comparing the number of colony units formed by DPSCs derived from 3 healthy and 3 carious teeth. The data are presented as the mean of biological replicates (n=3) ±SD. Data were analysed using Student's t test (P =0.0034).

4.2.2 Expression of stem cell surface markers in dental pulp cells isolated from carious versus healthy teeth

Flow cytometric analysis was used to characterize the surface phenotypic profile of cDPSCs versus hDPSCs. hDPSCs and cDPSCs were cultured at passage 4 under basal conditions (α-MEM, supplied with 20% (v/v) FBS, 100 unit/ml Pen Strep, and 200mM L-glutamine) then subjected to flow cytometric analysis to investigate the expression of stem cell surface markers on both hDPSCs and cDPSCs. The expression of putative stem cell markers was investigated in both cell types. Positive stem cell markers included CD146 (melanoma cell adhesion molecule, MCAM), CD90 (Thy-1, cell adhesion molecule), CD105 (Endoglin, cell adhesion molecule) and negative stem cell markers included CD45 (haematopoietic cell marker, leukocyte common antigen) and CD31 (platelet endothelial cell adhesion molecules, PECAM-1) (Table 12). An example of the gating strategy that has been used in the analysis of the collected data is demonstrated in Figure 14.

Fluorescence minus one (FMO) with antibody isotype controls were used as negative control in the analysis presented in this study (Chapter 3, Methods, Section 3.2.3.2.3). Negative control gate was set where 98% of the population falling beneath this gate, making any movement of the positive stain beyond this point represents more than three standard deviations away from the mean of the negative control, which considered statistically relevant.

Flow cytometric results showed that all positive stem cell markers (CD146, CD90 and CD105) appeared to be expressed by both hDPSCs and cDPSCs

but with variable percentages (Figure 15 , Figure 16, and Figure 17). Interestingly, the expression of CD146 was higher by 20% in cDPSCs (43.5% ±17.14) compared with hDPSCs (23.2% ±22.6) (Table 13). On the other hand, as shown in Table 13, flow cytometric analysis revealed almost the same expression level of CD90 positive populations in both hDPSCs (98.9% ±1.2) and cDPSCs (99.70 % ±0.36). Moreover, 99.60% ±0.08 of cDPSCs expressed CD105 compared to 97.8% ±1.6 of hDPSCs; giving approximately 2% increase in CD105 expression by cDPSCs compared with hDPSCs. Negative stem cell markers (CD45 and CD31) showed negative or very little expression in both cell populations. CD45 was expressed in about 5% ±4.94 of hDPSCs and 0.72% ±0.31 of cDPSCs, while CD31 was expressed only in 0.04% ±0.03 of hDPSCs and 0.10% ±0.05 of cDPSCs.

On an individual basis, two donors of hDPSCs expressed low levels of CD146 (H1- 3.76% and H3- 11%), while 54.9% of H2 expressed CD146. However, two donors of cDPSCs showed a moderate expression of CD146 (C2- 28.4% and C3- 34.7%), while C1 expressed 67.5% of CD146 (Figure 15). Interestingly, it is apparent from Figure 16 and Figure 17 that more than 95% of hDPSCs and cDPSCs expressed CD90 and CD105. What is more, a noted observation to emerge from these data was the expression of the leukocyte precursor marker CD45, which was expressed at a range of 0.85% to 12.1% in hDPSCs. While CD31 expressed at a range of 0% to 0.17% in both hDPSCs and cDPSCs. All in all, the results of this study did not show any significant differences between cells isolated from healthy and carious wisdom teeth in expressing positive and negative stem cell markers (Table 13).

In order to further analyse the stem cell population from both hDPSCs and cDPSCs, another gating strategy was used (Figure 20). In this strategy, dead cells were excluded using fixable viability dye, the CD90 positive subpopulation of DPSCs was gated, CD105 against CD45 was plotted from selected CD90+ subpopulation, then CD146 against CD31 was plotted from selected CD105+/CD45- subpopulation. The final gated population was assumed to be enriched stem cell population(s) (Figure 20). Flow cytometric results of the previous gating strategy showed that the cDPSCs expressed higher percentage of stem cell population (CD90+/CD105+/CD45-/CD146+/CD31-); about 34% ±16.6 compared with18.5% ±19.31 expressed by hDPSCs (Figure 21).

Regarding the individual analysis of both DPSC subpopulations using the previous gating strategy, hDPSCs showed low to moderate expression levels as H1 and H3 expressed 2.35% and 7.41% respectively, while H2 expressed about 45% of the same markers. However, cDPSCs showed moderate expression levels as C2 and C3 expressed 19.6% and 27% of stem cell markers respectively, while C1 expressed about 57.9% of the same markers (Figure 20).

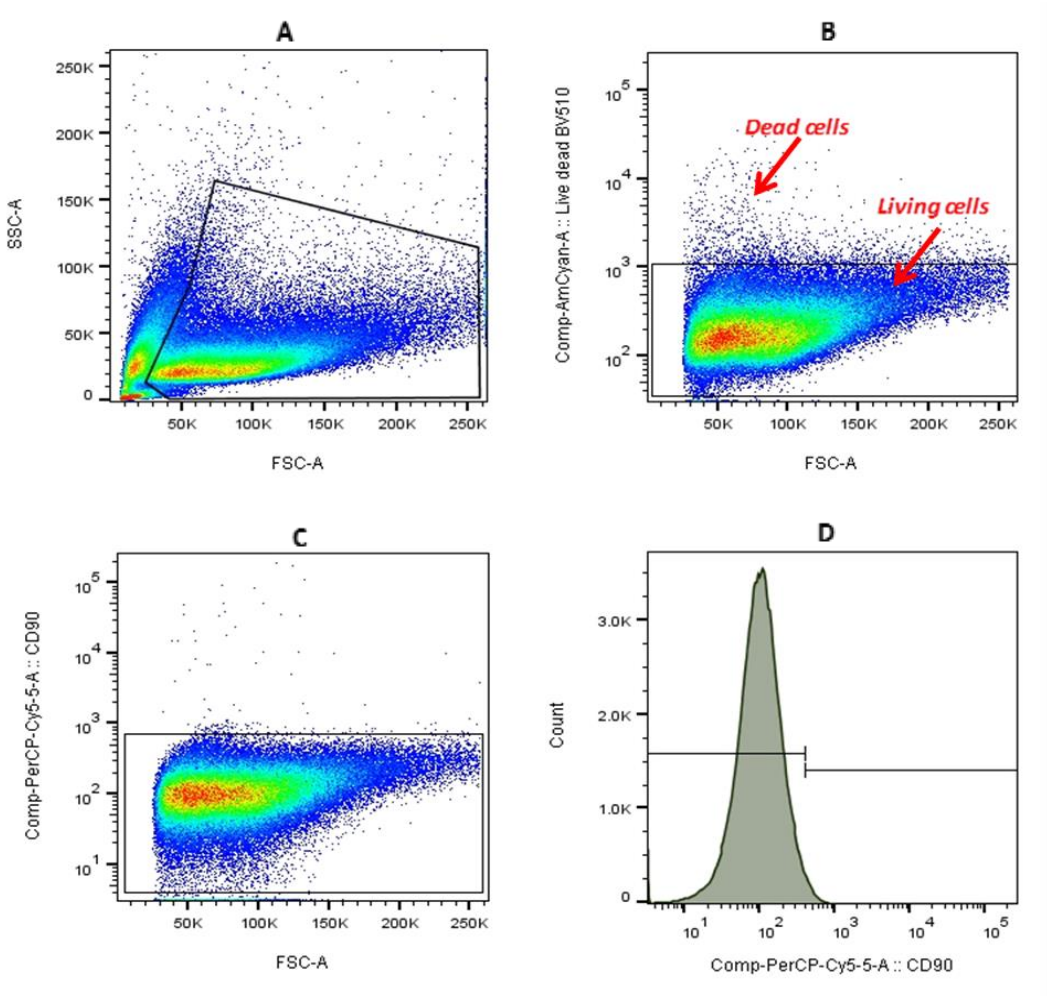


Figure 14: Example of gating strategy used to analyse single stem cell surface marker

Flow cytometry analysis of stem cell surface marker expression in DPSCs under basal conditions (CD90 was used as an example). (A) Representative dotplot of intact cellular bodies gating in DPSCs by excluding scattered very small nuclear debris . (B) Representative dotplot of living cells gating in DPSCs using FMO control relative to fixable viability dye (BV510). (C) Representative dotplot of the negative control (FMO + antibody isotype) relevant to CD90 (PerCP-Cy5.5). (D) Representative single-parameter histogram plot for the negative control (FMO + antibody isotype) relevant to CD90 (PerCP-Cy5.5), where the gate is positioned at 98% of the negative control and any movement of the positive stain beyond that point is statistically relevant .

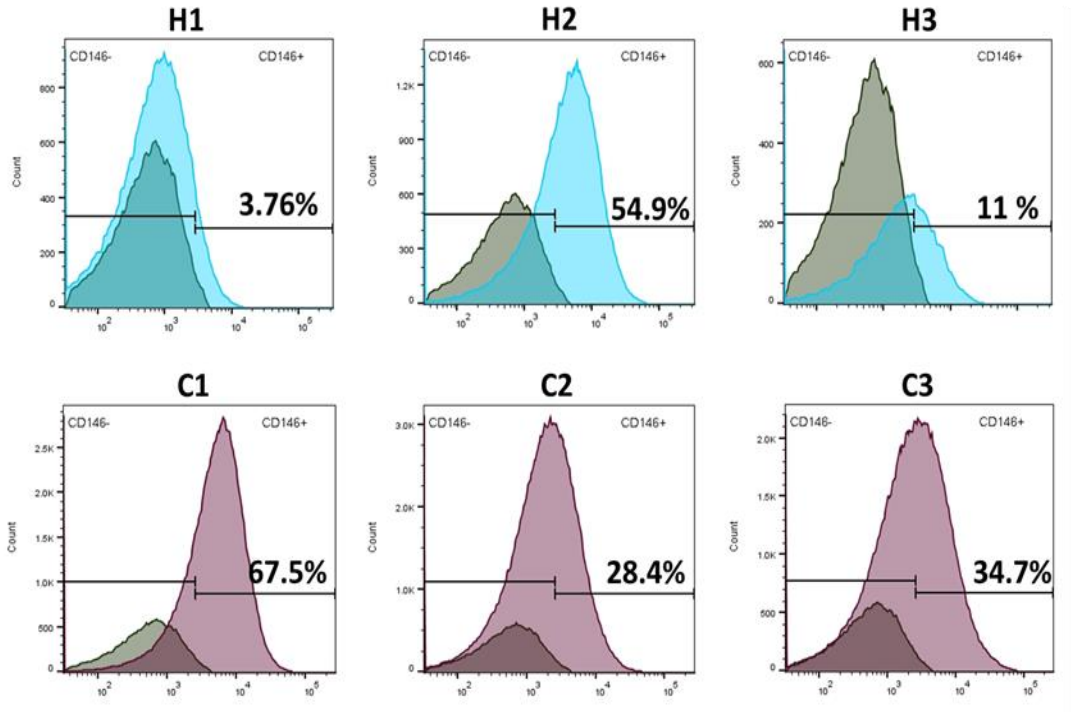


Figure 15: Expression of CD146 in hDPSCs and cDPSCs under basal conditions using flow cytometry.

Representative diagrams are single- parameter histograms showing the expression of CD146 in hDPSCs (H1, H2, H3) and cDPSCs (C1, C2, C3) cultures under basal conditions. The grey coloured histograms represent the negative control (FMO+ antibody isotype) relevant to CD146 (PE-Cy7), where the gate is positioned at 98% of the negative control and any movement of the positive stain beyond that point is statistically relevant. The blue coloured histograms represent positive CD146 population in hDPSCs and the bright red coloured histograms represent positive CD146 population in cDPSCs**.** * Gating strategy following what has been demonstrated in Figure 14.

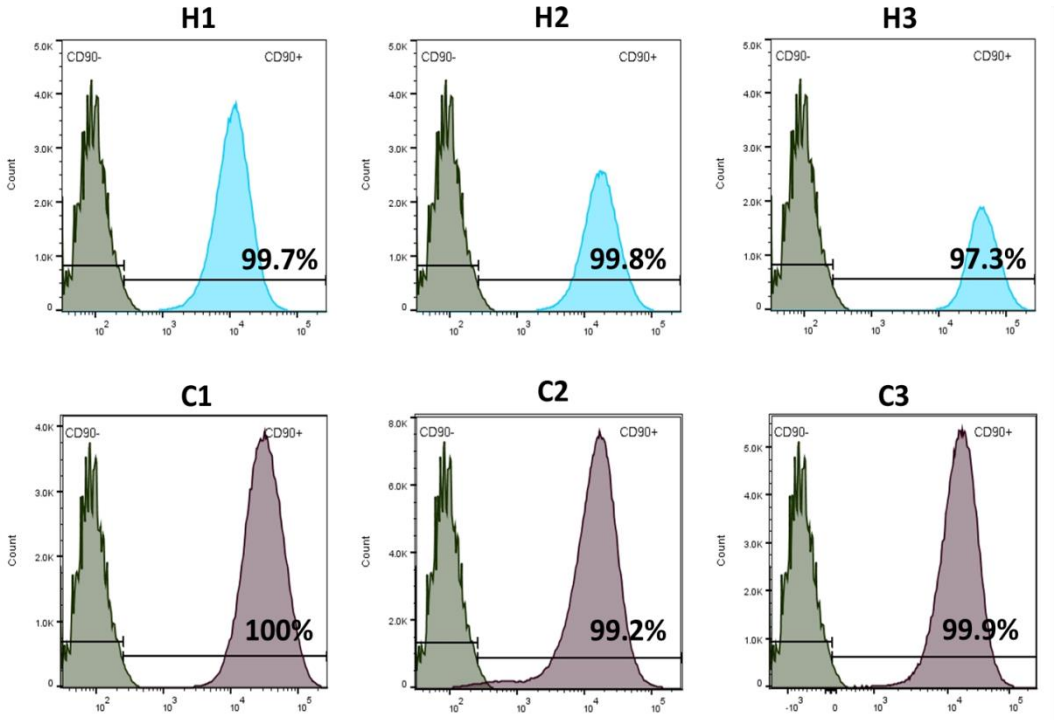


Figure 16: Expression of CD90 in hDPSCs and cDPSCs under basal conditions using flow cytometry.

Representative diagrams are single- parameter histograms showing the expression of CD90 in hDPSCs (H1, H2, H3) and cDPSCs (C1, C2, C3) cultures under basal conditions. The grey coloured histograms represent the negative control (FMO+ antibody isotype) relevant to CD90 (PerCP-5.5), where the gate is positioned at 98% of the negative control and any movement of the positive stain beyond that point is statistically relevant. The blue coloured histograms represent positive CD90 population in hDPSCs and the bright red coloured histograms represent positive CD90 population in cDPSCs. * Gating strategy following what has been demonstrated in Figure 14.

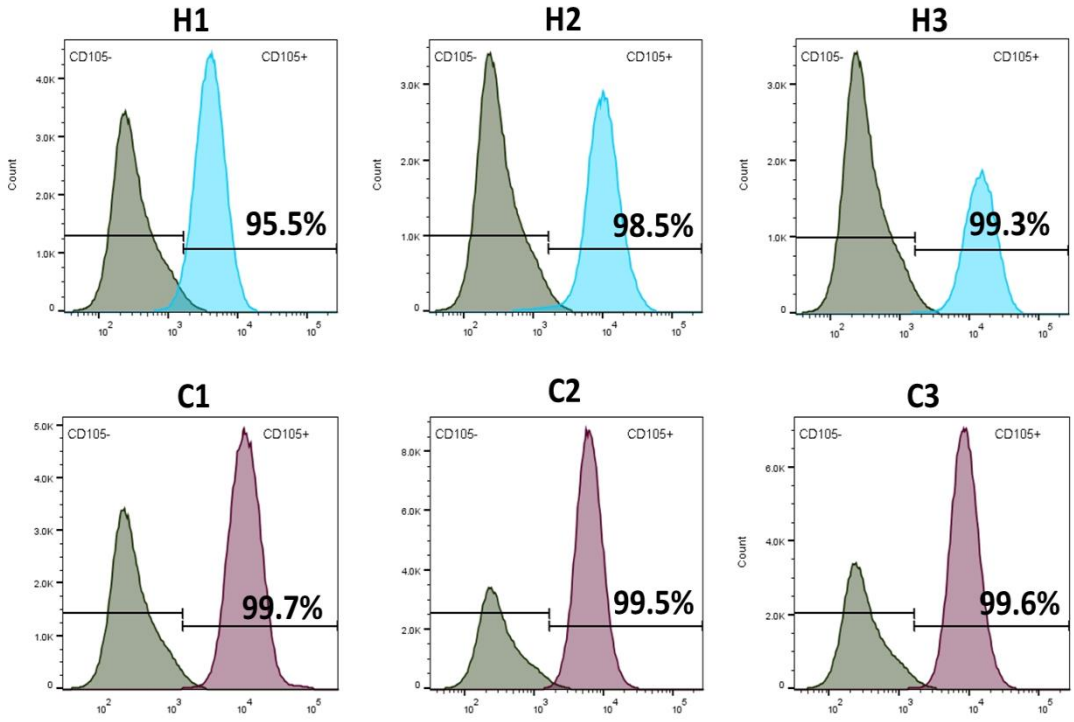


Figure 17: Expression of CD105 in hDPSCs and cDPSCs under basal conditions using flow cytometry.

Representative diagrams are single- parameter histograms showing the expression of CD105 in hDPSCs (H1, H2, H3) and cDPSCs (C1, C2, C3) cultures under basal conditions. The grey coloured histograms represent the negative control (FMO+ antibody isotype) relevant to CD105 (BV421), where the gate is positioned at 98% of the negative control and any movement of the positive stain beyond that point is statistically relevant. The blue coloured histograms represent positive CD105 population in hDPSCs and the bright red coloured histograms represent positive CD105 population in cDPSCs. * Gating strategy following what has been demonstrated in Figure 14.

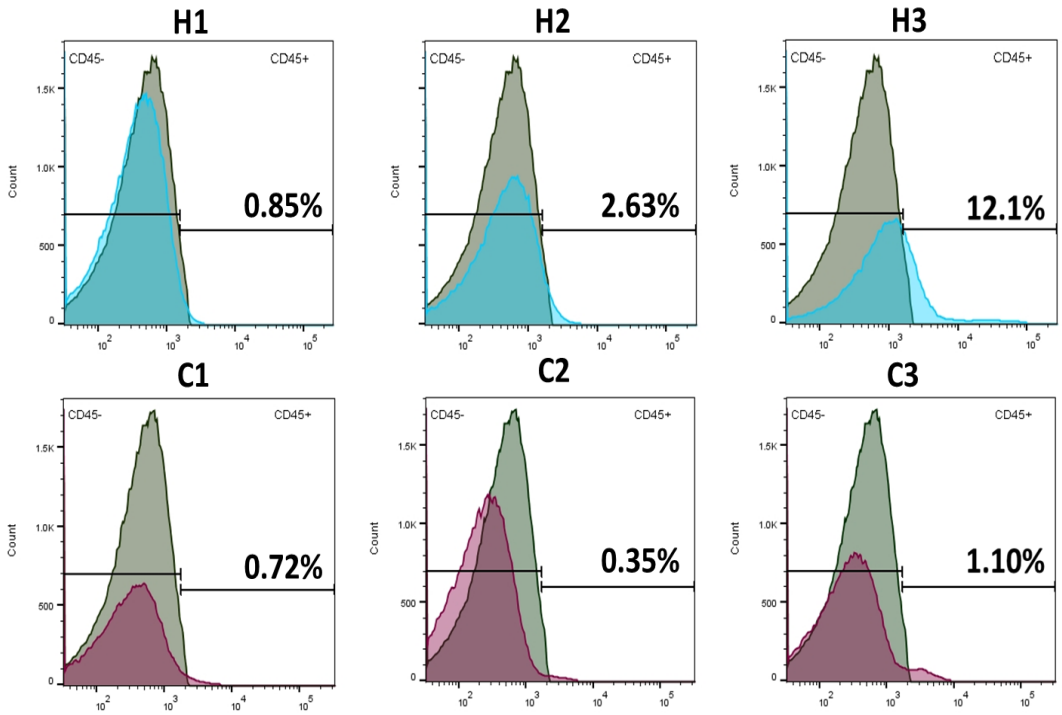


Figure 18: Expression of CD45 in hDPSCs and cDPSCs under basal conditions using flow cytometry.

Representative diagrams are single- parameter histograms showing the expression of CD45 in hDPSCs (H1, H2, H3) and cDPSCs (C1, C2, C3) cultures under basal conditions. The grey coloured histograms represent the negative control (FMO+ antibody isotype) relevant to CD45 (APC-Cy7), where the gate is positioned at 98% of the negative control and any movement of the positive stain beyond that point is statistically relevant. The blue coloured histograms represent positive CD45 population in hDPSCs and the red coloured histograms represent positive CD45 population in cDPSCs. * Gating strategy following what has been demonstrated in Figure 14.

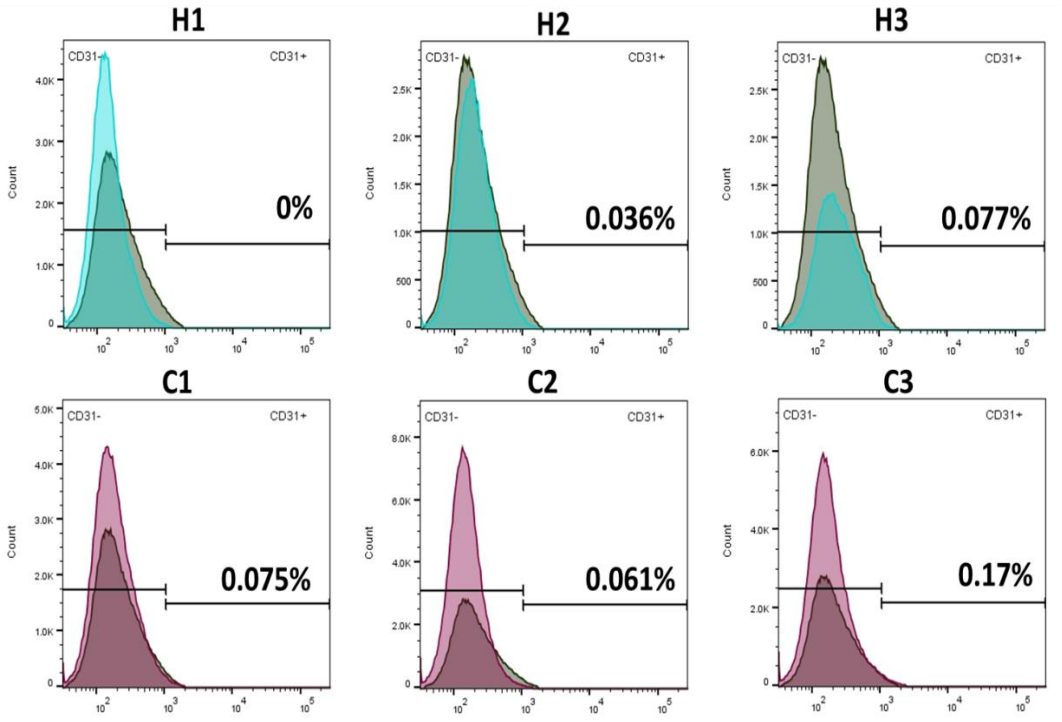


Figure 19: Expression of CD31 in hDPSCs and cDPSCs under basal conditions using flow cytometry.

Representative diagrams are single- parameter histograms showing the expression of CD31 in hDPSCs (H1, H2, H3) and cDPSCs (C1, C2, C3) cultures under basal conditions. The Grey coloured histograms represent the negative control (FMO+ antibody isotype) relevant to CD31 (FITC), where the gate is positioned at 98% of the negative control and any movement of the positive stain beyond that point is statistically relevant. The blue coloured histograms represent positive CD31 population in hDPSCs and the bright red coloured histograms represent positive CD31 population in cDPSCs. * Gating strategy following what has been demonstrated in Figure 14.

Table 13: Stem cell marker expression (%) in DPSCs isolated from healthy and carious teeth

Data presented as means of expression of the biological replicates (n=3) ± SD in each group. No statistical difference was observed in the expression of all markers between hDPSCs and cDPSCs using Student's t-test.

GROUP	Positive stem cell markers			Negative stem cell markers	
	CD146	CD105	CD90	CD45	CD31
hDPSCs	23.2% ±22.6	98.9% ±1.2	97.8% ±1.6	5.2% ±4.94	0.04% ±0.03
cDPSCs	43.5% ±17.14	99.70% ±0.36	99.60% ±0.08	0.72% ±0.31	0.10% ±0.05
P-value	(0.372)	(0.451)	(0.254)	(0.329)	(0.202)

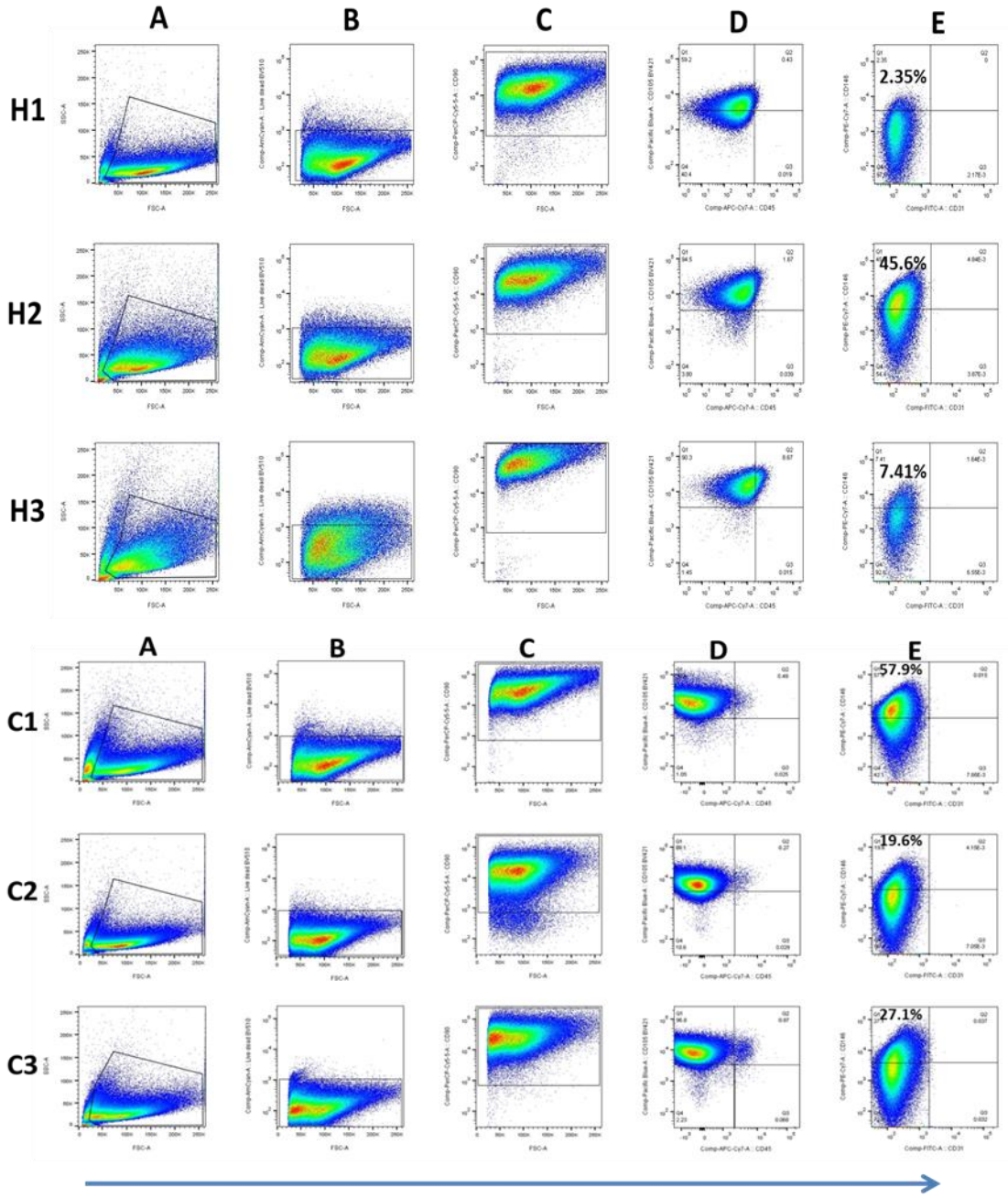


Figure 20: Stem cell population in dental pulp cells isolated from healthy and carious teeth

Flow cytometry analysis of stem cell surface markers expressed in DPSCs isolated from healthy (H1, H2, H3) and carious (C1, C2, C3) teeth under basal conditions. **(A)** Representative dotplots of intact cellular bodies gating in DPSCs. **(B)** Representative dot plots of living cells gating in DPSCs using fixable viability dye. **(C)** Representative dotplots of CD90+ cells gating in DPSCs. **(D)** Representative of dotplots of CD105 (Y axis) against CD45 (X axis) surface markers from selected CD90+ subpopulation. **(E)** Representative dotplots of CD146 (Y axis) against CD31 (X axis) surface markers from selected CD105+ /CD45- subpopulation in the previous plot (D).

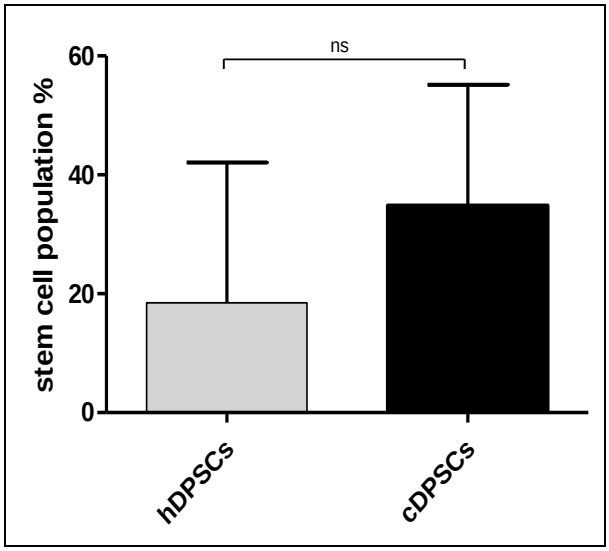


Figure 21: Stem cell population (%) in hDPSCs and cDPSCs.

The graph represent the mean percentage of stem cell population markers (CD90+/CD105+/CD45-/CD146+/CD31-) expressed in DPSCs derived from 3 healthy and 3 carious teeth. Data are presented as the mean of expressions of the biological replicates (n=3) ± SD in each group. Data were analysed using Student's t-test. P=0.414

4.3 Discussion

These results demonstrated the ability of both hDPSCs and cDPSCs to express mesenchymal stem cell surface markers, adhere to plastic surfaces, and their ability to form colonies. The behaviour of hDPSCs in this study is similar to that previously reported with regard to mesenchymal stem cell surface markers expression, colony forming and differentiation ability [151, 389, 396]. Colonies of cDPSCs occurred at higher frequency in comparison to hDPSCs, in agreement with previous observations showing a higher clonogenic potential on cDPSCs isolated from third molars (n=10) with deep caries [177]. Caries as a pathologic microenvironment is able to evoke different responses of the stem cells and other supportive cells in the pulp tissue through bacterial toxins that can reach the pulp through the dentinal tubules. Therefore, increased clonogenic potential of cDPSCs might possibly be a sign of early dentinal repair, in response to these pathological stimuli.

The surface markers on hDPSCs and cDPSCs have been analysed to confirm the presence of stem cells in these two groups and to characterise the different cell subpopulations within these mixed stromas based on the percentage of stem cell markers expression. In the present study, CD146 was expressed in 23.2% ±22.6 of the hDPSCs. This varied from the report of Bakopoulou *et al.* (2011), in which more than 80% of DPSCs expressed CD146 [406]. High expression of CD146 in their study may be related to the stage of root development as they isolated the DPSCs from impacted third molars of three donors aged 16-18 years. High vascularity was needed in

order to complete root formation in the eruption stage (18-25 years old) and CD146 is known to be expressed in perivascular MSCs [410]. CD90 and CD105 expressions in hDPSCs in the present study are largely in agreement with other studies, which reported high expression (>95%) of both CD90 and CD105 in dental pulp tissues isolated from normal healthy teeth [389, 407].

Interestingly, the present study showed the mesenchymal stem cell marker CD90 was comparably expressed in both hDPSCs (98.9% ±1.2) and cDPSCs (99.70% ±0.36). Similarly, CD105 showed also parallel expression in cDPSCs (99.60% ±0.08) compared with hDPSCs (97.8% ±1.6). Ma *et al.* (2012), demonstrated significantly higher expression of STRO-1, CD90, CD105 and CD29 in cDPSCs compared to hDPSCs [177], although the absolute levels of expression of both CD90 and CD105 in the previous study were far less compared to the current study. Ma *et al.* (2012), mentioned that they isolated hDPSCs and cDPSCs from pulp chambers only, while in the present study, DPSCs have been isolated from the pulp chamber and upper 2/3rd of the root canal, which might include different stem cell populations in the current study expressing higher percentages of stem cell markers. Furthermore, Ma *et al.* (2012), isolated DPSCs from teeth affected with deep caries as opposed to here, where DPSCs were isolated from teeth with shallow caries. In the case of deep caries with severe injury, odontoblasts can undergo cell death, perhaps leading to increased differentiation of stem cells into odontoblasts-like cells as a consequence of cariogenic challenge. This process depends on a cascade of events that involve stem cell proliferation, migration, and differentiation into odontoblasts-like cells [411]. Therefore, a more differentiated/lower stem cell pool in cells derived from

deep carious lesions may explain a lower expression of CD90 and CD105 compared with the current study where DPSCs have been isolated from shallow carious lesion; stem cell proliferation and migration may be occurring but differentiation may not yet be extensive. However this hypothesis clearly requires further experimental validation.

Another important study performed by Alongi *et al.* (2010), concluded that cells isolated from pulp tissue of third molars affected by irreversible pulpitis (DPSC-IPs) showed distinct characteristics compared with normal dental pulp stem cells (DPSC-NPs). The authors stated that: although *in vitro* some stem cell characteristics from DPSC-IPs pulp may be lost, these cells still demonstrated the capacity to form dentine/pulp complex *in vivo*. In addition, they claimed that DPSC-IPs might have the potential to be used as a cell source for pulp and dentine regeneration rather than discarding them as medical waste. Using immunohistochemistry, both DPSC-NPs and DPSC-IPs stained positively for STRO-1, CD90, CD105 and CD146. The results showed significantly higher antibody staining in the DPSC-IPs compared with DPSC-NPs [175]. Similarly, the current study showed a trend of cDPSCs to express higher levels of CD146 compared with hDPSCs (Table 13). However, the present study showed a higher expression levels of CD146, CD90 and CD105 in both groups compared with what has been demonstrated by Alongi *et al.* (2010). This might possibly be related to the criteria of irreversible pulpitis and degree of inflammation that were investigated in their study compared with shallow caries that has been targeted in the current study. Higher expression of CD146 in cDPSCs compared with hDPSCs can be attributed to the nature of CD146, which is

known to be associated with the blood vessels and higher vascularity that is expected to be associated with inflammation [412]. Additionally, CD146 showed strong staining during the initial inflammatory stages of dental pulp, which also may explain the higher expression of CD146 in cDPSCs compared to hDPSCs [412].

A comparison of DPSCs isolated from carious deciduous teeth (SCCD) with normal exfoliated deciduous teeth (SHED) indicated that both cell types positively expressed (>98%) CD29, CD73, and CD90. Furthermore, CD14, CD34, CD45 and HLA-DR were essentially negative in both populations [413]. Moreover, a similar study comparing SHED to deciduous teeth with inflamed pulps (SCIDs) showed no significant differences in proliferation, differentiation and MSC surface markers expression (CD146, CD90, CD105). SHED and SCIDs had a much higher CD146 (97.97% and 99.98%, respectively) expression compared with the current findings in hDPSCs and cDPSCs (23.2% and 43.5% respectively). However, SHED and SCIDs expressed less CD90 (54.56% and 78.17%, respectively) compared to hDPSCs and cDPSCs (98.9% and 99.70%, respectively) in the current study [414]. Kim *et al.* (2014), isolated DPSCs from inflamed pulp tissue of deciduous teeth and concluded that these cells had similar stem cell markers expression to those isolated from non-inflamed pulp tissue. Both populations showed positive expression of CD146 and CD90 while both were negative for CD45. The expression of CD146 was higher in both non-inflamed (70.54%) and inflamed (67.93%) populations [415], compared with the current study. Higher CD146 expressed by deciduous dental pulps compared with adult dental pulp tissues might be related to greater

proportion of stem cell population known to reside in deciduous teeth compared with adults [416]. However, a subsequent report suggested that cells isolated from inflamed pulp tissue of deciduous teeth (SCDIP) failed to show typical MSC characteristics compared with healthy pulp tissues (SCD). The authors reported that SCD derived cells showed faster proliferation rates and time to confluency compared with SCDIP during early passages. Interestingly, CD34 and CD45 were absence in both cell types and there was no much difference in the expression of CD73, CD90 and CD166 between SCD and SCDIP [417].

In the present study, both hDPSCs and cDPSCs demonstrated mild positivity for the hematopoietic stem cell marker CD45 (leukocyte common antigen). The average expression of CD45 in both hDPSCs and cDPSCs was 5.2% ±4.94 and 0.72% ±0.31, respectively. Previous data indicated that DPSCs were either negative for CD45 [3, 407, 418, 419] or expressed this marker at a very low concentration <1-2% of the cell population [389]. It was reported that leukocytes represent <1% of dental pulp cell populations after enzymatic digestion harvesting [420]. It might be that early passages (passage 4) of heterogeneous stromal cell populations of hDPSCs still contains traces of CD45 positive population. The DPSCs might be challenged by different stimuli through the apical foramen or even through the surrounding periodontal ligament, which might increase the leukocyte population in the dental pulp. hDPSCs and cDPSCs failed to react with endothelial cell marker CD31 and this agrees broadly with the findings of other studies [407, 421].

In the present study, flow cytometric results showed that cDPSCs expressed higher percentages (34.87% ±16.57) of stem cell population that co-expressed CD90, CD105, and CD146 ($CD90^+/CD105^+/CD146^+$), but not expressing CD45 and CD31 ($CD45^-/CD31^-$), compared with hDPSCs (18.45% ±19.31) (Figure 21). Other studies have reported various surface markers co-expression in DPSCs. For example, DPSCs co-expressed CD271 and CD90 ($CD271^+/CD90^+$) were represent 0.72% ±0.19% of total hDPSCs population isolated from normal impacted third molars [422]. Moreover, published data showed that 0.5% of hDPSCs negative for CD34 co-expressed with STRO-1 and c-kit (CD34-/STRO-1+/c-kit+), whereas 20% of hDPSCs positive for CD34 co-expressed with STRO-1 and c-kit (CD34+/STRO-1+/c-kit+) [423]. Caries-induced inflammation may be able to influence the stem cell characteristics of cDPSCs, which might have the potential to be used as future source of stem cells.

Chapter 5: Results

Expression of regenerative markers in dental pulp cells isolated from carious versus healthy teeth

5.1 Introduction

The dental pulp has a fundamental regenerative potential in response to noxious stimuli including caries-induced inflammation. Inflammatory processes induce mineralised tissue regeneration, and are critical for healing processes, which are regulated by wide range of signalling molecules and growth factors [21, 22]. Dental pulp stem cells (DPSCs) are multipotent and have a proven ability to differentiate down different lineages; including odontoblast and osteoblast phenotypes [74, 151, 424]. Both odontoblasts and osteoblasts are accountable for manufacturing hard tissues. Although dentine and bone are chemically similar, they are very different structurally. The ability of DPSCs to differentiate into functional osteoblasts and produce mineralised matrix have been reported in many *in vitro* studies [425, 426], whereas, *in vivo* studies reported that DPSCs were able to reconstruct bony structures [427, 428] with comparable behavior to human bone marrow stem cells [74]. It has been reported that dentine can be formed in *in vivo* models when subjected to reciprocal induction from epithelial cell sources (subcutaneous implantation, implantation within the kidney capsules) [157, 429]. In response to noxious stimuli in human teeth, DPSCs respond by differentiation into odontoblast-like cells and try to wall off the damage by forming reparative dentine, which is very similar in structure to woven bone.

Mineralised tissue regeneration requires vascularization [430]. With the dental pulp being essentially considered to be the neuro-vascular part of the tooth, and with its heterogeneous cell population, it is considered an ideal source for MSCs that can regenerate vascularized hard tissue [431]. The dental pulp MSCs population isolated from healthy (hDPSCs) and carious teeth (cDPSCs) have been successfully characterized and compared in Chapter 4 of this thesis.

The aim of this chapter was to evaluate the effect of inflammation on the regenerative potential of DPSCs and to investigate the alterations of the inflammatory environment during the osteogenic induction of cDPSCs. This may offer some understanding of how we can make use of low-grade inflammation in enhancing repair and regeneration of bone, dentine and other mineralised tissues. In order to achieve this, the expression of inflammatory markers were investigated in cDPSCs and hDPSCs cultured under basal and osteogenic culture conditions at different time points (1 and 3 weeks). These markers were: Toll-like receptors (*TLR-2 and TLR-4*) and their activation products, including IL-6 and IL-8. Furthermore, the expression of osteogenic and angiogenic markers were also investigated in both cell types under basal and osteogenic conditions. Correlation between the expression of these regeneration markers and the inflammatory markers was carried out in an attempt to deduce the effects that inflammation might have on the expression of these markers under different culture conditions.

5.2 Results

5.2.1 Confirmation of osteogenic differentiation in hDPSCs and cDPSCs using histochemical staining

Near confluent monolayers (80%) of hDPSCs and cDPSCs were cultured under osteogenic conditions as described in Chapter 3, Methods, Section 3.2.4; by treatment with osteogenic medium containing dexamethasone and ascorbic acid for 1 week and 3 weeks.

A: Alkaline phosphatase (ALP) staining of hDPSCs and cDPSCs cultured under basal and osteogenic conditions

Results of the current study showed positive ALP staining in cultures from all donors in hDPSCs and cDPSCs groups under basal and osteogenic conditions at 1 and 3 weeks (Figure 22). However, the results clearly indicated more intense ALP staining in all osteogenic cultures at 1 and 3 weeks compared with basal cultures (Figure 22). Interestingly, cDPSCs also showed increased ALP staining under osteogenic conditions at 1 and 3 weeks compared with hDPSCs under the same conditions (Figure 22). One donor from each group is presented in Figure 22 and similar results were obtained using DPSCs from other donors.

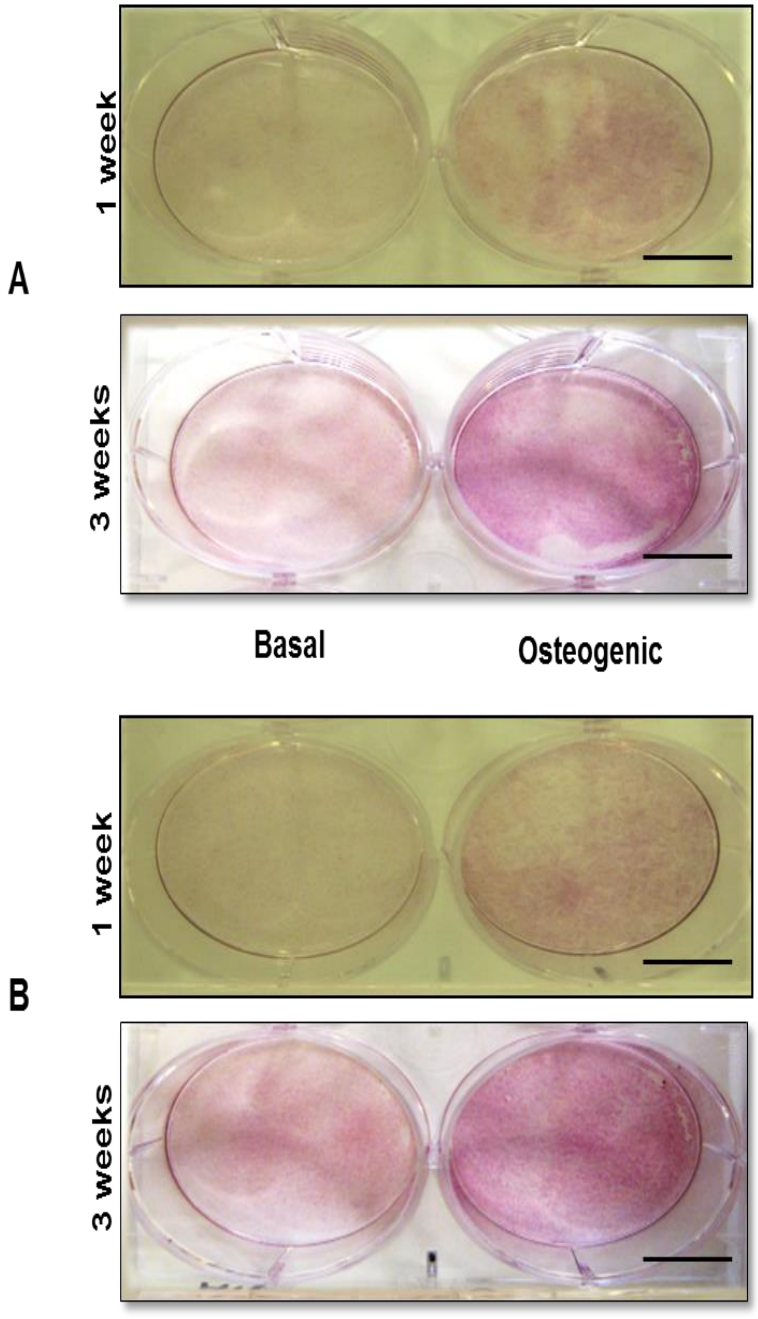


Figure 22: ALP staining of hDPSCs and cDPSCs

ALP staining of hDPSCs (A) and cDPSCs (B) cultured in monolayers under basal and osteogenic conditions for 1 and 3 weeks as labelled. Scale bar = 5 cm

B: Alizarin red staining of hDPSCs and cDPSCs cultured under basal and osteogenic conditions

Alizarin red positively stained mineralised nodules in both hDPSCs and cDPSCs cultured under osteogenic conditions for 1 and 3 weeks compared with basal cultures. This confirmed the ability of both DPSCs to form calcium nodules under osteogenic culture conditions. However, cDPSCs showed more Alizarin red stained nodules under osteogenic conditions at 1 and 3 weeks compared with hDPSCs under the same conditions (Figure 23). One donor from each group is presented in Figure 23 and similar results were obtained using DPSCs from other donors.

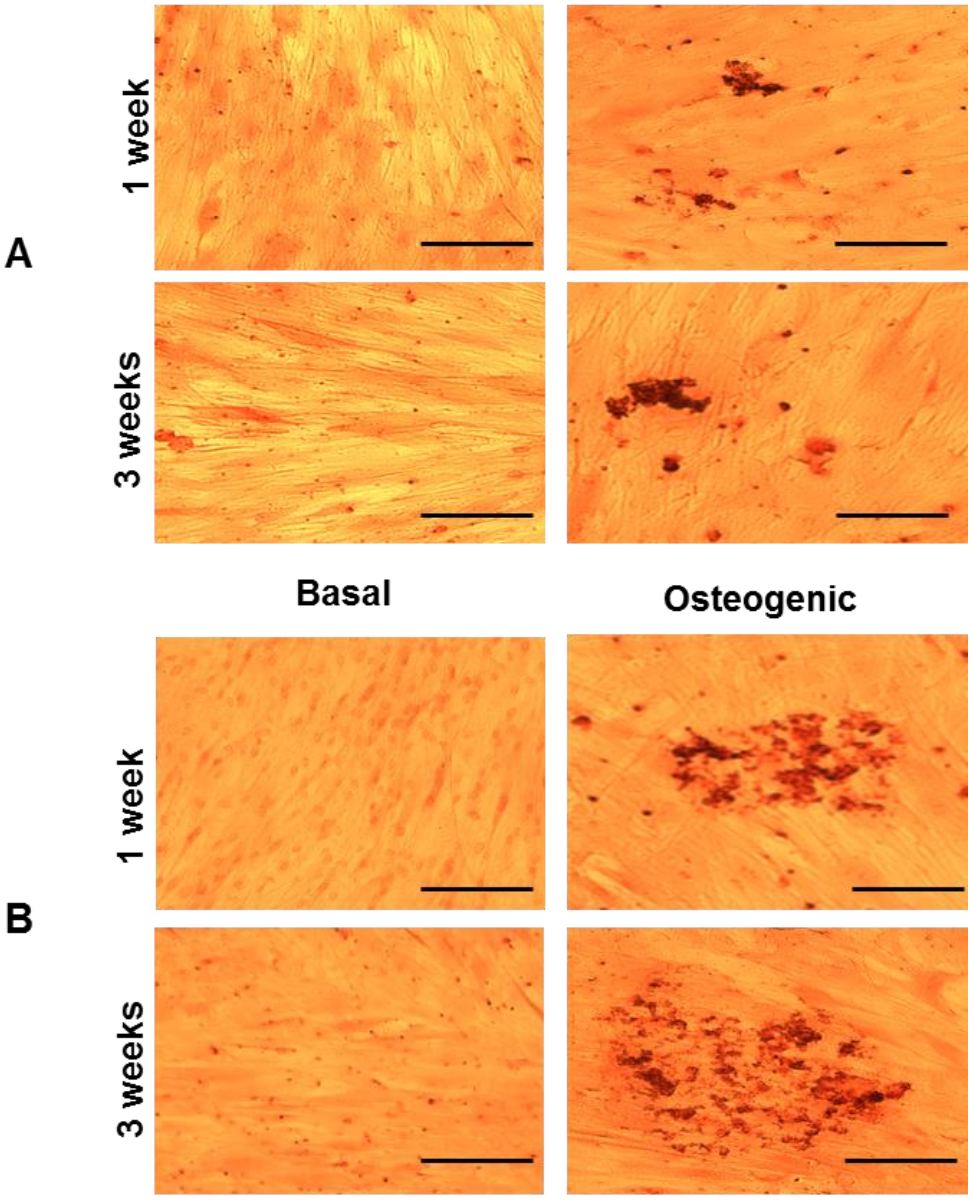


Figure 23: Alizarin red staining of hDPSCs and cDPSCs

Mineralised nodules are stained positively (red) with Alizarin red stain in hDPSCs (A) and cDPSCs (B) cultured in monolayers under osteogenic conditions for 1 and 3 weeks as labelled. Scale bar = 100 µm.

5.2.2 Gene expression

The changes in gene expression of osteogenic, angiogenic and inflammatory markers in hDPSCs and cDPSCs cultured under basal and osteogenic conditions for 1 and 3 weeks were investigated using qRT-PCR as previously described (Chapter 3, Methods, Section 3.2.5).

A: Baseline expression of osteogenic, angiogenic and inflammatory marker genes

The osteogenic gene markers investigated were alkaline phosphatase (*ALPL)*, osteocalcin (*OC)* and Runt-related transcriptional factor (*RUNX-2)*. Angiogenic markers were Vascular Endothelial Growth Factor Receptor-2 (*VEGFR-2*), and Platelet Endothelial Cell Adhesion Molecule-1 (*PECAM-1*). Inflammatory markers were Toll-Like receptors (*TLR-2, TLR-4*), and interleukins (IL-6, IL-8). The expression of these markers in hDPSCs and cDPSCs cultured under basal conditions were investigated to determine the baseline expression of those markers under healthy and inflamed conditions. The expression of the genes of interest in both hDPSCs and cDPSCs cultured under basal conditions were normalised to the house keeping gene GAPDH, and the results were presented as the means of ΔCt±SD to show the expression of these genes at the two time points (1 and 3 weeks). All experiments were repeated three times from three different donors in each group; hDPSCs and cDPSCs. Results from individual donors (individual analysis) as well as the average results of all donors together in each group (global analysis) were plotted for all genes.

B: Relative changes in the expression of osteogenic, angiogenic and inflammatory marker genes

The relative changes in the expression of osteogenic, angiogenic and inflammatory markers in both hDPSCs and cDPSCs cultured under osteogenic conditions were carried out to determine the changes in the expression of these genes in both cell types under osteogenic culture conditions, compared with their baseline expression under basal conditions. The expression of the genes of interest in both hDPSCs and cDPSCs cultured under osteogenic conditions were normalised to controls from the same cells, cultured under basal conditions. The ΔΔCt method was used to calculate the relative change in the gene expression (Chapter 3, Methods, Section 3.2.5.6). The means $2^{-\Delta\Delta ct}$ ±SD were plotted (Log 10 scale) to show the relative changes in gene expression at the both time points (1 and 3 weeks). Fold changes in gene expression were calculated. All experiments were repeated three times from three different donors in each group; hDPSCs and cDPSCs. Results from individual donors (individual analysis) as well as the average results of all donors together in each group (global analysis) were plotted for all genes of interest.

Statistical analysis:

Statistical analysis was carried out for individual donors and for global gene expression data using one way ANOVA followed by Bonferroni multiple comparison tests, using Graph Pad Prism software (v 6). Differences were considered significant when P values were <0.05.

5.2.2.1 Expression of osteogenic marker genes in hDPSCs and cDPSCs

The gene expression of bone markers (*ALPL, OC and RUNX-2*) were investigated in hDPSCs (n=3) and cDPSCs (n=3), both cultured in monolayers under basal and osteogenic conditions for 1 and 3 weeks.

5.2.2.1.1 Comparing the changes in *ALPL* gene expression in hDPSCs and cDPSCs

A: **Baseline expression of *ALPL* gene in hDPSCs and cDPSCs**

ALPL was expressed at moderate levels in both hDPSCs and cDPSCs cultured under basal conditions for 1 and 3 week time points (Figure 24). However at 1 week under basal conditions, cDPSCs from the first donor (C1) showed higher levels of *ALPL* expression compared with hDPSCs from the first and third donors (H1, H3) under the same conditions (Figure 24A). cDPSCs from the second donor (C2) showed higher levels of *ALPL* expression compared with hDPSCs from all three donors (Figure 24A).

At 3 weeks under basal conditions, cDPSCs from two out of three donors (C1, C2) showed higher levels of *ALPL* expression compared with hDPSCs from the first and second donors (H1, H2) under the same conditions (Figure 24A). The levels of *ALPL* expression in cDPSCs from the third donor (C3) was lower compared with hDPSCs from all three donors at 3 weeks under basal conditions, with only hDPSCs from the third donor (H3) reaching statistical significance (Figure 24A).

The levels of *ALPL* expression were higher in both hDPSCs and cDPSCs at 3 weeks compared with 1 week under basal conditions, in all three donors

(Figure 24A). Statistical significance between 1 and 3 weeks was demonstrated in hDPSCs isolated from the third donor (H3) and in two out of three donors in the cDPSCs group (C1, C2) under basal conditions (Figure 24A).

The global analysis showed that *ALPL* levels were significantly increased in hDPSCs and cDPSCs at week 3 compared with week 1 under basal conditions (Figure 24B). cDPSCs showed a slightly higher levels of *ALPL* expression at both time points compared with hDPSCs under basal conditions (Figure 24B).

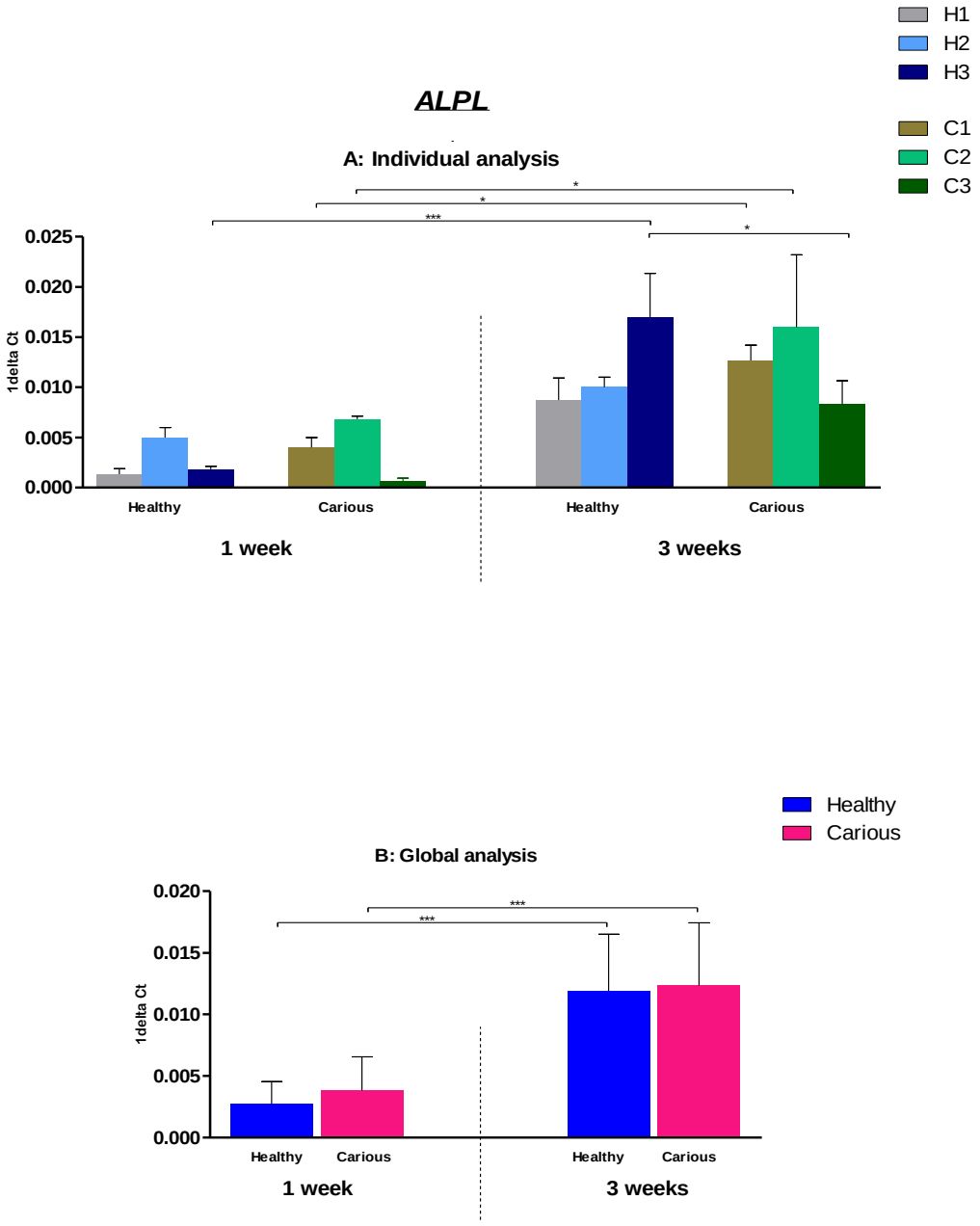


Figure 24: Baseline expression of *ALPL gene* in hDPSCs and cDPSCs cultured under basal conditions for 1 and 3 weeks

ALPL gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under basal conditions for 1 and 3 weeks. **A**: Individual analysis: showing the levels of *ALPL* gene expression in individual donors. The expression of *ALPL* was normalised to the house-keeping gene (GAPDH). Data are presented as means ΔCt ±SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged expression of *ALPL* from the three donors in each group of hDPSCs and cDPSCs. *P <0.05, **P <0.01, ***P <0.001.

B: Relative changes in *ALPL* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions

In hDPSCs and cDPSCs, the levels of *ALPL* expression were up-regulated at week 1 under osteogenic conditions compared with control cells cultured under basal conditions, in all three donors in both cell types (Table 14 and Figure 25A).

Comparing both cell types, the level of *ALPL* expression was higher at week 1 under osteogenic conditions, in hDPSCs from the first and third donors (H1, H3) compared with cDPSCs from the first and second donors (C1, C2), the only statistically significant difference was between hDPSCs from the first donor (H1) and cDPSCs from the second donor (C2). While the level of *ALPL* expression was higher at week 1 in cDPSCs from all donors compared with hDPSCs from the second donor (H2), only cDPSCs from the third donor (C3) reached statistical significance under osteogenic conditions (Table 14 and Figure 25A).

At 3 weeks, the levels of *ALPL* were down-regulated in hDPSCs from all three donors. Similarly, cDPSCs from the third donor (C3) showed down-regulation of *ALPL* under osteogenic conditions compared with basal conditions. However, cDPSCs from the first and second donors (C1, C2) showed up-regulation of *ALPL* under osteogenic conditions compared with basal controls (Table 14 and Figure 25A). There was a significant difference comparing cDPSCs cultured for 3 weeks with 1 week from the third donor (C3) (Table 14 and Figure 25A). *ALPL* levels were significantly higher at week 1 compared with week 3 in hDPSCs from all three donors under osteogenic conditions (Table 14 and Figure 25A).

The global analysis showed that *ALPL* levels were significantly down-regulated in hDPSCs compared with cDPSCs at week 3 under osteogenic conditions (Figure 25B). Furthermore, hDPSCs showed significant down-regulation of *ALPL* levels at week 3 compared with week 1 under osteogenic conditions (Figure 25B).

Table 14: Fold changes in *ALPL* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions compared with cells cultured under basal conditions at 1 and 3 weeks

Duration of cultures	Fold change in gene expression in DPSCs from donor [a]:					
	H1	H2	H3	C1	C2	C3
1 week	↑ [b] 3	↑ 1.1	↑ 2.5	↑ 1.7	↑ 1.5	↑ 2.5
3 weeks	↓1.7	↓ 2	↓ 1.3	↑ 1.4	↑ 1.2	↓1.1

a. hDPSCs donors (H1, H2, H3), cDPSCs donors (C1, C2, C3)

b. ↑ = up-regulation, ↓ = down-regulation

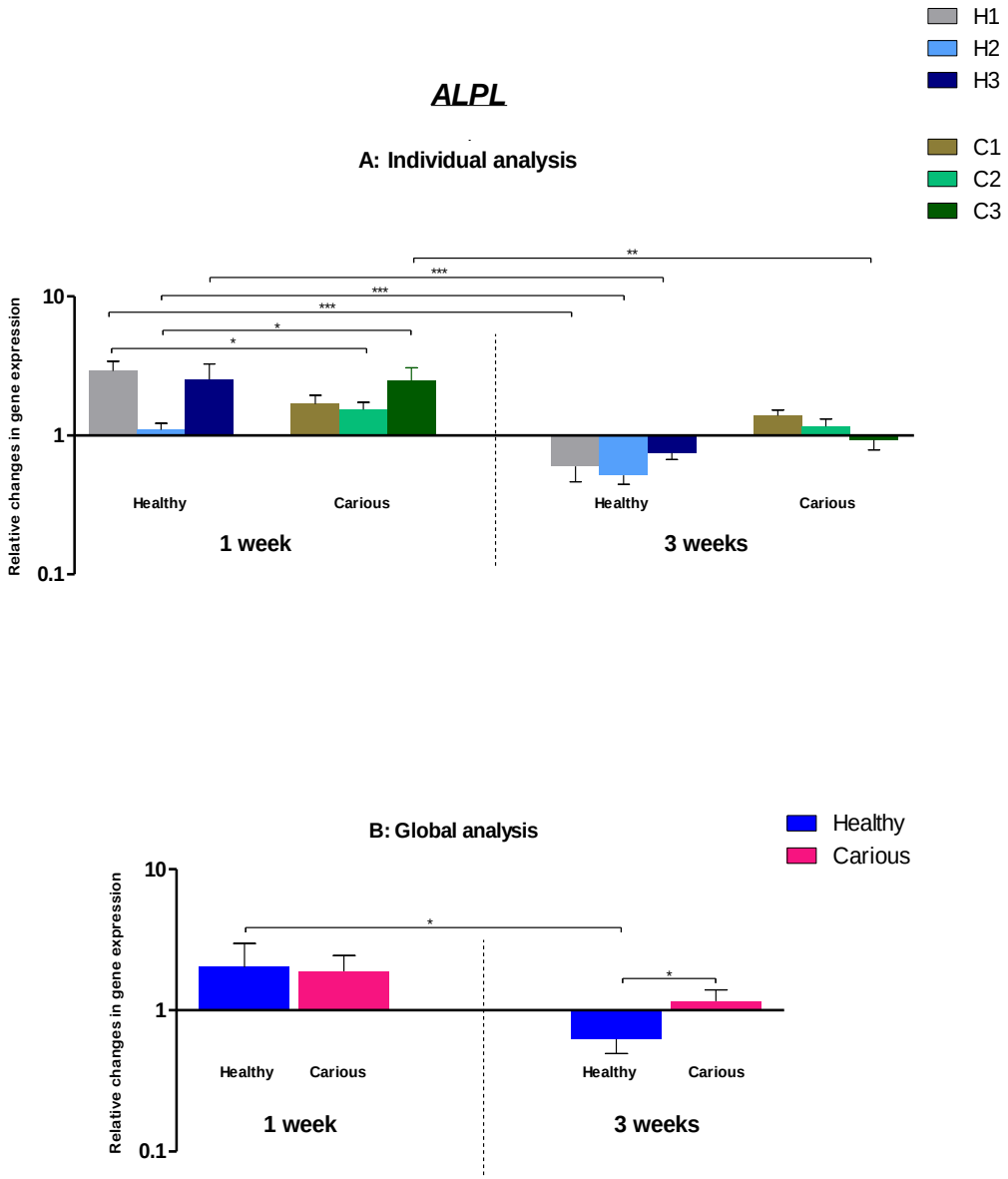


Figure 25: Relative changes in *ALPL* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions for 1 and 3 weeks

Relative changes in *ALPL* gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under osteogenic conditions for 1 and 3 weeks. **A:** Individual analysis: showing relative changes in *ALPL* gene expression in individual donors. The relative gene expression was normalised to corresponding control cultured under basal conditions. Data are presented as means $2^{-\Delta\Delta ct}$ ± SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged relative changes in *ALPL* gene expression from the three donors in each group of hDPSCs and cDPSCs. *P<0.05, **P<0.01, ***P <0.001.

5.2.2.1.2 Comparing the changes in *OC* gene expression in hDPSCs and cDPSCs

A: **Baseline expression of *OC* gene in hDPSCs and cDPSCs**

OC was expressed at moderate levels in both hDPSCs and cDPSCs cultured under basal conditions for 1 and 3 weeks (Figure 26). However at week 1 under basal conditions, cDPSCs from the first donor (C1) showed a trend to express higher levels of *OC* compared with hDPSCs from the first donor (H1) (Figure 26A). cDPSCs from the second donor (C2) showed a higher levels of *OC* expression compared with hDPSCs from all three donors at 1 week under basal conditions. The *OC* expression level at week 1 under basal conditions was higher in cDPSCs from the third donor (C3) compared with hDPSCs from the first and second donor (H1, H2) (Figure 26A).

At 3 weeks under basal conditions, two out of three donors in the cDPSCs group (C1, C2) showed higher *OC* expression levels compared with hDPSCs from the first and third donors (H1, H3) under the same conditions (Figure 26A). cDPSCs from the third donor (C3) showed a trend to express higher *OC* compared with hDPSCs from first donor (H1) at 3 weeks under basal conditions . Only differences comparing the first donor from each hDPSCs and cDPSCs group (H1 with C1) reached statistical significance at 3 weeks under basal conditions (Figure 26A).

The levels of *OC* expression were higher at 3 weeks culture compared with 1 week culture, in two out of three donors in both hDPSCs (H2, H3) and cDPSCs (C1, C2) under basal conditions (Figure 26A).

The global analysis showed a higher *OC* expression in cDPSCs compared with hDPSCs at both time points, and generally a higher expression at 3 weeks compared with 1 week under basal conditions. However, the results of global analysis did not reach statistical significance (Figure 26B).

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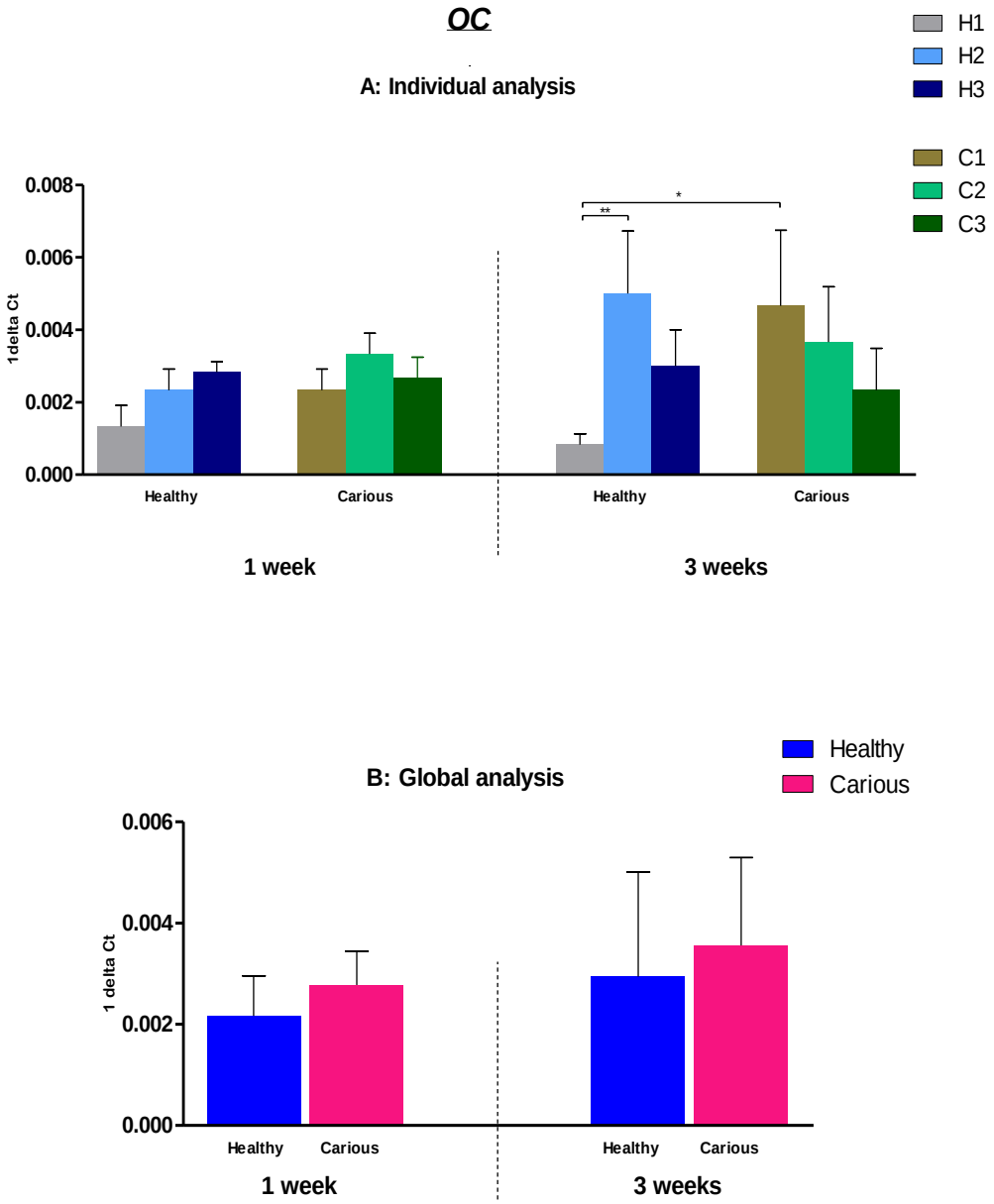

Figure 26: Baseline expression of *OC* gene in hDPSCs and cDPSCs cultured under basal conditions for 1 and 3 weeks

OC gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under basal conditions for 1 and 3 weeks. **A**: Individual analysis: showing the levels of *OC* gene expression in individual donors. The expression of *OC* was normalised to the house keeping gene (GAPDH). Data are presented as means ΔCt ±SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged expression of *OC* from the three donors in each group of hDPSCs and cDPSCs. *P <0.05, **P <0.01, ***P <0.001.

B: Relative changes in *OC* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions

At week 1, *OC* was up-regulated under osteogenic conditions compared with basal conditions in two out of three cDPSCs donors (C1, C3) and was down-regulated in two out three hDPSCs donors (H1, H2) (Table 15 and Figure 27A). At week 3, *OC levels were* up-regulated in both hDPSCs and cDPSCs cultured under osteogenic conditions compared with cells grown under basal conditions (Table 15 and Figure 27A). A general trend of higher *OC* expression was observed at both time points in cDPSCs compared with hDPSCs except in cDPSCs from the second donor (C2) at 1 week and from the first donor (C1) at 3 weeks. Statistical significance was demonstrated at the 3 week time point, comparing cDPSCs from the second donor (C2) and hDPSCs from the second and third donors (H2, H3) (Table 15 and Figure 27A).

The levels of *OC* expression under osteogenic conditions were higher at 3 weeks compared with 1 week, in hDPSCs from all three donors and in two out of three of cDPSCs donors (C2, C3), with only cDPSCs from the second donor (C2) reached statistical significance (Figure 27A).

The global analysis showed that *OC* expression levels were significantly higher at 3 weeks compared with 1 week in hDPSCs and cDPSCs grown under osteogenic conditions (Figure 27B). cDPSCs showed a trend to higher expression of *OC* compared with hDPSCs at both time points under osteogenic conditions (Figure 27B).

Table 15: Fold changes in *OC* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions compared to cells cultured under basal conditions at 1 and 3 weeks

Duration of cultures	Fold change in gene expression in DPSCs from donor [a]:					
	H1	**H2**	**H3**	**C1**	**C2**	**C3**
1 week	↓[b] 1.5	↓ 1.3	↓1.03	↑ 1.7	↓ 1.8	↑ 2
3 weeks	↑ 2.1	↑ 2	↑ 2	↑ 1.4	↑ 3.8	↑ 2.6

a. hDPSCs donors (H1, H2, H3), cDPSCs donors (C1, C2, C3)

b. ↑ = up-regulation, ↓ = down-regulation

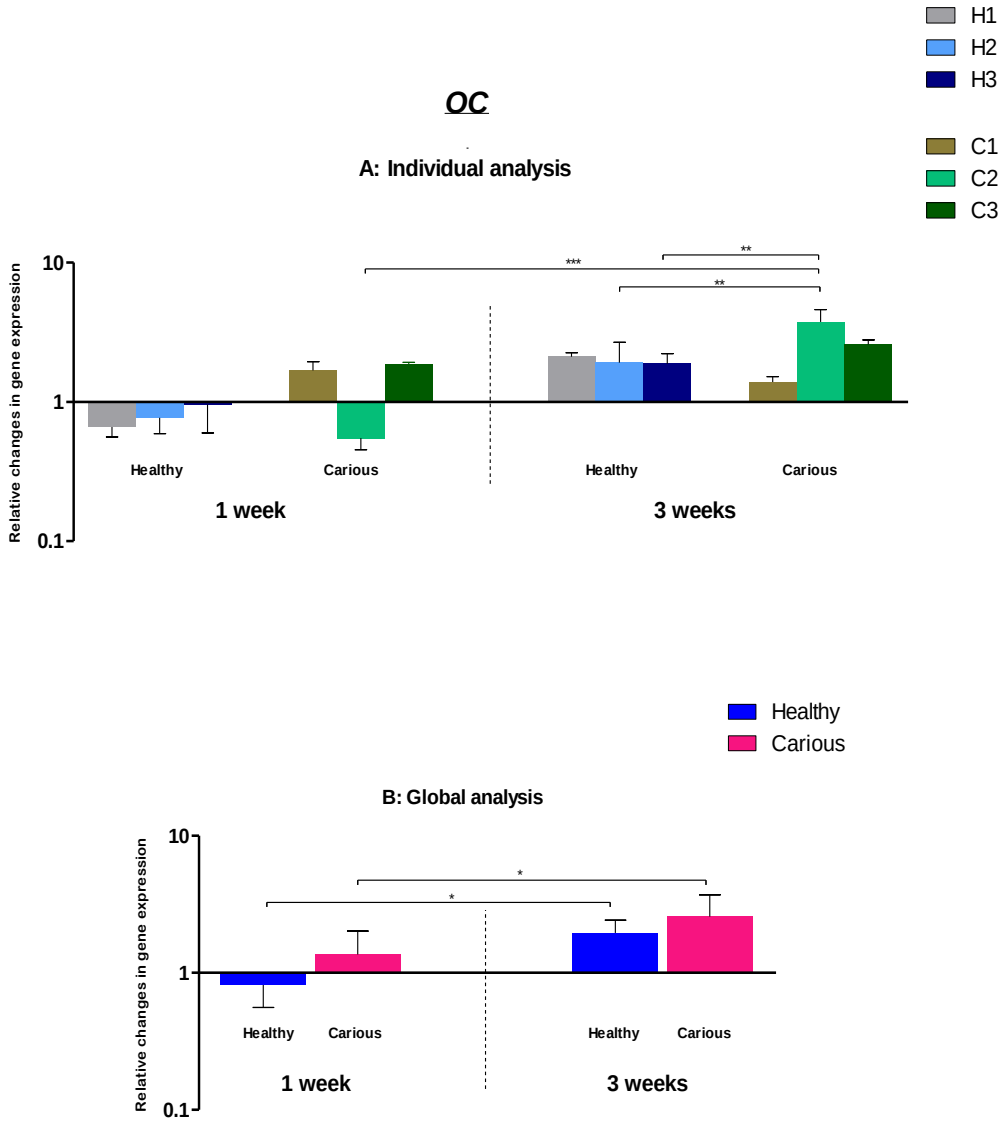

Figure 27: Relative changes in *OC* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions for 1 and 3 weeks

Relative changes in *OC* gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under osteogenic conditions for 1 and 3 weeks. **A:** Individual analysis: showing relative changes in *OC* gene expression in individual donors. The relative gene expression was normalised to corresponding control cultured under basal conditions. Data are presented as means $2^{-\Delta\Delta ct}$ ± SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged relative changes in *OC* gene expression from the three donors in each group of hDPSCs and cDPSCs. *P<0.05, **P<0.01, ***P <0.001.

5.2.2.1.3 Comparing the changes in RUNX-2 gene expression in hDPSCs and cDPSCs

A: **Baseline expression of *RUNX-2* gene in hDPSCs and cDPSCs**

RUXN-2 was expressed by both cell types at both time points under basal conditions, in cells from all donors (Figure 28). There was a notable variability in *RUNX-2* expression levels when comparing hDPSCs with cDPSCs. At 1 week under basal conditions, cDPSCs from two out of three donors (C1, C3) showed lower levels of *RUNX-2* expression compared with hDPSCs from all three donors (Figure 28A). However at 3 weeks under basal conditions, cDPSCs from the second donor (C2) showed significantly higher *RUNX-2* expression levels compared with hDPSCs from all three donors. While cDPSCs from the third donor (C3) showed a trend to express higher *RUNX-2* levels compared with hDPSCs from the first and third donors (H1, H3) (Figure 28A).

The levels of *RUNX-2* expression were higher at 1 week compared with 3 weeks in hDPSCs from all three donors under basal conditions (Figure 28A). However, in two out of three of cDPSCs donors (C2, C3), the levels of *RUNX-2* were higher at 3 weeks compared with 1 week expression under basal conditions, with only cDPSCs from the second donor (C2) reached statistical significance (Figure 28A).

The global analysis showed that *RUNX-2* baseline expression in cDPSCs was lower at 1 week culture and higher at 3 weeks culture compared with hDPSCs . In hDPSCs, the baseline expression of *RUNX-2* was higher at 1 week compared with 3 weeks, whereas in cDPSCs, the baseline expression

of *RUNX-2* was reversed. Nevertheless, none of these differences reached statistical significance (Figure 28B).

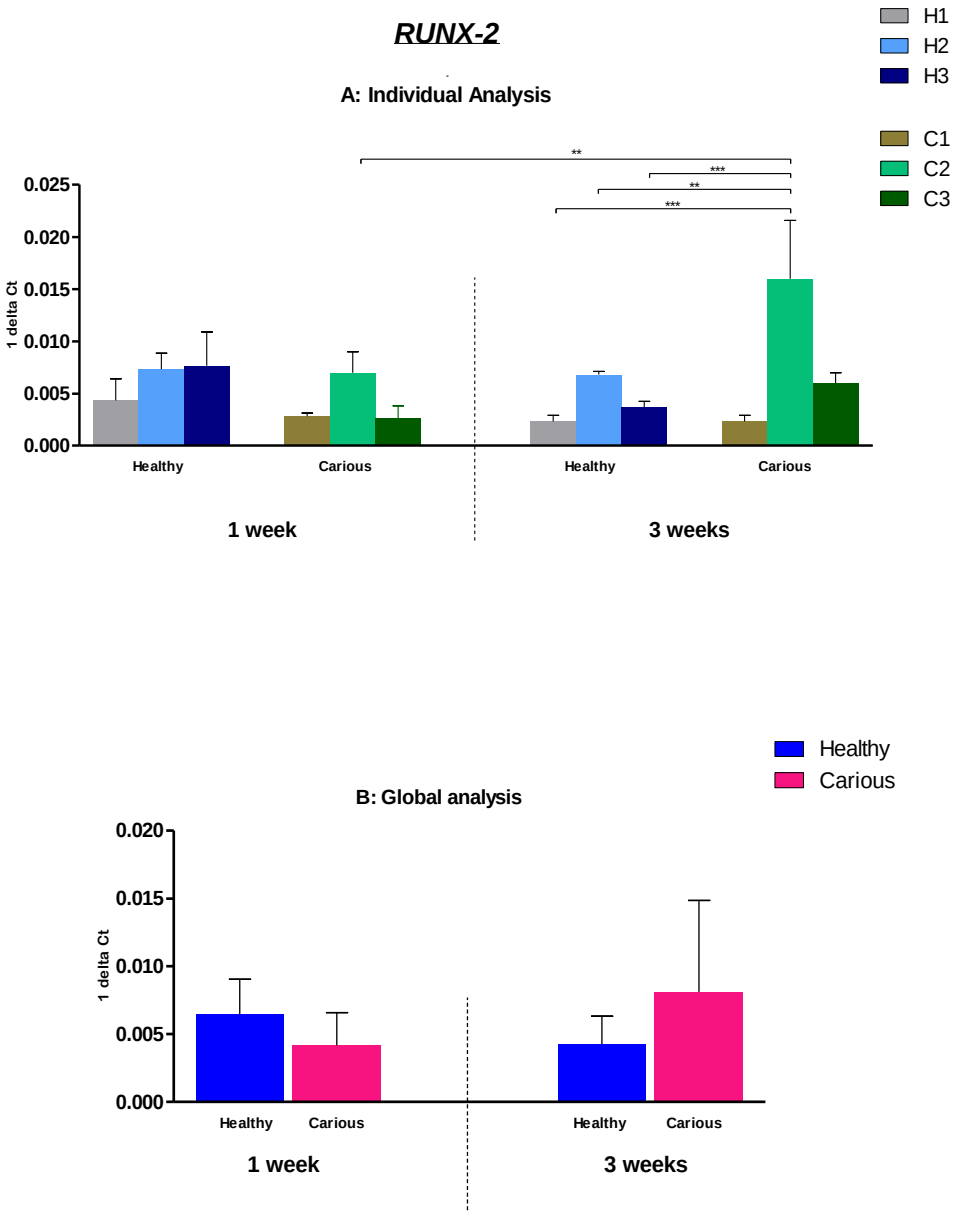

Figure 28: Baseline expression of *RUNX-2* gene in hDPSCs and cDPSCs cultured under basal conditions for 1 and 3 weeks

RUNX-2 gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under basal conditions for 1 and 3 weeks. **A**: Individual analysis: showing the levels of *RUNX-2* gene expression in individual donors. The expression of *RUNX-2* was normalised to the house keeping gene (GAPDH). Data are presented as means ΔCt ±SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged expression of *RUNX-2* from the three donors in each group of hDPSCs and cDPSCs. *P <0.05, **P <0.01, ***P <0.001.

B: Relative changes in *RUNX-2* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions

RUNX-2 was up-regulated in both cell types and at both time points in cells from all donors grown under osteogenic conditions compared with cells cultured under basal conditions (Table 16 and Figure 29A).

Interestingly under osteogenic conditions, cDPSCs from two out of three donors (C1, C3) showed higher levels of *RUNX-2* expression at 1 and 3 weeks compared with hDPSCs from all three donors. However, only comparing cDPSCs from the first donor (C1) with hDPSCs from the second donor (H2) reached statistical significance (Table 16 and Figure 29A).

However, the levels of *RUNX-2* expression in hDPSCs and cDPSCs were higher at 3 weeks compared with 1 week under osteogenic conditions with only first and second donor in cDPSCs group (C1, C2) showing statistical significance comparing 1 and 3 week time points (Table 16 and Figure 29A).

The global analysis showed that levels of *RUNX-2* were higher at 3 weeks compared with 1 week in both cell types, but only the difference comparing cDPSCs was significant. *RUNX-2* expression was higher in cDPSCs compared with hDPSCs at 1 and 3 weeks under osteogenic conditions compared with cells under basal conditions. However, these differences did not reach statistical significance due to variation between donors (Figure 29B).

Table 16: Fold changes in *RUNX-2* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions compared with cells cultured under basal conditions at 1 and 3 weeks

Duration of cultures	Fold change in gene expression in DPSCs from donor [a]:					
	H1	**H2**	**H3**	**C1**	**C2**	**C3**
1 week	↑[b] 1.4	↑ 2.02	↑ 1.3	↑ 3.8	↑ 1.2	↑ 2.5
3 weeks	↑ 3.02	↑ 4	↑ 2.5	↑ 21.8	↑ 2.5	↑ 5.6

a. hDPSCs donors (H1, H2, H3), cDPSCs donors (C1, C2, C3)

b. ↑ = up-regulation, ↓ = down-regulation

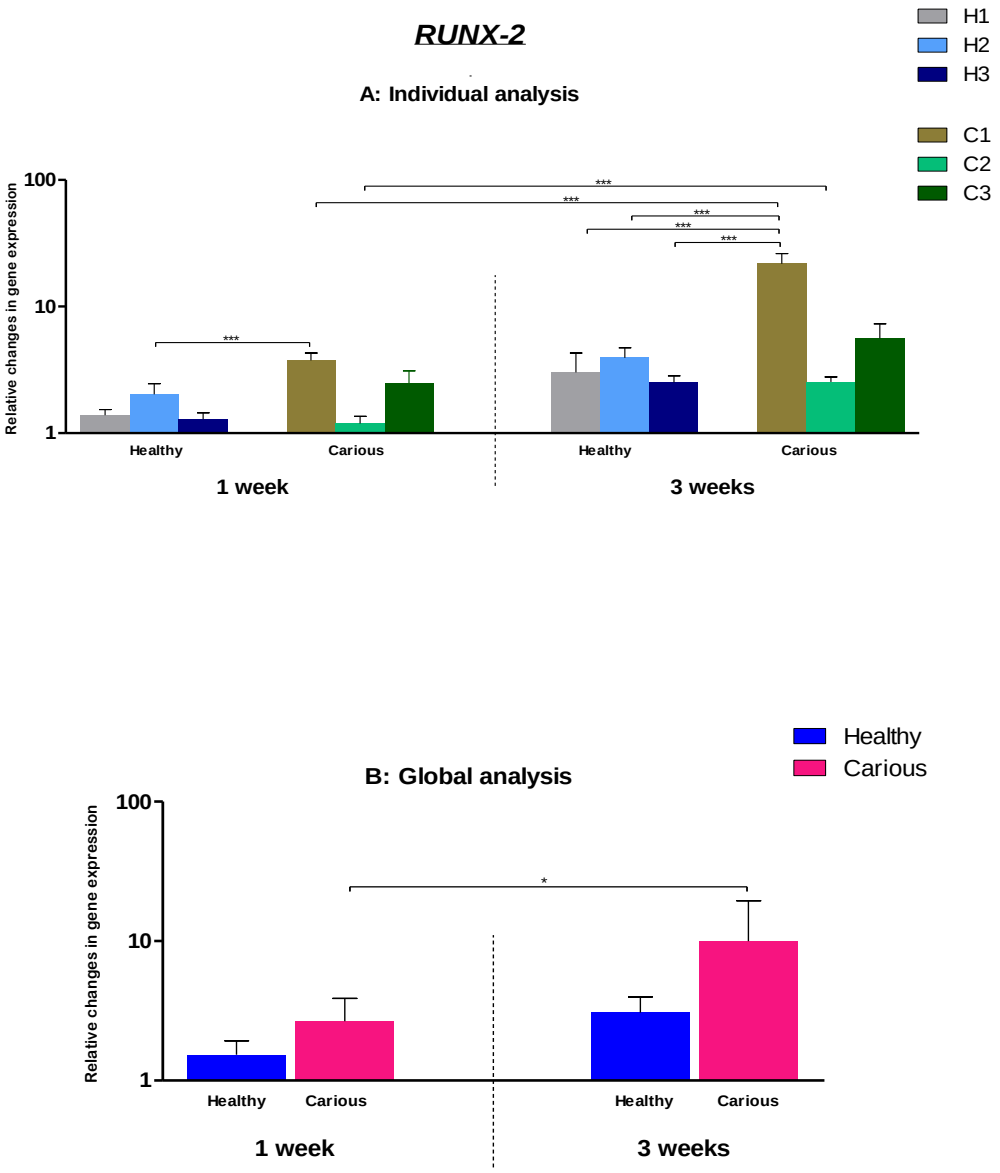

Figure 29: Relative changes in *RUNX-2* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions for 1 and 3 weeks

Relative changes in *RUNX-2* gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under osteogenic conditions for 1 and 3 weeks. **A:** Individual analysis: showing relative changes in *RUNX-2* gene expression in individual donors. The relative gene expression was normalised to corresponding control cultured under basal conditions. Data are presented as means $2^{-\Delta\Delta ct}$ ± SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged relative changes in *RUNX-2* gene expression from the three donors in each group of hDPSCs and cDPSCs. *P<0.05, **P<0.01, ***P <0.001.

5.2.2.2 Expression of angiogenic marker genes in hDPSCs and cDPSCs

The gene expression of angiogenic markers (VEGFR-2 and PECAM-1) were investigated in hDPSCs (n=3) and cDPSCs (n=3), both cultured in monolayers under basal and osteogenic conditions for 1 and 3 weeks.

5.2.2.2.1 Comparing the changes in *VEGFR-2* gene expression in hDPSCs and cDPSCs

A: **Baseline expression of *VEGFR-2* gene in hDPSCs and cDPSCs**

The baseline expression levels of *VEGFR-2* were very low in both hDPSCs and cDPSCs, after 1 and 3 weeks of basal culture (Figure 30), and compared with the levels of osteogenic markers discussed in the previous section. cDPSCs showed higher levels of baseline expression of *VEGFR-2* in all three donors at week 1, and in two out of three donors (C2, C3) at week 3 of culture, compared with hDPSCs. However results were only significant at week 3 when comparing cDPSCs from the third donor (C3) with hDPSCs from all three donors (Figure 30A).

Comparing the baseline expression of *VEGFR-2* at the two different time points of culture (1 and 3 weeks), within the same cell type; hDPSCs revealed no significant difference. In contrast, cDPSCs from the third donor (C3) showed significantly higher expression at 3 weeks compared with 1 week under basal conditions (Figure 30A).

The global analysis confirmed largely the individual analysis. It showed a higher baseline expression of *VEGFR-2* in cDPSCs compared with hDPSCs,

at both time points with this being statistically significant only at 3 weeks. cDPSCs showed a higher baseline expression of VEGFR-2 at week 3 compared with week 1, but this was not significant difference (Figure 30B).

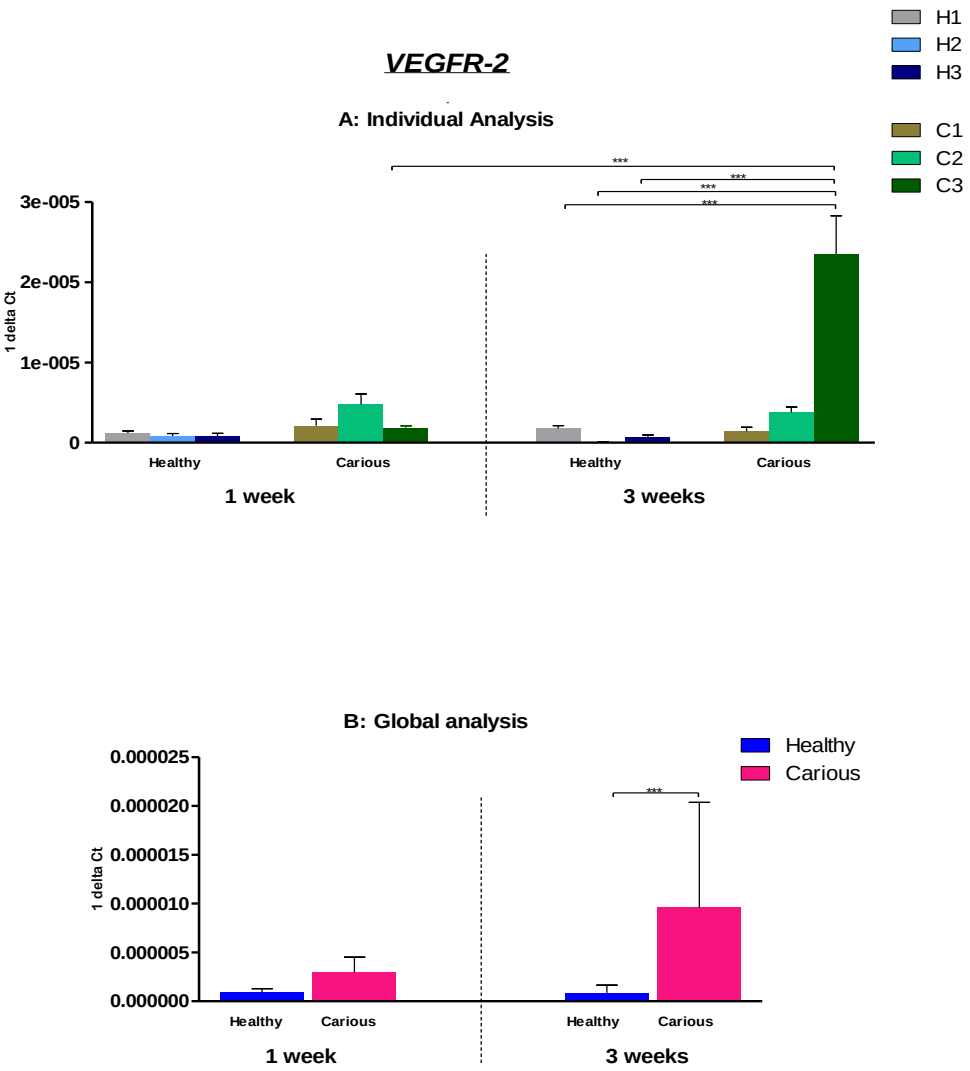

Figure 30: Baseline expression of *VEGFR-2* gene in hDPSCs and cDPSCs cultured under basal conditions for 1 and 3 weeks

VEGFR-2 gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under basal conditions for 1 and 3 weeks. **A**: Individual analysis: showing the levels of *VEGFR-2* gene expression in individual donors. The expression of *VEGFR-2* was normalised to the house keeping gene (GAPDH). Data are presented as means ΔCt ±SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged expression of *VEGFR-2* from the three donors in each group of hDPSCs and cDPSCs. *P <0.05, **P <0.01, ***P <0.001.

B: Relative changes in *VEGFR-2* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions

The expression of *VEGFR-2* in hDPSCs and cDPSCs from all donors, cultured under osteogenic conditions for 1 and 3 weeks was up-regulated compared with cells cultured under basal conditions (Table 17 and Figure 31A).

hDPSCs from the second donor (H2) showed significantly higher levels of *VEGFR-2* expression compared with cDPSCs from all three donors, after 1 and 3 weeks of culture under osteogenic conditions compared with cells cultured under basal conditions (Table 17 and Figure 31A). However, in two out of three donors in the cDPSCs group at 1 week (C1, C3), and at 3 weeks (C1, C2) showed higher levels of *VEGFR-2* expression compared with hDPSCs from the first and third donors (H1, H3) under osteogenic conditions (Table 17 and Figure 31A).

hDPSCs showed no significant difference in *VEGFR-2* expression comparing cells cultured for 1 week or 3 weeks. cDPSCs showed slightly higher levels of *VEGFR-2* expression at 3 weeks compared with 1 week, in two out of three donors (C1, C2) under osteogenic conditions (Table 17 and Figure 31A).

The global analysis showed that *VEGFR-2* expression was lower in cDPSCs compared to hDPSCs under osteogenic conditions compared with cells cultured under basal conditions at 1 and 3 weeks. This confirmed the results found in the individual comparisons of *VEGFR-2* expression at the different time points of culture, within the same cell type (Figure 31B).

Table 17: Fold changes in *VEGFR-2* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions compared with cells cultured under basal conditions at 1 and 3 weeks

Duration of cultures	Fold change in gene expression in DPSCs from donor [a]:					
	H1	H2	H3	C1	C2	C3
1 week	↑[b] 7.03	↑ 162.9	↑ 5.4	↑ 8.1	↑ 6.1	↑ 7.9
3 weeks	↑ 9.6	↑ 119.1	↑ 5.6	↑ 16.2	↑ 31.04	↑ 2.8

a. hDPSCs donors (H1, H2, H3), cDPSCs donors (C1, C2, C3)

b. ↑ = up-regulation, ↓ = down-regulation

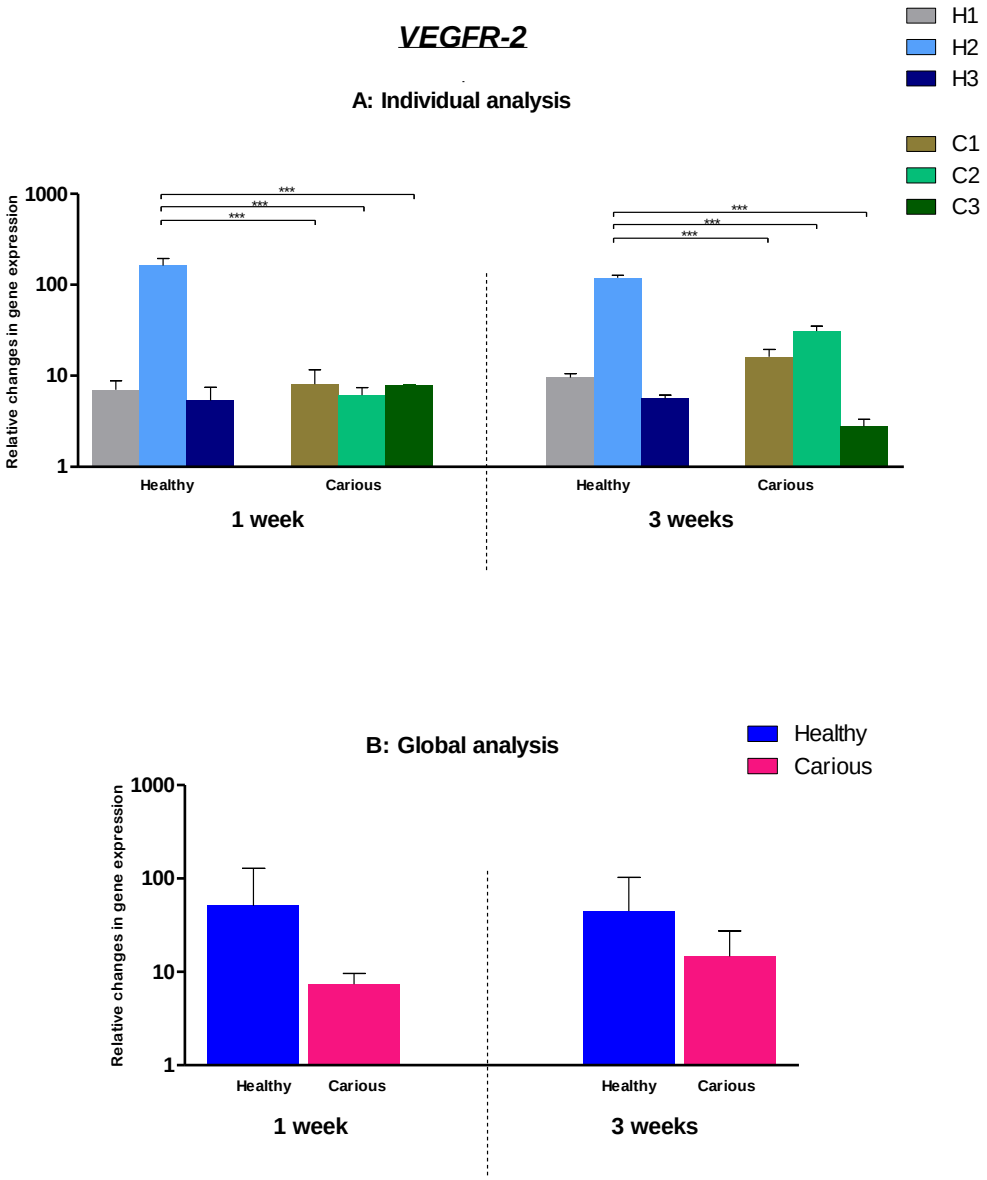

Figure 31: Relative changes in *VEGFR-2* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions for 1 and 3 weeks

Relative changes in *VEGFR-2* gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under osteogenic conditions for 1 and 3 weeks. **A:** Individual analysis: showing relative changes in *VEGFR-2* gene expression in individual donors. The relative gene expression was normalised to corresponding control cultured under basal conditions. Data are presented as means $2^{-\Delta\Delta ct}$ ± SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged relative changes in *VEGFR-2* gene expression from the three donors in each group of hDPSCs and cDPSCs. *P<0.05, **P<0.01, ***P <0.001.

5.2.2.2.2 Comparing the changes in *PECAM-1* gene expression in hDPSCs and cDPSCs

A: **Baseline expression of *PECAM-1* gene in hDPSCs and cDPSCs**

PECAM-1 expression levels were very low in both hDPSCs and cDPSCs cultured under basal conditions at 1 and 3 week time points (Figure 32). The baseline expression of *PECAM-1* at 1 week was higher in cDPSCs from all three donors compared with hDPSCs from the first and third donors (H1, H3) (Figure 32A).

However, at 3 weeks the baseline expression of *PECAM-1* was higher in two out of three donors of cDPSCs (C1, C3) compared with hDPSCs from all donors, with only cDPSCs from the third donor (C3) showing statistically significant differences (Figure 32A).

There was no significant difference in the baseline levels of *PECAM-1* expression comparing 1 and 3 weeks cultures within the same cell type, except in cDPSCs from the third donor (C3), which showed significantly higher expression of *PECAM-1* at week 3 compared with week 1 (Figure 32).

Global analysis showed a trend for cDPSCs to express higher levels of *PECAM-1* compared with hDPSCs at 3 weeks under basal conditions (Figure 32B). Also, cDPSCs showed higher baseline expression levels of *PECAM-1* at week 3 compared with week 1 (Figure 32B).

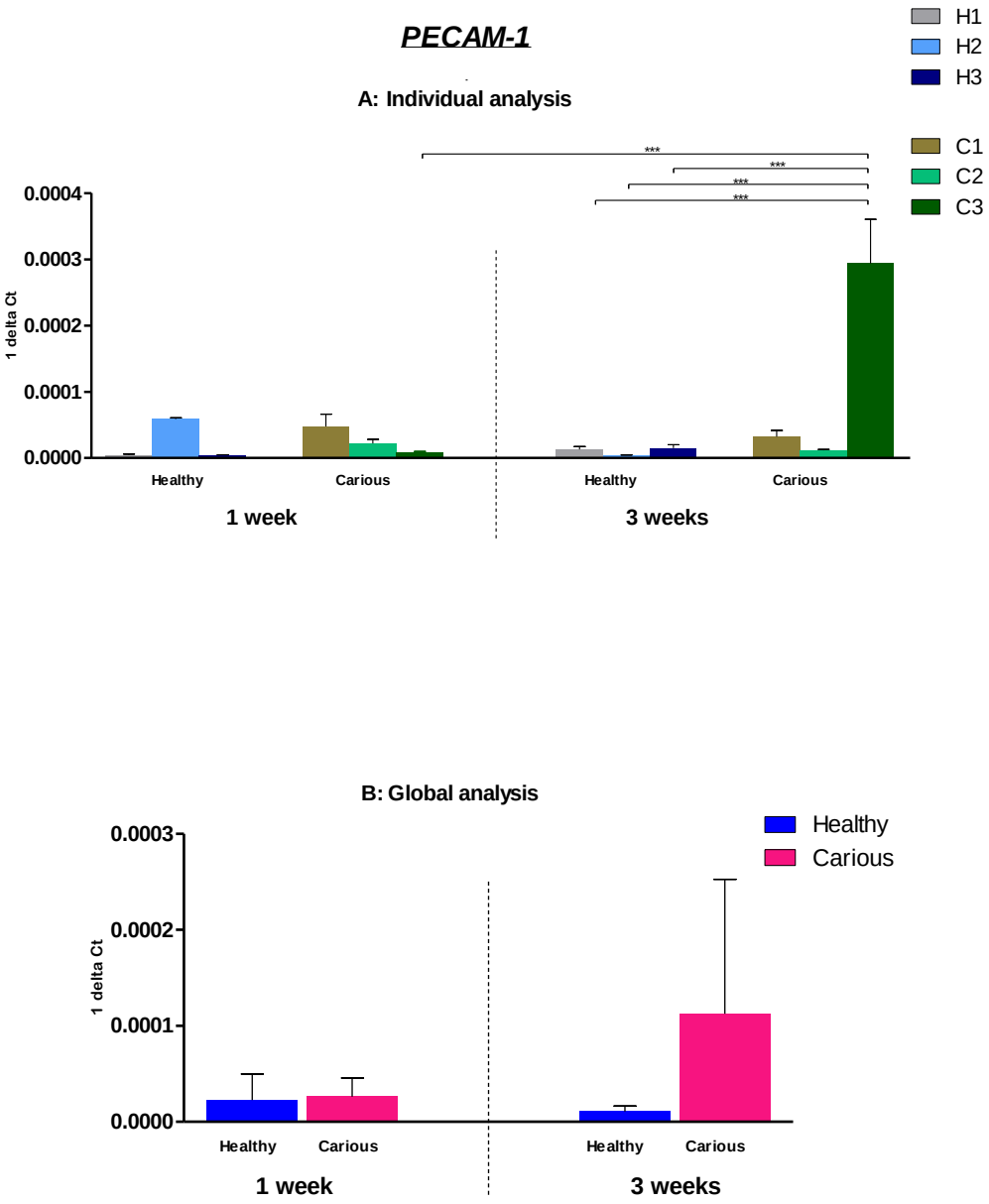

Figure 32: Baseline expression of *PECAM-1* gene in hDPSCs and cDPSCs cultured under basal conditions for 1 and 3 weeks

PECAM-1 gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under basal conditions for 1 and 3 weeks. **A**: Individual analysis: showing the levels of *PECAM-1* gene expression in individual donors. The expression of *PECAM-1* was normalised to the house keeping gene (GAPDH). Data are presented as means ΔCt ±SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged expression of *PECAM-1* from the three donors in each group of hDPSCs and cDPSCs. *P <0.05, **P <0.01, ***P <0.001.

B: Relative changes in *PECAM-1* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions

The levels of PECAM-1 were up-regulated in both hDPSCs and cDPSCs from all donors at 1 and 3 weeks under osteogenic culture conditions. The only exception was the cDPSCs from the third donor (C3) at week 3, which demonstrated a down-regulation in *PECAM-1* levels under osteogenic culture conditions (Table 18 and Figure 33A).

At 1 week under osteogenic conditions, cDPSCs from two out of three donors (C1, C3) showed higher levels of *PECAM-1* expression compared with expression by hDPSCs from all three donors (Table 18 and Figure 33A). However, at 3 weeks under osteogenic conditions, cDPSCs from two out of three donors (C1, C3) showed lower level of *PECAM-1* expression compared with hDPSCs (H1, H3) cultured under the same conditions, only cDPSCs from the third donor (C3) showed a statistically significant difference in comparison with hDPSCs from the first donor (H1) (Table 18 and Figure 33A).

The levels of *PECAM-1* gene expression were higher at week 3 compared with week 1 in all hDPSCs donors. However, in cDPSCs, two out of three donors (C1, C3) showed higher levels of *PECAM-1* expression at 1 week under osteogenic conditions compared with 3 weeks, with only third donor (C3) showing statistical significance (Table 18 and Figure 33A). In contrast, cDPSCs from the second donor (C2) showed significantly lower levels of *PECAM-1* expression at 1 week compared to 3 weeks under osteogenic conditions (Table 18 and Figure 33A).

The global analysis showed higher levels of *PECAM-1* expression in cDPSCs at 1 week under osteogenic conditions compared with hDPSCs (Figure 33B), while at 3 weeks cDPSCs showed slightly lower levels of *PECAM-1* compared with hDPSCs under osteogenic conditions (Figure 33B). The level of *PECAM-1* was higher in hDPSCs at 3 weeks compared to 1 week under osteogenic conditions (Figure 33B).

Table 18: Fold changes in *PECAM-1* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions compared with cells cultured under basal conditions at 1 and 3 weeks

Duration of cultures	Fold change in gene expression in DPSCs from donor [a]:					
	H1	H2	H3	C1	C2	C3
1 week	↑[b] 1.4	↑ 1.04	↑ 1.5	↑ 3	↑ 1.1	↑ 2.8
3 weeks	↑ 3.2	↑ 1.7	↑ 2.6	↑ 1.7	↑ 4	↓ 4.1

a. hDPSCs donors (H1, H2, H3), cDPSCs donors (C1, C2, C3)
b. ↑ = up-regulation, ↓ = down-regulation

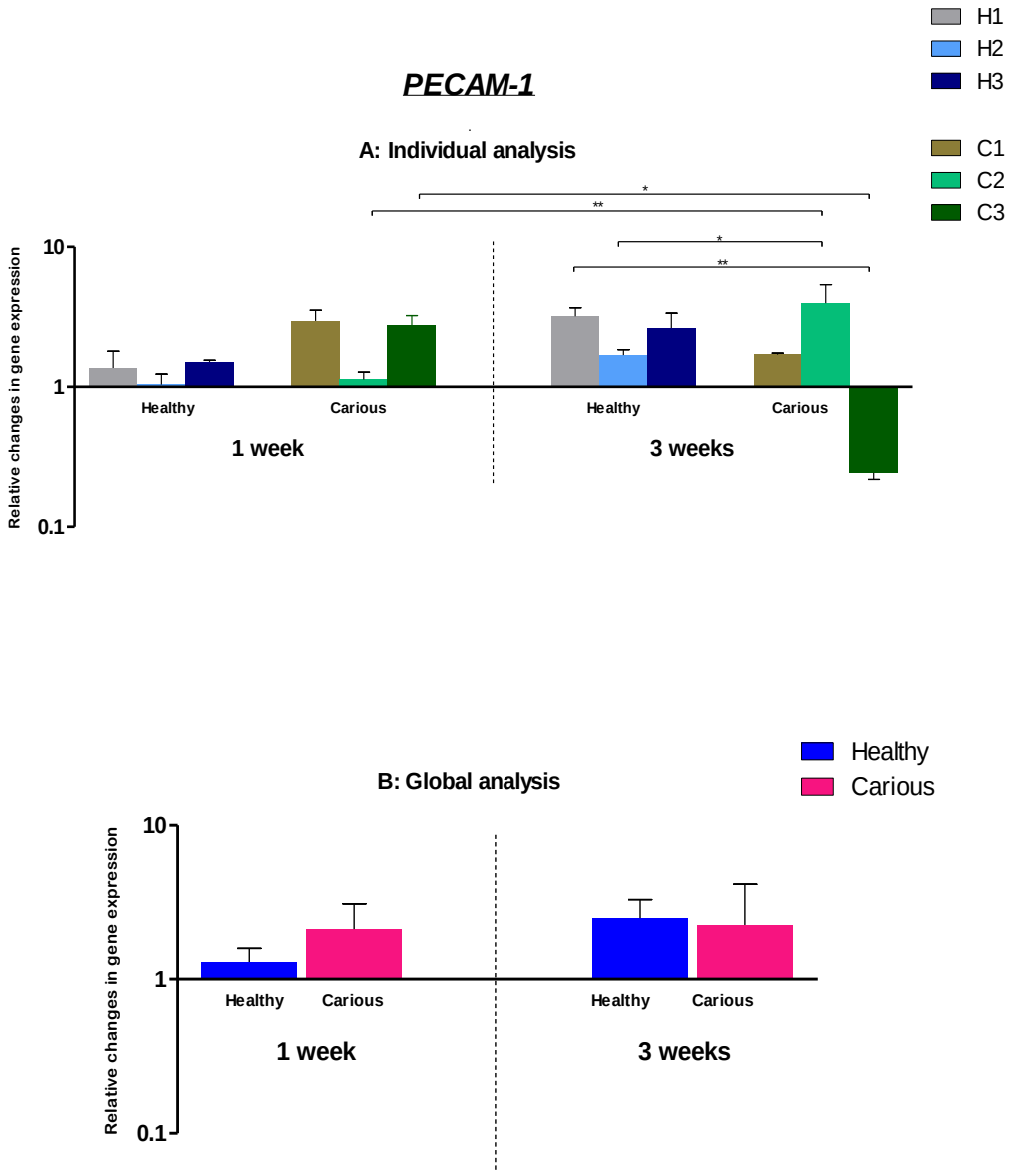

Figure 33: Relative changes in *PECAM-1* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions for 1 and 3 weeks

Relative changes in *PECAM-1* gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under osteogenic conditions for 1 and 3 weeks. **A:** Individual analysis: showing relative changes in *PECAM-1* gene expression in individual donors. The relative gene expression was normalised to corresponding control cultured under basal conditions. Data are presented as means $2^{-\Delta\Delta ct}$ ± SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged relative changes in *PECAM-1* gene expression from the three donors in each group of hDPSCs and cDPSCs. *P<0.05, **P<0.01, ***P <0.001.

5.2.2.3 Expression of inflammatory marker genes in hDPSCs and cDPSCs

The gene expression of inflammatory markers (*TLR-2* and *TLR-4*) were investigated in hDPSCs (n=3) and cDPSCs (n=3), both cultured in monolayers under basal and osteogenic conditions for 1 and 3 weeks.

5.2.2.3.1 Comparing the changes in TLR-2 gene expression in hDPSCs and cDPSCs

A: **Baseline expression of *TLR-2* gene in hDPSCs and cDPSCs**

The baseline expression of *TLR-2* was low to moderate in both hDPSCs and cDPSCs, cultured under basal conditions at 1 and 3 weeks (Figure 34). cDPSCs from all three donors showed significantly higher levels of *TLR-2* at both time points, compared with hDPSCs under the basal culture conditions. The only exception was in cDPSCs from the first donor (C1) compared with hDPSCs from the second donor (H2) at 1 week under basal conditionswhere there was no significant difference (Figure 34A)

The baseline levels of *TLR-2* expression showed a trend to higher expression at 3 weeks compared with 1 week in hDPSCs from the first donor (H1) and in cDPSCs from the first and third donor (C1, C3), with only differences comparing cDPSCs from the first donor (C1) showing statistical significance (Figure 34A).

Global analysis confirmed that baseline levels of *TLR-2* expression were significantly higher in cDPSCs compared with hDPSCs at both time points. However, there was no significant difference in the baseline expression

between week 1 and 3 within the same cell type cultured under basal conditions (Figure 34B).

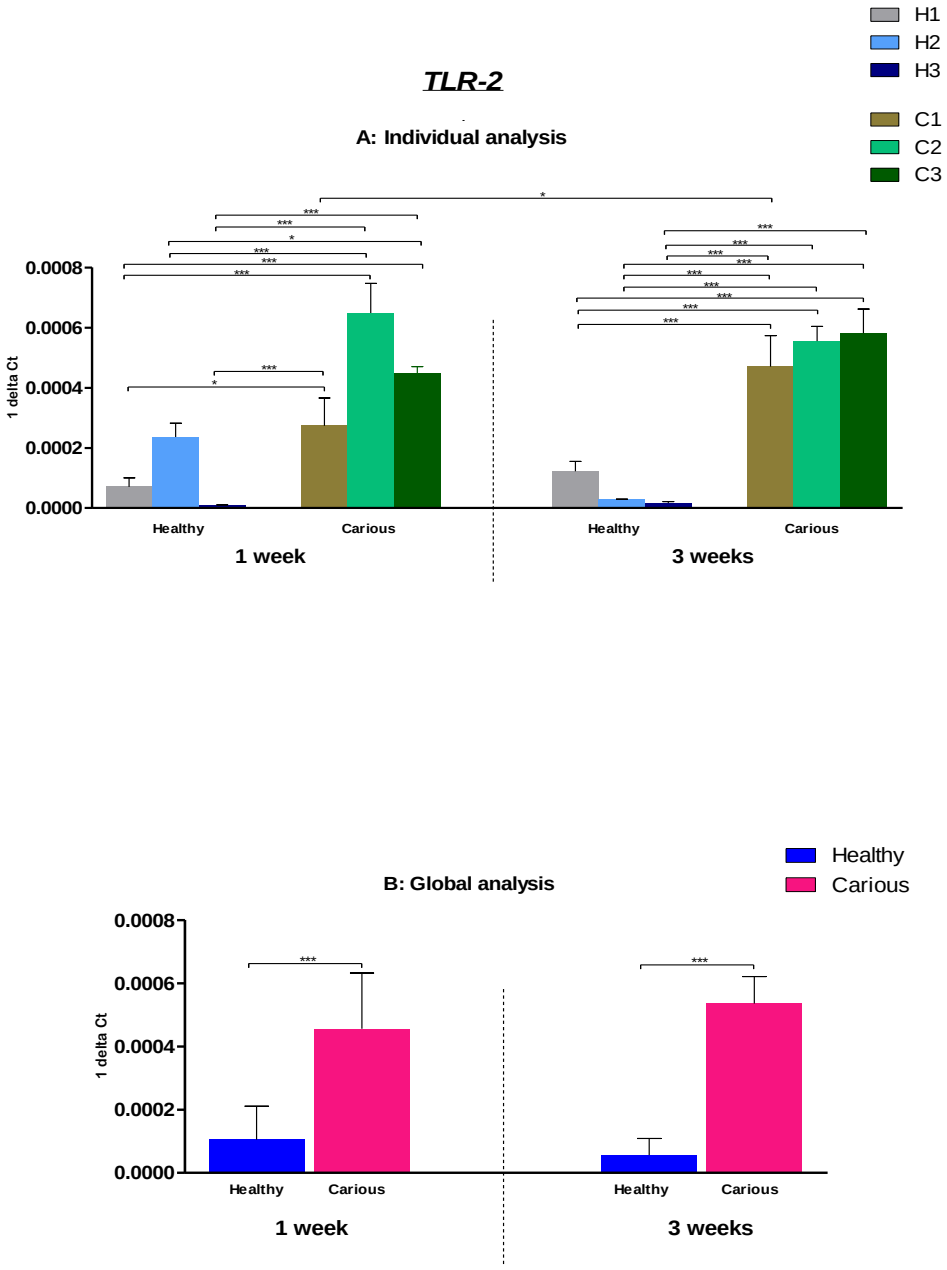

Figure 34: Baseline expression of *TLR-2* gene in hDPSCs and cDPSCs cultured under basal conditions for 1 and 3 weeks

TLR-2 gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under basal conditions for 1 and 3 weeks. **A**: Individual analysis: showing the levels of *TLR-2* gene expression in individual donors. The expression of *TLR-2* was normalised to the house keeping gene (GAPDH). Data are presented as means ΔCt ±SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged expression of *TLR-2* from the three donors in each group of hDPSCs and cDPSCs. *P <0.05, **P <0.01, ***P <0.001.

B: Relative changes in *TLR-2* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions

For hDPSCs from all three donors, the level of *TLR-2* expression was up-regulated at 1 and 3 weeks under osteogenic conditions compared with cells cultured under basal conditions (Table 19 and Figure 35A). The only exception was for hDPSCs from the third donor (H3), in which the level of *TLR-2* expression was down-regulated at 1 week under osteogenic conditions compared with basal conditions (Table 19 and Figure 35A).

For cDPSCs from all three donors, the level of *TLR-2* expression was up-regulated at 1 and 3 weeks under osteogenic conditions compared with cells cultured under basal conditions (Table 19 and Figure 35A). The only exception was for cDPSCs from the first donor (C1), in which the level of *TLR-2* expression was down-regulated at 1 week under osteogenic conditions compared with basal conditions (Table 19 and Figure 35A).

hDPSCs from two out of three donors (H1, H2) showed higher levels of *TLR-2* expression compared with cDPSCs from all three donors at 1 week under osteogenic conditions, with only cDPSCs from the first and third donors (C1, C3) showing statistical significance (Figure 35A). However at 3 weeks under osteogenic conditions, the level of *TLR-2* was significantly higher in hDPSCs from the second donor (H2) compared with cDPSCs from all donors (Figure 35A).

The levels of *TLR-2* were higher at 3 weeks compared with 1 week, in two out of three of hDPSCs (H2, H3) and cDPSCs (C2, C3) under osteogenic conditions, with only hDPSCs from the second donor (H2) showing statistical significance (Figure 35A).

Although differences were not statistically significant, the global analysis showed that *TLR-2* expression was lower in cDPSCs compared with hDPSCs at 1 and 3 weeks under osteogenic conditions compared with cells cultured under basal conditions. The *TLR-2* levels were slightly higher at 3 weeks compared with 1 week, in hDPSCs and cDPSCs cultured under osteogenic conditions compared with basal controls (Figure 35B).

Table 19: Fold changes in *TLR-2* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions compared with cells cultured under basal conditions at 1 and 3 weeks

Duration of cultures	Fold change in gene expression in DPSCs from donor [a]:					
	H1	H2	H3	C1	C2	C3
1 week	↑[b] 3.9	↑ 5.1	↓ 1.6	↓ 4.03	↑ 2.8	↑ 1.3
3 weeks	↑ 2.7	↑ 8.1	↑ 2.04	↑ 1.001	↑ 3.2	↑ 2.1

a. hDPSCs donors (H1, H2, H3), cDPSCs donors (C1, C2, C3)
b. ↑ = up-regulation, ↓ = down-regulation

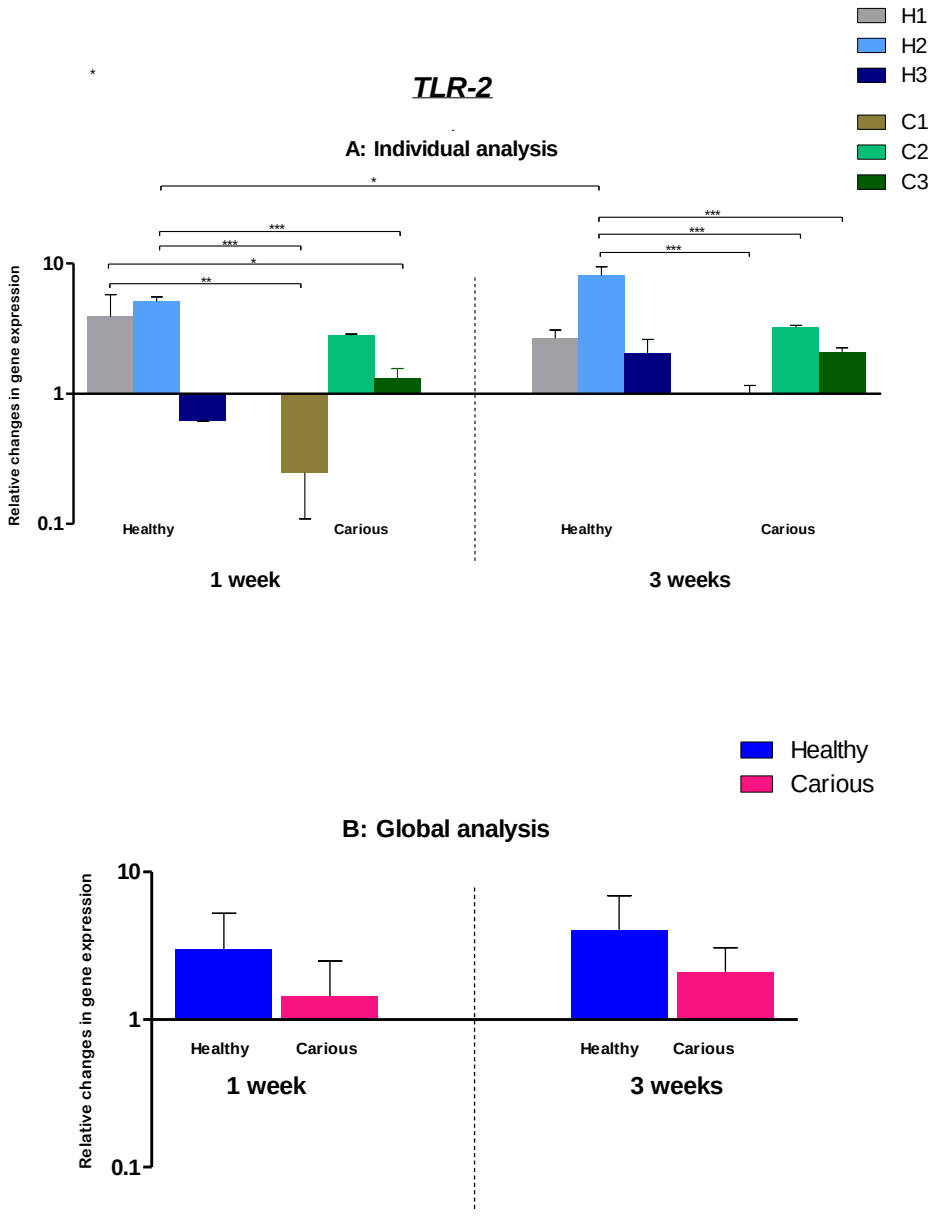

Figure 35: Relative changes in *TLR-2* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions for 1 and 3 weeks

Relative changes in *TLR-2* gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under osteogenic conditions for 1 and 3 weeks. **A:** Individual analysis: showing relative changes in *TLR-2* gene expression in individual donors. The relative gene expression was normalised to corresponding control cultured under basal conditions. Data are presented as means $2^{-\Delta\Delta ct}$ ± SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged relative changes in *TLR-2* gene expression from the three donors in each group; hDPSCs and cDPSCs. *P<0.05, **P<0.01, ***P <0.001.

5.2.2.3.2 Comparing the changes in TLR-4 gene expression in hDPSCs and cDPSCs

A: **Baseline expression of *TLR-4* gene in hDPSCs and cDPSCs**

TLR-4 baseline levels of expression were low to moderate in both hDPSCs and cDPSCs, cultured under basal conditions at 1 and 3 weeks (Figure 36). cDPSCs showed significantly higher baseline levels of *TLR-4* expression from all three donors at both time points compared with hDPSCs cultured under the same conditions. The only exception was in cDPSCs from the first and second donors (C1, C2) compared with hDPSCs from the third donor (H3) at 3 weeks under basal conditions (Figure 36A). The baseline levels of *TLR-4* expression were significantly higher at 3 weeks compared with 1 week , in hDPSCs (H3) and cDPSCs from the third donor (C3) (Figure 36A).

The global analysis showed significantly higher baseline levels of *TLR-4* in cDPSCs compared with hDPSCs (Figure 36B). It also showed no significant difference between 1 and 3 weeks within the same cell type (Figure 36B).

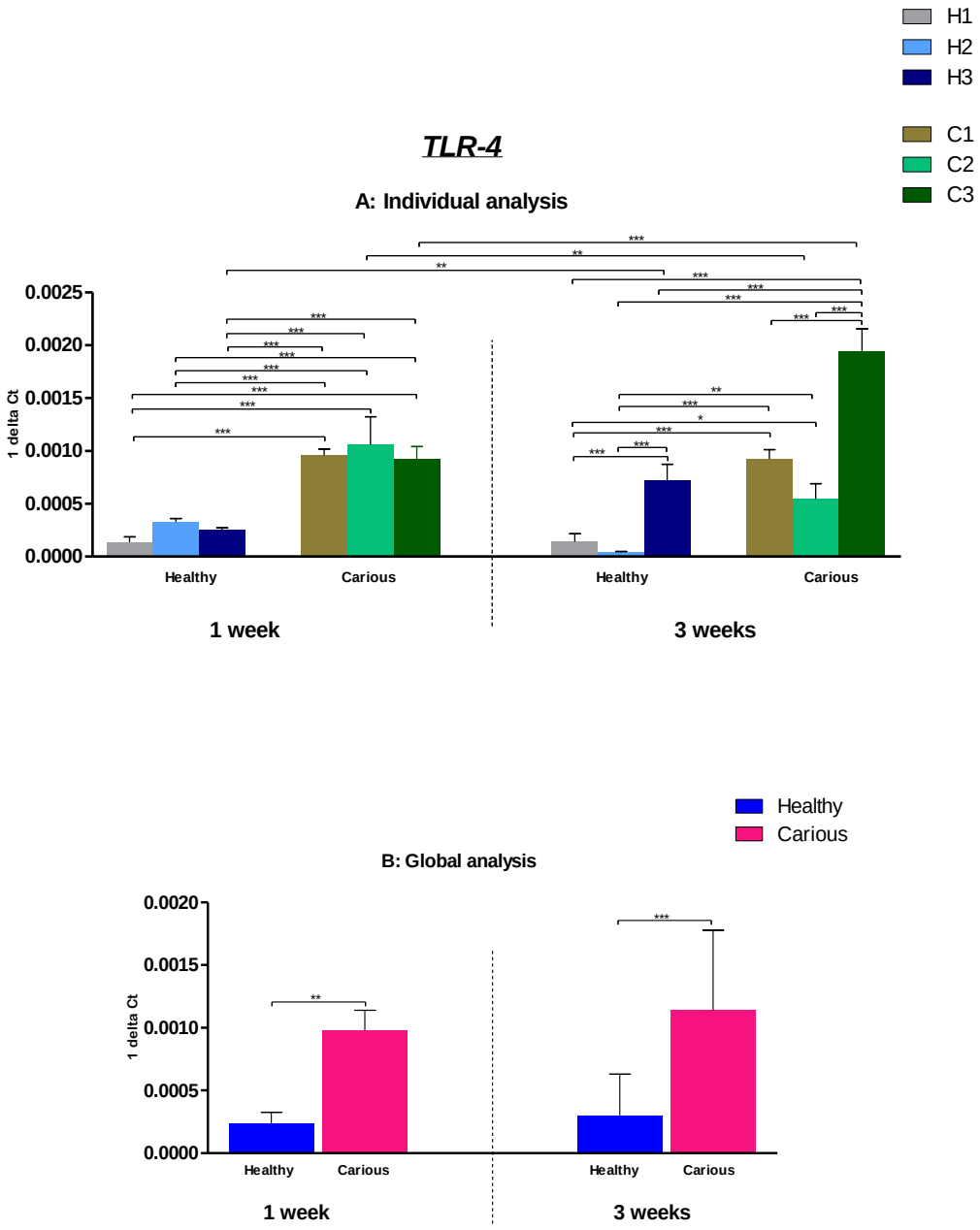

Figure 36: Baseline expression of *TLR-4* gene in hDPSCs and cDPSCs cultured under basal conditions for 1 and 3 weeks

TLR-4 gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under basal conditions for 1 and 3 weeks. **A**: Individual analysis: showing the levels of *TLR-4* gene expression in individual donors. The expression of *TLR-4* was normalised to the house keeping gene (GAPDH). Data are presented as means ΔCt ±SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged expression of *TLR-4* from the three donors in each group of hDPSCs and cDPSCs. *P <0.05, **P <0.01, ***P <0.001.

B: Relative changes in *TLR-4* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions

For hDPSCs from all three donors, the level of *TLR-4* expression was up-regulated at 1 and 3 weeks under osteogenic conditions compared with cells cultured under basal conditions (Table 20 and Figure 37A). For cDPSCs from all three donors, the level of *TLR-4* expression was up-regulated at 1 week and 3 weeks under osteogenic conditions compared with cells cultured under basal conditions (Table 20 and Figure 37A). The only exception was the third donor (C3) where the level of *TLR-4* expression was slightly down-regulated at 1 week under osteogenic conditions compared with basal conditions (Table 20 and Figure 37A). hDPSCs from two out of three donors (H1, H2) at 1 week showed higher levels of *TLR-4* expression compared with cDPSCs from the first and third donors (C1, C3) under osteogenic conditions compared with basal controls, with only cDPSCs from the third donor (C3) showing statistical significance (Table 20 and Figure 37A). However, at 3 weeks under osteogenic conditions, hDPSCs from the first and third donors (H1, H3) showed higher levels of *TLR-4* expression compared with cDPSCs from all donors, with only hDPSCs from the first donor (H1) reached statistical significance (Table 20 and Figure 37A).

The levels of *TLR-4* expression were significantly higher at 3 weeks compared with 1 week in two out three of hDPSCs donors (H1, H3) (Table 20 and Figure 37A). The level of *TLR-4* expression was significantly higher at 1 week compared with 3 weeks in cDPSCs from the second donor (C2) (Table 20 and Figure 37A).

The global analysis confirmed that cDPSCs showed lower levels of expression of *TLR-4* under osteogenic conditions compared with hDPSCs, with statistical significance only at week 3 time point. There was no significant difference in levels of *TLR-4* levels between 1 and 3 weeks under osteogenic conditions within the same cell type (Figure17B).

Table 20: Fold changes in *TLR-4* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions compared with cells cultured under basal conditions at 1 and 3 weeks

Duration of cultures	Fold change in gene expression in DPSCs from donor [a]:					
	H1	H2	H3	C1	C2	C3
1 week	↑[b] 5.6	↑ 4.4	↑ 2.8	↑ 2.9	↑ 5.8	↓ 1.1
3 weeks	↑ 9.5	↑ 2.2	↑ 10.7	↑ 2.9	↑ 2.1	↑ 2.6

a. hDPSCs donors (H1, H2, H3), cDPSCs donors (C1, C2, C3)
b. ↑ = up-regulation, ↓ = down-regulation

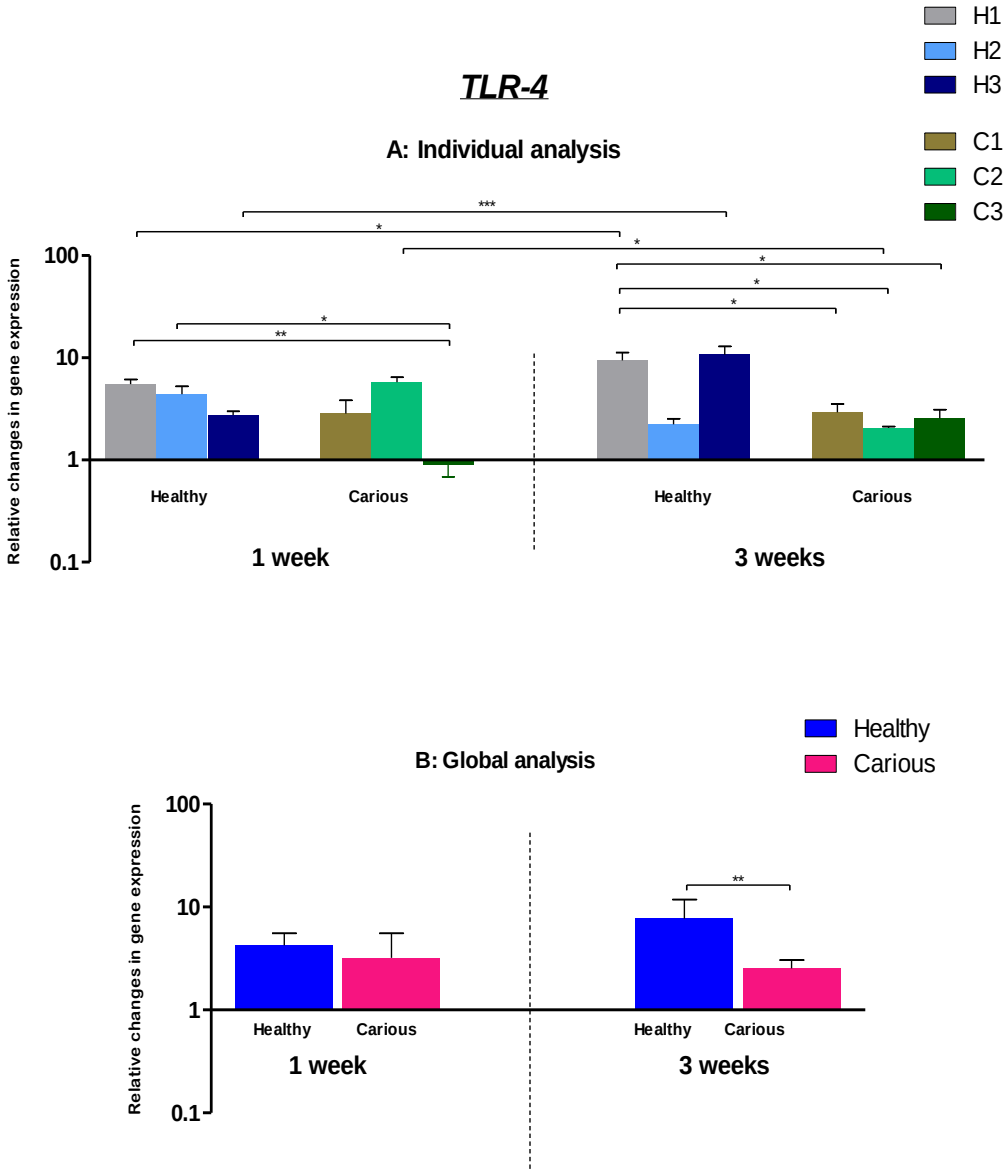

Figure 37: Relative changes in *TLR-4* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions for 1 and 3 weeks

Relative changes in *TLR-4* gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under osteogenic conditions for 1 and 3 weeks. **A:** Individual analysis: showing relative changes in *TLR-4 gene* expression in individual donors. The relative gene expression was normalised to corresponding control cultured under basal conditions. Data are presented as means $2^{-\Delta\Delta ct}$ ± SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged relative changes in *TLR-4* gene expression from the three donors in each group of hDPSCs and cDPSCs. *P<0.05, **P<0.01, ***P <0.001.

5.2.3 Protein expression

Further investigations were carried out to study the protein expression levels of the interleukins IL-6 and IL-8, which are known to be released following TLR activation. hDPSCs (n=3) and cDPSCs (n=3) were cultured under both basal and osteogenic conditions for 1 and 3 weeks.

Conditioned medium (1mL) was collected from each culture at 1 and 3 weeks. IL-6 and IL-8 concentrations in conditioned media were determined using the Cytometric Beads Array as described (Chapter 3, Methods, Section 3.2.6.2). The data were analysed using the flow cytometry analysis program (FCAP). Statistical analysis was carried out using one way ANOVA followed by Bonferroni multiple comparison tests, using Graph Pad Prism software (v 6). Differences were considered significant when P values were <0.05.

5.2.3.1 Comparing IL-6 concentration in media conditioned by hDPSCs and cDPSCs cultured under basal and osteogenic conditions

IL-6 was expressed in media conditioned by both hDPSCs and cDPSCs, cultured under basal conditions at 1 and 3 week time points (Figure 38). Media conditioned by cDPSCs from all three donors showed higher levels of IL-6 at both time points under basal conditions compared with hDPSCs under the same conditions (Figure 38A).

Absolute values of IL-6 protein concentration (pg/mL) were significantly higher in basal media collected at 3 weeks compared with those collected at 1 week, in hDPSCs from the first donor (H1), and cDPSCs from the first and third donors (C1, C3) cultured under basal conditions (Figure 38A).

The global analysis confirmed that concentration of IL-6 was significantly higher in cDPSCs compared with hDPSCs at 1 and 3 weeks under basal conditions (Figure 38B). The baseline levels of IL-6 secretion were higher at 3 weeks compared with 1 week within both cell types, but the differences were not statistically significant (Figure 38B).

Osteogenic conditioned media showed much lower levels of IL-6 compared with basal media conditioned by hDPSCs and cDPSCs. These findings were confirmed in all three donors within each cell type at 1 and 3 week time points (Figure 38A). Figure 38A demonstrated the levels of IL-6 in osteogenic media collected from the different donors in both cell types, and between the two different time points.

The global analysis showed that there was no significant difference between media collected at 1 week compared with those collected at 3 weeks from both types of cells under osteogenic culture conditions (Figure 38B). However, there were statistically significant differences in IL-6 expression between cDPSCs cultured under basal conditions and cDPSCs cultured under osteogenic conditions at 1 and 3 week time points (Figure 38B)

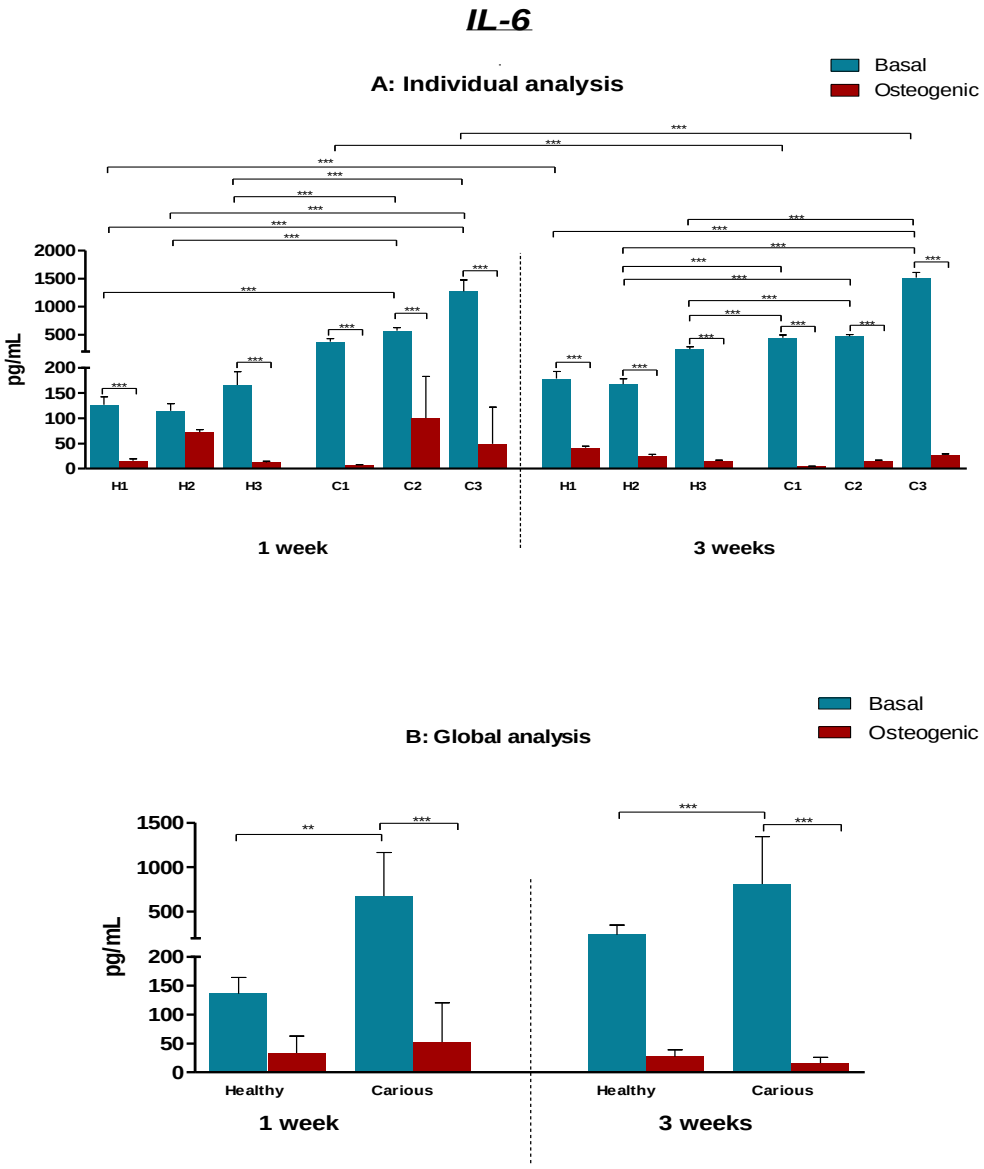

Figure 38: IL-6 protein concentration in media conditioned by cDPSCs and hDPSCs

IL-6 protein concentration in media conditioned by hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under basal (blue) and osteogenic (red) conditions for 1 and 3 weeks were determined by CBA. **A**: Individual analysis: showing IL-6 concentrations secreted by cells from individual donors. Data are presented as means ±SD of IL-6 concentrations (pg/mL) from three technical replicates from each donor. **B:** Global analysis: showing the average IL-6 concentrations (pg/mL) from the three donors in each group of hDPSCs and cDPSCs. *P<0.05, **P<0.01, ***P<0.001

5.2.3.2 Comparing IL-8 concentration in media conditioned by hDPSCs and cDPSCs cultured under basal and osteogenic conditions.

IL-8 was expressed in media conditioned by both hDPSCs and cDPSCs , cultured under basal conditions for 1 and 3 weeks (Figure 39). Media conditioned by cDPSCs under basal conditions from all three donors contained higher IL-8 protein concentrations at both time points, compared with hDPSCs from the first and third donors (H1, H3) under the same conditions (Figure 39A).

Absolute values of IL-8 protein concentration (pg/mL) were higher in basal media collected at 3 weeks compared with those collected at 1 week, in hDPSCs and cDPSCs, from all three donors. However, differences were only significant for cDPSCs from the first and second donors (C1, C2) (Figure 39A).

The global analysis demonstrated that cDPSCs showed a trend to higher baseline expression of IL-8 compared with hDPSCs at 1 and 3 weeks. In addition, the results confirmed that IL-8 levels were higher in 3 weeks culture compared with 1 week culture in both cell types (Figure 39B).

Osteogenic conditioned media showed higher levels of IL-8 compared with basal media conditioned by hDPSCs and cDPSCs. These findings were confirmed in all three donors within each cell type (Figure 39A). Figure 39A demonstrated the levels of IL-8 in osteogenic media collected from the different donors in both cell types, and between the two different time points.

The global analysis confirmed that the levels of IL-8 were higher in osteogenic conditioned media compared with the basal conditioned media,

however, only findings at 3 weeks in both hDPSCs and cDPSCs cultures reached statistical significance (Figure 39B). The lL-8 levels were significantly higher in osteogenic conditioned media collected at 3 weeks compared with those collected at 1 week in both cell types (Figure 39B).

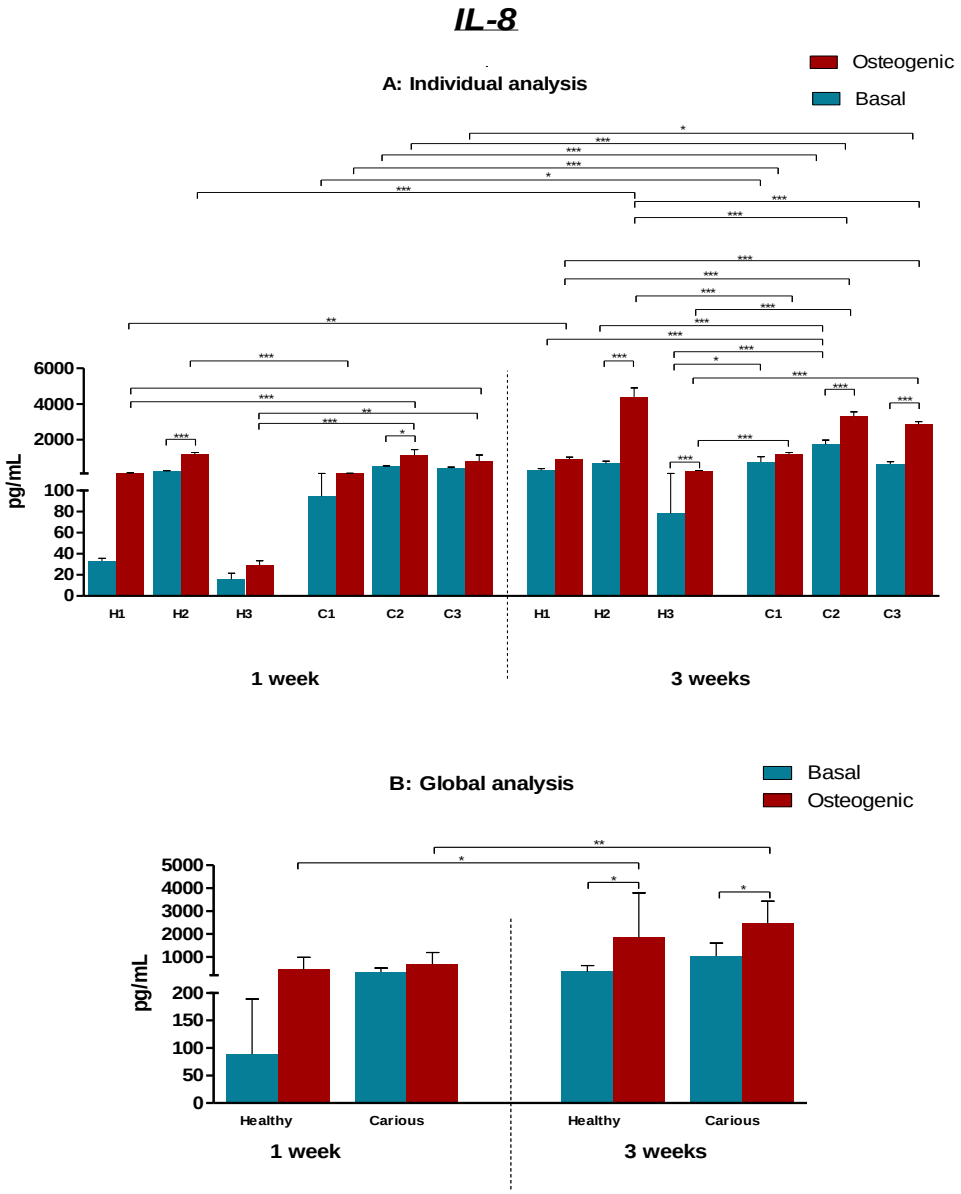

Figure 39: IL-8 protein concentration in media conditioned by cDPSCs and hDPSCs

IL-8 protein concentration in media conditioned by hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under basal (blue) and osteogenic (red) conditions for 1 and 3 weeks were determined by CBA. **A**: Individual analysis: showing IL-8 concentrations secreted by cells from individual donors. Data are presented as means ±SD of IL-8 concentrations (pg/mL) from three technical replicates from each donor. **B:** Global analysis: showing the average IL-8 concentrations (pg/mL) from the three donors in each group of hDPSCs and cDPSCs. *P<0.05, **P<0.01, ***P<0.001.

5.3 Discussion

In this chapter hDPSCs and cDPSCs were cultured under basal and osteogenic conditions and the ability of these cells to differentiate to an osteogenic phenotype was confirmed by the use of histochemical stains and the expression of osteogenic markers. In addition, the expression of angiogenic and inflammatory markers in hDPSCs and cDPSCs isolated from teeth with shallow caries were successfully investigated under basal and osteogenic conditions. To date, only limited studies have been performed on the regulation of regenerative markers in DPSCs isolated from teeth affected by shallow caries. The current work examined DPSCs isolated from shallow caries as dental pulp tissue under these conditions is capable of initiating the innate immune responses and reparative processes following bacterial invasion. In active or deep carious lesions, reparative processes might be absent [432].

Prior to enamel cavitation and dentine involvement caused by bacterial infection, various changes have been observed in the dental pulp [433, 434]. In the case of caries-induced inflammation, bacterial products diffuse through dentinal tubules to further stimulate the dental pulp [267].

In the current study no noticeable morphological changes were apparent in the fibroblast-like dental pulp cells beneath shallow caries. In contrast, an earlier study reported that there were morphological changes in odontoblasts beneath carious enamel [433]. This earlier study used computerised histomorphometry to measure the cytosolic: nuclear volume ratio of odontoblasts, the odontoblast cell: dentinal tubule ratio and the adjacent pre-

dentine area. In the current study only phase-contrast light microscopy was used to highlight the general morphology of hDPSCs and cDPSCs.

This study examined the ability of cDPSCs versus hDPSCs to differentiate down an osteogenic lineage using qualitative and quantitative analyses. Osteogenic induction was carried out by culture in media containing dexamethasone and ascorbic acid. This has been used successfully in earlier studies [63, 435-437]. Other induction factors have been used, which were also successful in inducing osteogenic differentiation in DPSCs; these included enamel matrix derivatives [438], β-glycerophosphate [423, 439, 440], epidermal growth factor [441], 1,25-dihydroxyvitamin D3 [442, 443], human serum [425], TNF-α [444], TGF-β [445, 446] and bone morphgenic proteins [447, 448].

The findings of this study showed that alkaline phosphatase (Figure 22) and Alizarin red staining (Figure 23) were more positive in both hDPSCs and cDPSCs under osteogenic conditions compared with basal cultures. This confirms that these cells could differentiate under the osteogenic conditions used in the current study. However, stronger staining was observed in cDPSCs compared with hDPSCs (Figure 22 and Figure 23). Other studies have also found that hDPSCs have the ability to differentiate down the osteogenic lineage [74, 151, 157, 220], while controversial data have been published regarding cDPSCs isolated from permanent teeth [175, 417, 449-451], and deciduous teeth [177, 415] affected by deep caries.

ALPL, *OC* and *RUNX-2* are essential markers involved in osteogenesis. They were used as potentially indicative of active osteogenic differentiation

and mineralisation [63]. The results of the current study clearly demonstrated that *ALPL* was expressed in hDPSCs and cDPSCs (Figure 24), confirming several previous studies that reported the expression of alkaline phosphatase in dental pulp cells [452-457]. Alkaline phosphatase is normally found in matrix vesicles of mineralised tissues and plays a role in initial formation as well as early mineralisation of these tissues [458, 459]. Additionally, its expression in dental pulp cells is associated with the function of these cells in terms of dentine bio-mineralisation, where phosphate ions are fundamentally essential for nucleation of hydroxyapatite and crystal growth [460]. Furthermore, *ALPL* levels were increased with confluent cultures at the 3 week time point compared to 1 week. This suggests that increased culture duration and cell density established higher levels of *ALPL* expression which may also be associated with lower proliferation of dental pulp cells [460, 461].

Interestingly, cDPSCs from two out of three donors showed higher levels of *ALPL* under basal conditions at both time points compared to hDPSCs under the same conditions (Figure 24). Previous observations have suggested the role of alkaline phosphatase in the beginning of pulp response to the stimulus [462, 463]. For example, an increase in ALP activity was reported in reversible pulpitis although decreased ALP activity was associated with irreversible pulpitis [462, 464]. Different levels of ALP activity were attributed to cellular infiltrates, and the influx of inflammatory mediators associated with advanced stages of inflammation that have an inhibitory effect on the expression of ALP [461].

ALPL is useful osteogenic marker for *in vitro* cell cultures [151, 465-467]. It is related to mineralisation and is considered as an early osteoblastic marker [61, 468]. In the current study, *ALPL* expression in hDPSCs and cDPSCs was up-regulated at 1 week under osteogenic conditions but returned to basal levels or just below at 3 weeks (Figure 25). This temporal pattern of ALP activity in DPSCs has been identified previously [469].

The results of the current study showed that *OC* demonstrated a lower degree of expression in hDPSCs and cDPSCs under basal conditions at both 1 and 3 week time points compared with other osteogenic markers (Figure 26). Low levels of *OC* expression have previously been reported in hDPSCs [467]. However, in impacted third molar teeth where the odontoblastic layer was not included, no OCN expression was reported [470]. Juliana *et al.* (2009), suggested that the expression of *OC* in human dental pulp isolated from healthy premolar teeth indicated that primitive human dental pulp tissue already contains mature osteoblast cells irrespective of any differentiation treatment [471].

Interestingly, cDPSCs from two out of three donors appeared to express slightly higher *OC* under basal conditions compared to hDPSCs at 1 and 3 week time point (Figure 26). Previous observations indicated that this reparative molecule is involved in the repair during pulp injury [470, 472] and the inflammatory-stimulated pulp response [473]. Another study reported higher OCN expression levels in reversible and irreversible pulpitis tissues compared with healthy tissues, although OCN levels in reversible pulpitis were found to be higher compared with irreversible pulpitis [470]. This may

be attributed to the decreased number of cells associated with irreversible pulpitis [474]. Macrophages, which are predominantly present in reversible pulpitis [474], are known to express *OC* during the final stages of differentiation into osteoblasts [475]. In another study, OCN was positively stained in cells located at the periphery of inflamed tissue and around the blood vessels, where stem cells are known to reside [470]. This might be an indication of differentiation of stem cells in response to inflammation and the initiation of a repair/regeneration process.

OC is also widely used as an osteogenic marker [151, 476], and the up-regulation that is reported in the current study has been reported previously during osteogenic differentiation of DPSCs [467]. *OC* is reported to be a marker for mature osteoblasts [477], and is considered a late marker for odontoblast differentiation [478]. This agrees with the data reported in this current study in that *OC* showed a trend of higher expression at 3 weeks compared with 1 week under osteogenic conditions (Figure 27). Such time dependant increases of *OC* expression have been observed previously in hDPSCs [467] and may be associated with elevated levels of matrix mineralisation and lower osteoblast proliferation [479].

Odontoblasts are reported to express *RUNX-2* at variable levels [480, 481]. This current study showed that *RUNX-2* was expressed in hDPSCs and cDPSCs under basal conditions at early and later time points (Figure 28). Furthermore, *RUNX-2* levels were higher in cells from two out of three of donors in the hDPSCs group at 1 week compared with 3 weeks under basal conditions (Figure 28). Contrary to our findings, a separate study showed

increased *RUNX-2* expression in hDPSCs at days 4, 7 and decreased expression at days 14 and 28 in these cells [481].

RUNX-2 expression has been investigated in this study as it is a key player in the differentiation of odontoblasts and osteoblasts [482]. It is a transcription factor whose expression is regulated by Wnt signalling during osteoblast differentiation [483], and β-catenin stimulates the odontoblastic differentiation of dental pulp cells by stimulating *RUNX-2* activity [484]. This study showed a consistent pattern of *RUNX-2* up-regulation at both 1 and 3 week time points under osteogenic culture conditions (Figure 29). However, *RUNX-2* demonstrated higher levels of expression at the 3 week time point compared with 1 week expression under osteogenic culture conditions (Figure 29). This suggests that both hDPSCs and cDPSCs might be still in an active differentiation stage, as *RUNX-2* controls immature osteoblast production and bone formation [485], and its expression is up-regulated until complete maturation of functioning osteoblasts, which are able to produce extracellular matrix [486]. In agreement with this, up-regulation of *RUNX-2* in *in vitro* stromal cell cultures resulted in matrix mineralisation [487]. Interestingly, two donors out of three in the cDPSCs group appeared to express higher osteogenic markers compared with hDPSCs at 1 and 3 week time points, although that applied only at 3 week time point for *ALPL* expressed under osteogenic culture conditions (Figure 25, Figure 27 and Figure 29). This might be due to the active reparative stage of cDPSCs, which is enhanced by an inflammatory environment [488].

Although endothelial cells express VEGFR-1, -2 and -3, angiogenic activity is mediated mainly through VEGFR-2 [489, 490]. VEGF is an important growth factor involved in vasculogenesis (*de novo* formation of new blood vessels) and angiogenesis (the process of forming new blood vessels from pre-existing capillaries and vasculature) [356, 491, 492],. VEGFR expression is required for endothelial cell differentiation during vasculogenesis and angiogenesis, and it activates the migration and proliferation of these cells [493].

This study demonstrated that *VEGFR-2* was expressed at a very low levels in both hDPSCs and cDPSCs under basal conditions (Figure 30). Low levels of *VEGFR-2* gene expression were reported previously in neonatal mouse molar-derived dental pulp [494], and both VEGF and VEGFR-2 proteins were found in dental pulp isolated from healthy third molars, suggesting that both proteins can be produced locally in pulp tissue even with no signs of inflammation [495]. VEGF is secreted by DPSCs, and it was suggested that this was important to the ability of these cells to repair induced myocardial infarction in a rat model by causing an increased number of vessels and a reduction in the infarct size [496]. Further studies suggested that VEGF caused this effect through activation of the PI3K-AKT and MEK-ERK pathway [368]. Using immunohistochemistry, VEGFR-2 was located in endothelial cells of the dental pulp of healthy primary teeth close to the sub-odontoblastic layer, and in healthy incisor and premolar pulp tissue; in endothelial cells and throughout the pulp tissue [497]. The expression of VEGF and VEGFR-2 in odontoblasts and inner enamel epithelium of developing teeth suggests that they might have a role in maturation of the

odontoblasts and ameloblasts [498], although their expression throughout endothelial and stromal cells suggests a more widespread role in pulp angiogenesis [499].

The current study showed that cDPSCs, from two out of three donors, showed higher expression of *VEGFR-2* compared with hDPSCs grown under basal conditions (Figure 30). The inflammatory stimuli and their associated chemical mediators associated with carious lesions are known to modulate the microcirculatory hemodynamic and may result in a greater blood supply to the affected area [500]. In this regard it has been reported that lipopolysaccharides (LPS) derived from Gram negative bacteria [501, 502], and lipoteichoic acid (LTA) derived from Gram positive bacteria induced VEGF expression in macrophages and pulp cells [503]. High VEGFR expression was demonstrated in the inflammatory cell infiltrate in teeth affected by irreversible pulpitis, although expression was decreased significantly in pulp stromal cells under the same culture conditions [495, 499] . An earlier study also found that VEGF is expressed in dentine matrix and suggested that this molecule may be released slowly during dentine injury to play a role in reparative processes [504, 505].

Vasculogenesis has been reported to enhance differentiation of DPSCs into odontoblasts [360], and data from the current study suggest an increased expression of *VEGFR-2* under osteogenic conditions in both hDPSCs and cDPSCs (Figure 31). VEGF is known to be highly expressed in osteoprogenitors, mesenchymal stem cells and in osteoblasts themselves [195, 506-508]. Some studies have even shown that osteoblasts release

higher levels of VEGF than endothelial cells [509]. VEGFR-2 is also known to act in both osteoblast and endotheliocyte differentiation, and it is essential to achieve complete differentiation and formation of mature bone [510, 511], although the full significance of VEGF and VEGFR-2 in the differentiation and formation of bone remains controversial [356, 512]. Studies continue into the dual angiogenic and osteogenic functions of this growth factor [172, 184, 205, 220, 427, 512].

PECAM-1 was expressed at very low levels under basal conditions in both hDPSCs and cDPSCs (Figure 32). However, cDPSCs from two out of three donors showed higher gene expression of *PECAM-1* under basal conditions compared with hDPSCs under the same conditions (Figure 32). PECAM-1 is an adhesion molecule highly expressed in endothelial cells, and plays a key role in intercellular junctions, and in interaction of endothelial cells with leukocytes [513, 514]. Immunohistochemically, PECAM-1 has been identified in healthy dental pulp and in inflamed pulp derived from cases of periodontitis [515]. Supportive to our study, immunohistochemical staining demonstrated that the number of vessels showing positive PECAM-1 expression was higher and more intense in inflamed pulps compared with healthy pulps [515]. Healthy pulps showed staining for PECAM-1 in many blood vessels and throughout the odontoblast layer, sub-odontoblast layer as well as in the pulp core [515].

Under the osteogenic conditions used in this study, levels of *PECAM-1* expression were up-regulated in hDPSCs from all three donors and cDPSCs from two out of three donors (Figure 33). An earlier study reported that

DPSCs isolated from healthy permanent teeth expressed *VEGFR-2* and *PECAM-1* during osteogenic differentiation of these cells in 3D cultures [220]. This 3D experimental model leads to the generation of structures similar to human adult bone with integral blood supply [220]. Simultaneous up-regulation of *VEGFR-2* and *PECAM-1* (as also found in the current study) under osteogenic culture conditions may indicate cell-cell communication between osteoprogenitors and endothelial cells [184].

Toll-like receptors (TLRs) play a pivotal role in inflammatory processes and are activated in DPSCs during bacterial challenge [516, 517]. The expression of *TLR-2* and *TLR-4* in hDPSCs and cDPSCs, and the effect of osteogenic culture conditions on the expression of these receptors were examined in the current study (Figure 34, Figure 35, Figure 36and Figure 37).

The findings presented in Figure 34 and Figure 36 confirm earlier studies showing *TLR-2* and *TLR-4* expression by dental pulp cells isolated from healthy non-erupted third molars [155]. A separate study confirmed *TLR-2* expression in healthy dental pulp, although no protein was identified. This may be due to the sensitivity threshold of immunhistochmical methods used [89], and a subsequent study detected TLR-2 protein in healthy pulp from third molars [156]. For TLR-4 there is more consensus in the literature, and several studies have demonstrated TLR-4 gene and protein expression in healthy dental pulp cells [310, 311, 362, 518].

cDPSCs examined in this study showed higher levels of *TLR-2* and *TLR-4* expression compared with hDPSCs at 1 and 3 weeks under basal conditions (Figure 34 and Figure 36). Generally, TLR expression remains low in healthy cells and would be expected to change in caries [318]. In the early development of caries, Gram positive bacteria infect the dentine, and as the lesion develops towards the pulp, the number of aerobic/facultatively anaerobic Gram positive bacteria decreases and the number of anaerobic Gram negative bacteria increases [517]. Relevant to this is that aerobic/facultatively anaerobic Gram positive bacteria are known to be key player microorganisms in shallow caries lesions [267]. TLR-2 and TLR-4 recognize LTA and LPS respectively, which are produced by the bacteria [400, 519]. Increased expression of *TLR-2* was reported in odontoblasts isolated from mouse molar pulps which were challenged by Gram positive bacteria [306]. The findings of the current study are in agreement with recent results on the expression of TLRs in DPSCs under basal and inflammatory culture conditions which showed differential expression of the TLR family in DPSCs under basal conditions and up-regulation of TLR-2, -3, -4, -5, and -8 following challenge with an inflammatory medium [298].

TLR-2 and *TLR-4* expression levels were up-regulated in both hDPSCs and cDPSCs, in two out of three donors, at 1 and 3 weeks under osteogenic culture conditions (Figure 35 and Figure 37). Up-regulation of *TLR-2* and *TLR-4* is perhaps not unexpected given that osteoblasts are known to express *TLR-2* and *TLR-4* [520, 521]. It is encouraging to compare the results of the current study with those of another study, which demonstrated that prolonged exposure of osteoprogenitors to microbial infections resulted

in down-regulation of TLRs expression [520]. Shallow caries may indicate short period of exposure of DPSCs to microbial infection. This could explain to some extent the up-regulation of both *TLR-2* and *TLR-4* in cDPSCs under osteogenic culture conditions used in this study. However, there are some controversial data concerning TLRs expression and osteogenesis/ odontogenesis. TLR-2 activation up-regulated osteogenic mediators in human primary osteoblasts [522] and osteogenesis in human bone marrow MSCs [336, 520]. Moreover, it has been reported that TLR-4 activation induces dentine synthesis, enhances DPSCs differentiation to odontoblasts [297], and mineralisation [523]. It also promotes bone marrow MSCs proliferation and osteogenic differentiation via Wnt3a and Wnt5a signalling [524]. Similarly, LPS-induced Wnt5a expression mediated through the TLR-4/Myd88/PI3-kinase/AKT pathway [525], and Wnt5a participated in the induction of MSC osteogenesis [526]. Fischer *et al.* (2006), observed that Pam3Cys (a synthetic lipopeptide and TLR-2 ligand) enhances ALP activity and extra-cellular calcium deposition in bone marrow MSCs, although this differentiation was weak and sporadic compared with MSCs cultured under osteogenic induction conditions [527]. This may be related to the heterogeneity of the MSC population, containing only minor cell subpopulations able to differentiate to an osteoblasts phenotype. Activation of TLR-2 stimulates IL-6 secretion which may itself affect osteogenic differentiation [527].

TLRs are reported to have non-pathological activity in tooth development. For example, activation of TLR-4 enhanced ameloblast differentiation although it inhibited subsequent mineralisation of enamel and dentine [335].

Similarly TLR-4 down-regulated the levels of osteogenic mediators and osteogenesis in osteoblasts [522]. In this context, TLR-4 activation may decrease the ALP activity and in infected pulps, LPS may bind the Ca^{2+} decreasing its availability for enamel and dentine mineralisation [334, 528]. In human PDL stem cells, LPS decreased osteogenic differentiation through activation of TLR-4, which regulated the NF-kB pathway in these cells [291]. Li *et al*. (2014), found that blocking of TLR-4 or the NF-kB signalling pathway led to reversed or decreased osteogenic potential [291]. Additionally, studies reported that TLR-2 and TLR-4 in bone marrow MSCs activated NF-kB dependant signalling [527]; this pathway was observed to inhibit the differentiation of bone marrow MSCs into osteoblasts under osteogenic differentiation conditions [529, 530]. Also, it has been reported that LPS from Porphyromonas gingivalis mediated TLR-2 activation, which resulted in poor differentiation of osteoblasts [531].

Pattern recognition receptors such as TLRs expressed on odontoblast and play a crucial role in innate immunity. One major consequence of TLRs stimulation is the activation of the NF-kB and p38 mitogen activated protein (MAP) kinase intracellular signalling cascades. As a result, subsequent pro-inflammatory mediator expression increases, including that of inflammatory cytokines which in turn influence the target immune cells [271, 274, 285, 532].

Cytokines are molecules that mediate inflammatory responses as a consequence of their biological effects on target cells. Many cytokines and chemokines (including IL-6 and IL-8) have been well studied in relation to

the inflammatory response to bacterial infection as they are expressed by a variety of cells, in addition to immune cells [533]. IL-6 and IL-8 proteins were secreted into media conditioned by all three donors from each group; hDPSCs and cDPSCs. Interestingly, there was a trend for higher concentrations of IL-6 and IL-8 in media conditioned by cDPSCs compared with hDPSCs under basal conditions (Figure 38 and Figure 39). These findings are supported by earlier studies, which found that *IL-6* and *IL-8* gene expression was higher in carious pulp tissue compared with healthy pulp tissue isolated from human premolar and molar teeth [380, 534, 535]. In addition, *TLR-4*, *IL-6* and *IL-8* gene expression were each found to be up-regulated in DPSCs isolated from third molars after treatment with LPS and extracts from Streptococcus mutans (a Gram positive bacterium strongly associated with caries) [536], and increased IL-6 protein levels were found in inflamed pulp with a presenting periapical lesion compared to healthy pulp tissue isolated from third molars [537]. The expression of IL-6 and IL-8 were reported to be the highest in deep caries compared with shallow caries [380].

The results of this study demonstrated that IL-6 levels were dramatically down-regulated in all three donors from both hDPSCs and cDPSCs at 1 and 3 weeks under osteogenic conditions (Figure 38). Data with respect to IL-6 and osteogenic differentiation are conflicted. IL-6 enhances the differentiation of pre-osteocytes within the bone marrow MSCs population [527], and is up-regulated in association with late bone remodelling following fracture and secondary bone formation [538]. IL-6 also enhanced osteoblastic differentiation during the process of distraction osteogenesis

[539]. However, it has been shown that the *in vivo* effect of IL-6 on bone homeostasis is mainly towards bone resorption rather than bone formation [540]. Interestingly, treatment with the synthetic glucocorticoid dexamethasone inhibited the endogenous production of IL-6 [271, 537, 541]. This may be pertinent to our study as we used dexamethasone in our osteogenic medium and it would be interesting in the future to use dexamethasone-free osteogenic induction medium and examine whether IL-6 expression remains down-regulated.

In contrast to IL-6, IL-8 secretion into conditioned medium increased under osteogenic conditions (Figure 39). Increase IL-8 expression has been reported previously under osteogenic conditions for human MSCs, progenitor cells, and osteoblasts [542]. IL-8 expression was also up-regulated in bone marrow MSCs during osteogenic differentiation using bone morphogenic proteins [543]. IL-6 and IL-8 activate the NF-kB signalling pathway, which activates the osteogenic differentiation of several stem cells including those derived from adipose tissue MSCs [345] and valve interstitial cells [544, 545]. However, the association between the NF-kB pathway and the osteogenic differentiation of MSCs is still controversial [546-550].

Changes in the pulp microenvironment due to inflammatory stimuli are associated with the expression of several chemical mediators [551]. Restricting the responses of dental pulp cells to inflammatory stimuli using; for example, steroidal anti-inflammatory drugs inhibits the expression of pro-inflammatory genes and increases the expression of anti-inflammatory

genes as well as mineralisation related genes including *OC* and *RUNX-2* [552].

During caries injury, demineralisation of dentine leads to mobilization of proangiogenic growth factors including VEGF [504]. Local increase in pulp vasculature has a crucial role to support reparative/regenerative processes including survival in a hypoxic environment [553], nutrition supply, gaseous exchange during the post-differentiation stages of DPSCs [554] as well as pulp cell homing [555]. Angiogenesis events can be stimulated by inflammatory molecules such TLR-4 mediated NF-kB and MAPK signalling in DPSCs, which induces VEGF expression in these cells [310, 362]. IL-6 and IL-8 are essential in the inflammatory response, and are also pro-angiogenic [556-559]. In addition, such inflammatory cytokines were shown to be generated in response to demineralised dentine matrix [271].

Pulp response to different stimuli including dental caries suggested that the inflammatory process and its subsequent signaling might be essential initiators for dental repair [322, 488]. Shallow caries is capable of initiating changes in expression of regenerative molecules including osteogenic, angiogenic and inflammatory markers in DPSCs under different culture conditions.

Chapter 6: Results

IGF axis expression in dental pulp cells

6.1 Introduction

The insulin-like growth factor (IGF) axis comprises two polypeptide growth factors (IGF-1 and IGF-2), two cell surface receptors (IGF-1R and IGF-2R) and six high affinity, soluble binding proteins (IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6) [560]. The IGF axis is known to play a role in the differentiation of stem and progenitor cells into skeletal and dental mineralised tissue [561-563]. This molecular axis is also involved in induction of enamel bio-mineralisation [564], differentiation of dental pulp cells [565], and reparative dentinogenesis [566]. IGF-1 is important for osteogenesis, and when delivered by liposomes into the tooth socket [563], it enhanced deposition of osteodentine-like matrix around implants in combination with calcium hydroxide [221]. IGF-1 in combination with platelet derived growth factor (PDGF) and calcium hydroxide, improved healing of apical tooth perforations in a canine model [567]. Further evidence suggested that IGF-1 regulated the balance between odontogenesis and osteogenesis in apical papilla stem cells [568]. IGF-1 is expressed by dental pulp cells and enhances odontogenic differentiation and deposition of extracellular matrix [565, 569]. IGF-2 was also expressed by dental pulp cells both gene and protein levels, although its function in this tissue is still largely unknown [570].

IGF-1R showed higher expression in teeth with incomplete root development, suggesting the role of IGF-1 in root development [212]. IGFBP-1, -3, -5, and -6 have also been detected in DPSCs isolated from healthy premolars and third molars [571]. IGF axis expression is influenced by different culture conditions including treatment with pharmacological doses of dexamethasone [572], 3D culture conditions [573], exogenous treatment by antagonistic analogues of growth hormone releasing hormone (GHRH) [574], and different concentrations of glucose and amino-acid supplements [575].

In this chapter, the changes in the IGF axis gene and protein expression in dental pulp stromal/stem cells isolated from healthy (hDPSCs) and carious (cDPSCs) teeth grown under osteogenic culture conditions *in vitro* were successfully investigated. In addition, expression data were used to test the hypothesis that members of IGF axis were causally involved in the osteogenic differentiation of hDPSCs.

6.2 Results

6.2.1 Gene expression

The changes in the gene expression of IGF axis members in hDPSCs and cDPSCs cultured under basal and osteogenic conditions for 1 and 3 weeks, were investigated using qRT-PCR as previously described (Chapter 3, Methods, Section 3.2.5). The relative changes in the expression of the IGF axis genes in both hDPSCs and cDPSCs cultured under osteogenic conditions were carried out to determine the changes in the expression of this molecular axis in both cell types under osteogenic culture conditions, compared with their baseline expression under basal conditions. The expression of the genes of interest in both hDPSCs and cDPSCs cultured under osteogenic conditions were normalized to controls from the same cells cultured under basal conditions. The ΔΔCt method was used to calculate the relative change in the gene expression (Chapter 3, Methods, ,Section 3.2.5.6). The mean $2^{-\Delta\Delta ct}$ ±SD were plotted (log 10 scale) to show the relative changes in gene expression at the two time points (1 and 3 weeks). Fold changes in the gene expression were calculated. All experiments were repeated three times from three different donors in each group; hDPSCs and cDPSCs. Results from individual donors (individual analysis) as well as the average results of all donors together in each group (global analysis) were plotted for all genes of interest.

Statistical analysis

Statistical analysis was carried out for individual donors and for global gene expression data using one way ANOVA followed by Bonferroni multiple

comparison tests, using Graph Pad Prism software (v 6). Differences were considered significant when P values were <0.05.

6.2.1.1 Relative changes in the expression of IGF axis genes in hDPSCs and cDPSCs under osteogenic culture conditions

In hDPSCs, expression of *IGF-1* and *IGFBP-1* genes was down-regulated by ~2-fold at week 1 and showed up-regulation by ~34-fold and ~2-fold, respectively, at week 3 under osteogenic culture conditions compared with the same cells cultured under basal conditions (Figure 40). *IGF-2* and *IGFBP-4* levels were up-regulated by ~2-fold and ~4-fold respectively at week 1, and were down-regulated by ~1-fold and ~2-fold, respectively, at week 3 under osteogenic culture conditions compared with the same cells cultured under basal conditions. *IGF-1R* and *IGF-2R* gene levels were up-regulated at both 1 and 3 weeks by ~2-fold under osteogenic culture conditions compared with the same cells cultured under basal conditions (Figure 40). *IGFBP-2* gene expression was up-regulated at both1 week (~4-fold) and 3 weeks (~6-fold) (Figure 40). In contrast, the expression of *IGFBP-3*, *BP-5* and *BP-6* were down-regulated by ~16-fold, ~3-fold and ~2-fold, respectively, at week 1. However, less down regulation was demonstrated at week 3 under osteogenic culture conditions compared with the same cells cultured under basal conditions. The fold changes at 3 weeks under osteogenic culture conditions compared with the same cells cultured under basal conditions were as follows; for *IGFBP-3*: ~11-fold; *IGFBP-5:* ~2-fold; and *IGFBP-6:* ~1-fold (Figure 40).

In cDPSCs, *IGF-1* and *IGFBP-4* gene expressions were down-regulated by ~2-fold at 1 week and showed up-regulation by ~13-fold *(IGF-1)* and ~1-fold *(IGFBP-4)* at week 3 under osteogenic culture conditions compared with the cells cultured under basal conditions (Figure 40). *IGF-1R* and *IGFBP-6* gene expression were down-regulated by ~2-fold at 1 week and were up-regulated by ~5-fold at 3 weeks under osteogenic culture conditions compared with the same cells cultured under basal conditions (Figure 40). The levels of *IGF-2R* and *IGFBP-5* were down-regulated by ~1-fold and ~8-fold, respectively, at week 1 and were up-regulated at week 3 by ~4-fold and ~1-fold, respectively, under osteogenic culture conditions compared to the same cells cultured under basal conditions (Figure 40). On the other hand, *IGF-2*, *IGFBP-1*, and *BP-2* expressions were up-regulated by ~3-fold, ~1-fold and ~3-fold, respectively, at week 1 under osteogenic conditions. However, greater increases were demonstrated in the gene expression of *IGF-2*, *IGFBP-1* and *BP-2* at week 3 (~27-fold, ~6-fold, ~11-fold, respectively) under osteogenic culture conditions compared with the same cells cultured under basal conditions (Figure 40). In cDPSCs, all members of the IGF axis were up-regulated at the 3 week time point compared with 1 week expression, except for *IGFBP-3*. *IGFBP-3* was down-regulated at week 1 (~18-fold) and week 3 (~3-fold) under osteogenic culture conditions (Figure 40).

IGF-1 displayed its highest level of gene expression in hDPSCs at week 3 under osteogenic culture conditions compared with cDPSCs. *IGFBP-2* and *IGFBP-3* were the only two members of the IGF axis which showed consistent pattern of expression with changing culture conditions and

changing time points (1 and 3 weeks) in both hDPSCs and cDPSCs (Figure 40 red and blue columns, respectively).

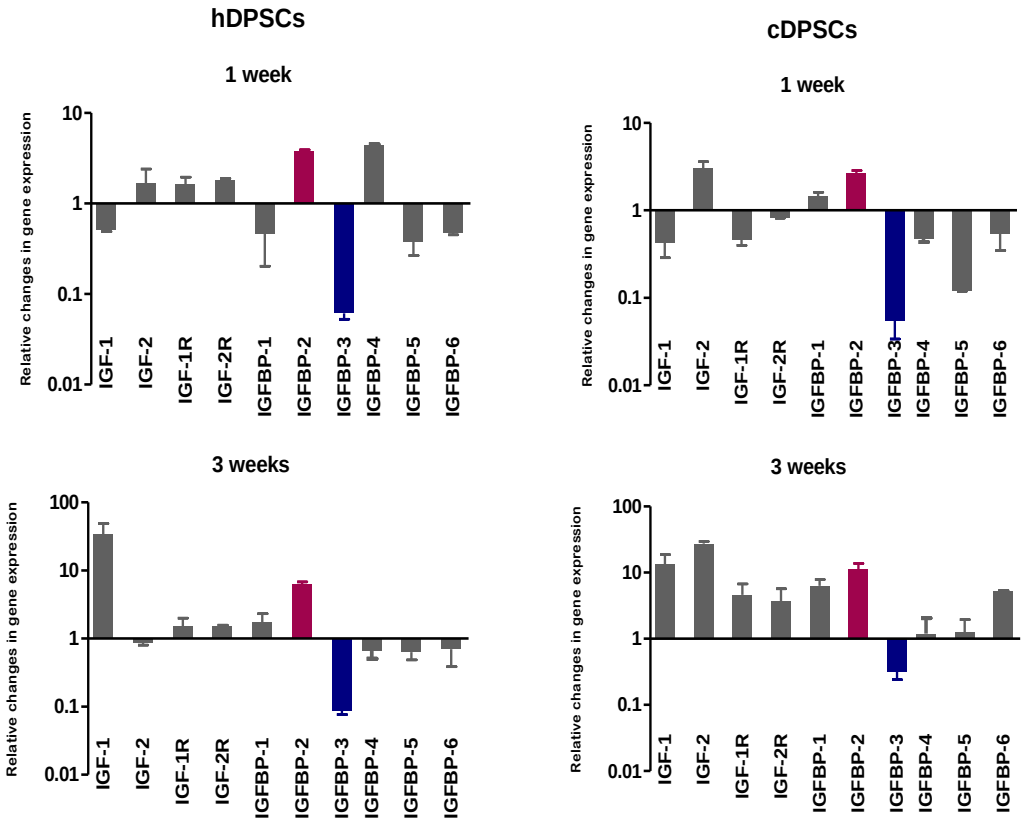

Figure 40: Relative changes in the expression of IGF axis genes in hDPSCs and cDPSCs cultured under osteogenic conditions for 1 and 3 weeks

Relative changes in the expression IGF axis genes in hDPSCs (n=1) and cDPSCs (n=1) cultured in monolayers under osteogenic conditions for 1 and 3 weeks. The relative gene expression was normalised to corresponding control cultured under basal conditions. Data are presented as mean $2^{-\Delta\Delta ct}$ ± SD for three technical replicates.

6.2.1.2 Comparing the changes in *IGFBP-2* and *IGFBP-3* gene expression in hDPSCs and cDPSCs under osteogenic culture conditions

The results in the previous section have shown reproducible and consistent up-regulation of *IGFBP-2* and down-regulation of *IGFBP-3* gene expression in both hDPSCs and cDPSCs under osteogenic culture conditions compared with the basal culture conditions at 1 and 3 week time points. Therefore, further investigations were carried out to confirm and compare the changes in the gene expression of *IGFBP-2* and *IGFBP-3* in DPSCs from six different donors, hDPSCs (n=3) and cDPSCs (n=3), both cultured in monolayers under basal and osteogenic conditions for 1 and 3 weeks.

A: Relative changes in *IGFBP-2* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions

For hDPSCs cultured under osteogenic conditions at 1 and 3 weeks, all three donors showed up-regulation of *IGFBP-2* expression compared with cells cultured under basal conditions (Table 21 and Figure 41A). However, the levels of *IGFBP-2* expression were higher at week 3 compared with week 1 under osteogenic conditions (Table 21 and Figure 41A). The increases were statistically significant comparing the two time points under osteogenic conditions in hDPSCs from the third donor (H3) (Table 21 and Figure 41A).

For cDPSCs cultured under osteogenic conditions for 1 and 3 weeks, all three donors showed up-regulation of *IGFBP-2* levels compared with cells cultured under basal conditions (Table 21 and Figure 41A). The levels of

IGFBP-2 expression appeared to be higher at week 3 compared with week 1 under osteogenic conditions, in cDPSCs from two (C1, C2) out of three donors, with only cDPSCs from the first donor (C1) showing statistically significant difference (Table 21 and Figure 41A).

Comparing both cell types cultured under osteogenic conditions, the levels of *IGFBP-2* expression were higher in cDPSCs from two out of three donors at 1 week (C2, C3), and 3 weeks (C1, C2), compared with hDPSCs from the first and second donors (H1, H2) under the same culture conditions (Table 21 and Figure 41A). The levels of *IGFBP-2* expression were higher at both time points under osteogenic conditions in hDPSCs from the third donor (H3) compared with cDPSCs from all three donors under the same culture conditions. However, results were significant in cDPSCs from the first donor (C1) at 1 week, and in cDPSCs from all three donors at 3 weeks under osteogenic conditions when comparing cDPSCs with hDPSCs (Table 21 and Figure 41A).

Global analysis showed a trend for cDPSCs to express lower *IGFBP-2* levels compared with hDPSCs at 1 and 3 week under osteogenic conditions (Figure 41B). The levels of *IGFBP-2* expression were higher within the same cell type at 3 weeks compared with 1 week under osteogenic conditions. Nevertheless, none of these differences reach statistical significance (Figure 41B).

Table 21: Fold changes in *IGFBP-2* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions compared with cells cultured under basal conditions at 1 and 3 weeks

Duration of culture	Fold change in gene expression in DPSCs from donor [a]:					
	H1	H2	H3	C1	C2	C3
1 week	↑[b] 3.7	↑ 3.2	↑ 12	↑ 2.6	↑ 7.6	↑ 7.3
3 weeks	↑ 6.2	↑ 4.2	↑ 19.6	↑ 11.4	↑ 8.2	↑ 2.1

a. hDPSCs donors (H1, H2, H3), cDPSCs donors (C1, C2, C3)

b. ↑ = up-regulation

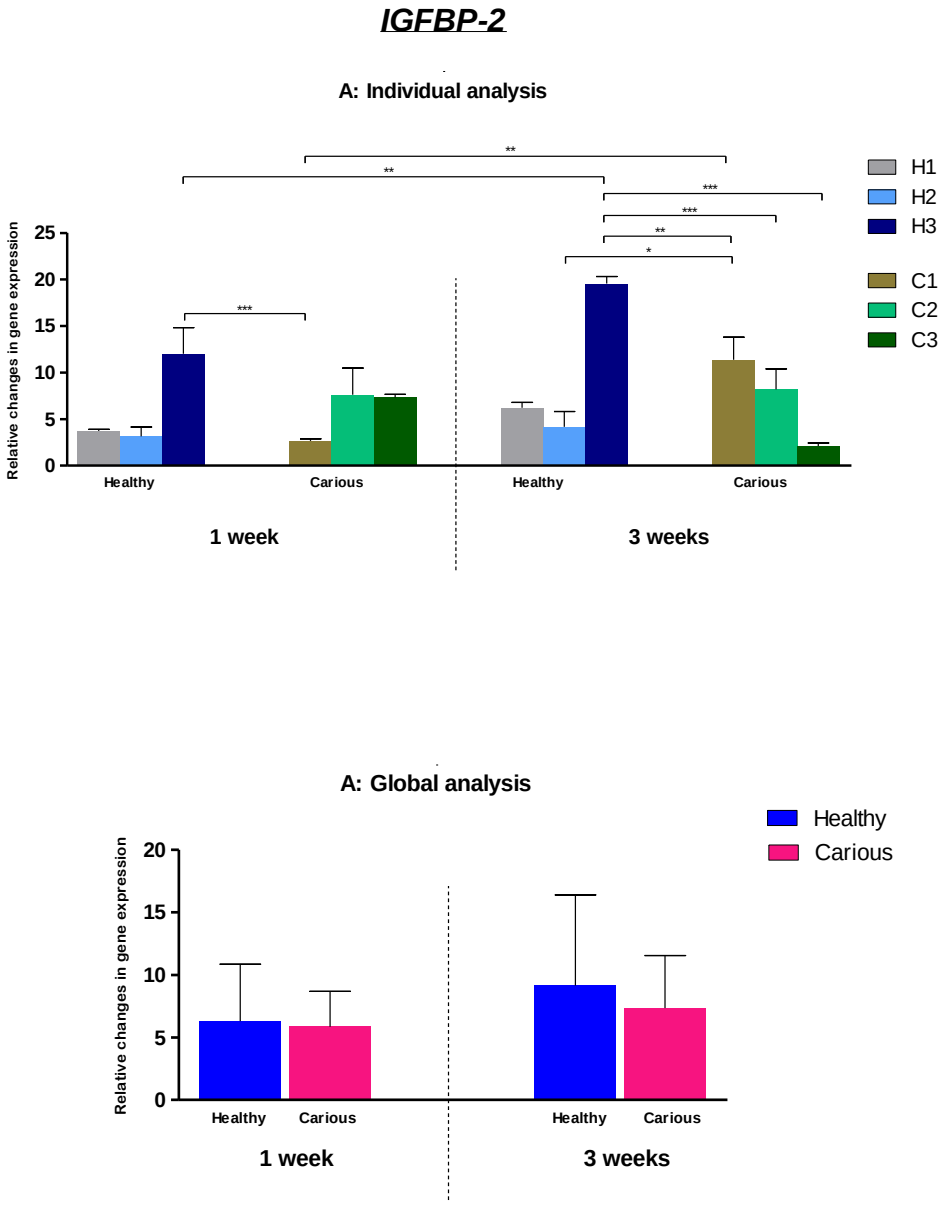

Figure 41: Relative changes in *IGFBP-2* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions for 1 and 3 weeks

Relative changes in *IGFBP-2* gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under osteogenic conditions for 1 and 3 weeks. **A:** Individual analysis: showing relative changes in *IGFBP-2* gene expression in individual donors. The relative gene expression was normalised to corresponding control cultured under basal conditions. Data are presented as means $2^{-\Delta\Delta ct}$ ± SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged relative changes in *IGFBP-2* gene expression from the three donors in each group; hDPSCs and cDPSCs. *P<0.05, **P<0.01, ***P <0.001.

B: Relative changes in *IGFBP-3* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions

For hDPSCs cultured under osteogenic conditions at 1 and 3 weeks, all three donors showed down-regulation of *IGFBP-3* expression compared with cells cultured under basal conditions (Table 22 and Figure 42A). However, the levels of *IGFBP-3* gene expression appeared to be lower in hDPSCs at 1 week compared with 3 weeks under osteogenic culture conditions (Table 22 and Figure 42A).

For cDPSCs cultured under osteogenic conditions at 1 and 3 weeks, all three donors showed down-regulation of *IGFBP-3* gene expression compared with cells cultured under basal conditions (Table 22 and Figure 42A). However, the levels of *IGFBP-3* expression were lower at 3 weeks compared with 1 week under osteogenic conditions, in cDPSCs from two (C2, C3) out of three donors, with only cDPSCs from the second donor (C2) showing statistically significant difference (Table 22 and Figure 42A)

Comparing both cell types, the levels of *IGFBP-3* expression were significantly lower at 1 week under osteogenic conditions, in hDPSCs from all three donors compared with cDPSCs from two out of three donors (C2, C3). The only exception was when comparing the *IGFBP-3* levels in cells from the second donor in each group (H2 and C2), the difference did not reach statistical significance (Table 22 and Figure 42A). At 3 weeks under osteogenic conditions, the levels of *IGFBP-3* were lower in hDPSCs from the first and third donors (H1, H3) compared with cDPSCs from all three donors (Table 22 and Figure 42A). However, the levels of *IGFBP-3* in hDPSCs from the second donor (H2) was higher compared with cDPSCs from the first and

second donors (C1, C2) at 3 weeks under osteogenic conditions, with only cDPSCs from the second donor (C2) reached statistical significance (Table 22 and Figure 42A).

The global analysis showed a trend for cDPSCs to express higher *IGFBP-3* levels compared with hDPSCs at 1 and 3 weeks under osteogenic conditions (Figure 42B). However, the results of the global analysis did not reach statistical significance comparing 1 and 3 weeks within the same cell type (Figure 42B).

Table 22: Fold changes in *IGFBP-3* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions compared with cells cultured under basal conditions at 1 and 3 weeks

Duration of culture	Fold change in gene expression in DPSCs from donor [a]:					
	H1	H2	H3	C1	C2	C3
1 week	↓[b] 18.3	↓ 3.2	↓ 10	↓18.2	↓ 2	↓ 1.2
3 weeks	↓ 11.4	↓ 2	↓ 7.3	↓ 3.2	↓ 7	↓ 1.3

a. hDPSCs donors (H1, H2, H3), cDPSCs donors (C1, C2, C3)
b. ↓ = down-regulation

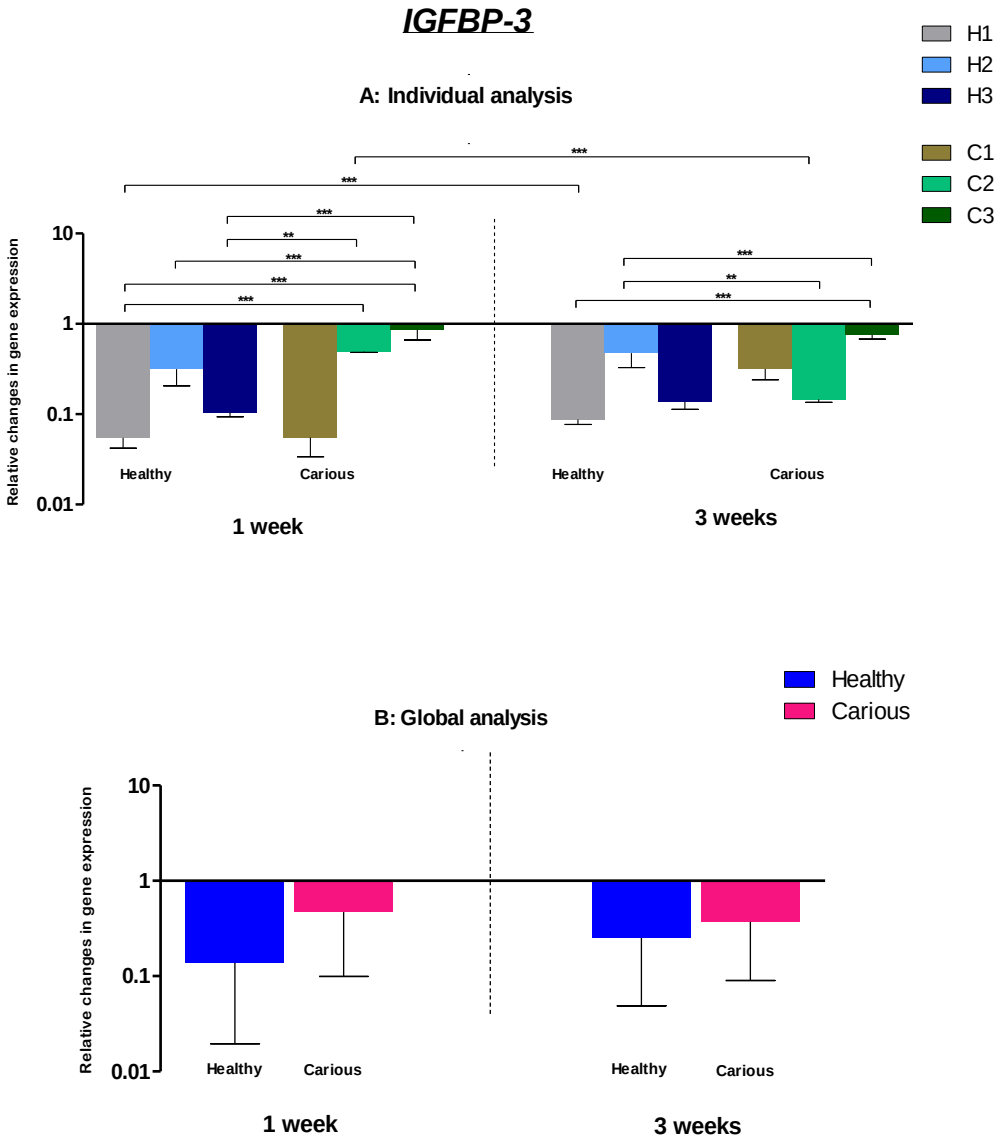

Figure 42: Relative changes in *IGFBP-3* gene expression in hDPSCs and cDPSCs cultured under osteogenic conditions for 1 and 3 weeks

Relative changes in *IGFBP-3* gene expression in hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under osteogenic conditions for 1 and 3 weeks. **A:** Individual analysis: showing relative changes in *IGFBP-3* gene expression in individual donors. The relative gene expression was normalised to corresponding control cultured under basal conditions. Data are presented as means $2^{-\Delta\Delta ct}$ ± SD of three technical replicates from each donor. **B:** Global analysis: showing an averaged relative changes in *IGFBP-3* gene expression from the three donors in each group; hDPSCs and cDPSCs. *P<0.05, **P<0.01, ***P <0.001.

6.2.2 Protein expression

The results in the previous section have shown reproducible and consistent up-regulation in *IGFBP-2* expression and down-regulation in *IGFBP-3* expression in both hDPSCs (n=3) and cDPSCs (n=3) under osteogenic conditions compared with controls from the same cells, cultured under basal conditions for 1 and 3 week time points. Therefore, further investigations were carried out to confirm and compare the changes in the protein levels of IGFBP-2 and IGFBP-3 in DPSCs from six different donors, hDPSCs (n=3) and cDPSCs (n=3).

Conditioned medium (1mL) was collected from each culture at 1 and 3 weeks. IGFBP-2 and IGFBP-3 concentrations in conditioned media were determined using ELISA as described (Chapter 3, Methods, Section 3.2.6). Statistical analysis was carried out using one way ANOVA followed by Bonferroni multiple comparison tests, using Graph Pad Prism software (v 6). Differences were considered significant when P values were <0.05.

A: Comparing IGFBP-2 concentration in media conditioned by hDPSCs and cDPSCs cultured under basal and osteogenic conditions

For hDPSCs and cDPSCs, IGFBP-2 protein concentration (ng/mL) were higher in media conditioned by cells cultured under osteogenic conditions compared with basal cultures at both time points in cells from all three donors (Figure 43A). Absolute values of IGFBP-2 protein concentration were significantly higher in media conditioned by hDPSCs and cDPSCs from all donors cultured under basal and osteogenic conditions at 3 weeks

compared with 1 week in culture. The only exception was for cDPSCs from the second donor (C2), which showed no statistical significant difference comparing the two time points under basal conditions (Figure 43A). The levels of IGFBP-2 in basal and osteogenic media collected from the different donors in both cell types, and comparing the two different time points are demonstrated in Figure 43A.

The global analysis showed that the levels of IGFBP-2 were higher in osteogenic media compared with basal media conditioned by hDPSCs and cDPSCs at 1 and 3 weeks, with only 3 weeks data reached statistical significance (Figure 43B). The levels of IGFBP-2 were significantly higher in osteogenic media conditioned by cDPSCs compared with hDPSCs at 3 week time point (Figure 43B). However, within the same cell type, the levels of IGFBP-2 in both basal and osteogenic conditioned media were higher at 3 weeks compared with 1 week cultures, with only osteogenic media conditioned by cDPSCs reached statistical significance (Figure 43B).

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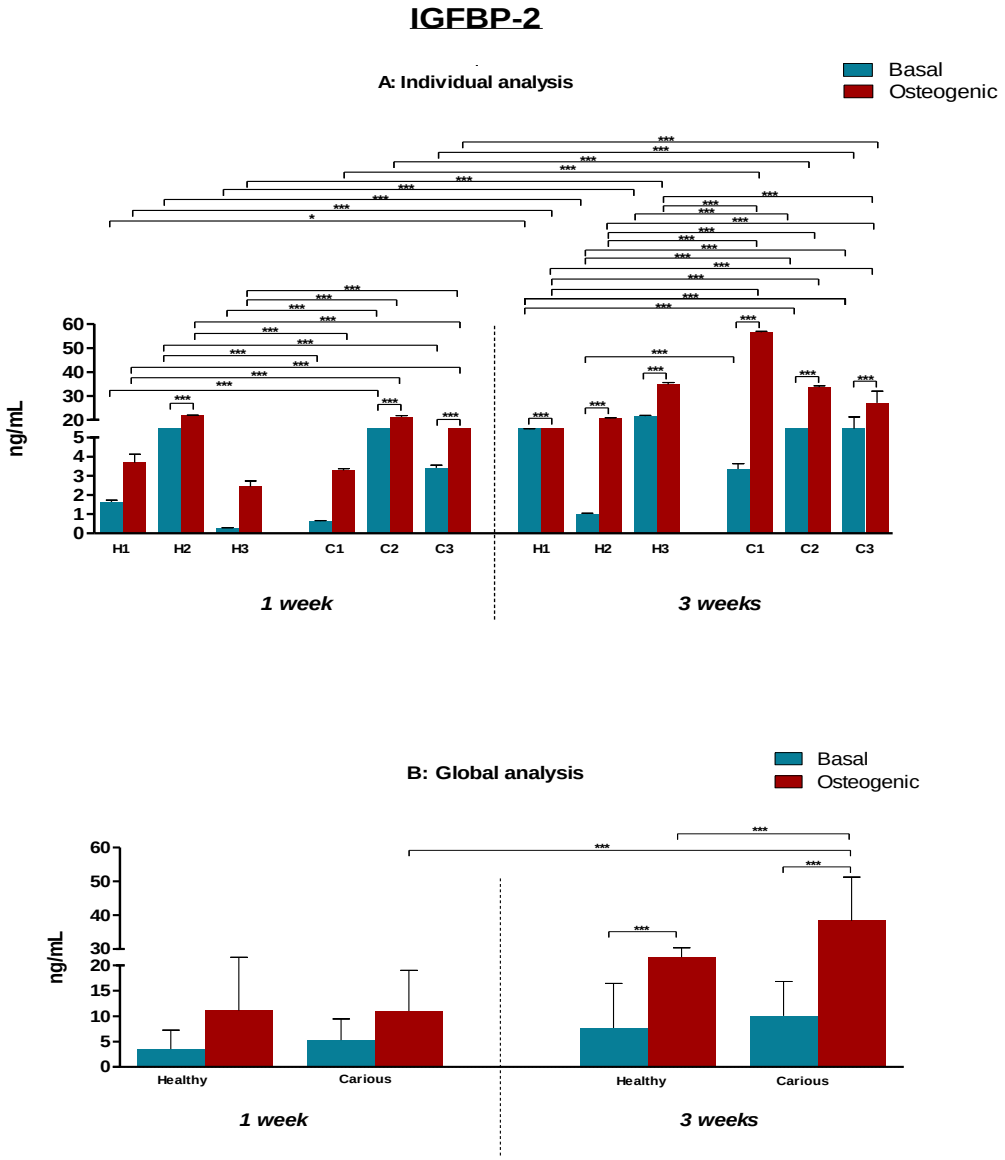

Figure 43: IGFBP-2 protein concentration in media conditioned by cDPSCs and hDPSCs

IGFBP-2 protein concentration in media conditioned by hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under basal (blue) and osteogenic (red) conditions for 1 and 3 weeks were determined by ELISA. **A**: Individual analysis: showing IGFBP-2 concentration in individual donors. Data are presented as means ±SD of IGFBP-2 concentrations (ng/mL) from three technical replicates from each donor. **B:** Global analysis: showing the average IGFBP-2 concentrations (ng/mL) form the three donors in each group. *P<0.05, **P<0.01,***P<0.001

B: Comparing IGFBP-3 concentrations in media conditioned by hDPSCs and cDPSCs cultured under basal and osteogenic conditions

For hDPSCs and cDPSCs, IGFBP-3 concentrations (ng/mL) were lower in media conditioned by these cells cultured under osteogenic conditions compared with basal cultures at both time points, in cells from all donors (Figure 44A). Absolute values of IGFBP-3 concentrations were higher in media conditioned by hDPSCs and cDPSCs from all donors, cultured under basal and osteogenic conditions at 3 weeks compared with 1 week cultures (Figure 44A). The levels of IGFBP-3 in basal and osteogenic media collected from the different donors in both cell types, and comparing the two different time points are demonstrated in Figure 44A.

The global analysis showed that the levels of IGFBP-3 were lower in osteogenic media compared with basal media conditioned by hDPSCs and cDPSCs at 1 and 3 weeks, with only cDPSCs at 3 weeks reached statistical significance (Figure 44B). However, the levels of IGFBP-3 within the same cell type were higher at 3 weeks compared with 1 week in both basal and osteogenic conditioned media, with only basal media conditioned by cDPSCs reached statistical significance (Figure 44B).

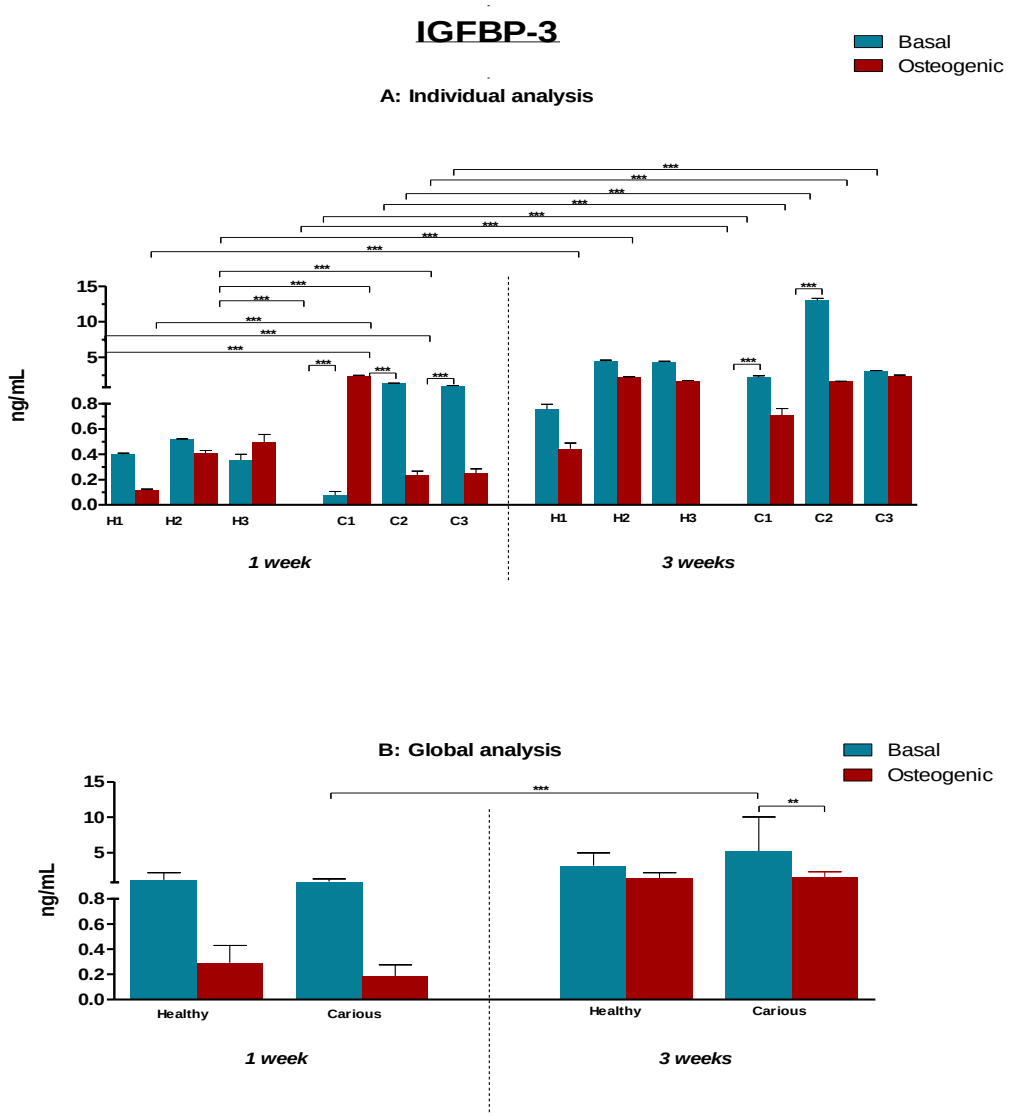

Figure 44: IGFBP-3 protein concentration in media conditioned by cDPSCs and hDPSCs

IGFBP-3 protein concentration in media conditioned by hDPSCs (n=3; H1, H2, H3) and cDPSCs (n=3; C1, C2, C3) cultured in monolayers under basal (blue) and osteogenic (red) conditions for 1 and 3 weeks were determined by ELISA. **A**: Individual analysis: showing IGFBP-3 concentration in individual donors. Data are presented as means ±SD of IGFBP-3 concentrations (ng/mL) from three technical replicates from each donor. **B:** Global analysis: showing the average IGFBP-3 concentrations (ng/mL) form the three donors in each group. *P<0.05, **P<0.01,***P<0.001

6.2.3 IGFBP-2 and IGFBP-3 biological activity

Although we have shown reproducible and reciprocal changes in IGFBP-2 and IGFBP-3 mRNA and protein expression following osteogenic differentiation of hDPSCs. The hypothesis that either/both of these proteins played a causative role in the osteogenic differentiation process of DPSCs has also been tested in the current study. IGFBPs can have a direct IGF-independent effect on cell physiology or an IGF dependant effect where they act to inhibit or enhance the activity of IGF(s). To take account of this we examined the effects of IGFBP-2 and IGFBP-3 when co-incubated at a fixed concentration (10nM) with varying concentrations (0-100nM) of IGF-1 or IGF-2. As a marker for osteogenic differentiation, ALP enzyme activity was investigated as it showed up-regulation under osteogenic culture conditions used in the current study (Chapter 5).

Figure 45 shows that ALP activity is increased after 7 days. Differential ALP activity under basal and osteogenic conditions was maximal at 14 days which was the final time point of this assay. Accordingly in the current study, a 14 day time point was used to examine the effect of IGFs ± IGFBP-2/-3 on osteogenic differentiation of hDPSCs. The results of such an experiment are shown in Figure 46. For IGF-1 (upper panel); data shows that co-incubation with IGFBP-2 enhances the osteogenic activity of IGF-1 whereas co-incubation with IGFBP-3 has an inhibitory effect on IGF-1 action. At the highest concentrations of IGF-1 (100 nM), ALP activity reaches the highest level and there is no enhancing or inhibitory effect(s) of IGFBP-2 or IGFBP-3 respectively. hDPSCs appear to be less sensitive to IGF-2 with respect to

increased osteogenesis and there is no effect on of co-incubation with either IGFBP-2 or IGFBP-3 on IGF-2 osteogenic activity.

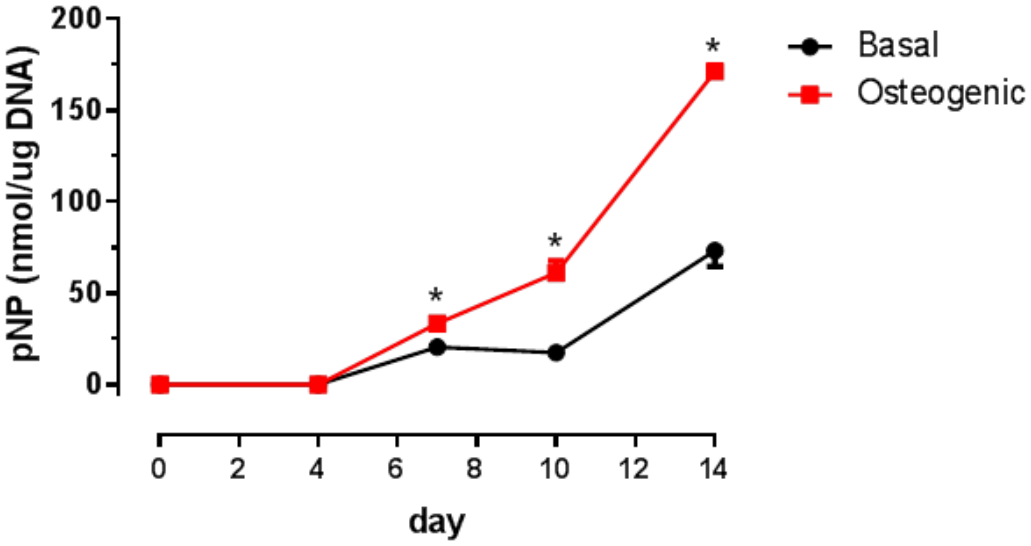

Figure 45: ALP enzyme activity

ALP enzyme activity was monitored over the period 0-14 days in hDPSCs treated under osteogenic (red) or basal (black) conditions (red). Data are presented nmol/µg DNA and represent the mean ± SD of three technical replicates of duplicate wells at each time point for both basal and osteogenic cultures. **P ≤ 0.01 basal v osteogenic.

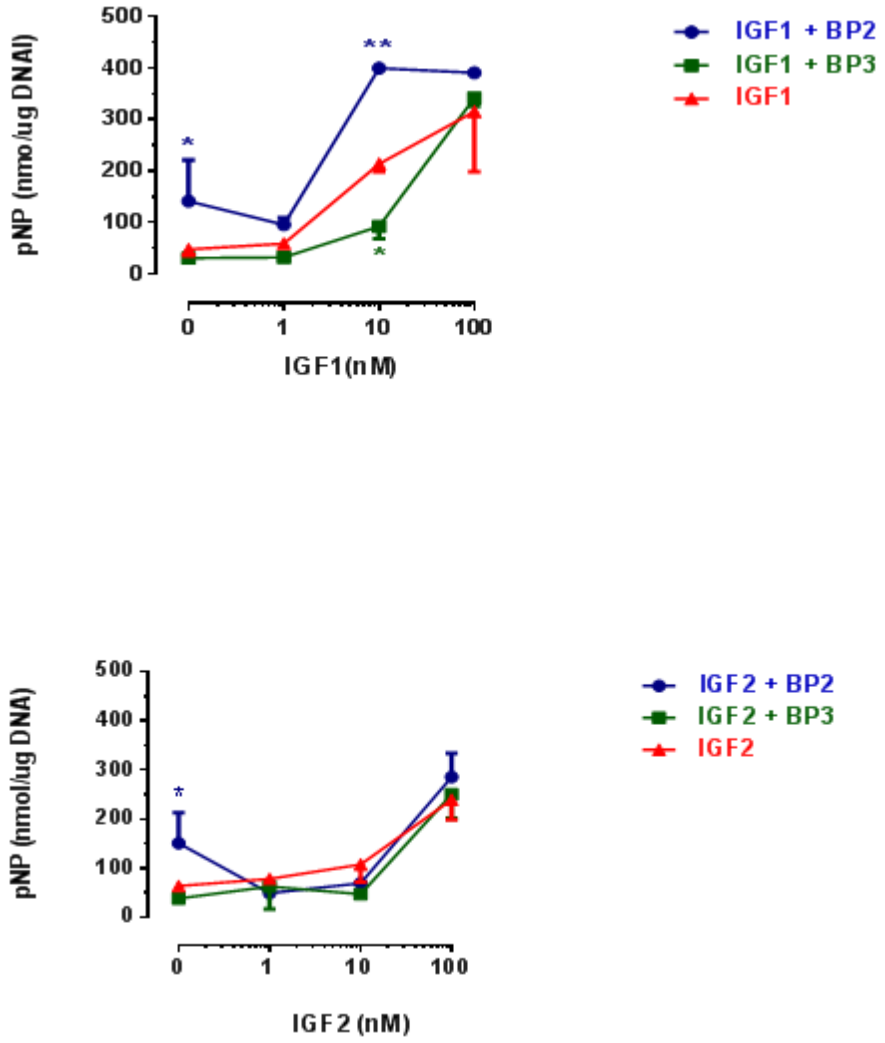

Figure 46: Effect of co-incubation with IGFBP-2 or IGFBP-3 on the osteogenic activity of IGF-1 and IGF-2

Effect of co-incubation with IGFBP-2 (blue) or IGFBP-3 (green) on the osteogenic activity of IGF-1 (red – top panel) or IGF-2 (red- bottom panel). DPSCs were incubated in osteogenic medium containing IGFs (0-100nM) ± IGFBPs at a fixed concentration of 10nM. Incubations were conducted for 14 days before assay of ALP activity. Data are presented as mean ± SD nmol/µg DNA and represent technical triplicate repeats from duplicate wells for each condition. *P<0.05, **P<0.01.

6.3 Discussion

In this study, hDPSCs and cDPSCs have been cultured under basal and osteogenic conditions; the ability of both cells to differentiate into osteogenic lineage has been confirmed in Chapter 5 of this thesis. In the current chapter, the IGF axis profile has been investigated in hDPSCs and cDPSCs cultured in monolayers under basal and osteogenic conditions. The expression of the entire IGF axis under osteogenic conditions in both hDPSCs and cDPSCs has not yet been fully investigated in dental pulp cells.

The current study showed that *IGF-1* was down-regulated in both hDPSCs and cDPSCs at 1 week, although it was up-regulated at 3 weeks under osteogenic conditions compared with the same cells cultured under basal conditions (Figure 40). An earlier study investigated *IGF-1* expression *in vitro* in differentiated normal rat osteoblasts and reported that *IGF-1* gene expression increased from day 5 in culture, reached a maximum level at day 11 and declined thereafter [576]. A subsequent study showed a biphasic change in *IGF-1* expression as the osteoblast cultures progressed through the developmental stages of proliferation, matrix maturation, and mineralisation [577]. Decreased levels of *IGF-1* were also observed prior to mineralisation of the cultures [577].

IGF-2 and *IGFBP-1* demonstrated consistent up-regulation in cDPSCs under osteogenic conditions at 1 and 3 week time points compared with inconsistent patterns in hDPSCs under the same conditions (Figure 40). IGF-2 is known to enhance cell proliferation and differentiation [578], and is particularly active during embryonic development [579]. This growth factor

may be sequestered in dentine matrix during dentine formation and released during demineralisation of dentine. This may to some extent explain the pattern of IGF-2 gene expression in cDPSCs observed in the current study. Supportive of the current results, the expression *IGF-2* and *IGFBP-1* genes were highly expressed in dental pulp cells isolated from deciduous teeth where the pulp was exposed during the removal of proximal caries compared to the level of expression in healthy premolar teeth [580].

IGFs act through cell surface IGF receptors which are expressed in osteoblasts and play a role in osteoblastic differentiation in combination with other members of the IGF axis [581]. The current study showed that IGF-1R and IGF-2R were both up-regulated in hDPSCs at both time points (1 and 3 weeks) under osteogenic conditions compared with basal conditions. These findings confirm to some extent previous reports by Reichenmiller *et al.* (2004), reporting an association between mineralised nodule formation and increased expression of IGF-1R [582].

IGFBP-5 is one of the most abundant IGFBPs in bone. In our study, *IGFBP-5* gene expression was down-regulated in hDPSCs and cDPSCs cultured under osteogenic conditions at the 1 week time point compared with the basal conditions. IGFBP-5 has been implicated as a pro-osteogenic factor in several studies but, conversely, it has been shown to act as an inhibitor of bone formation, as it might interfere with IGF actions on osteoblasts [244]. In contrast to the findings of the current study, Mori *et al.* (2011), demonstrated that the *IGFBP-5* gene was up-regulated during the osteogenic differentiation of dental pulp cells isolated from healthy third molars. They

used microarray analysis to demonstrate an 8-fold increase in IGFBP-5 gene expression in dental pulp cells following 10 days osteogenic treatment [583] . Although their methods were almost similar to here, they isolated pulp tissue from the crown and root of the tooth through cutting around the cementum–enamel junction, which might lead to possible contamination by cells from the gingiva and periodontal ligament. Such contamination of the cell population might have led to the variation in the outcomes of that study compared to the current study [583]. In primary osteoblast cultures, IGFBP-5 is secreted by pre-osteoblasts but decreases in abundance during their differentiation and maturation [577]. *IGFBP-5* gene expression is induced by IGF-1 in cultured osteoblasts [584]. In the current study, though *IGF-1* gene levels were up-regulated in hDPSCs under osteogenic conditions at 3 weeks, *IGFBP-5* gene levels were down-regulated at both time points. IGFBP-5 may be regulated by proteolysis before binding to IGF-1 under osteogenic conditions [585] and the results of this study also showed that *IGFBP-5* level was lower in cDPSCs compared with hDPSCs after 1 week in culture. Indeed, an earlier study revealed that *IGFBP-5* expression was inhibited in stem cells isolated from inflamed periodontal tissue compared with healthy periodontal tissue. This suggested that inflammation itself might potentially down-regulate the local expression of *IGFBP-5* [586].

It has been clearly demonstrated in the results of this study, that there was consistent, reproducible up-regulation of *IGFBP-2* gene expression in both hDPSCs and cDPSCs under osteogenic conditions at 1 and 3 week time points. These findings were mirrored in the results for protein concentrations in conditioned medium. The first report describing *IGFBP-2* gene and protein

expression in osteogenic cultures was in osteoblast-like cells isolated from neonatal rat calvariae [587]. In later studies, IGFBP-2 expression at protein level was reported in both human and mouse osteoblast cell lines [588, 589]. Involvement of IGFBP-2 in enhanced osteogenesis was suggested by the observation of skeletal thickening associated with elevated serum IGFBP-2 levels in hepatitis-associated osteosclerosis [590]. Despite two reports of inhibitory effects of IGFBP-2 on bone size, mass and proliferation of rat calvarias derived cells [591, 592], consensus opinion appears to favour an anabolic role of IGFBP-2 in osteogenesis either complexed with IGFs or acting independently of the growth factor [593, 594].

It has been clearly demonstrated in the results from this part of the study that there was reproducible down-regulation of *IGFBP-3* at gene and protein levels of in both hDPSCs and cDPSCs under osteogenic conditions at 1 and 3 week time points. IGFBP-3 is a key component of the IGF axis and is the most abundant IGFBP in serum [595]. Although IGFBP-3 has been reported to have a direct role on bone formation at the growth plate [595], there remains a considerable debate concerning the effects of IGFBP-3 in bone [596]. *IGFBP-3* overexpression was observed to be associated with enhanced osteogenesis and decreased osteoclastogenesis [597, 598]. Additionally, systematic treatment of ovariectomized rats with recombinant human IGF-1 (rhIGF-1)/IGFBP-3 complexes led to enhanced cortical bone formation [599]. Furthermore, Lee *et al.* (2014), demonstrated a reduction in bone marrow derived macrophage osteoclastogenesis when rhIGFBP-3 was used independently of IGF-1 [598]. IGFBP-3 and a non-IGF binding mutant IGFBP-3 reduced the osteoclast numbers and suppressed cell activity in

mice with collagen induced arthritis [598]. In contrast to these reports, overexpression of *IGFBP-3* (in the presence of IGF-1) in transgenic mice led to increase in osteoclast activity and bone resorption together with diminished osteoblast proliferation and led eventually to decreased cortical and trabecular bone mineral density. This suggests that expression of IGFBP-3 that is largely bound to IGF-1, might sequester the IGF-1 from being delivered to the tissue, which can impair its osteogenic function [596].

Early studies suggested that the expression of IGFBP-3 can be regulated by glucocorticoids such as dexamethasone. Glucocorticoids inhibit IGFBP-3 expression in hepatocytes [600] and fibroblasts [601], although these steroids are also reported to up-regulate *IGFBP-3* expression *in vivo* [602]. Based on these reports, the levels of IGFBP-3 in the current study might be down–regulated due to the effect of dexamethasone in the osteogenic differentiation cultures. Another group who investigated the IGFBP-3 gene expression in an odontoblast-like cell line cultured in non- dexamethasone containing medium confirmed the expression of IGFBP-3 at later stages (25 days) of differentiation [603]. However, Jia *et al.* (2002), reported that *IGFBP-3* gene expression was lower in dexamethasone-treated cultures at day 20, while at days 8 and 14, IGFBP-3 expression levels were higher in dexamethasone-treated cultures compared to non- dexamethasone controls in osteoprogenitor cells derived from the vertebral explants [604]. The use of dexamethasone free differentiation medium may, therefore, be indicated for future experiments and it would be interesting to compare the results of such experiments with those reported in the current study.

In contrast to the current findings, Reichenmiller *et al.* (2004), reported that IGFBP-2 concentrations decreased slightly, while IGFBP-3 increased during differentiation of human pulp cells isolated from healthy third molars [582]. There are several possible explanations for this contradiction, Reichenmiller *et al.* (2004) assayed the cell extracts (and thus intracellular IGFBPs) to determine the protein concentration of IGFBP-2 and IGFBP-3, whilst in the current study, these proteins were measured in conditioned media. IGFBPs are essentially secreted proteins and therefore media conditioned by dental pulp cells are considered more appropriate sources to investigate IGFBP-2 and IGFBP-3 concentrations *in vitro*. Additionally, Reichenmiller *et al*. (2004), did not treat the cells with dexamethasone, or ascorbic acid but simply allowed confluent cells to differentiate in basal medium [582]. Furthermore, the current findings regarding IGFBP-2 (protein and gene) up-regulation in osteogenic differentiation medium containing dexamethasone was confirmed by a previous study [604].

In the current study, media conditioned by cDPSCs showed higher levels of IGFBP-2 and IGFBP-3 compared with hDPSCs in two out of three donors (Figure 41, Figure 42, Figure 43 and Figure 44). Inflammatory processes might explain such findings as it is considered a key player in cDPSCs. The predominant inflammatory environment could activate NF-kB [605]. NF-kB translocation into the nucleus leads to expression of numerous target genes including IGFBPs [606].

However the most exciting and novel data described in this chapter of the thesis relates to the functional interaction of IGFs and IGFBP-2/-3 and how this affects osteogenic differentiation of hDPSCs (Figure 46). IGF-1 clearly stimulated ALP activity in a dose dependant manner and this function in hDPSCs is enhanced by IGFBP-2 or inhibited by IGFBP-3 (Figure 46). The significance in these observations is that they integrate with the expression data of IGFBP-2 and IGFBP-3 in this chapter, which showed up-regulation of IGFBP-2 and down-regulation of IGFBP-3 under osteogenic culture conditions. These experiments designed where the ratio of IGFs: IGBPs would vary as 0.1, 1 and 10 thus allowing study of IGFBP effects at below equimolar, at equimolar and above equimolar concentrations with respect to IGFs. The enhancing and or inhibiting actions of IGFBP-2 and -3 respectively are not seen at the highest ratios of IGF: IGFBP and this was interpreted simply due the fact that the large excess of IGF-1 present under these conditions allows maximum levels of stimulation to be achieved irrespective of the presence of either IGFBP (Figure 46). hDPSCs appear to be less sensitive to the action IGF-2, although ALP stimulation still occurs at the highest concentration of this growth factor (Figure 46).

In conclusion, this part of the current study showed consistent changes in gene and protein expression of IGFBP-2 and IGFBP-3 under osteogenic conditions compared with basal conditions, indicating that they could be key players in the osteogenic differentiation process of DPSCs, and have a role in the regenerative process.

Using an *in vitro* functional assay the effects of IGFBPs were confirmed and consistent with the changes in gene and protein expression seen under

osteogenic culture conditions in hDPSCs. The regulatory roles of IGF axis in osteogenic/odontogenic differentiation processes and the interactions of IGF axis with other hormones, growth factors, extracellular matrix and inflammatory molecules raises more questions that need to be answered in context of basic and clinical research. In the next Chapter, details are provided for initial and preliminary experiments aimed at knocking out *IGFBP-2* expression in hDPSCs in an attempt to further confirm a role of this gene in osteogenic differentiation of these cells.

Chapter 7: Results

Role of the IGFBP-2 in osteogenesis
IGFBP-2 Knock down

7.1 Introduction

The results outlined in earlier chapters have clearly demonstrated that IGFBP-2 was consistently up-regulated in both hDPSCs and cDPSCs under osteogenic conditions compared with similar cells cultured under basal conditions. This opened a question to whether these changes in IGFBP-2 profile were causally associated with the osteogenic differentiation of DPSCs, or whether they could be regarded as the result of differentiation to an osteogenic lineage. Evidence was presented in Chapter 6, Section 6.2.3 that demonstrated IGFBP-2 did indeed play a functional role in the enhancement of the osteogenic activity of IGF-1. To further investigate the role of IGFBP-2 in osteogenic differentiation of hDPSCs, a gene knock down strategy was employed. These experiments were designed to determine whether IGFBP-2 knock-down in hDPSCs would down-regulate the ability of these cells to go down the osteogenic differentiation lineage. A short or small hairpin RNA (shRNA)-based knock down strategy was employed (Methods, Chapter 3, Section 3.2.8) with puromycin selection of stably transfected cell colonies.

7.2 Results

7.2.1 Lipid-based transfection

Determination of the optimum puromycin concentration for selection of stably transfected clones was performed for hDPSCs at passage 4, in 6 well plates and a range of puromycin concentrations of 0-10 µg/mL (Figure 47); cDPSCs were not included in this experiment. The results demonstrated that the minimal concentrations of puromycin required to achieve 100% cell death in hDPSCs were between 4 and 6µg/mL. This range was therefore used for all subsequent lipid-based transfection experiments.

In order to knock down IGFBP-2 gene expression in hDPSCs at passage 4, transfections of target and control plasmids were performed in 6 well plates, in which the concentration of plasmid was held constant at 1µg, while the ratio of concentration of transfection reagent varied between 1:1 and 1:6. Scrambled sequence shRNA was used as a negative control. The experiment was performed according to the manufacturer's instructions (Methods, Chapter 3, Section 3.2.8). However, puromycin resistance could not be achieved by hDPSCs after three weeks. Observations showed that there was no indication of cell growth or proliferation using light microscopy (Figure 48). The puromycin optimisation experiment was therefore repeated to include a lower range of puromycin concentrations, 0-2.5 µg/mL. In a repeat experiments, it was demonstrated that the minimum concentration of puromycin required to achieve 100% cell death was between 1 and 1.5µg/mL, and this range was therefore used in all subsequent lipid-based transfection experiment. Then the knock down experiment was repeated as

described previously, and the observations again confirmed that hDPSCs showed no sign of growth or proliferation using light microscopy.

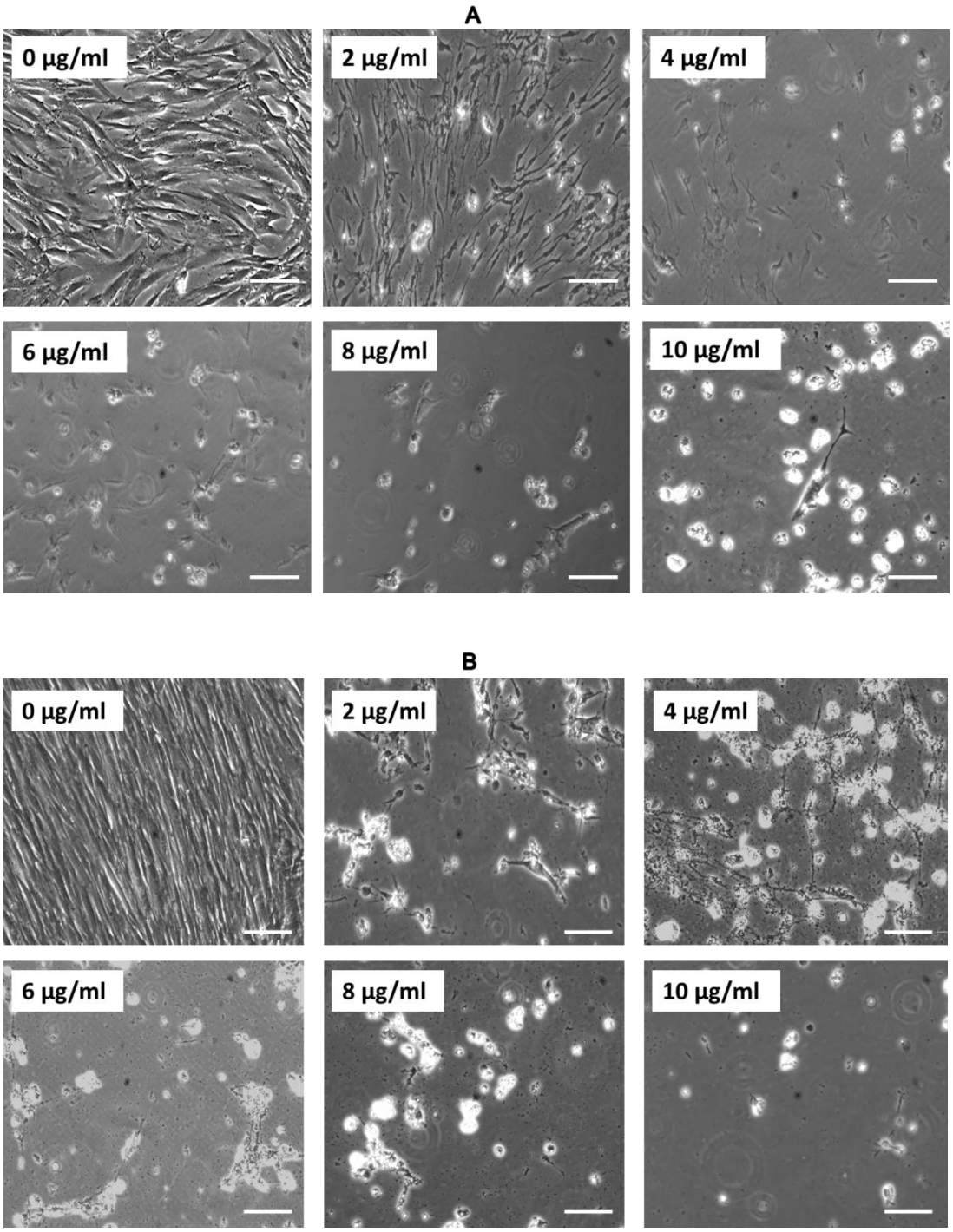

Figure 47: Puromycin optimization

hDPSCs were treated with different concentrations of puromycin within the range 0-10 µg/ml. The images show the appearance of hDPSCs after treatment with different concentrations of puromycin for (A) two days and (B) eight days hDPSCs were cultured in monolayers under basal conditions. Scale bar =100 µm

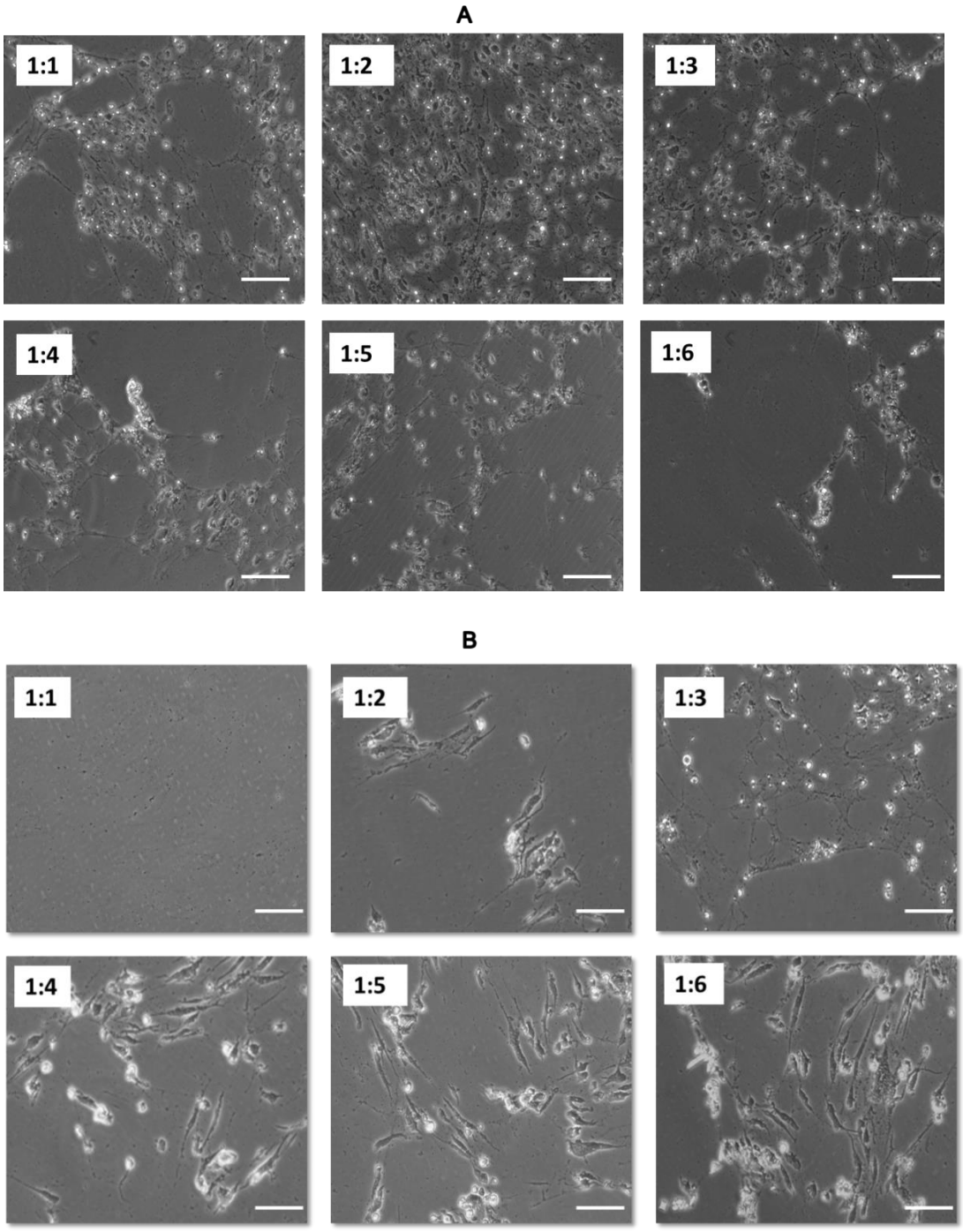

Figure 48: Knock down of IGFBP-2 in 4µg/mL puromycin containing medium

hDPSCs were treated with (A) control scrambled shRNA or (B) IGFBP-shRNA in transfection medium. Transfections of IGFBP-2 shRNA plasmid and control plasmid were performed in 6 well plates in which the concentration of plasmid was held constant at 1µg, while the concentration of transfection reagent varied between a ratio of 1:1 and 1:6. ,Scale bar= 100 µm

7.2.2 Electroporation-based transfection

Previous observations suggested that lipid-based transfection methodologies were unsuccessful to transfect hDPSCs. As an alternative, electroporation-based methodology was used. Electroporation is known to create transient pores in the cellular membrane and these permit nucleic acid to pass into the cells [387]. copGFP (green fluorescent protein cloned from copepod *Pontellina plumata*) control plasmid was used in this experiment as a positive control.

Determination of the optimum puromycin concentration for selection of stably transfected clones was repeated for hDPSCs at passage 4 in 6 well plates and a range of puromycin concentrations of 0-2.5 µg/mL. The cells were exposed to different concentrations of puromycin after they had been exposed to an optimised electric voltage to demonstrate any changes can be caused by electroporation to the optimum puromycin concentration. The minimal concentrations of puromycin required to achieve 100% cell death in hDPSCs were between 1 and 1.5 µg/mL. These concentrations were used in all subsequent electroporation-based transfection experiments.

In electroporation-based transfection experiment, hDPSCs at passage 4 were used with either positive control (copGFP control plasmid), negative control (control shRNA plasmid) or IGFBP-2 shRNA plasmid in electroporation buffer. The protocol was adapted from a previous study, which optimised the electroporation conditions for transfection of dental pulp stem cells [388] (Chapter 3, Methods, Section 3.2.8). After 24 and 48 hours, the cells were checked using fluorescence microscopy and the results were completely negative, as the green fluorescence of copGFP (positive control)

could not be detected inside the cells, indicating failure of the electroporation process to transfect this plasmid into the cells.

7.3 Discussion

In order to investigate whether the alteration in IGFBP-2 expression under osteogenic conditions in hDPSCs was causal or as a result of osteogenic differentiation, a number of attempts were carried out to knock down the gene expression of *IGFBP-2* in hDPSCs. This part of the study aimed to generate a stably transfected cell line in which *IGFBP-2* was targeted to be knocked down using a well-established route. Gene silencing allows investigation of gene function in primary cells [607, 608], and shRNA technology used in the current study has previously been successfully used to knock down *IGFBP-2* in breast cancer cells [609]. Studies have shown for the first time that RNA interference is an active pathway in mammals [610], and this suggested a reverse genetic approach using RNA interference in mammalian systems. The RNA interference approach depends on the formation of double-stranded RNA, in which the antisense strand is complementary to the transcript of the gene of interest. Figure 49 illustrates how RNA silencing becomes functional using stem loop constructs encoding hairpin RNA, resulting in intracellular production of siRNA-like species [608].

Lipid-based chemical transfection is commonly used, in which a lipid transfection reagent allows the shRNA plasmid to pass into the cells. Among gene delivery systems, viral-based vector methods are known to be highly efficient in stable transfection over a prolonged period. Although chemical methods result in lower transfection efficiency, these are simpler to use and are considered biologically safe in comparison to virus-based vectors [611-613].

To select a stably transfected cell line, an antibiotic (puromycin) resistance system has been used in the present study as a selection marker introduced to the cells after transfection. Only those cells which have integrated the plasmid survive, and these contain the drug-resistant gene.

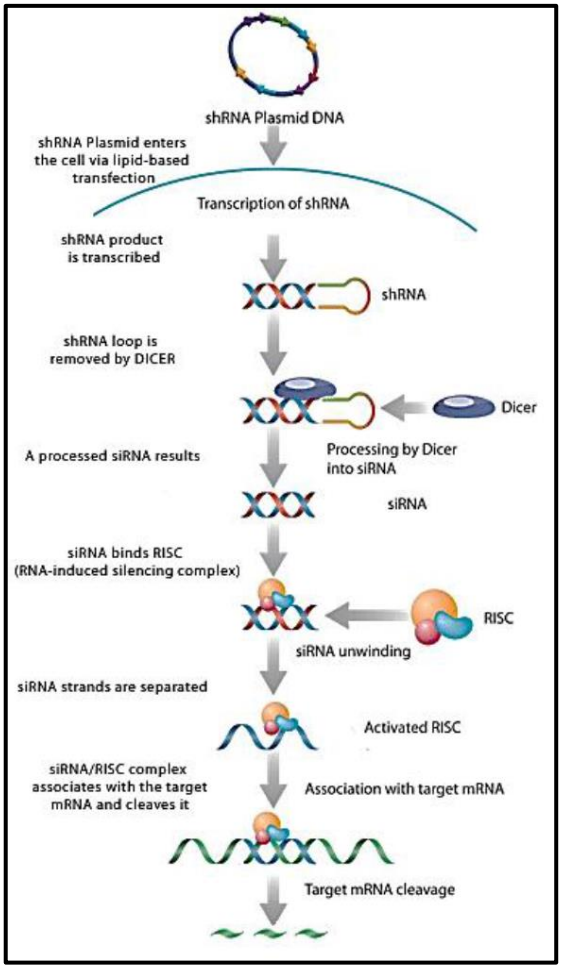

Figure 49: Mechanism of shRNA based gene silencing

Gene silencing using micro RNA designed hairpin RNA. An enzyme known as Dicer cleaves the hairpin RNA and generates small RNA called short interference molecule. The RNA-induced silencing complex (RISC) uses siRNA as a guide in the sequential specific cleavage of the target RNA transcript.
(Santa Cruz Biotechnology, RNA interference, www.scbt.com/gene_silencers.html)

This current study has demonstrated the difficulty in transfecting dental pulp cells as a mixed primary population using a lipid-based chemical transfection technique. hDPSCs in the present study were unable to develop puromycin resistance after lipid-based transfection, indicating the failure of these cells to take up the plasmid. Although chemical-based transfection techniques are simple to apply and require no special equipment, they are relatively expensive and showed insufficient transfection of primary cells in our hands. This might be related to the sensitivity of hDPSCs and the problem of the cytotoxicity of such reagents also remains an issue [614].

It was therefore decided to use the alternative transfection technique of electroporation [615]. This is based on the application of high voltage pulses which can cause transient pores in the cellular membrane and allow the transfection materials to access the intracellular compartment [387, 616]. This technique has been successful to transfect MSCs from different sources [617]. In addition, other groups have studied the difference between electroporation and alternative commercially available transfection reagents [613], based on the human dental follicle cells isolated from human impacted third molars, and have concluded that electroporation results in relatively higher transfection efficiency, with higher cell viability compared with chemical transfection techniques [613].

In the current study, the optimised electroporation parameters have been adapted from the studies of Rizk *et al.* (2012), who examined dental pulp stem cells isolated from sound healthy premolars. They reported that the highest transfection efficiency was achieved under 100V, 20msec and one-pulse square-wave conditions [388]. In contrast to the studies of Ahmed Rizk

et al. (2012), the current observations showed no evidence of successful transfection proved by using copGFP as positive control. Although similar conditions to those described by Rizk *et al.* (2012), have been used, transfection of hDPSCs was not successful in the current study. There may be several reasons why this is the case; for example, this inconsistency may be the result of differences in the electroporation buffer or culture conditions. The degree of permeablisation can be manipulated by the ionic strength of the buffer in which the cells are electroporated [618]. Commonly, a high ionic strength buffer (low resistance) is used in electroporation [387], and serum-free growth media have been used in the present study as electroporation buffer. However, we cultured the cells after electroporation in complete growth medium (containing 20% FBS), although the earlier study did not indicate the culture conditions following electroporation. Studies on transfection of dental follicle cells using electroporation have demonstrated that adding BSA or FBS in the electroporation buffer significantly improved cell viability and increased the number of transfected cells [619]; this could be worth considering in the transfection of DPSCs in the future.

When a specific cell type is difficult to transfect, this might indicate that its membrane is difficult to penetrate. The properties and composition of the cell membrane show considerable variation in different types of cells. The charge offered by the plasma membrane of a given cell type, its thickness and the orientation of its molecules, together with its chemical interaction with the transfection reagent, will all have a role in the efficiency of transfection. For example, Glycocalyx, which is glycoprotein-polysaccharide located in the outer cell membranes of certain bacteria, epithelia and other

cells [620], has a negative charge, and this plays an effective role in the uptake of lipoplexes into the cells [621]. It has however been reported that cationic species can readily pass through the negatively charged cell membrane [621]. This can serve to explain why certain virus protein coat which is simply a sequence of positively charged amino acids can successfully perform cell transfection. Cationic liposome-based gene delivery has proved successful in cell transfection [621]. Lipoplexes larger than 200nm may internalize through caveolin-mediated endocytosis, although smaller complexes can also be internalized via endocytosis [622]. These methodologies may be worth further investigation to achieve successful transfection of hDPSCs. The lipid structure may also play a role in achieving a high level of effectiveness in transfection [623]. Additionally, helper lipids can be used in lipofection, and are responsible for forming the inverted hexagonal structure in liposomes which results in the release of DNA from lipoplex into the cytoplasm [624]. This also promotes optimum fluidity of bilayer of lipoplexes that improves the interaction of vector with cell membrane [625].

Interestingly, a recently developed nucleofection protocol has been used successfully to deliver transfection reagent to the nucleus. Nucleofection is a physical method based on electroporation and uses a combination of electrical parameters applied by a nucleofector with specific reagents [626]. It can therefore be used for non-dividing cells such as neurons [627] and resting blood cells, in addition to other cells known to be difficult to transfect such as human umbilical vein endothelial cells [628].

It might be necessary to optimise the electroporation parameters on a sorted population of dental pulp cells to achieve an adequate level of transfection efficiency including cell concentration, electroporation media, DNA concentration, pulse type and voltage [387, 388, 613]. The differences in published electroporation protocols suggest that minor variations need to be adapted to each type of cell .

Developing techniques to transfect dental pulp stromal/stem cells as a mixed primary population is an important step in the further investigation of gene function for future tissue engineering.

Chapter 8: General Discussion

8.1 General discussion

Dental caries and the consequent pulp inflammation cause major changes in the dental pulp tissue caused by bacterial invasion into the dentine-pulp complex [175, 177, 462, 629, 630]. It is now well-acknowledged that DPSCs can be successfully isolated and cultured from teeth with deep caries, although there are controversial data published regarding their ability to retain stem cell surface marker expression and tissue regeneration potential [175, 177]. However, none of the previous studies have performed thorough investigations of DPSCs isolated from teeth with shallow caries (i.e. cDPSCs), including the expression of stem cell markers, osteogenic, angiogenic and inflammatory markers, as well as the expression of IGF axis. This project set out to better characterise and identify changes in the expression of stem cell and other selected markers in cDPSCs under basal and/or mineralisation conditions compared with hDPSCs. The IGF axis, which plays a crucial role in mineralised tissue formation, was profiled for the first time in this study in cDPSCs. All markers were investigated in each donor in the cDPSCs group and then compared with the donors in the hDPSCs group, which were cultured under exactly the same conditions. Understanding of the inflammation-regeneration processes might help to determine whether the cDPSCs can be used for mineralised tissue regeneration, either *in situ* for dentine/pulp complex, or as a source of autologous stem cells for bone regeneration

In this study, hDPSCs and cDPSCs showed the classic features of MSCs; they were found to express the following stem cell markers: $CD146^{+}$, $CD105^{+,}$ $CD90^{+}$, and were negative for the following markers: $CD45^{-}$ and $CD31^{-}$. They both demonstrated remarkable colony-forming ability, plastic adherence and osteogenic differentiation potential. An earlier studies observed that cDPSCs isolated from teeth with deep caries expressed comparable stem cell surface markers compared with DPSCs isolated from healthy teeth [175, 177, 414]. The multi-lineage differentiation potential of these cells has also been investigated earlier [175].

The findings of the current study concluded that cDPSCs exhibited a higher potential to differentiate into an osteogenic lineage compared with hDPSCs. This was confirmed in two out of three donors in almost all marker genes. Although the pattern of changes in the selected markers under basal and mineralisation culture conditions was comparable in both hDPSCs and cDPSCs, the level of expression in cDPSCs were apparently affected by the inflammatory micro-environment. Two members of the IGF axis; IGFBP-2 and IGFBP-3, demonstrated consistent changes under mineralisation culture conditions in both hDPSCs and cDPSCs; however, the expression of these members appeared to be influenced by inflammation in cDPSCs cell cultures. Interestingly, earlier studies observed that early pro-inflammatory cytokines promoted the mineralisation of DPSCs [631]. Dentine extracellular matrix expresses a range of bioactive molecules that have previously been reported to influence differentiation of dental pulp cells and mineralisation [632-635]. These bioactive dentine extracellular matrix molecules are released as a result of degradation of dentine extracellular matrix due to the

action of acids and proteolytic enzymes derived from both invading bacteria and immune response cells [636, 637]. Dentine extracellular matrix breakdown products have been previously shown to be essential and involve powerful signalling molecules such as basic fibroblast growth factor (bFGF), Insulin-like growth factor (IGF), transforming growth factor-β (TGF-β) [205, 638-640]. The ability of different molecules to stimulate dentine formation *in vitro* and *in vivo* has been proven earlier [108]. The expression of angiogenic factors in dental pulp cells was up-regulated during pulp inflammation. It has been suggested that this observation is due to the role of inflammatory cytokines that are released into the pulp interstitial fluid during inflammation [641, 642]. In addition, activation of TLRs has been observed to stimulate the neoangiogenesis processes along with inflammatory responses [274, 285, 325]. The angiogenic growth factor; VEGF [643, 644], was observed to be expressed by inflammatory cell infiltrates, including neutrophils, eosinophils and lymphocytes in profound dental pulp inflammation [499], and it was expressed by monocytes and macrophages [645]. An earlier study suggested that VEGF is potentially valuable in repairing inflamed pulp tissue through angiogenic activity, which is required at the sites of injury [499].

Tissue reparative events will favourably occur when the infection and inflammation are under control, either due to an effective immune response that eliminates the infection or as a result of clinical treatment that aims to resolve the disease and restore the function of the tissue [25]. Fine-tuning and balance between defence and repair processes is basically the key factor in regulating such complex events [25]. Interesting studies demonstrated that the immune- and repair-related stem/progenitor cells

expressed the same receptors; for example, C-X-C receptor 4 (CXC4) was expressed on both cell types [646, 647], stromal cell derived factor-1 (SDF-1) and its ligand CXCL12 were reported to be expressed within the dentine-pulp complex [648]. Sharing of receptors between immune and repair-related cells can be explained by the parallel needs of both immune and stem cells during caries infection, in attempt to repair the damaged tissue [649].

The cross talk between inflammation and mineralised tissue formation is a controversial and interesting area to discuss. Lipopolysaccharide- activated TLR-4 can promote odontogenic differentiation of human DPSCs isolated from healthy teeth via ERK and p38 MAPK signalling pathways but not NF-kB signalling [297]. On the other hand, another study reported that NF-kB potentially activates the osteogenic transition and promotes the ectopic mineralisation of aortic valves [544]. It has also been reported that TLR-4 signalling was down-regulated during the mineralisation stage of murine odontoblast-like cells [650]. TLR-4 inhibits bone marrow MSCs differentiation into osteoblasts through its inhibitory effect on the Wnt signalling pathway [651]. The inflammatory cytokines as well demonstrated controversial role during mineralised tissue repair. For example, IL-6 levels were increased in response to mechanical strain, indicating that this cytokine might have an anabolic effect during distraction osteogenesis, although it was also reported to have a catabolic effect during fracture repair [539].

8.2 Conclusion

This research characterised DPSCs isolated from teeth with shallow caries. The data generated confirmed that cDPSCs exhibited a higher potential than hDPSCs to differentiate into an osteogenic lineage, with a higher trend to express stem cell, osteogenic, angiogenic and inflammatory markers under basal culture conditions. These findings (along with the observed differences in the expression of selected markers in cDPSCs under mineralised culture conditions compared with hDPSCs) may shed light on the cDPSC . These cells in particular are almost always extracted and discarded; however, they may prove potential for future applications for mineralised tissue repair and regeneration. Changes in the expression of osteogenic, angiogenic and inflammatory markers in both hDPSCs and cDPSCs under basal and osteogenic conditions are summarized in Table 23 and Table 24 .

8.3 Limitations

Variability of cell behaviour and response may due to donor variation as well as to true differences between cells. Unfortunately, it is almost impossible to isolate healthy and carious DPSCs from the same patient and same type of tooth. Furthermore, the available samples for hDPSCs and cDPSCs were generally collected from the tissue bank with very restricted information about patients' medical or dental history. These are important features in examining and interpreting the behaviour of the cells. Efforts were made to restrict the variability within DPSCs population by including only Wisdom teeth. However, there still remained concerns of donor variation in respect of

age, gender and patient's history, which might explain some reported variations in the gene and protein expression seen across the results from all three donors in each group. Nonetheless, the findings presented in this study demonstrated to a great extent very good reproducibility between the donors in each group, as at least two out of three donors showed the same pattern of gene and protein expression in each category under both culture conditions and different time points.

Finally, this study showed unsuccessful attempts to establish stably transfected DPSC lines. The development of stably transfected cell lines is a pre-requisite for our mechanistic studies, due to the lengthy time course(s) associated with most differentiation protocols and particularly with the use of cells in tissue engineering protocols; it is believed that such studies are worth revisiting drawing on the expertise of members within the group, department and indeed elsewhere.

8.4 Future work

A more extensive use of dental tissue material to include stem cells isolated from PDL or SHED may prove informative and it would be interesting to compare the behaviour of cells isolated from these tissues with DPSCs reported in the current study. The development of methodologies to screen larger groups of the population would be useful, particularly if combined with 3D culture methodologies.

The use of flow cytometry as an analytical tool in experiments with DPSCs has been demonstrated in the current study. An access to FACS also as a

preparative tool (via a cell sorter) is also available and the use of the FACS data in this thesis as guide to design an appropriate cell sorting experiments will lead to the isolation of enriched stem cell populations, which can led to more successful transfection experiments. An enriched or purified stem cell population will permit testing the hypothesis that subpopulations of cells may show a preference for differentiation down a particular phenotypic lineage and may be a good basis for future investigation, which would allow development of customised tissue engineering strategies.

Table 23: Summary for regenerative marker expression in both hDPSCs (H1, H2, H3) and cDPSCs (C1, C2, C3) under basal conditions

+ , + + and **+ + +** indicate low, moderate and high level of expression ↑ and ↓ indicate increase or decrease with time

		Osteogenic markers			Angiogenic markers		Inflammatory markers			
		ALPL	***OC***	***RUNX-2***	***VEGFR-2***	***PECAM-1***	***TLR-2***	***TLR-4***	**IL-6**	**IL-8**
H1	***1W***	+ +	+ +	+ +	+	+	+	+	+	+
	3W	↑	↓	↓	↑	↑	↑	+	↑	↑
H2	***1W***	+ +	+ +	+ +	+	+	+	+	+	+ +
	3W	↑	↑	↓	↓	↓	↓	↓	↑	↑
H3	***1W***	+ +	+ +	+ +	+	+	+	+	+	+
	3W	↑	↑	↓	+	↑	↑	↑	↑	↑
C1	***1W***	+ +	+ +	+ +	+	+	+ +	+ +	+ +	+ +
	3W	↑	↑	↓	↓	↓	↑	↓	↓	↑
C2	***1W***	+ +	+ +	+ +	+	+	+ +	+ +	+ +	+ +
	3W	↑	↑	↑	↓	↓	↓	↓	↓	↑
C3	***1W***	+ +	+ +	+ +	+	+	+ +	+ +	+ +	+ +
	3W	↑	↓	↑	↑	↑	↑	↑	↑	↑

Table 24: Summary for regenerative marker expression in both hDPSCs (H1, H2, H3) and cDPSCs (C1, C2, C3) under osteogenic conditions.

▲ and ▼ indicate up-regulation and down-regulation under osteogenic conditions

		Osteogenic markers			Angiogenic markers		Inflammatory markers			
		ALPL	***OC***	***RUNX-2***	***VEGFR-2***	***PECAM-1***	***TLR-2***	***TLR-4***	**IL-6**	**IL-8**
H1	***1W***	▲	▼	▲	▲	▲	▲	▲	▼	▲
	3W	▼	▲	▲	▲	▲	▲	▲	▼	▲
H2	***1W***	▲	▼	▲	▲	▲	▲	▲	▼	▲
	3W	▼	▲	▲	▲	▲	▲	▲	▼	▲
H3	***1W***	▲	▼	▲	▲	▲	▼	▲	▼	▲
	3W	▼	▲	▲	▲	▲	▲	▲	▼	▲
C1	***1W***	▲	▲	▲	▲	▲	▼	▲	▼	▲
	3W	▲	▲	▲	▲	▲	▲	▲	▼	▲
C2	***1W***	▲	▼	▲	▲	▲	▲	▲	▼	▲
	3W	▲	▲	▲	▲	▲	▲	▲	▼	▲
C3	***1W***	▲	▲	▲	▲	▲	▲	▼	▼	▲
	3W	▼	▲	▲	▲	▼	▲	▲	▼	▲

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Publications

Review articles:

H. Al-Kharobi · R. El-Gendy · D. A. Devine · J. Beattie
The role of the insulin-like growth factor (IGF) axis in osteogenic and odontogenic differentiation. Cellular and Molecular Life Sciences 2013

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Research articles:

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Deregulation of IGF-binding proteins -2 and -5 contributes to the development of endocrine resistant breast cancer in vitro. Oncotarget, Advance Publications 2016

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IGFBP-2 and -3 regulate the osteogenic differentiation of human dental pulp stromal cells. manuscript number SCR-D-16-00187. Stem Cell Research

Presentations

Poster Presentation:

H. Al-Kharobi · R. El-Gendy · D. A. Devine · J. Beattie

IGF Axis Expression during Osteogenic Differentiation of Dental Pulp Cells

Tissue and Cell Engineering Society (TCES) 2014, Newcastle upon Tyne, UK, 2-4 July 2014

Oral Presentations:

H. Al-Kharobi · R. El-Gendy · D. A. Devine · J. Beattie

IGF Axis Expression during Osteogenic Differentiation of Dental Pulp Cells

British Society of Oral and Dental Research (BSODR), Bath, UK, 9-10 September 2013

H. Al-Kharobi · R. El-Gendy · D. A. Devine · J. Beattie

Cross Talk Between Inflammation And Regeneration in Dental Pulp Cells

International Association of Dental research (IADR) +American Association of Dental Research (AADR), Boston, United States, 11-14 March 2015

H. Al-Kharobi · R. El-Gendy · D. A. Devine · J. Beattie

Cross Talk Between Inflammation And Regeneration in Dental Pulp Cells

World Congress of Dental Research, Dubai, United Arab Emirates, 23-25 November 2015

H. Al-Kharobi · R. El-Gendy · D. A. Devine · J. Beattie

Characterization of Human Dental Pulp Cells Derived from Carious Teeth

2016 IADR/APR General Session & Exhibition, Seoul, Republic of Korea, 22-25 June 2016